U Mass. Analytical Methods for Algal Assay ✓

NONE 23

Determination of Ortho Phosphate and Total Phosphate in Water and Sea Water

(Heteropoly Blue-Ascorbic Acid Spectrophotometric Method)

Scope

This method is applicable for the determination of ortho and total phosphate in water and sea water.

Principle of the Method

Ortho Phosphate - The sample of water* is treated with a composite reagent containing molybdic acid, ascorbic acid, and trivalent antimony. The absorbance of the reduced heteropoly acid is measured spectrophotometrically at 885 mμ.

Total Phosphate - The sample is boiled with acid and potassium persulfate to convert all phosphorus to ortho phosphate. The phosphate is determined spectrophotometrically in the same manner as ortho phosphate.

Concentration Range

The range extends from 0.3 to 13 micrograms of P in 100 ml of solution using 10-cm cells. The colored system follows Beer's law and is stable for at least two hours.

Interferences (Reference 1)

The following concentrations of ions do not interfere with 3 micrograms of P: copper (II), 500 μg; iron, 2500 μg; silicate, 500 μg; arsenate, 1 μg.

Sodium chloride in sea water causes less than 1 percent error.

Organic phosphorus compounds such as sodium glycerophosphate, inositol hexaphosphoric acid, lecithin, and diphenylamino phosphate do not react under the conditions of the test for ortho phosphate.

Apparatus

Spectrophotometer capable of measuring absorbance of solutions at 885 mμ.

Solutions and Reagents

Ammonium Molybdate Solution - Dissolve 15 g of ammonium molybdate $(NH_4)_6Mo_7O_{24}\cdot 4H_2O$ in 500 ml of distilled water. Store in plastic bottle out of direct sunlight. The solution is stable indefinitely.

Sulfuric Acid for composite reagent - 140 ml conc. H_2SO_4/900 ml H_2O (about 5 N).

Ascorbic Acid - Dissolve 27 grams of ascorbic acid in 500 ml of distilled water. The solution should not be used after standing for a week at 4^oC.

Potassium Antimony Tartrate Solution - Dissolve 0.34 gram of potassium antimony tartrate $K(SbO)\ C_4H_4O_6\cdot 1/2\ H_2O$ in 250 ml of distilled water, warming if necessary. The solution is stable for many months, if stored in dark plastic bottle at 4^oC.

Sulfuric Acid Solution (about 11 N) for oxidation of unreactive P. Cautiously add 300 ml of H_2SO_4 to 600 ml of water. Cool and dilute with distilled water to one liter for oxidation step.

Potassium Persulfate - Analytical reagent grade.

Composite Reagent Solution - Mix together 50 ml ammonium molybdate, 125 ml sulfuric acid (7:45 or 1.4:9), 50 ml ascorbic acid and 25 ml potassium antimonyl-tartrate solutions (total volume about 250 ml). Prepare fresh before use. 250 ml does approximately 30 replicate samples.

1°Stock Standard P Solution - (1 ml contains 40 µg P) - Dissolve 0.1757 g of oven dried (110°C) potassium dihydrogen phosphate (KH_2PO_4) and dilute to 1 liter in a volumetric flask. Preserve with 1 ml chloroform.

2°Dilute Standard Phosphate Solution - (1 ml contains 0.4 µg P) - Transfer 10.0 ml of the stock standard P solution to a 1-liter volumetric flask and dilute to the mark with phosphate-free distilled water. Preserve with 1 ml chloroform.

Preparation of Calibration Graph**

A) Ortho phosphate P. Transfer 0.5-, 1-, 2-, 3-, 5-, and 7-ml aliquots of the P solution (0.4 µg P/ml) to clean 125-ml Erlenmeyer flasks (or 50-ml Nessler tubes). Add distilled water from a buret to bring the volume to 40 ml.

Add 4.0 ml of the composite reagent solution and mix immediately. If Nessler tubes are not provided with glass stoppers mix by pouring the solution into another clean glass container. After 10 minutes and before two or three hours measure the absorbance in a 10-cm cell against distilled water at maximum absorption which occurs near 885 mµ.

Plot absorbance versus concentration of P (µg PO_4-P/l).

B) Total P. Transfer 0.5-, 1-, 2-, 3-, 5-, and 7-ml aliquots of the P solution (0.4 µg P/ml) to clean (6 N HCl washed and distilled water rinsed) 50-ml culture tubes. Add appropriate amounts of distilled water (using a 10 ml graduated cylinder) to make the final volume 20-ml. Proceed by adding 0.4 ml of 11 N sulfuric acid and 0.2 g of potassium persulfate. Carry

along a blank. Cap the culture tubes and autoclave for at least 30 minutes at 15 pounds of pressure.

Cool, add 1 drop phenolphthalein indicator and then adjust to a faint pink color with 4 N sodium hydroxide solution - use vortex mixer. Adjust carefully to clear color with 1 N sulfuric acid (until the pink color just disappears).

Mix well.

Transfer sample to a measuring cylinder, rinsing the tube with distilled water, and dilute with distilled water to 40 ml. Add 4.0 ml composite reagent and transfer back to the empty culture tube. (The transferring action mixes the solution of sample and composite reagent.)

Measure the absorbance as described under Preparation of the Calibration Graph for ortho phosphate P. Plot absorbance versus concentration of P (µg P/l).

Procedure for Ortho Phosphate

Measure 40.0 ml of filtered sample (note 1) in a measuring cylinder. Add 4.0 ml of the composite reagent and transfer to an acid washed (6 N HCl) and distilled water rinsed 50 ml glass culture tube. (The transferring action mixes the solution of sample and composite reagent.) Carry along a blank.

Measure the absorbance as described under Preparation of the Calibration Graph.

Refer to the calibration graph for concentration of the phosphate in the sample.

Procedure for total Phosphate

Collect a 20.0 ml water sample (note 1) in an acid washed (6 N HCl) and distilled water rinsed 50 ml culture tube. Add 0.4 ml of 11 N sulfuric acid and 0.2 gm of potassium persulfate. Carry along blanks. Proceed as described under the procedure for preparation of the total P calibration curve. Refer to the calibration graph for total P for measuring the concentration of total P in the sample. Please see note 2.

*0.45 μ Millipore filtered. Filters must be prewashed with excess distilled water.

**If samples have been preserved with $HgCl_2$ + Cl then your standards (curves) should contain the same amount of preservative.

Notes

1. The sample volume should be adjusted so that the P content is within 0.3 to 13 micrograms using 100 ml volumes and 10-cm cells. For higher concentrations vary the sample volume of use 1-cm cells.
2. If an unfiltered (turbidity containing), well mixed sample was taken for analysis, the sample aliquot should be filtered through a 0.45 μm membrane filter after cooling and before neutralizing with 4 M NaOH. This step is necessary to prevent readsorption and loss of phosphate on the suspended particulate matter during the neutralization adjustment. Also if a clean sample develops turbidity after color developing it must be discarded. Find out if turbidity was developed after the addition of $NaOH-H_2SO_4$: in this case you must filter the $NaOH-H_2SO_4$ treated sample before developing color.

3. If samples have color or turbidity a turbidity or color blank should be carried along.

Ascorbic Acid can be frozen

Combined Reagent - H_2SO_4, Ammonium Molybdate, K Antimony tartrate - can be frozen also

NH_3 - Scaled-down Indophenol Method

1. All glassware is washed in 10 percent HCl prior to use to remove ammonical impurities.

Reagents - refrigerate at 4° but bring to room temperature before using.

1. Phenol solution - dissolve 20 g crystalline analytical grade phenol in 200 ml of 95 percent ethyl alcohol.

2. Sodium nitrogen prusside solution - 1.0 g sodium nitroprusside in 200 ml ammonia free distilled water. This solution is stable at least one month.

3. Alkaline reagent - dissolve 100 g sodium citrate and 5 g analytical grade NaOH in 500 ml deionized H_2O. This solution is stable indefinitely.

4. Oxidizing solution - prepared daily with 10 ml reagent grade sodium hypochloride* diluted to 50 ml with alkaline stock solution. *or chlorox bleach - keep refrigerated

Procedure

Preparation of Calibration Curve

Stock ammonia solution (1,000 mg/L N; stable at least six months) is prepared by dissolving 3.810 g NH_4 Cl (previously dried at least one hour at 100°C and allowed to cool in a dessicator) in deionized water or distilled water and diluting to one liter.

Intermediate ammonia solution (20 mg/L; stable four days) is made by diluting 2 ml stock ammonia solution to 100 ml with distilled H_2O.

Standard ammonia solutions containing 0.1, 0.2, 0.3, 0.4, 0.5, 0.6, 0.8, and 1.0 mg/L are prepared by dilution of 0.5, 1.0, 1.5, 2.0, 2.5, 3.0, 4.0, and 5.0 ml of Intermediate ammonia solution to 100 ml with distilled water.

Samples

Acid washed 20 ml Pyrex test tubes are filled with distilled water and capped with aluminum foil prior to use to lessen contamination by atmospheric ammonia. This water is discarded immediately prior to use for a determination. Five ml of sample or standard are pipetted into each test tube. The following solutions are sequentially added with automatic pipets to samples, 2 blanks and 8 standard solution. The test tubes are vortex mixed after each addition:

0.2 ml phenol solution
0.2 ml nitroprusside solution
0.5 ml oxidizing reagent

The test tubes are then capped with foil and allowed to stand at room temperature for one hour for complete color development. Absorbance is read at 635 nm against the reagent blank. 0.5 in* cells are used to measure the absorbance on the Perkin-Elmer spectrophotometer in the instrumentation room. If absorbance is at the extreme end of the absorbance dial, a 1 cm cuvette should be used in place of the 10 cm cells and all standards and the blank must be read with the 1 cm cuvette also.

Sources:

Strickland and Parsons, A Practical Handbook of Sewager Analysis, Fisheries Board of Canada, 1972.

Neil Ram's PhD thesis, Nitrogenous Organic Compounds in Aquatic Sources, 1979.

Total Organic Nitrogen
Kjeldahl Method

Reagents

1. Digestion mixture

   Dissolve 0.1 g of analytical reagent quality selenium dioxide in 500 ml distilled water. Add 500 ml of concentrated sulfuric acid (reagent grade) and bring to one liter in a one liter volumetric flask. Allow to cool (solution will shrink a little) and bring up to one liter again with distilled water. Add 20 g K_2SO_4 and dissolve. Store in a tightly stoppered glass bottle.

2. Sodium Hydroxide Solution - 4M NaOH

   Rinse 80 g of analytical grade NaOH in pellet form, very rapidly with about 20 ml of deionized water and bring volume to 500 ml in a volumetric flask. Store in a well stoppered polyethylene bottle.

3. Dilute Sulfuric Acid

   Add 50 ml H_2SO_4 to 500 ml of deionized H_2O. Store in a well-stoppered glass bottle and transfer a few milliliters at a time to a clean glass dropping bottle for use.

4. Biomothymol Blue Indicator

5. Boiling beads

   Use four small acid washed beads for each Kjeldahl flask.

Procedure

1. Pipette 25 ml of sample directly into a Kjeldahl flask. Add 2.0 ml of digestion mixture from a pipette or burette and four glass beads.

2. Heat the flasks in the hood on the Kjeldahl heating rack until all the water is removed (when fuming starts). Place glass funnels on mouths of the Kjeldahl flasks to help prevent loss during digestion. The temperature should be adjusted so that the acid refluxes near the top bulb of the flasks with very little loss of vapor. Reflux for 1.0 hour.

3. Cool the flasks and add 10 ml deionized water and transfer to 100 ml volumetrics. Sequential (repeated) rinsing with deionized water assures a complete transfer. Add deionized water to about 75 mls.

4. Add one drop Bromothymol Blue indicator.

5. Titrate the solution in the flask with 4M NaOH. Approach the end point (yellow to faint blue) slowly so as not to overshoot end point. If you overshoot back titrate with dilute H_2SO_4. Dilute to 100 ml w/deionized water.

6. Five ml aliquots are then analyzed for ammonia using the scaled down Indophenol method described earlier.

Sources:

Neil Ram's thesis, Nitrogenous Organic Compounds in Aquatic Sources, 1979.

Strickland and Parsons, A Practical Handbook of Seawater Analysis, Fisheries Research Board of Canada, 1972.

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

1. Objective:

   To determine the nitrate-nitrite nitrogen and the nitrate nitrogen content of an effluent.

2. Brief Description of Analysis:

   The procedure converts nitrate nitrogen to nitrite nitrogen when the nitrate is passed through a column containing copper-cadmium granules. Nitrate is almost quantitatively reduced to nitrite by this process. The resulting nitrite is determined by reacting the effluent with sulfanilamide and coupling with N - (1-napthyl) - ethylenediamine dihydrochloride to form a highly colored dye which can then be determined colorimetrically. A correction must be made for any nitrite initially present in the sample since the method determines total nitrite. The concentration of nitrite originally present in a sample can be determined by omitting the initial copper-cadmium reduction and carrying out the remainder of the procedure. Separate nitrate-nitrite values for a sample may be obtained by analyzing two aliquots of the sample; one with the copper-cadmium reduction step and one without the initial reduction step.

3. Applicability of this Procedure:

   a. Range of Concentration:

      0.01 to 1.0 mg NO_3-NO_2 N/liter

      (The range may be extended for samples by dilution.)

   b. Pretreatment of Samples:

      The Federal Register Guidelines do not specify any pretreatment.

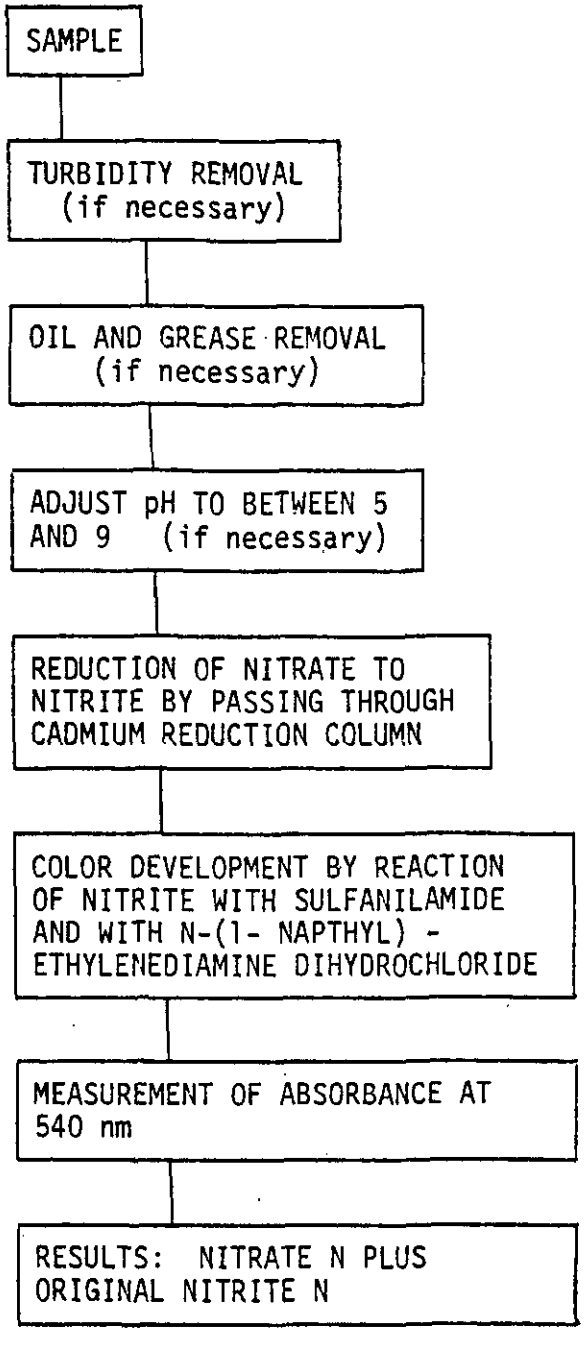
   c. Treatment of Interferences in Samples:

      This procedure includes directions for removal of turbidity and/or of grease and oil from samples. It also includes addition of EDTA to eliminate interferences from metals. No other interferences are noted in the Source of Procedure.*

* Source of Procedure: Methods for Chemical Analysis of Water and Wastes, 1979 U.S. Environmental Protection Agency, Enivornmental Monitoring and Support Laboratory, Cincinnati, Ohio, page 353.3-1 (Issued 1974).

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

FLOW SHEET:



The above procedures determine nitrate N plus nitrite N. The initial nitrite concentration of the samples could be determined without reduction. Thus, the nitrate concentration can be determined by:

Nitrate N = Total Nitrite N - Nitrite N without reduction

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

Equipment and Supply Requirements

A. Capital Equipment:

1. Balance, analytical, 160 g capacity, precision ± 0.1 mg
2. Balance, triple beam, 500 g capacity, precision ± 0.25 g
3. pH meter/combination electrode, range 0-14 pH
4. Refrigerator, temperature range 2° - 10°C
5. Spectrophotometer, wave length range 325-825 nm
6. Still and de-ionizing cartridges (or other means of distilling and de-ionizing water)

B. Reusable Supplies:

1. One apron, laboratory
2. One 100 ml beaker
3. Four 250 ml beakers (3 for buffer solutions)
4. One 400 ml beaker
5. One 1 liter beaker
6. One 2 liter beaker
7. Two bottles, Barnes with stoppers and two droppers, small gauge
8. One 150 ml bottle, dropper
9. One 250 ml bottle, plastic wash
10. One 100 ml bottle, storage with screw-on cap (storage of 6N HCl)
11. Seven 1 liter bottles, storage, brown with screw-on caps or rubber stoppers
12. Two 5 gallon bottles, water with bottom spout
13. One brush, camel hair (cleaning analytical balance)
14. Two brushes, bottle (cleaning glassware)
15. One bulb, propipet type
16. One buret holder, double clamps (reduction column support)
17. Two columns, reduction (see Figure 1 at the end of this section)
18. Three cuvettes
19. One 25 ml cylinder, graduated
20. One 50 ml cylinder, graduated
21. One 100 ml cylinder, graduated
22. One 500 ml cylinder, graduated
23. One 1 liter cylinder, graduated
24. One 50 ml flask, volumetric with stopper (dilution of sample)
25. Twelve 100 ml flasks, volumetric with stoppers (for standards)
26. X 100 ml flasks, volumetric with stoppers (for samples - 1 flask per sample)
27. Twelve 250 ml flasks, Erlenmeyer with stoppers (for standards)
28. X 250 ml flasks, Erlenmeyer with stoppers (for samples-1 flask p[illegible] s[illegible]
29. One 1 liter flask, Erlenmeyer, or a large, empty chemical bott[illegible] (for Cd washings)
30. Three 1 liter flasks, volumetric with stoppers
31. Two 2 liter flasks, volumetric with stoppers
32. One filter funnel for 0.45 μ filter (turbidity removal)

B. Reusable Supplies (Continued)

33. One funnel, powder
34. One funnel, large powder with large filter paper (for Cd washings)
35. One 250 ml funnel, separatory (oil and grease removal)
36. One pair glasses, safety
37. Two hoses, rubber, 3" strip, 4 cm I.D. with screw type clamp
38. One notebook (recording data)
39. Two 100 ml volumetric pipets (construction of reduction columns)
40. One 0.5 ml pipet, volumetric
41. One 1 ml pipet, volumetric
42. One 2 ml pipet, volumetric
43. One 5 ml pipet, volumetric
44. One 10 ml pipet, volumetric
45. One 25 ml pipet, volumetric
46. One 50 ml pipet, volumetric
47. One rod, stirring (6" or 12")
48. One sieve, 40 mesh
49. One sieve, 60 mesh
50. One spatula (scoopula)
51. Two stands, ring (support funnel, and reduction column)
52. One support, ring, small (support funnel)

C. Consumable Supplies:

1. Glasswool, wad
2. Membrane filter, 0.45 μ
3. Notebook (recording data)
4. Pen or pencil (recording data, marking flasks)
5. Soap
6. Sponges (for cleaning)
7. Tissues, soft (wiping cuvettes and electrodes)
8. Towels, paper
9. Twelve weighing boats
10. 26 g ammonium chloride, NH_4Cl

*11. 100 ml ammonium hydroxide, NH_4OH

*12. 150 ml buffer solution, STD pH 4
*13. 600 ml buffer solution, STD pH 7
*14. 450 ml buffer solution, STD pH 10
**15. 25 g cadmium granules, 40-60 mesh
16. 55 ml chloroform, $CHCl_3$ (Freon or another non-polar solvent may be used.)
17. 20 g copper sulfate, pentahydrate, $CuSO_4 \cdot 5H_2O$
18. 3.4 g disodium ethylenediamine tetraacetate, $C_{10}H_{14}N_2Na_2O_8$
19. 1 g N-(1-napthyl) - ethylenediamine dihydrochloride, $C_{12}H_{14}N_2 \cdot 2HCl$

*20. 200 ml hydrochloric acid, concentrated, HCl
21. 100 ml hydrochloric acid, dilute (6N), HCl
22. 100 ml phosphoric acid, concentrated, H_3PO_4

*23. Potassium dichromate (cleaning solution), $K_2Cr_2O_7$

24. 7.218 g potassium nitrate, KNO_3

C. Consumable Supplies (Continued)

25. 6.072 g potassium nitrite, KNO_2
26. 240 g sodium hydroxide, pellets, NaOH
27. 10 g sulfanilamide, $C_6H_8N_2O_2S$
*28. Sulfuric acid, concentrated, (cleaning solution) H_2SO_4
29. 100 g zinc sulfate, heptahydrate, $ZnSO_4 \cdot 7H_2O$
30. Labels, package, 1 1/2 x 1 inch
31. Paper, graph 8 1/2 x 11, package

All reagents should be reagent grade.

The above amounts do not allow for spillage or mistakes.

*These amounts will vary

**MCB Reagents . . .

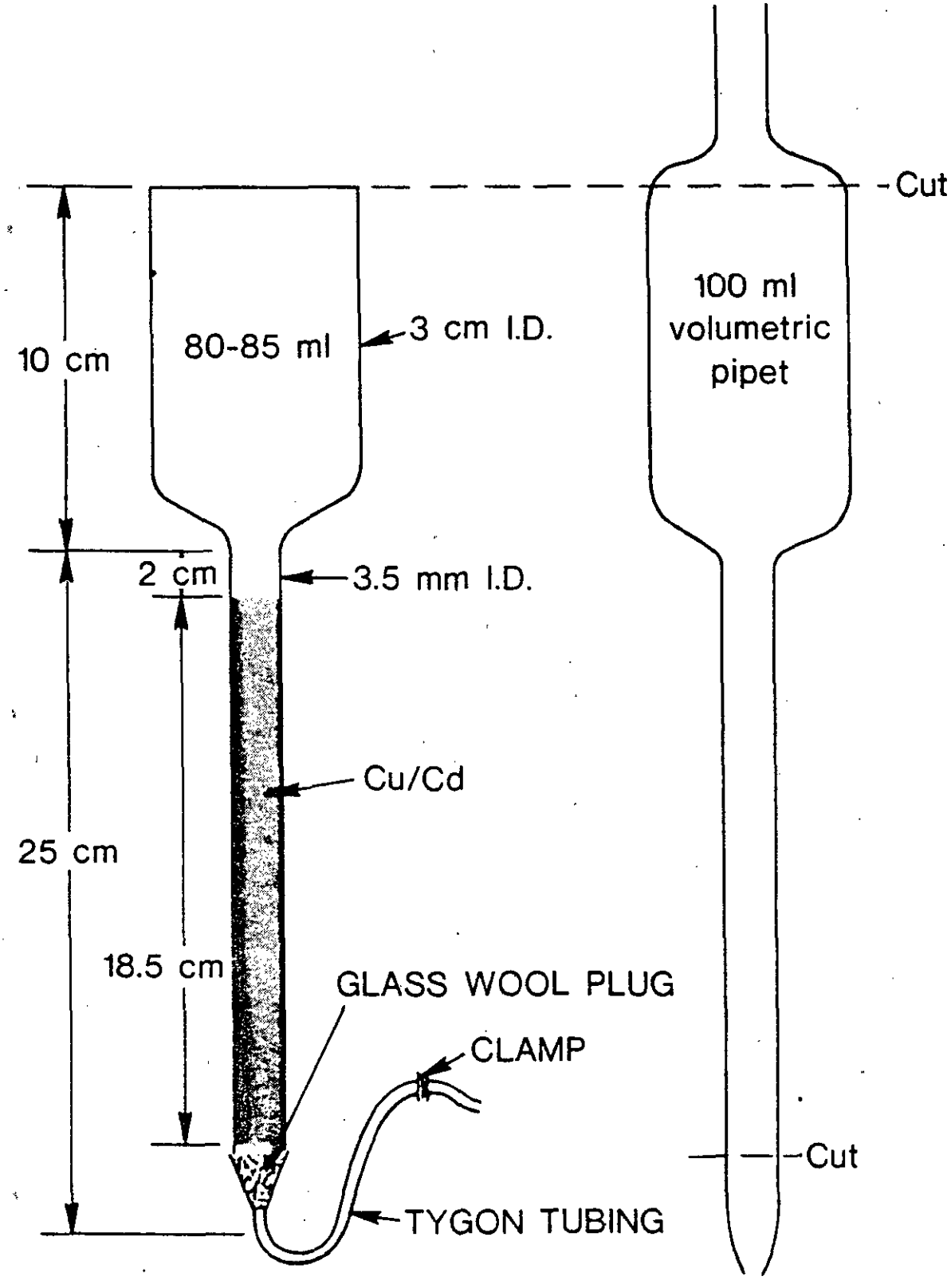


Figure 1. Reduction column

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
DETERMINATION OF NITRATE-NITRITE NITROGEN AND OF NITRATE NITROGEN, mg/liter			I (p. 41)
A. Equipment Preparation			
1. Glassware Wash-Up	1. Clean all glassware in suitable detergent.	1a. Distilled water drains without leaving any droplets on surfaces. 1b. Use chromerge if necessary.	
2. Balance Inspection	1. Clean balance.	1a. Free of dust and dirt.	
3. Spectrophotometer Inspection	1. Clean spectrophotometer.	1a. Free of dust and dirt.	
	2. Turn power on by rotating the power control clockwise.	2a. Pilot lamp on. 2b. Directions are for Spectronic 20.	
	3. Select wavelength by rotating the wavelength control knob either direction until the proper wavelength is reached.	3a. 540 nm on the wavelength scale.	
	4. Zero the instrument by bringing the meter needle to "0" on the percent transmittance scale.	4a. Meter needle reads zero.	
	5. Use an empty cell and adjust the light control to 100% T.	5a. To be sure that the instrument can achieve 100% T.	

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation			
1. Distilled Water	1. Prepare approximately ten (10) liters of highly pure water.	1a. An ion exchange column in conjunction with a still provides an adequate source of highly pure water. 1b. This water will be used for all reagent preparation and washing of equipment. 1c. The pH of the water must be between 5.5-7.5.	
2. Concentrated Ammonium Chloride EDTA Solution	1. Weigh 26 g of ammonium chloride, NH_4Cl, in a weighing boat and wash into 2.0 liter graduated beaker.	1a. Distilled water should be used for all phases of solution preparation including water used in washing a solid into a container.	
	2. Weigh 3.4 g of disodium ethylenediamine tetra-acetate, $C_{10}H_{14}N_2Na_2O_8$, and wash into the same beaker.		
	3. Add enough distilled water to bring the total volume to approximately 1800 ml.		
	4. Use a pH meter to adjust the pH of the solution to 8.5 by the dropwise addition of concentrated ammonium hydroxide, NH_4OH.	4a. Mix the solution thoroughly by stirring, after the addition of each drop of NH_4OH.	
	5. After the pH has been adjusted, transfer the solution to a 2 liter volumetric flask.	5a. Whenever a solution is transferred, the container from which the transfer is made should be washed and the washings added to the container to which the transfer was made.	

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)	6. Dilute to volume with distilled water.	6a. The solution is stable for several months.	
	7. Label the bottle in which the solution is stored.	7a. Include the name of the solution, your name and the date of preparation.	
3. Dilute Ammonium Chloride EDTA Solution	1. Measure 300 ml of the concentrated ammonium chloride-EDTA solution into a one liter graduated cylinder.		
	2. Add distilled water to bring the volume to 500 ml in the cylinder.		
	3. Swirl to mix the solution.		
	4. Store in a labeled container.	4a. This dilute ammonium chloride-EDTA solution is stable for several months.	
4. Color Reagent	1. Add 800 ml of distilled water to a 1 liter flask.	1a. Use a graduated cylinder. 1b. Use a 1 liter volumetric flask.	
	2. Add 100 ml of concentrated phosphoric acid, H_3PO_4, to the same flask.		
	3. Mix thoroughly.		
	4. Weigh 10 g of sulfanilamide ($C_6H_8N_2O_2S$) in a weighing boat.		

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)	5. Use a wash bottle and funnel to wash the sulfanilamide into the 1 liter flask containing phosphoric acid solution.		
	6. Weigh 1 g N-(1-napthyl)-ethylenediamine dihydrochloride, Marshall's Reagent, and wash into same flask.		
	7. Dilute to volume with distilled water.		
	8. Store in a labeled container.	8a. Container should be dark 1 liter plastic reagent bottle. 8b. Store at 4°C when not in use. 8c. Use at room temperature. 8d. The solution is stable for several months. 8e. A very faint pink color may show up in this color reagent. You may still use the reagent. If a precipitate forms in the reagent, though, discard it.	
5. Zinc Sulfate Solution	1. Weigh 100 g of zinc sulfate heptahydrate, $ZnSO_4 \cdot 7H_2O$, in a weighing boat.	1a. This reagent is used if flocculation is employed as an alternative to filtration if the sample requires removal of turbidity.	
	2. Wash into a 1 liter flask using a wash bottle and a funnel.	2a. Use a volumetric flask.	
	3. Add sufficient distilled water to dissolve all of the solid.		

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)	4. Dilute to volume with distilled water.		
	5. Store in a labeled container.	5a. This solution is stable for at least one year.	
6. Sodium Hydroxide Solution (6N)	1. Rapidly weigh 240 g of solid sodium hydroxide, NaOH, pellets in a 1 liter graduated beaker.	1a. This reagent is used if flocculation is employed as an alternative to filtration if the sample requires removal of turbidity. 1b. Sodium hydroxide picks up moisture from the air quite readily.	
	2. Add 500 ml distilled water to dissolve the sodium hydroxide.	2a. The water should be added with constant swirling to avoid fusing. CAUTION: Heat is liberated. Place Beaker in a pan of cold water.	
	3. Dilute to a total volume of 1 liter.	3a. The solution should be allowed to cool to room temperature before the dilution is made.	
	4. Store in a glass bottle or jug and stopper with a rubber stopper.	4a. Sodium hydroxide slowly etches glass causing glass stoppers to stick. 4b. The solution is stable for at least a year.	
	5. Label the container.		
7. Ammonium Hydroxide	1. A 100 ml supply should be available.	1a. Drop quantities may be required for pH adjustment.	
	2. Place in a Barnes (dropper) bottle.		
8. Hydrochloric Acid, (6N)	1. Add 50 ml of distilled water to a 400 ml beaker.	1a. A 100 ml graduated cylinder is suitable for measuring the volume of the distilled water.	

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)	2. Slowly add 50 ml of concentrated hydrochloric (HCl) acid (12 N) to the same beaker.	2a. Measure the acid in a 100 ml graduated cylinder.	
	3. Mix thoroughly.		
	4. Store in a 100 ml bottle.		
	5. Label the container.		
9. Copper Sulfate Solution (2%)	1. Weigh 20 g of copper sulfate pentahydrate, $CuSO_4 \cdot 5H_2O$, in a weighing boat.		
	2. Wash copper sulfate into a two liter beaker.		
	3. Add 500 ml distilled water and swirl to dissolve the solid.	3a. Use a graduated cylinder to measure 500 ml.	
	4. Add 500 ml distilled water and swirl to mix.		
	5. Store in a labeled container.	5a. This solution is stable for at least one year.	
✓10. Nitrate Stock Solution	1. Carefully weigh 7.218 g of potassium nitrate, KNO_3, in weighing boat.	1a. An analytical balance should be used.	
	After drying and dessicating		

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)	2. Transfer the solid to a 1 liter volumetric flask equipped with a powder funnel.	2a. This is best achieved by washing the solid onto the funnel with a wash bottle.	
	3. Use wash bottle to wash the solid into the flask.	3a. The weighing boat should be rinsed three times and all of the rinse water should be added to the flask.	
	4. Add sufficient distilled water to dissolve the solid.	4a. About 500 ml is sufficient.	
	5. Dilute to volume with distilled water and thoroughly mix.		
	6. Store in a labeled glass bottle.		
	7. Preserve the solution by adding 2 ml of chloroform, $CHCl_3$.	7a. The solution prepared, stored and preserved in this manner should be stable for at least 6 months. 7b. The nitrate stock solution contains 1.00 mg of nitrate nitrogen (NO_3-N) in each 1.00 ml of solution.	
11. Nitrate Standard Solution	1. Carefully pipet 10.0 ml of nitrate stock solution into a 1 liter volumetric flask.	1a. This nitrate standard solution should be prepared fresh for each use. 1b. The nitrate stock solution should be at room temperature before using. 1c. Use a 10 ml volumetric pipet.	
	2. Dilute to volume with distilled water.		

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation *(Continued)*	3. Store in a labeled container.	3a. Use within two hours of preparation. 3b. The nitrate standard solution contains 0.01 mg of nitrate nitrogen (NO_3-N) in each 1.0 ml of solution.	
12. Nitrite Stock Solution	1. Weigh 6.072 g of potassium nitrite, KNO_2, in a weighing boat.	1a. An analytical balance should be used for all weighings involving standards.	
	2. Transfer the solid to a 1 liter volumetric flask using a powder funnel.		
	3. Use wash bottle to wash the solid into the flask.	3a. The weighing boat should be washed three times and the washings added to the flask.	
	4. Add sufficient distilled water to dissolve the solid.	4a. About 500 ml is sufficient.	
	5. Dilute to volume and mix thoroughly.		
	6. Store in a labeled glass bottle.		
	7. Preserve the solution by adding 2 ml of chloroform for each 1 liter of solution and refrigerate when not in use.	7a. The solution should be stable for at least 3 months when preserved this way and stored at about 4°C when not in use. 7b. The nitrite stock solution contains 1.00 mg of nitrite nitrogen (NO_2-N) in each 1.0 ml of solution.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
B. Reagent Preparation (Continued)			
13. Nitrite Standard Solution	1. Pipet 10.0 ml of nitrite stock solution into a 1 liter volumetric flask.	1a. This nitrite standard solution should be prepared fresh for each use. 1b. The nitrite stock solution should be at room temperature before using. 1c. Use a 10 ml volumetric pipet.	
	2. Dilute to volume with distilled water.		
	3. Store in a labeled container.	3a. Use within two hours of preparation. 3b. The nitrite standard solution contains 0.01 mg of nitrite nitrogen (NO_2-N) in each 1.0 ml of solution.	
C. Reduction Column Preparation			
1. Preparation of the Glass Column	1. Construct a glass column by joining a 10 cm length of 3 cm ID glass tubing with a 25 cm length of 3.5 mm ID tubing using figure 1 as a guide.	1a. Figure 1 is at the end of the Equipment and Supply Requirements Section. 1b. The column shown in Figure 1 was constructed by cutting both ends off a 100 ml volumetric pipet as indicated. 1c. Fire polish all cut surfaces.	
	2. Loosely plug the delivery tip of the column with glass wool.	2a. The plug must be firm enough to hold the cadmium granules in the column, but not so firmly packed as to slow down the later flow of solutions through the column.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
C. Reduction Column Preparation (Continued)			
2. Preparation of Copperized Cadmium for Packing the Glass Column	1. Weigh about 25 g of cadmium granules in a weighing boat.	1a. This will be enough for one column. 1b. Granulated cadmium (40-60 mesh) can be purchased. 1c. Alternatively, file sticks of pure cadmium metal (reagent grade) with a coarse metal hand file (about second cut) and collect the fraction which passes a sieve with 10 mesh openings and is retained on sieves with 40, then 60 mesh openings. 1d. Handling cadmium is hazardous, thus filing should be conducted under a hood using rubber gloves and mask.	VIII.C.2.1d (p. 46)
	2. Transfer the cadmium to a 400 ml beaker.	2a. A scupula and wash bottle with water is good for this.	
	3. Add enough dilute (6N) hydrochloric acid to cover the granules.		
	4. Swirl the contents of the beaker.		
	5. Pour off the acid while retaining the granules in the beaker. The cadmium should be silver.	5a. All decanting should be done into a container equipped with a large funnel and filter paper so as to catch all the small cadmium particles. 5b. Use this filter paper for any subsequent cadmium washings.	
	6. Add enough distilled water to cover the granules.		

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
C. Reduction Column Preparation (Continued)	7. Pour off the water while retaining the granules in the beaker.		
	8. Repeat steps 6 and 7, above, two more times so that the granules receive a total of three distilled water washings.		
	9. Add 100 ml of the 2% copper sulfate solution to the granules and swirl for five minutes or until the blue color of the copper sulfate fades.	9a. A brown colloidal (very fine) precipitate of metallic copper should form.	
	10. Carefully decant off the solution leaving the copperized cadmium granules in beaker.	10a. Also decant off through the filter paper any precipitate that formed. 10b. The cadmium should have a black color.	
	11. Repeat steps 9 and 10 until a brown colloidal (very fine) precipitate of metallic copper does form.	11a. If a brown colloidal precipitate is formed in step 9, and the cadmium is black, do not repeat steps 9 and 10.	
	12. Wash the copper-cadmium at least 10 times with distilled water.	12a. All of the brown precipitated copper should be removed by washing 10 times but continue to wash if any remains.	
	13. Place the washed copper-cadmium on the 60 mesh sieve.		

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
C. Reduction Column Preparation (Continued)	14. Pour water over the granules at least three times so that all the small particles will wash through the 60 mesh screen.	14a. Hold the sieve over the filter paper during these washings.	
	15. Return meshed granules to the beaker.	15a. Use a scupula and the wash bottle.	
	16. Decant off excess water used to transfer the cadmium.		
	17. Close the clamp on the column delivery tube.		
	18. Fill the column with liquid, using about 60 ml DILUTE ammonium chloride-EDTA solution.	18a. Use a graduated cylinder and very slowly pour the solution down the inside wall of this column so air pockets do not form.	
	19. Loosely fill the reduction column with copper cadmium granules to a level about 2 cm below the broad, cup-like section as shown in Figure 1 page 9.	19a. Avoid tight packing of granules by allowing the granules to "float" down through the solution of ammonium chloride-EDTA. 19b. A glass stirring rod may be used to transfer the cadmium to the column.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
C. Reduction Column Preparation (Continued)	20. Open the screw clamp and measure the flow rate of ammonium chloride-EDTA solution through the column. The flow rate must be between 7 ml and 10 ml/minute before you go to the next step. Keep a record if you add more dilute ammonium chloride-EDTA solution.	20a. To calculate the flow rate, place a 50 or 100 ml graduated cylinder under column and measure the amount of fluid collected in one minute 20b. The flow rate should be between 7 ml and 10 ml/minute. DO NOT let the column go dry. 20c. If the flow rate is too fast, tighten the screw clamp. If the clamp must be so tight that control is lost, add more copper-cadmium granules to the column. 20d. If the flow rate is too slow, decrease the length of the copper-cadmium column until a flow rate of 7-10 ml/minute is achieved.	
	21. Rinse the column with up to 140 ml dilute ammonium chloride-EDTA solution, draining until the solution is about 2.5 cm above the top of the granules. Then close the screw clamp.	21a. There is to be a 200 ml rinse with this solution. You used about 60 ml in Step 18 and may have added more in Step 20. Now add the balance to total 200 ml. 21b. It is convenient to add a second clamp to shut off the flow so the flow-regulating clamp can remain undisturbed. 21c. When the column is not in use, the granules should be covered with solution so they do not dry out.	
D. Removal of Interferences			VI.D (p. 42)
1. Turbidity Removal (If necessary)	1. Prior to analysis, remove turbidity from samples by filtering through a 0.45 µ membrane filter.	1a. If the turbidity is not removed by filtration, proceed as follows: Add 1 ml of the zinc sulfate solution to 100 ml of sample. Add enough 6 N sodium hydroxide to bring the pH to 10.5 (about 8 to 10 drops is usually sufficient). Let the treated sample stand for 15 minutes. Filter through a 0.45 µ membrane filter. 1b. Suspended solids can clog the reduction column.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
D. Removal of Interferences (Continued)			
2. Oil and Grease Removal (If Necessary)	1. Prior to analysis, measure 100 ml of the sample (filtered sample if the original sample was turbid) into a 400 ml beaker.	1a. Oil and grease can clog the reduction column and coat the Cu/Cd granules.	
	2. By dropwise addition, add sufficient concentrated hydrochloric acid (12 N) to bring the pH down to 2.	2a. Use a pH meter in adjusting the pH to 2. 2b. Standardize using standard buffer of pH = 4.00.	
	3. Place the sample in a 250 ml separatory funnel.		
	4. Add 25 ml of chloroform.	4a. Freon or another non-polar solvent may be used.	
	5. Shake gently to extract the oils and grease into the chloroform layer.	5a. Carefully release the pressure after shaking gently so that no sample is lost. This can be accomplished by inverting the separatory funnel and slowly opening the stopcock away from face and other people.	
	6. Allow the separatory funnel to stand until all of the chloroform layer settles to the bottom.	6a. Place funnel in ring stand. 6b. Remove stopper while layer is settling.	
	7. Open the stopcock and allow the bottom (chloroform) layer to pass into a 400 ml beaker.	7a. Grease and oils are extracted into chloroform layer leaving a grease-oil free sample which is used for analysis.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
D. Removal of Interferences (Continued)	8. Repeat steps 4, 5, 6, and 7 with 25 ml of fresh chloroform.	8a. The second chloroform extract is added to the same beaker as the first extract.	
E. Preparation of Nitrate Working Standards			
1. Nitrate Working Standards	1. Prepare nitrate working standards by respectively pipetting the following volumes of nitrate standard solution into each of six 100 ml volumetric flasks.	1a. Label flasks. 1b. Use appropriate volumetric pipets (0.5 ml, 1.0 ml, 2.0 ml, 5.0 ml, 10.0 ml). 1c. The 0.00 solution which contains no nitrate (or nitrite) serves as the reagent blank for the nitrate samples and standards which are passed through the reduction column.	

To Flask No.	Add This Volume of Nitrate Standard Solution	For This Concentration of NO_3-N in mg/l
1	0.0 ml	0.00
2	0.5 ml	0.05
3	1.0 ml	0.10
4	2.0 ml	0.20
5	5.0 ml	0.50
6	10.0 ml	1.00

2. Dilute each of the flasks to volume with distilled water.

3. Stopper and mix thoroughly by inversion.

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
E. Preparation of Nitrate Working Standards (Continued)	4. Use the working standards immediately after their preparation.		
F. Reduction of Nitrate to Nitrite			
1. Adjustment of pH	1. Use a pH meter to adjust the pH of each of the working standards to between 5 and 9 either with concentrated hydrochloric acid or with concentrated ammonium hydroxide.	1a. Use a beaker small enough for this volume of standard to cover the pH electrode(s). 1b. Make sure that the pH meter is calibrated within this range. 1c. Use buffer solutions pH 4, pH 7, pH 10 to calibrate and check the meter. 1d. This pH adjustment is necessary to insure that the pH is approximately 8.5 (No pH adjustment is necessary if the pH is already between 5 and 9.)	
2. Activation of Column	1. Pipet 25.0 ml of working standard #6 to a small Erlenmeyer flask.	1a. Activation of column is necessary to prepare surfaces of Cu-Cd granules for reduction process. 1b. This standard is 1.00 mg NO_3-N/liter concentration. 1c. A 250 ml flask is good for this purpose.	
	2. Add 75 ml of the CONCENTRATED ammonium chloride-EDTA solution to the same flask.	2a. A 100 ml graduated cylinder is good for this purpose.	
	3. Mix the working standard thoroughly by swirling the contents of the flask.		
	4. Place a 250 ml beaker under the reduction column.		

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
F. Reduction of Nitrate to Nitrite (Continued)	5. Check that the level of ammonium chloride-EDTA solution in the column is near to the top of the granules.	5a. If the level is too high, drain the excess into the beaker.	
	6. Pour the prepared nitrate working standard into the reduction column.	6a. Since the column will not hold the total amount, add the solution in portions.	
	7. Using the screw clamp (see Figure 1) adjust the collection rate to 7-10 ml per minute.	7a. The clamp should be slowly opened until a collection rate of 7-10 ml per minute is achieved. 7b. A collection rate of 7-10 ml of solution per minute should be carefully maintained throughout the collection process to assure complete reduction of nitrate in the sample.	
	8. Collect the reduced working standard until the level of solution is one cm above the top of the granules.		
	9. Close the screw clamp to stop the flow.		
	10. Discard the entire reduced working standard.	10a. The column is now activated.	
	11. Measure about 40 ml of DILUTE ammonium chloride-EDTA solution.		
	12. Pour the 40 ml into the column.		

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
F. Reduction of Nitrate to Nitrite (Continued)	13. Collect the solution until the level of the solution is one cm above the top of the granules. Then close the screw clamp to stop the flow.	13a. You can check for "carry over" by collecting about 5 to 10 ml of the final effluent in a clean receiver and adding a few drops of the nitrite color reagent to verify that no color develops after 10 minutes. (A very faint pink color is negligible). Repeat steps 11 through 13 if significant color develops in this check procedure.	
	14. The column should be ready to use.		
3. Reduction of Working Standards	1. Pipet 25.0 ml of the lowest concentration of nitrate working standard into a small Erlenmeyer flask.	1a. A 250 ml flask is good for this purpose. 1b. Label the flask. 1c. Begin with the 0.00 mg/liter solution.	
	2. Add 75 ml of the CONCENTRATED ammonium chloride-EDTA solution to the same flask.	2a. Use a 100 ml graduated cylinder.	
	3. Mix nitrate working standard thoroughly by swirling the contents of the flask.		
	4. Place a short graduated cylinder under the reduction column.	4a. You need to measure 25 ml of solution in the graduate.	
	5. Pour the prepared nitrate working standard into the reduction column.	5a. Since the column will not hold the total amount, add the solution in portions.	
	6. Using the screw clamp (see Figure 1) adjust the collection rate to 7-10 ml per minute.	6a. The clamp should be slowly opened until a collection rate of 7-10 ml per minute is achieved. 6b. A collection rate of 7-10 ml of solution per minute should be carefully maintained throughout the collection process to assure complete reduction of the nitrate in the nitrate working standard.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
F. Reduction of Nitrate to Nitrite (Continued)	7. Discard the first 25 ml of solution which is collected.	7a. This discard portion serves to "wash off" solution remaining in the column from any previous pass-through.	
	8. Replace the graduate with the rinsed, air-dried flask used for this standard.	8a. The solution originally in the flask should now be in the column so you can thoroughly rinse it. A different flask may also be used.	
	9. Collect the remaining portion of the reduced standard in the original flask.	9a. Close the screw clamp when the level of solution is about one cm above the granules. 9b. About 70 ml should be in the flask.	
	10. Analyze the reduced standard IMMEDIATELY after collection from the reduction column.	10a. While one solution is passing through the column you should proceed to color development of the previous solution that has already been reduced. Color development (Section G) MUST BEGIN WITHIN 15 MINUTES after reduction.	
	11. Repeat steps 1 through 10 for each of the prepared working nitrate standards.	11a. Proceed from the least concentrated to the most concentrated standard. 11b. Label each receiver flask.	
G. Color Development of Reduced Nitrate Working Standards	1. Use a 50.0 ml pipet to remove a 50.0 ml aliquot from flask #1 (0.00 mg/liter NO_3-N).	1a. By using a propipet the aliquot can remain in the pipet during the next two steps. 1b. Aliquots of each of the working standards should have been passed through the reduction column as described in the previous section (Section F). 1c. The reduced working standards should be analyzed as soon as possible after the reduction and in no case should they be allowed to stand for more than 15 minutes after reduction before color development is begun.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
G. Color Development of Reduced Nitrate Working Standards (Continued)	2. Discard the remainder of the nitrate reduced working standard.		
	3. Shake flask dry.	3a. Do not rinse the flask.	
	4. Add the 50.0 ml working standard back to same flask from which it was removed.	4a. If you find the technique in steps 1-4 too difficult, transfer the 50.0 ml to a different flask.	
	5. Add 2.0 ml of the color reagent to the 50.0 ml of working standard.	5a. Use a 2.0 ml volumetric pipet.	
	6. Mix thoroughly by swirling.		
	7. Allow the working standard to stand until color develops.	7a. The reduced working standard should be allowed to stand for at least 10 minutes but NOT MORE THAN TWO HOURS before doing Procedure L, Spectrophotometric Measurements.	
	8. Repeat steps 1 through 7 for each of the reduced working standards.	8a. Start with least concentrated solution and proceed to most concentrated. 8b. Rinse the 50.0 ml pipet thoroughly after each standard.	
H. Analysis of Samples for Nitrate Reduced to Nitrite			
1. Dilution of Samples (if necessary)	1. Pipet 25.0 ml of unknown sample into 50 ml volumetric flask.	1a. Potable water samples will usually require no dilution, while sewage samples may require dilution.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
H. Analysis of Samples for Nitrate Reduced to Nitrite (Continued)	2. Dilute to volume with distilled water.	2a. If you need to dilute a sample, you must apply a dilution factor to the concentration found from a standard curve.	VII.H.1.2a (p. 44)
2. Adjustment of pH	1. Use a pH meter to adjust the pH of each sample to between 5 and 9 either with concentrated hydrochloric acid or with concentrated ammonium hydroxide.	1a. Put the 50 ml of sample in a small beaker so the pH electrode(s) is covered with solution. 1b. Make sure that pH meter is calibrated within this range. 1c. Use buffer solutions pH 4, pH 7, pH 10 to calibrate and check the meter. 1d. This pH adjustment is necessary to insure that the pH is approximately 8.5 (No pH adjustment is necessary if the pH is already between 5 and 9.)	
3. Reduction of Nitrate to Nitrite in Samples	1. Aliquots of each of the samples should be passed through the reduction column as described in Procedure F.3, "Reduction of Working Standards."		
4. Color Development in Samples	1. Follow the steps in Procedure G, "Color Development."		
I. Preparation of Nitrite Working Standards			
1. Nitrite Working Standards	1. Prepare nitrite working standards by respectively pipetting the following volumes of nitrite standard solution into each of six 100 ml volumetric flasks.	1a. Label flasks. 1b. Use appropriate volumetric pipets (0.5 ml, 1.0 ml, 2.0 ml, 5.0 ml, 10.0 ml). 1c. The 0.00 solution which contains no nitrite (or nitrate) serves as the reagent blank for the nitrite standards and samples that are <u>not</u> passed through the column.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
I. Preparation of Nitrite Working Standards (Continued)	To Flask No. / Add This Volume of Nitrite Standard Solution / For This Concentration of NO_2-N in mg/l 1 / 0.0 ml / 0.00 2 / 0.5 ml / 0.05 3 / 1.0 ml / 0.10 4 / 2.0 ml / 0.20 5 / 5.0 ml / 0.50 6 / 10.0 ml / 1.00 2. Dilute each of the flasks to volume with distilled water. 3. Use the working standards immediately after their preparation.		
2. Adjustment of pH	1. Use a pH meter to adjust the pH of each of the working standards to between 5 and 9 either with concentrated hydrochloric acid or with concentrated ammonium hydroxide.	1a. Use a beaker small enough for this volume of standard to cover the pH electrode(s). 1b. Make sure that pH meter is calibrated within this range. 1c. Use buffer solutions pH 4, pH 7, pH 10 to calibrate and check the meter. 1d. This pH adjustment is necessary to insure that the pH is approximately 8.5 (No pH adjustment is necessary if the pH is already between 5 and 9.)	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
J. Color Development of Nitrite Working Standards	1. Pipet 25.0 ml of each of the nitrite working standards into each of six clean 250 ml Erlenmeyer flasks.	1a. Use a 25.0 ml volumetric pipet. 1b. Label each flask. 1c. The nitrite working standards are <u>not</u> passed through the reduction column.	
	2. Add 75 ml of CONCENTRATED ammonium chloride-EDTA solution to each of the nitrite working standards.	2a. Use a 100 ml graduated cylinder.	
	3. Mix each thoroughly by swirling each flask.		
	4. Use a 50.0 ml pipet to remove a 50.0 ml aliquot from flask #1 (0.00 mg/liter NO_2-N).	4a. By using a propipet the aliquot can remain in the pipet during the next two steps.	
	5. Discard the remainder of the standard from the flask.		
	6. Shake the flask dry.	6a. Do not rinse the flask.	
	7. Add the 50.0 ml nitrite working standard back to the same flask from which it was removed.	7a. If you find the techniques in steps 4-7 too difficult, transfer the 50.0 ml to a different flask.	
	8. Add 2.0 ml of the color reagent to each nitrite working standard.	8a. Use a 2.0 ml volumetric pipet.	
	9. Mix thoroughly by swirling.		

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
J. Color Development of Nitrite Working Standards (Continued)	10. Allow the working standards to stand until color develops.	10a. At least 10 minutes but NO MORE THAN 2 HOURS should be allowed before doing Procedure L, Spectrophotometric Measurements.	
	11. Repeat steps 4 through 10 for each of the nitrite standards.	11a. Proceed from the least concentrated to the most concentrated standard. 11b. Rinse the 50.0 ml pipet thoroughly after each standard.	
K. Analysis of Non-reduced Samples for Nitrite			
1. Dilution of Samples (if necessary)	1. Pipet 25.0 ml of unknown sample into 50 ml volumetric flask.	1a. NOTE: Potable water samples will usually require no dilution, while sewage samples may require dilution.	
	2. Dilute to volume with distilled water.	2a. If you need to dilute a sample, you must apply a dilution factor to get a final answer.	VII.K.1.2a (p. 44)
2. Adjustment of pH	1. Use a pH meter to adjust the pH of each sample to between 5 and 9 either with concentrated hydrochloric acid or with concentrated ammonium hydroxide.	1a. Put the 50 ml of sample in a small beaker so the pH electrode(s) is covered with solution. 1b. Make sure that pH meter is calibrated within this range. 1c. Use buffer solutions pH 4, pH 7, pH 10 to calibrate and check the meter. 1d. This pH adjustment is necessary to insure that the pH is approximately 8.5. (No pH adjustment is necessary if the pH is already between 5 and 9)	
3. Color Development	1. Pipet 25.0 ml of sample into a clean 250 ml Erlenmeyer flask.	1a. Use a 25.0 ml volumetric pipet. 1b. Label the flask. 1c. The sample is not passed through the reduction column.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
K. Analysis of Non-reduced Samples for Nitrite (Continued)	2. Add 75 ml of the concentrated ammonium chloride-EDTA solution to the same flask.	2a. Use a 100 ml graduated cylinder.	
	3. Mix the sample thoroughly by swirling.		
	4. Use a 50.0 ml pipet to remove a 50.0 ml aliquot from flask.	4a. By using a propipet the aliquot can remain in the pipet during the next two steps.	
	5. Discard the remainder of the solution from the flask.		
	6. Shake flask dry.	6a. Do not rinse the flask.	
	7. Add the 50.0 ml of sample back to same flask from which it was removed.		
	8. Add 2.0 ml of the color reagent to the same flask.	8a. Use a 2.0 ml volumetric pipet.	
	9. Mix the sample thoroughly by swirling.		
	10. Allow the sample to stand until color develops.	10a. At least 10 minutes but NO MORE THAN 2 HOURS should be allowed before doing Procedure L, Spectrophotometric Measurements.	
	11. Repeat steps 1 through 10 for each sample.	11a. Rinse the 50.0 ml pipet thoroughly after each sample.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
L. Spectrophotometric Measurements			
1. Adjusting the Instrument	1. Consult the manufacturer's instructions for calibrating your particular instrument.	1a. Instrument must be warmed up for at least 10 minutes. 1b. There is an EMP on "Use of a Spectrophotometer."	
	2. Adjust the wavelength to 540 nm.		
	3. Check to make sure that the instrument reads infinite absorbance with no sample cell in the instrument.	3a. If it does not read infinite absorbance with no sample cell in it, adjust the instrument so that it does read infinite absorbance (see manufacturer's instructions). 3b. Use calibration knob to calibrate infinite absorbance.	
2. Reduced Nitrate Standards and Sample(s)	1. Use the reduced nitrate reagent blank to adjust the instrument to zero absorbance.	1a. Use 0.00 nitrate working standard reagent blank which has been passed through the column. 1b. Adjust to zero absorbance using the calibration knob.	
	2. Measure and record the absorbance of each reduced nitrate working standard.	2a. Use the nitrate working standards which have been passed through the column. 2b. Use data sheet provided.	IX.L.2.2b (p. 47)
	3. Measure and record the absorbance for each reduced sample.	3a. Use data sheet provided.	
3. Non-reduced Nitrite Standards and Sample(s)	1. Use the nitrite reagent blank (non-reduced) to adjust the instrument to zero absorbance.	1a. Use 0.00 nitrite working standard reagent blank. 1b. Adjust to zero absorbance using the calibration knob.	

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OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
L. Spectrophotometric Measurements (Continued)	2. Measure and record the absorbance of each non-reduced nitrite working standard.	2a. Use data sheet provided.	IX.L.3.2a (p. 47)
	3. Measure and record the absorbance for each non-reduced sample.	3a. Use data sheet provided.	
M. Preparation of Calibration Curve	1. Obtain an 8 1/2 x 11 inch piece of graph paper.		
	2. Label the longer side as the concentration axis.	2a. See Training Guide for an example of labeling the axis on a calibration curve.	VII.M.2a (p. 45)
	3. Label the shorter side as the absorbance axis.		
	4. Use the absorbance value and its corresponding nitrate concentration for each of the nitrate working standards to make a plot of absorbance versus concentration.	4a. Use the absorbances and concentrations recorded on the data sheet in Column B, "Total NO_2+NO_3-N." 4b. This will be the standard curve for reduced samples.	IX (p. 48)
	5. On another piece of graph paper follow steps 1, 2, 3, and 4 using absorbance values and the corresponding nitrite concentrations for each of the nitrite working standards.	5a. Use the absorbances and concentrations recorded on the data sheet in Column D, "NO_2-N." 5b. This will be the standard curve for non-reduced samples.	IX (p. 48)

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
N. Checking Column Efficiency	1. Divide the absorbance value for the 1.00 mg/liter NITRATE (NO_3) working standard by the absorbance for the 1.00 mg/liter NITRITE (NO_2) working standard to obtain the column efficiency as follows:	1a. The abbreviation, abs is used to stand for absorbance.	
	$\frac{\text{abs of 1.00 mg/liter } NO_3 \text{ std}}{\text{abs of 1.00 mg/liter } NO_2 \text{ std}} \times 100 = \%$ efficiency		
	2. Divide the absorbance values for each of the other NITRATE (NO_3) working standards by the absorbance value for the corresponding NITRITE (NO_2) working standard to obtain a column efficiency value in each case as was done in the previous step.	2a. At least one reduced nitrate standard should be compared to a nitrite standard of the same concentration to check column efficiency, calculated as given in Step 1. If series of the standards are run, you can calculate the average column efficiency using this Step 2 and then Step 3.	
	3. Calculate the average value for the column efficiency.	3a. The average value for the column efficiency should be between 96% and 104%. If the average % efficiency does not fall in this range, another cadmium reduction column should be prepared and tested until the average column efficiency does fall in this range. 3b. For regeneration of a column, see Training Guide.	VII.N.3b (p. 43)

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
O. Determination of mg/liter Nitrite Nitrogen Plus Nitrate Nitrogen in a Sample	1. Use the absorbance for the reduced sample and the standard curve for reduced samples ("Total NO_2+NO_3-N") to obtain the mg/liter of nitrite-N plus nitrate-N in the sample and record it in Column (A) on the data sheet provided.	1a. If the sample was not diluted (25 ml of sample is used), the mg/liter result is read directly from the nitrate standard curve. 1b. If the concentration of nitrate in the sample is too high for analysis, the sample must be diluted. The procedure is described in H.1 and involves diluting the sample to a 50 ml volume. In this case, the mg/liter result from the nitrate standard curve must be multiplied by a dilution factor which would be: $\text{Dilution Factor} = \frac{50\ \text{ml}}{\text{ml sample used in dilution}}$ 1c. The reduction process converts the nitrate-N initially present in the sample to nitrite nitrogen and the species analyzed is nitrite nitrogen. 1d. Any nitrite nitrogen initially present in the sample remains as nitrite nitrogen after the reduction. Thus the total nitrite analyzed is the sum of the nitrite initially present and the nitrite which has been formed by reduction of nitrate.	IX.O.1a (p. 47) VII.O.1b (p. 44)
P. Determination of mg/liter Nitrite Nitrogen in a Sample	1. Use the absorbance for the non-reduced sample and the standard curve for non-reduced samples ("NO_2-N") to obtain the mg/liter of nitrite-N in the sample and record it in Column (C) on the data sheet provided.	1a. If the sample was not diluted (25 ml of sample is used), the mg/liter result is read directly from the nitrite standard curve. 1b. If the sample was diluted to a 50 ml volume (as given in K.1), the mg/liter result read from the nitrite standard curve must be multiplied by a dilution factor which would be: $\text{Dilution Factor} = \frac{50\ \text{ml}}{\text{ml sample used in dilution}}$	IX.P.1a (p. 47) VII.P.1b (p. 44)

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

OPERATING PROCEDURES	STEP SEQUENCE	INFORMATION/OPERATING GOALS/SPECIFICATIONS	TRAINING GUIDE NOTES
Q. Calculation of mg/liter Nitrate Nitrogen in a Sample	1. Subtract the mg/liter of nitrite-N in the sample from the mg/liter of nitrite-N plus nitrate-N in the sample to obtain the concentration of nitrate-N. 2. Record the answer in Column (E) on the data sheet provided.	1a. Since the procedure measures the total nitrite concentration in a sample, the nitrite concentration of samples must be determined with reduction and without reduction. The nitrate concentration of a sample is then determined by: $NO_3\text{-}N = (NO_2+NO_3\text{-}N)_{\text{TOTAL WITH REDUCTION}} - (NO_2\text{-}N)_{\text{WITHOUT REDUCTION}}$ These concentrations were recorded on the data sheet in Columns (A) and (C) respectively.	IX.Q.1a (p. 47)
R. Calculation of mg/liter Nitrate in Sample	1. Multiply the value found for nitrate-nitrogen (NO_3-N) by a factor of 4.43. 2. Record the answer in Column (F) on the data sheet provided.	1a. (NO_3-N) x (4.43) = mg/liter Nitrate in sample. 1b. NO_3-N value was calculated in Procedure Q and recorded in Column (E).	IX.R.1b (p. 47)
S. Calculation of mg/liter Nitrite in Samples	1. Multiply the value found for nitrite-nitrogen (NO_2-N) by a factor of 3.29. 2. Record the answer in Column (G) on the data sheet provided.	1a. (NO_2-N) x (3.29) = mg/liter Nitrite in sample. 1b. NO_2-N value is found by using the calibration curve for non-reduced samples as in Procedure P and recorded in Column (C).	IX.S.1b (p. 47)

TRAINING GUIDE

SECTION	TOPIC
I*	Introduction
II	Educational Concepts - Mathematics
III	Educational Concepts - Science
IV	Educational Concepts - Communications
V	Field and Laboratory Equipment
VI*	Field and Laboratory Reagents
VII*	Field and Laboratory Analysis
VIII*	Safety
IX*	Records and Reports

Training guide materials are presented here under the headings marked. These standardized headings are used throughout this series of procedures.

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and Nitrate Nitrogen, Cadmium Reduction Method

INTRODUCTION — Section I

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
	The cadmium reduction procedure for nitrate-nitrite nitrogen provides a sensitive method for the determination of nitrate singly, or nitrite and nitrate combined in drinking, surface, and saline waters. The method is commonly used to determine both nitrate-N and nitrite-N in water samples.	
	The procedure described in this EMP is applicable for range of 0.01 to 1.0 mg/liter of nitrate-nitrite nitrogen. However, the range may be extended by appropriate sample dilution.	1. Methods for Chemical Analysis of Water and Wastes, 1979, EPA-EMSL, Cincinnati, Ohio 45268, p. 353.3-1.
	The test described in this instruction can be found in the 1979 EPA Methods Manual on p. 353.3-1, entitled Nitrogen, Nitrate-Nitrite (Cadmium Reduction Method). Another reference which contains an acceptable test for NPDES monitoring is on page 423 of the 14th edition of Standard Methods.	2. Standard Methods for the Examination of Water and Wastewater, 14th ed., 1976, APHA, New York, New York, p. 423.
	The major sources of nitrogen entering the environment are: through the heavy application of nitrogenous fertilizers which cause agricultural runoffs, as the end products of aerobic stabilization of organic nitrogen, in domestic sewage, through animal and plant processing wastes, in animal manure, through the atmosphere and in various types of industrial effluents.	3. Federal Water Pollution Control Administration Water Quality Criteria, U.S. Government Printing Office, Washington, D.C. 1968.
	While nitrogen is essential to our survival (as in the make-up of amino acids and proteins), when it exists as nitrate and nitrite it can be toxic. A limit of 10 mg/l nitrate-N and 1 mg/l nitrite-N is recommended for public water sources. The desirable criteria is virtually 0 mg/liter.	
	In ruminant animals (i.e. cows) nitrates may be internally reduced by bacteria present in the rumen to nitrites. The nitrites have been found to be toxic to these animals. Dr. Joptha E. Campbell, (Chief, Food Chemistry Unit, Milk and Food Research, Environmental Sanitation Program, Public Health Service, U.S. Department of H.E.W., Cincinnati, Ohio, 1968) has reported methemoglobinemia in cattle receiving water containing 2.790 mg/liter of nitrate.	
	Nitrates in high concentrations have also been found to stimulate vegetative growth under favorable conditions. Heavy undesirable growth in fresh water can lead to eutrification of important waterways.	

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and Nitrate Nitrogen, Cadmium Reduction Method

FIELD AND LABORATORY REAGENTS — Section VI

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
D.	Samples should be analyzed for nitrate nitrogen as soon as possible after sampling to avoid any change in nitrogen balance due to biological activity. If analysis can be made within 24 hours, the sample should be preserved by refrigeration at 4°C. Samples should be preserved with sulfuric acid if they are to be held more than 24 hours. To preserve samples for analysis, add 2.0 ml of concentrated sulfuric acid per liter of sample and store at 4°C.	

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
N.3b	Check the column efficiency when it is suspected that column efficiency is decreasing, as indicated by suspected low concentration levels. Prepare working standard nitrate solutions, and pass them through the column. (Begin at E. Preparation of Nitrate Working Standards.) If the absorbance for the known concentration does not give an average between 96% and 104% of your standard curve value for reduced nitrate standards of equivalent concentration, the column must be reactivated. REACTIVATION OF COLUMN 1. Empty cadmium granules from column into a clean beaker. 2. Wash with distilled water 3 times. 3. Add enough dilute HCl to cover granules. 4. Swirl contents. 5. Decant HCl. 6. Wash with distilled water 3 times. 7. Add 100 ml $CuSO_4$ solution to granules. 8. Swirl contents of beaker for approximately 5 minutes until the blue color fades to colorless. 9. Decant liquid leaving the granules. 10. Repeat steps 7, 8, and 9 until a very fine brown-red precipitate forms. 11. Wash granules with distilled water (approximately 10 times) until precipitate is removed. 12. Place granules on the 60 mesh sieve. 13. Shake to remove the small particles (the particles which remain on the sieve are the ones you want.) 14. Repack column (packing must be loose). 15. Activate the column (See F.2). 16. Standard curve using nitrate working stadards must be re-established. 17. Check column efficiency as described in N, Checking Column Efficiency.	

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
H.1.2a K.1.2a O.1b P.1b	Since a dilution is only part sample, when the absorbance reading obtained for it is converted to a concentration using a calibration curve, the concentration obtained is only that of the dilution. To obtain the mg/liter concentration of the sample, the mg/liter concentration of the dilution must be multiplied times the amount of dilution (must be multiplied times the dilution factor). For a 1/2 dilution (25 ml sample/50 ml total volume) the dilution factor would be 2 (the dilution is only half sample). For a 1/5 dilution (10 ml of sample/50 ml total volume) the dilution factor would be 5. Below is a table of some dilution factors when the sample is diluted to a 50 ml volume.	

ml of Sample per 50 ml Total Volume	Amount of Dilution	Dilution Factor
→ 25	1/2	2
10	1/5	5
5	1/10	10
1	1/50	50
0.5	1/100	100
0.05	1/1000	1000

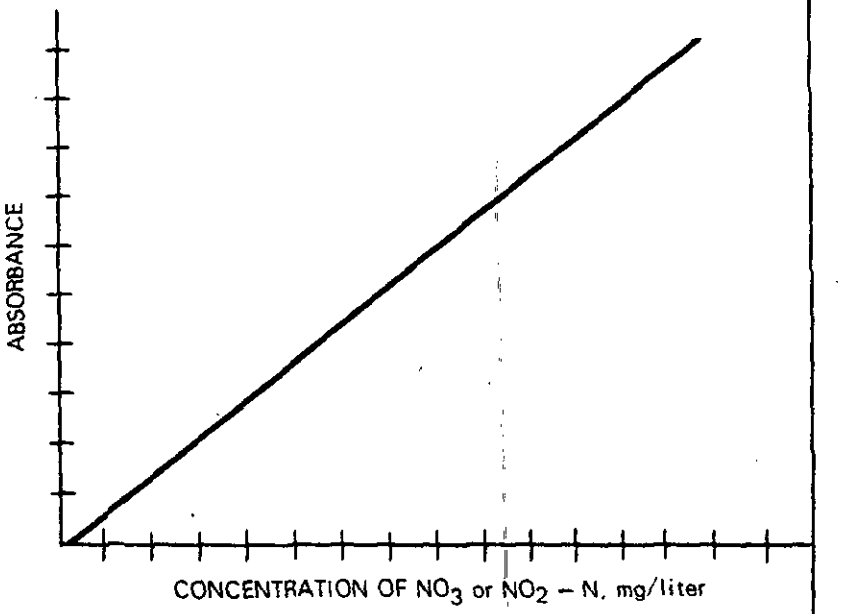
The dilution factor for any dilution may be calculated by dividing the ml of sample used in the dilution into 50:

$$\text{Dilution Factor} = \frac{50 \text{ ml}}{\text{ml sample used in dilution}}$$

Ex. 2 ml of sample diluted to 50 ml

$\frac{50}{2} = 25$

The dilution factor for this dilution would be 25.

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
M.2a	*A calibration curve is prepared by plotting the* measured absorbance of each of the working standard versus the concentration in the working standard as shown below. 	

EFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and Nitrate Nitrogen, Cadmium Reduction Method

SAFETY		Section VIII
	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
C.2.1d	Cadmium metal is highly toxic thus caution must be exercised in the use of cadmium. Cadmium metal should never be handled directly since cadmium has been shown to have cumulative effects. Rubber gloves should be used whenever cadmium must be handled. A mask should be worn during the filing of cadmium and the filing should be done in a hood. The waste cadmium should be disposed of in an appropriate manner which conforms to Federal, State and local pollution control regulations.	

	TRAINING GUIDE NOTE	REFERENCES/RESOURCES
	You will need the following Key to use the Example Data Sheet found on the next page:	
	KEY TO DATA SHEET	
L.2.2b M.4a	(B) Record the absorbances of the column-reduced nitrate working standards and of the column-reduced sample(s) in Column (B).	
L.3.2a M.5a	(D) Record the absorbances of the non-reduced nitrite working standards and of the non-reduced sample(s) in Column (D).	
O.1a	(A) Read the mg/liter (concentration) of Total NO_2+NO_3-N in the column-reduced sample(s) from the corresponding calibration curve and record the answer(s) in Column (A).	
P.1a	(C) Read the mg/liter (concentration) of NO_2-N in the non-reduced sample(s) from the corresponding calibration curve and record the answer(s) in Column (C).	
Q.1a	(E) Subtract: Value (A) - Value (C) = Value (E)	
R.1b	(F) Multiply: Value (E) x 4.43 = Value (F)	
S.1b	(G) Multiply: Value (C) x 3.29 = Value (G)	

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and Nitrate Nitrogen, Cadmium Reduction Method

RECORDS AND REPORTS — Section IX

EXAMPLE DATA SHEET

See Key on Page No. 7-47

SAMPLE NUMBER	mg/liter TOTAL NO_2+NO_3-N (A)	ABSORBANCE OF TOTAL NO_2+NO_3-N (B)	mg/liter NO_2-N (C)	ABSORBANCE NO_2-N (D)	mg/liter NO_3-N (E)	mg/liter NO_3 (F)	mg/liter NO_2 (G)
Reduced Nitrate Working Standards							
2	0.05				0.05	0.22	
3	0.10				0.10	0.44	
4	0.20				0.20	0.89	
5	0.50				0.50	2.22	
6	1.00				1.00	4.43	
Reduced Sample(s)							
Non-reduced Nitrite Working Standards							
2			0.05				0.16
3			0.10				0.33
4			0.20				0.66
5			0.50				1.65
6			1.00				3.29
Non-reduced Sample(s)							

EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

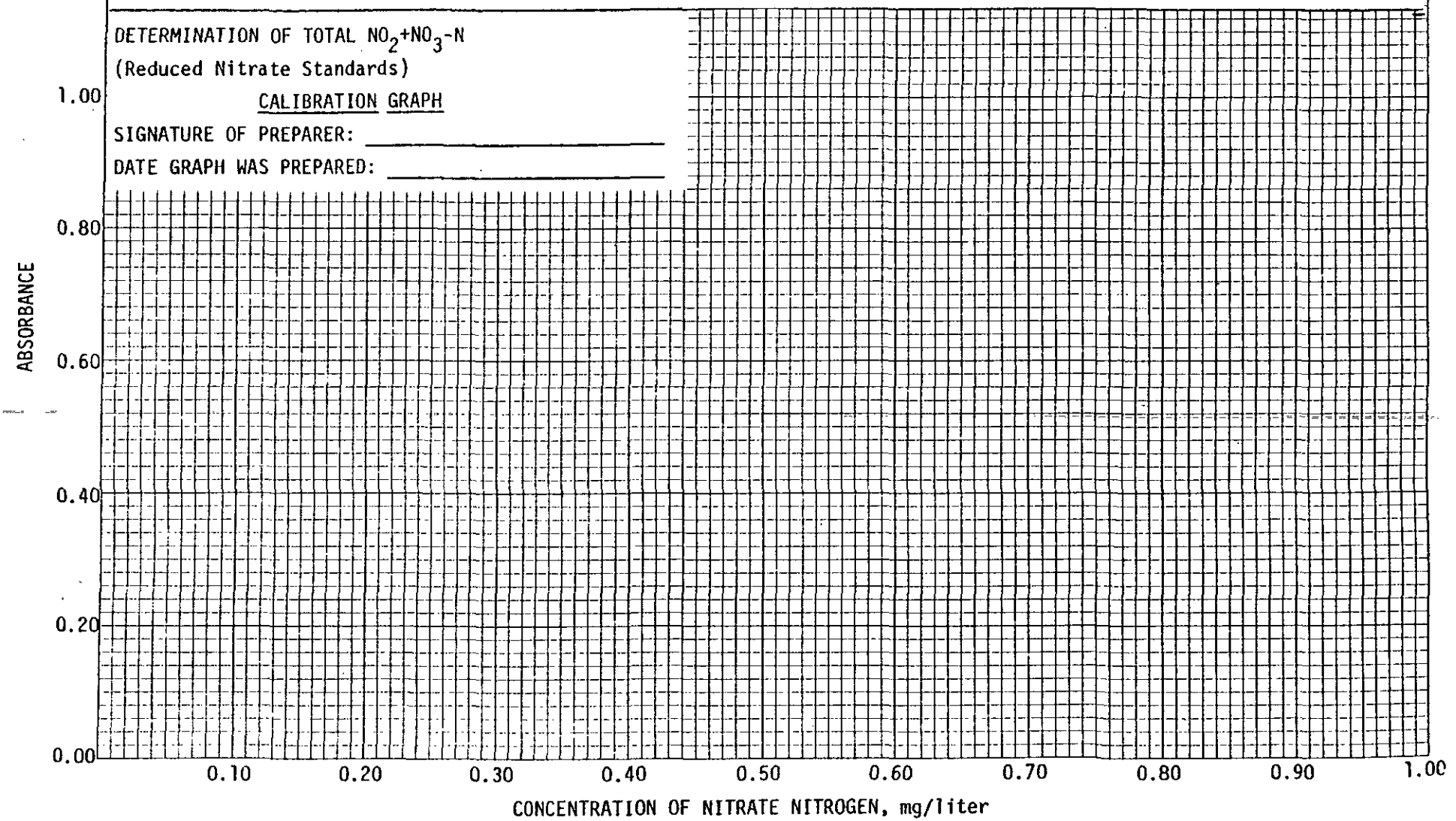
DETERMINATION OF TOTAL NO_2+NO_3-N

(Reduced Nitrate Standards)

CALIBRATION GRAPH

SIGNATURE OF PREPARER: _____

DATE GRAPH WAS PREPARED: _____



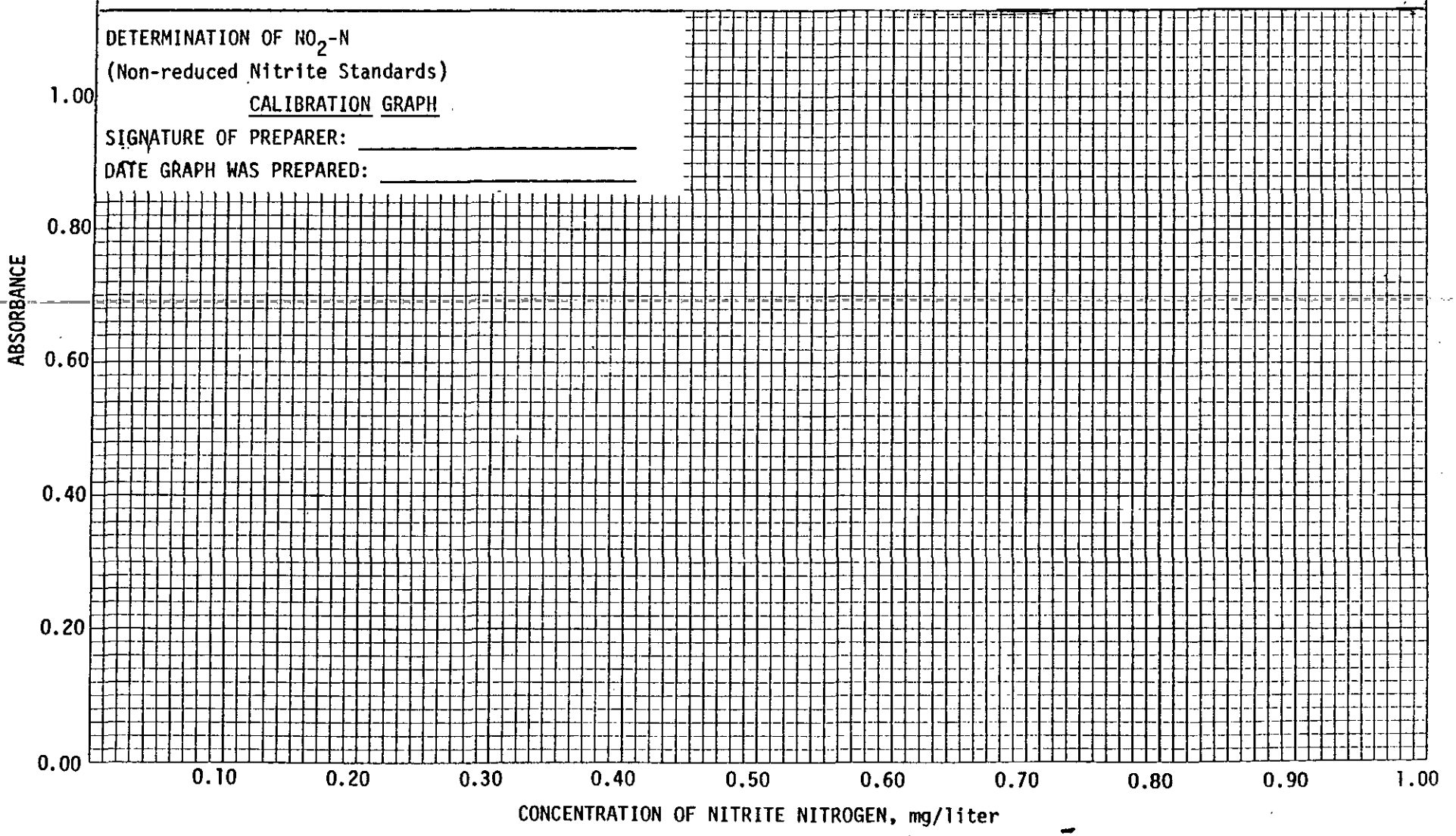
EFFLUENT MONITORING PROCEDURE: Determination of Nitrate-Nitrite Nitrogen and of Nitrate Nitrogen, Cadmium Reduction Method

RECORDS AND REPORTS SECTION IX

DETERMINATION OF NO_2-N
(Non-reduced Nitrite Standards)
CALIBRATION GRAPH

SIGNATURE OF PREPARER: _____

DATE GRAPH WAS PREPARED: _____



IV.3. PIGMENT ANALYSIS

IV.3.I. SPECTROPHOTOMETRIC DETERMINATION OF CHLOROPHYLLS AND TOTAL CAROTENOIDS

INTRODUCTION

At present the only rapid chemical method known for estimating living plant matter in the particulate organic matter of sea water is to determine the characteristic plant pigments — the chlorophylls, carotenes and xanthophylls. Unfortunately the amount of organic substance associated with a given quantity of plant pigment is very variable, depending upon the class of the phytoplankter and its state of nutrition. (The factor for converting chlorophyll *a* to total plant carbon can vary between about 25 and 100.) The method described below determines the three chlorophylls commonly found in planktonic algae, chlorophylls *a*, *b*, and *c*. The carotenoid pigments (the carotenes and xanthophylls) are only estimated collectively in somewhat arbitrary units. If the plant population contains many myxophyceae some forms of phycobilin pigments may extract and interfere with all determinations except that of chlorophyll *a*. Fortunately this occurs only rarely in truly marine waters.

The following technique is taken largely from the method described by Richards (Richards with Thompson, *J. Marine Res.*, 11: 156, 1952) with later modifications (Creitz and Richards, *J. Marine Res.*, 14: 211, 1955) and a few minor changes by the present authors. The "specific plant unit" (SPU) defined by Richards has now attained almost international acceptance. This unit, used for chlorophyll *c* and the carotenoids, *approximates* to 1 g of dry pigment. The Richards equations, however, are capable of improvement in the light of more recent research. As well as the original equations we include modified versions given by Parsons and Strickland (*J. Marine Res.*, 21: 155, 1963), and the SCOR/UNESCO Working Group on photosynthetic pigments (*Monographs on oceanographic methodology*, Publ. Unesco, 1966). These lead to somewhat lower values for chlorophyll *a* and express the concentration of chlorophyll *c* in terms of milligrams of pigment rather than "specific plant units." It must be stressed that these and other equations are still liable to change according to which specific extinction values in the literature are considered most authoritative and whether or not values for "dried" or "undried" chlorophyll are used (*refer to* Parsons and Strickland, *J. Marine Res.*, 21: 155, 1963). Determinations of chlorophyll *c* by a trichromatic method will never be satisfactory when dealing with a low standing crop of phytoplankton, and a separate routine method for this pigment is desirable (*see* Sect. IV.3.III). The equations given by Parsons and Strickland also express carotenoid pigments in arbitrary units but factors are chosen according to whether the plant population is predominantly composed of members of the Chlorophyta and/or Cyanophyta or is dominated by species in the Chrysophyta and/or Pyrrophyta. This choice of factors enables a much better estimate of the total weight of carotenoid to be made than heretofore.

We have not included any calculations for astacin or animal pigments as suggested initially by Richards. In our experience artifacts are possible when using these calculations with a phytoplankton crop rich in dinoflagellates and the relationship between the apparent astacin content of the water and the planktonic invertebrate population is too tenuous for much useful ecological application.

An extraction with 90% acetone under the conditions described in the method which follows has been considered satisfactory by most workers for many years. We believe this still to be the case for most seawater samples, having regard to the accuracy considered adequate for most investigations of marine ecology. However, results are undoubtedly low in many instances because of the presence of plant cells that are not fully extracted. With certain species 50% or more of the pigments may be left behind in the cell. A change of solvent may be beneficial but will rarely ensure complete extraction so it is probably not worth the trouble to re-establish extinction coefficients for other solvents or solvent mixtures. The use of a sonic disintegrator has been recommended but we have not found sufficient improvement to merit the application of such equipment on a routine basis. The use of a tissue grinder, such as that recommended by Yentsch and Menzel (*Deep-Sea Res.*, 10: 221, 1963), is relatively convenient and improves results on many natural populations but even this approach fails to give complete extraction in a reasonable time with certain species. Fortunately bad cases are generally found only among the Chlorophyta and some benthic species, and reasonable results will be obtained with open-sea samples for much of the time, even without grinding. For work in lakes or shallow estuaries, the adequacy of extraction must be carefully checked.

The sensitivity of the method described below is adequate except where sample volumes are restricted or where the chlorophyll content of the water is below about 0.2 mg/m³. The precision decreases appreciably with concentrations below this level, becoming very poor if less than 0.1 mg/m³ is present. In these circumstances a fluorimetric determination is recommended. Such a method requires a sensitive fluorimeter and a suitable technique is described in Sections IV.3.IV and V.

Finally mention should be made of chlorophyll degradation products. The presence of chlorophyllide will go undetected and this pigment will be reported as an equivalent weight of chlorophyll. If phaeophytin or phaeophorbide are present in samples the extinction of 6650 Å (*see later*) will decrease and these pigments will go undetected and will be reported as if about half the amount of chlorophyll were present. Some idea of the amounts of phaeo-pigments present in a sample may be obtained by measuring extinctions (or fluorescence) before and after the acidification of extracts. Chlorophyll degradation products in samples of sea water are best determined by chromatography but this approach is lengthy and not suitable for routine application. The presence of such compounds can generally be ignored but large amounts may be found if bottom deposits are disturbed, if there has been very heavy grazing by zooplankton, or if samples are taken from just below the euphotic zone in the open sea. For this reason a procedure for the determination of phaeo-pigments has been added at the end of this section.

METHOD

A. Capabilities

The limit of detection of plant pigments in sea water cannot be simply stated. An unlimited volume of water may be filtered for analysis; however, in practice, the volume filtered will rarely exceed 10 liters. The lower limit of detection for the filtration of 10 liters has not been statistically determined but appears to be of the

order of 0.02 mg/m³ for chlorophyll *a* and 0.04 mg/m³ for most other pigments except chlorophyll *c*.

1. CHLOROPHYLL *a* PRECISION AT THE 5 μG LEVEL
The correct value lies in the range:

Mean of n determinations $\pm 0.26/n^{\frac{1}{2}}$ μg chlorophyll *a*.

2. CHLOROPHYLL *b* PRECISION AT THE 0.5 μG LEVEL
The correct value lies in the range:

Mean of n determinations $\pm 0.21/n^{\frac{1}{2}}$ μg chlorophyll *b*.

3. PLANT CAROTENOIDS PRECISION AT THE 1.5 μ-SPU LEVEL
The correct value lies in the range:

Mean of n determinations $\pm 0.15/n^{\frac{1}{2}}$ μ-SPU.

The precision of chlorophyll *c* determinations is variable and very poor, anywhere between ±10 and ±30% of the amount being measured, and results are not *accurate*, almost always being too *high*.

B. Outline of Method

The larger zooplankters are removed by straining a sample of sea water through a nylon net of about 300-μ mesh size and then the phytoplankters are filtered onto a Millipore AA filter or a glass filter. Pigments are extracted from the algae cells for estimation spectrophotometrically.

C. Special Apparatus and Equipment

Millipore filtration equipment designed to hold 47-mm diam membrane filters.

One 300-ml polyethylene wash bottle.

Stoppered graduated centrifuge tubes of 15-ml capacity having both glass and polyethylene stoppers.

"Small volume" spectrophotometer cells having a path length of 10 cm but holding 10 ml or less of solution.

D. Sampling Procedure and Sample Storage

Adequate sampling of the euphotic zone or detrital layers for phytoplankton is a subject which is outside the scope of the present method. Once obtained, the final sample (generally 500 ml–5 liters in volume) is filtered through a small piece of clean 0.3-mm mesh nylon netting to remove the larger zooplankton. For open sea samples filtration of small volumes through a 0.15-mm mesh net will still not retain significant amounts of phytoplankton. The required volume of this filtrate should be measured by a polyethylene measuring cylinder into a polyethylene bottle. Two or three drops (*ca.* 0.1–0.2 ml) of magnesium carbonate suspension (*see* Sect. E.2) are added. The sample may then be stored in a cool dark place for a maximum of about 8 hr. It is desirable, however, that samples be filtered through a membrane filter at the time of collection.

Membrane filters can be stored by folding them in half (with the plankton

innermost) and storing them *in the dark* in a desiccator frozen to −20 C but only for a few weeks. This procedure almost always leads to low results and makes the extraction of chlorophyll more difficult; filters should be extracted without delay if at all possible.

E. Special Reagents

1. Special reagents

Distill reagent grade acetone over about 1% of its weight of both anhydrous sodium carbonate and anhydrous sodium sulphite. Collect the fraction boiling at a constant temperature near 56.5 C (uncorrected). 100 ml of water is pipetted into a liter volumetric flask and acetone added to make the volume to exactly 1000 ml. The redistilled acetone should be stored in a tightly stoppered dark glass bottle and the 90% reagent prepared in moderately small amounts (say 1 liter at a time) for use. This reagent is conveniently dispensed from a polyethylene wash bottle which should be kept nearly full. If good quality reagent acetone is available, it should be shaken with a little granular anhydrous sodium carbonate and decanted directly for use.

2. Magnesium carbonate suspension

Add approximately 1 g of finely powdered magnesium carbonate (light weight or "Levis" grade) of analytical reagent quality to 100 ml of distilled water in a stoppered Erlenmeyer flask. Shake vigorously to suspend the powder *immediately before use.*

F. Experimental

1. Invert the polyethylene bottle containing the sample (Sect. D) into the funnel of the Millipore filter equipment fitted with either a 47-mm diam Millipore AA filter or a 4.5-cm Whatman GF/C glass filter paper (Note *a*). The bottle need not be rinsed but the contents should be shaken vigorously, before filtration is commenced. If not added previously, introduce about 1 ml of magnesium carbonate suspension to the last few hundred milliliters of sample being filtered (Note *b*).

2. Drain the filter thoroughly under suction before removing it from the filtration equipment and if a Millipore filter is used trim away the peripheral excess of unstained membrane with clean scissors (Note *c*). Store the filter if necessary but if possible extract the pigment without delay (Sect. D).

3. Place the filter in a 15-ml stoppered graduated centrifuge tube. If a Millipore filter was used add approximately 8 ml of 90% acetone, stopper the tube, and dissolve the filter by shaking the tube vigorously. If a glass paper was used add approximately 10 ml of 90% acetone, stopper the tube, and disperse and disintegrate the paper by shaking the tube vigorously (Note *d*). Allow the pigments to be extracted by placing the tube in a refrigerator in *complete darkness* for about 20 hr (Notes *e* and *f*). It is good practice to shake the tubes vigorously once more after *they have been 1 or 2 hr in the refrigerator.*

4. Remove tubes from the refrigerator and let them warm up in the dark nearly to room temperature. Add 90% acetone to make the extracts from Millipore filters up to exactly 10.0 ml and those from glass filters to exactly 12.0 ml (Note *g*).

Centrifuge the content of the tubes for 5–10 min (Note *h*) having replaced the glass stoppers on the centrifuge tubes with plastic stoppers to prevent breakage during centrifugation.

5. Decant the clear supernatant liquid into a 10-cm-path-length spectrophotometer cell designed to hold 10 ml or less of liquid. In the event of extinction values exceeding about 1.3 the measurements described below should be made with 2.5-cm or 1-cm cells and the extinction values multiplied by 4 or 10, respectively, to normalize them to the values expected with a 10-cm cell. If 12 ml of acetone is used with glass papers multiply the extinction values by 1.2 to normalize them to the values expected from 10 ml of extract.

6. Without delay measure the extinction of the solution against a cell containing 90% acetone (Note *i*) at 7500, 6650, 6450, 6300, and 4800 Å (Note *j*). If the Richards equations are to be used for carotenoids (*see below*) a further measurement at 5100 Å is required, and if the SCOR/UNESCO equations are used the measurement at 6650 Å should be replaced by one at 6630 Å. Record the extinction values to the nearest 0.001 unit in the range 0–0.4 and the nearest 0.005 for extinctions exceeding about 0.4. Correct the extinctions at each wavelength by the procedure described in Section G below.

7. Calculate the concentration of pigments in sea water from the equation

$$\text{mg (or m-SPU) pigment/m}^3 = \frac{C}{V}$$

where C is a value obtained from the following equations and V is the volume of sea water filtered in liters. When the Parsons-Strickland equations are used values for chlorophylls *a*, *b*, or *c* will be in mg/m³ and those for carotenoids in a millispecified plant pigment unit approximating to the milligram. If the "classical" Richards equations are used values are in mg/m³ only for chlorophylls *a* and *b*. The Richards m-SPU is used for chlorophyll *c* and is considerably greater than the milligram. The m-SPU is considerably smaller than the milligram if carotenoids are mainly fucoxanthin or peridinin which are present in Chrysophyta or Pyrrophyta.

8. Formulae: (R = Richards, P.S. = Parsons and Strickland, S.U. = SCOR/UNESCO. E stands for the extinction values, at wavelengths indicated by the subscripts, measured in 10-cm cells *after* correcting for a blank as described in Section G.2 below.)

R. $C\text{ (chlorophyll } a) = 15.6\,E_{6650} - 2.0\,E_{6450} - 0.8\,E_{6300}$
P.S. $C\text{ (chlorophyll } a) = 11.6\,E_{6650} - 1.31\,E_{6450} - 0.14\,E_{6300}$
S.U. $C\text{ (chlorophyll } a) = 11.64\,E_{6630} - 2.16\,E_{6450} + 0.10\,E_{6300}$

R. $C\text{ (chlorophyll } b) = 25.4\,E_{6450} - 4.4\,E_{6650} - 10.3\,E_{6300}$
P.S. $C\text{ (chlorophyll } b) = 20.7\,E_{6450} - 4.34\,E_{6650} - 4.42\,E_{6300}$
S.U. $C\text{ (chlorophyll } b) = 20.97\,E_{6450} - 3.94\,E_{6630} - 3.66\,E_{6300}$

R. $C\text{ (chlorophyll } c) = 109\,E_{6300} - 12.5\,E_{6650} - 28.7\,E_{6450}$
P.S. $C\text{ (chlorophyll } c) = 55\,E_{6300} - 4.64\,E_{6650} - 16.3\,E_{6450}$
S.U. $C\text{ (chlorophyll } c) = 54.22\,E_{6300} - 14.81\,E_{6450} - 5.53\,E_{6630}$

[1] Filtration, using Whatman GF/C discs in all-glass apparatus may be easier than centrifugation.

R. C (Plant carotenoids) $= 7.6\ (E_{4800} - 1.49\ E_{5100})$, without regard to nature of crop.

P.S. C (Plant carotenoids) $= 4.0\ E_{4800}$, if crop predominately Chlorophyta or Cyanophyta.

$= 10.0\ E_{4800}$, if crop predominately Chrysophyta or Pyrrophyta.

When large numbers of samples are involved, considerable time can be saved by converting the simultaneous equations into a nomographic form (Duxbury and Yentsch, *J. Marine Res.*, 15: 92–101, 1956). The nomographs given in Table XV are for use with the revised equations for ascertaining chlorophylls as discussed by Parsons and Strickland (*J. Marine Res.*, 21: 155–163, 1963) and have been taken from Stephens (*J. Fish. Res. Bd. Canada*, 22: 1575, 1966).

The following description for the use of the nomographs is given for chlorophyll *a*. By using the appropriate nomographs, the estimation of chlorophylls *b* and *c* follow a similar procedure. The nomographs are set up using mμ as units, i.e., E_{645} (nomograph) $\equiv E_{6450}$ (P.S. equations above).

Corrected optical densities at 6450 and 6300 Å (E_{6450} and E_{6300}) are plotted on their respective lines and joined with a straight edge. The intercept on Line Q is then lined up with the corrected optical density at 6650 Å (E_{6650}). The intercept on the C_a line then gives the concentration of pigment.

NOTES

(*a*) Millipore filters have the advantage that they dissolve in acetone completely, give no complications at the centrifugation stage, and require no particular precautions during filtration. However, unless great care is taken, undesirably high blanks will occur when using Millipore filters, making the determination of small concentrations of carotenoids difficult. These filters are expensive. Glass filters are cheaper and their use results in practically no blank. They are recommended if a cell grinding step is required to give better extraction (Note *d*), although care must be taken when filtering samples through the comparatively coarse glass filters and trouble is experienced at the centrifugation stage. A manostat (there are several cheap commercial laboratory units based on the cartesian diver) must be used with glass papers to ensure that the suction never exceeds ½–¾ atm or else pigment may pass through the filters. *Millipore filters must be used if chlorophyll c is to be determined on the same extract by method IV.3.III.*

(*b*) The magnesium carbonate is added at this stage to ensure that the phytoplankton chlorophyll is prevented from becoming acid with the resulting decomposition to give phaeophytin pigments. We have some doubts of the efficacy of such an addition compared with, say, the addition of a completely soluble organic base but the use of magnesium carbonate is established practice and doubtlessly has some value as a precautionary measure. Care should be taken to see that Millipore filtration equipment, centrifuge tubes, and spectrophotometer cells are kept free from acid and that the filter is not touched with acidic fingers.

(*c*) The troublesome blank, measured at 7500 Å (Sect. G), found with Millipore filters is caused almost entirely by the salt left in the filter at this stage which subsequently "salts out" membrane material from the acetone. The blank can be greatly reduced if filters are sucked dry of sea water very thoroughly at this stage and as much as possible of the unwanted peripheral filter is cut away.

(*d*) If poor extraction is anticipated use a glass filter and after filtration push this to the bottom of a "Potter" type grinder holding about 20 ml. Following Yentsch and Menzel we have used the Arthur H. Thomas grinder No. 4288-B fitted to an ordinary laboratory stirring motor. About 2 ml of 90% acetone should be added and the grinder run for 1–2 min *in subdued light.*

The tube should be pushed up and down the pestle during the extraction but for much of the time the pestle should be hard against the bottom of the tube. After use, the pestle is rinsed into the tube with a few milliliters of 90% acetone which is also used to transfer the contents of the grinder tube to a 15-ml centrifuge tube. The total volume in the centrifuge tube should not exceed 10 ml. The contents should be left in the dark for a few hours to ensure the complete removal of all extractable pigments.

(*e*) During the extraction period pigments are very photosensitive and neither extracts nor the unextracted filters should be exposed to strong sunlight or else chlorophyll values will be reduced to a small fraction of their initial level in less than 1 hr. Tubes must be stored in complete darkness.

(*f*) The period of extraction should be about 15–20 hr. After this time the rate of further extraction is too slow for an extension to be merited. Pigment extracts should preferably be kept chilled but they can be kept at room temperature for many hours without deterioration. If cells are pretreated in a grinder (Note *d*) any further extraction is slow, but for safety, tubes should be stored for a few hours to complete the leaching of cell fragments.

(*g*) The use of 10 ml of solution in a 10-cm-path-length cell is recommended for maximum sensitivity. Greater sensitivity can be obtained by using 10-cm cells containing less than 10 ml but this is scarcely great enough to warrant the increased manipulative difficulties. The ultimate sensitivity is, in practice, more dependent on the size and reproducibility of blanks. Glass filters disintegrate to pulp, instead of dissolving in acetone, and the pulp retains at least 1 ml of solvent. To ensure enough extract to fill a 10-cm cell, therefore, 12 ml of acetone, instead of 10 ml, should be used.

(*h*) Centrifugation should be as efficient as possible when Millipore filters are used. In most small centrifuges 3000–4000 rpm for about 10 min is generally satisfactory but the efficiency should be tested with each instrument used. Difficulties may be encountered when centrifuging down the glass pulp from glass filters. Tubes should be centrifuged for 1–2 min to pack most of the fibers to the bottom. The centrifuge is then stopped, the tubes removed, and glass fibers adhering to the walls of the tubes above the level of the solvent are taken down into the bulk of the liquid by gently splashing the walls by flicking the tubes. The tubes are then returned to the centrifuge and spun for about 5 min. If this precaution is not taken some fibers held above the solvent layer may enter the spectrophotometer cell.

(*i*) These extracts should not be allowed to evaporate and should be exposed only to subdued light for the briefest possible period. The measurement of extinction against acetone (instead of against water) is recommended as acetone has markedly less absorption in a 10-cm cell at 7500 A than has distilled water.

(*j*) The wavelength setting of the spectrophotometer used should be checked against a standard hydrogen or neon line source as the precision of the present method depends upon settings being correct to better than 20–30 Å. With quartz prisms at wavelengths exceeding 6000 Å very slight movements of the optical system, brought about by vibrations, etc., can easily result in errors of 50 Å or more in wavelength settings. If a suitable lamp is not available check the extinction of a suitably concentrated plant extract and adjust the spectrophotometer, if necessary, until a maximum extinction is obtained at 6630 Å.

G. Determination of Blank

1. Cell-to-Cell Blanks

As the precise values of comparatively small extinctions have to be measured, corrections for all optical inequalities become important. Fill both spectrophotometer cells with 90% acetone and find the "cell-to-cell" blank of the sample cell against the reference cell *at all wavelengths used in the method*. Correct *all* extinction values by this cell-to-cell blank which may amount to 0.01 or more.

2. Turbidity Blanks

If glass papers are used there should be only a very small blank. This is

measured by the spectrophotometer reading at 7500 where there is known to be no absorption of light from pigments. We have sometimes found a small negative blank for reasons which are not clear. In any case the value positive or negative should not exceed about 0.002 and may be corrected for cell-to-cell blank and used for the extinctions at all wavelengths.

A certain amount of colloidal material remains after the solution of an AA Millipore filter, even after centrifugation. The extinction from this material depends on the wavelength of light used, increasing at shorter wavelengths because of light scattering effects.

The extinction at 7500 Å is corrected for any cell-to-cell blank at this wavelength and the resulting extinction (E_b) is multiplied by a factor f to give the turbidity blank extinction to be used with spectrophotometer readings at other wavelengths.

$$\text{Total blank correction} = \text{cell-to-cell blank} + (f \times E_b)$$

where f has the values shown below:

Wavelength	f
6650	1
6450	1
6300	1
5100	2
4800	3

It must be stressed that these values for f are *very approximate*. Extinction values at 4800 Å should undoubtedly be corrected by a greater blank than the one obtained at 7500 Å but the value of 3 is so approximate that there is no substitute for having low E_b values. If a good correction is required E_b must not exceed about 0.02.

ADDENDUM TO IV.3.I. SPECTROPHOTOMETRIC DETERMINATION OF PHAEO-PIGMENTS

INTRODUCTION

Chlorophyll degradation products may at times constitute a significant fraction of the total green pigments in sea water. These degraded forms of inactive chlorophyll interfere with the spectrophotometric determination of chlorophylls because they absorb light in the same region of the spectrum as chlorophyll. Pigment samples from the aphotic zone, sediments, and samples from areas of high zooplankton grazing are particularly likely to contain inactive chlorophyll products. Chemically these may consist predominantly of phaeophytin and phaeophorbide (phaeo-pigments) but sometimes large quantities of chlorophyllide may also be present. In the following method it is possible to obtain a measure of the total quantity of chlorophyll *a* and phaeophytin *a* plus phaeophorbide *a*, but not of chlorophyllide *a* or the phaeophytins and phaeophorbides of other chlorophylls. For a complete analysis of all chlorophylls and their degradation products there is probably no alternative to chromatographic methods which are generally too tedious for the routine analysis of a large number of samples. For a routine observation, however, it is often sufficient to obtain a measure of the amount of non-active chlorophyll *a* in terms of the quantity of phaeo-pigments present. Two similar procedures have been described for this determination (Moss, *Limnol. Oceanog.*, 12: 335, 1967; Lorenzen, *Limnol. Oceanog.*, 12: 343, 1967). The procedure employed here is written as an addendum to the procedure for plant pigment analysis (IV.3.I) and employs equations in the second of the two references given above.

METHOD

A. Capabilities

1. PHAEOPHYTIN *a* PRECISION AT THE 0.5 μG LEVEL

The correct value lies in the range:

Mean of n determinations $\pm 0.05/n^{\frac{1}{2}}$ μg phaeophytin *a*.

2. LIMIT OF DETECTION

The limit of detection will depend on the total amount of sea water filtered but for all measurements the initial extinction at 6650 Å should be greater than 0.2.

B. Outline of Method

The extinction of an acetone extract of plant pigment is measured before and after treatment with dilute acid. The change following acidification is used as a measure of the quantity of phaeo-pigments in the original sample.

C. Special Apparatus and Equipment

See Section IV.3.I,C.

D. Sampling Procedure and Sample Storage

See Section IV.3.I,D.

E. Special Reagents

Reagents 1 and 2, see Section IV.3.I,E.

3. HYDROCHLORIC ACID

Dilute 50 ml of concentrated hydrochloric acid to 100 ml with distilled water.

F. Experimental

PROCEDURE

1. Carry out procedure in Section IV.3.I,F., procedures 1–5.

2. Measure the extinction of the extract at 6650 and 7500 Å. Add two drops of dilute hydrochloric acid to the cuvette (Note *a*), mix (Note *b*), and remeasure the extinction at 6650 and 7500 Å.

3. Subtract each 7500 Å reading from the corresponding 6650 Å extinction and use the following equations to calculate the concentration of chlorophyll *a* and phaeo-pigments in the sample (Note *c*):

$$\text{Chl } a \text{ (mg/m}^3) = \frac{26.7(665_o - 665_a) \times v}{V \times l}$$

$$\text{Phaeo-pigments (mg/m}^3) = \frac{26.7(1.7[665_a] - 665_o) \times v}{V \times l}$$

where 665_o *is the extinction at 6650 Å before acidification,* 665_a *the extinction at* 6650 Å after acidification, v the volume of acetone used for extraction (ml), V the volume of water filtered (liters), and l the path length of the cuvette (cm).

NOTES

(*a*) If the extraction of pigment has been made using acetone soluble filters, the addition of a small amount of hydrochloric acid will cause a transient turbidity which disappears on mixing.

(*b*) The samples are best mixed by holding a small piece of aluminum foil over the mouths of the cuvettes and inverting them several times. The destruction of chlorophyll *a* to phaeophytin is not instantaneous and the sample should be allowed to stand for 4–5 min before being measured again. Rinse the cuvette thoroughly with 90% acetone after each determination to ensure that no acid is carried over when the next 665_o reading is taken.

(*c*) For convenience, the corresponding equation for the determination of chlorophyll *a* has been included here. The value for the specific absorption coefficient for chlorophyll *a* is the same as is employed in Section IV.3.I,F (Parsons and Strickland equations).

IV.3.II. SCOR/UNESCO PROCEDURE FOR CHLOROPHYLLS

INTRODUCTION

The following procedure was agreed to by a SCOR/UNESCO working group and has been published by Unesco in *Monographs on Oceanographic Methodology*.

METHOD

CONCENTRATION OF SAMPLE

Use a volume (Note *a*) of sea water which contains about 1 μg chlorophyll *a*. Filter (Note *b*) through a filter (Note *c*) covered by a layer of $MgCO_3$ (Note *d*).

STORAGE

The filter can be stored in the dark over silica gel at 1 C or less for 2 months but it is preferable to extract the damp filter immediately and make the spectrophotometric measurement without delay.

EXTRACTION

Fold the filter (plankton inside) and place it in a small (5–15 ml) glass, pestle-type homogenizer. Add 2–3 ml 90% acetone. Grind 1 min at about 500 rpm. Transfer to a centrifuge tube and wash the pestle and homogenizer 2 or 3 times with 90% acetone so that the total volume is 5–10 ml. Keep 10 min in the dark at room temperature. Centrifuge (Note *e*) for 10 min at 4000–5000 *g* (Note *f*). Carefully pour into a graduated tube so the precipitate is not disturbed and if necessary dilute (Note *g*) to a convenient volume (Note *h*).

MEASUREMENT

Use a spectrophotometer with a band-width of 30 Å or less, and cells with a light path of 4–10 cm (Note *i*). Read the extinction (optical density, absorbance) at 7500 (Note *j*), 6630, 6450, and 6300 Å against a 90% acetone blank.

CALCULATION

Subtract the extinction at 7500 Å from the extinctions at 6630, 6450, and 6300 Å. Divide the answers by the light path of the cells in centimeters. If these corrected extinctions are E_{6630}, E_{6450}, and E_{6300} the concentrations of chlorophylls in the 90% acetone extract as μg/ml are given by the SCOR/UNESCO equations (*refer to* Section IV.3.I). If the values are multiplied by the volume of the extract in milliliters and divided by the volume of the seawater sample in liters, the concentration of the chlorophylls in the sea water is obtained as μg/liter (= mg/m^3).

NOTES

(*a*) The amount of chlorophyll *a* should be less than 10 μg, otherwise a second extraction with 90% acetone might be necessary. With ocean water about 4–5 liters of sample should be used; with coastal and bay waters, sometimes one tenth of this amount is sufficient.

(*b*) Use no more than two thirds of full vacuum.

(*c*) Satisfactory filters include paper (Albet), cellulose (Cella "grob"), and cellulose ester (0.45–0.65 μ pore-size); the filter should be 30–60 mm in diameter. If these filters clog with inorganic detritus, use Schleicher & Schüll 575.

(*d*) Add about 10 mg $MgCO_3$/cm² filter surface, either as a powder or as a suspension in filtered sea water.

(*e*) A swing-out centrifuge gives better separation than an angle centrifuge.

(*f*) If a stoppered, graduated centrifuge tube is used, the extract can be made up to volume and the supernatant carefully poured or pipetted into the spectrophotometer cell.

(*g*) If turbid, try to clear by adding a little 100% acetone or distilled water or by centrifuging again.

(*h*) This depends on the spectrophotometer cell used. The volume should be read to 0.1 ml.

(*i*) Dilute with 90% acetone if the extinction is greater than 0.8.

(*j*) If the 7500 Å reading is greater than 0.005/cm light path, reduce the turbidity as in Note *g*.

IV.3.III. DETERMINATION OF CHLOROPHYLL *C*

INTRODUCTION

The trichromatic method, described in IV.3.I, has, inherently, a positive error and a poor precision for chlorophyll *c*, which becomes worse the smaller the extinctions being measured and the larger the blanks. A separate method for chlorophyll *c* is required and the present method is based on the procedure given by Parsons (*J. Marine Res.*, 21: 164, 1963). It is somewhat lengthy and tedious for routine work but can be used conveniently for small numbers of samples and is designed specifically for samples of low pigment content where a precise chlorophyll *c* value is required.

The method described below has the advantage that it may be used in conjunction with method IV.3.I using acetone extracts after the other pigments have been determined.

METHOD

A. Capabilities

The lower limit of detection, with the filtration of 10 liters, has not been statistically determined but will be about 0.05 mg/m³.

PRECISION AT THE 5 μG LEVEL

Mean of n determinations $\pm 1.5/n^{\frac{1}{2}}$ μg chlorophyll *c*.

Unlike the trichromatic method, we believe that there is no systematic error in this method although certain interferences are possible (*see later*).

B. Outline of Method

The acetone extract of the phytoplankton in a water sample is treated with a dilute sodium chloride solution and extracted with *n*-hexane. The extinction of the aqueous acetone layer containing the chlorophyll *c* is then measured by a spectrophotometer at 4500 Å before and after adding acid, which converts the chlorophyll *c* to the phaeo-pigment. The chlorophyll *c* content of the sample is calculated from the resulting decrease of extinction.

C. Special Apparatus and Equipment

See IV.3.I, Section C.

60-ml pear-shaped separatory funnels with the stems cut short. Do not grease the taps but grind them into place with a little fine emery and water, if necessary, to ensure a snug fit.

D. Sampling Procedure and Sample Storage

See IV.3.I, Section D.

E. Special Reagents

See IV.3.I, Section E, for reagents 1 and 2. In addition a little 100% acetone, purified as described in IV.3.I, is required.

3. *n*-HEXANE

Use analytical quality material and, for safety, redistill the solvent for use from a little sodium carbonate.

4. SODIUM CHLORIDE SOLUTION

Dissolve 0.5 g analytical reagent quality sodium chloride in 1000 ml of distilled water.

F. EXPERIMENTAL

PROCEDURE

1. Determine the pigments exactly as described in IV.3.I, Section F 1–5 using Millipore AA filters. The measurement of extinctions to obtain values for chlorophylls and carotenoids is, of course, not essential to the *present* method but may as well be done as the required extracts will have been prepared. The whole 10 ml should be drained from the centrifuge tube into the spectrophotometer cell.

2. Transfer the 10 ml of 90% acetone extract from the cell into a clean *dry* separatory funnel. Drain the cell thoroughly but *do not* rinse.

3. Add 3.5 ml of sodium chloride solution from a 5-ml graduated pipette and 13.5 ml of hexane from a 20- or 25-ml graduated pipette. Shake the funnel gently for 1 min (Note *a*).

4. Run off exactly 8.5 ml of the lower aqueous–acetone phase into a 15-ml graduated centrifuge tube. Add 100% acetone to make the volume to exactly 10.0 ml and centrifuge if necessary (Note *b*). Decant the clear liquid into an *acid free* 10-cm-path-length spectrophotometer cell designed to hold 10 ml or less of liquid (Note *c*). Work only in diffuse light.

5. Without delay measure the extinction against a cell containing 90% acetone at 4500 Å (E_1). Add one *small* drop (*ca.* 0.02 ml) of concentrated hydrochloric acid to the extract, stopper the cell, and invert it several times to mix the acid and acetone. Re-read the extinction *immediately* at 4500 Å (E_2) (Note *c*).

6. Calculate the amount of chlorophyll *c* present from the formula (Note *d*):

$$\text{mg chlorophyll } c/\text{m}^3 = \frac{17.5\ (E_1 - E_2)}{V}$$

where V is the volume of sea water filtered in liters.

NOTES

(*a*) The addition of this saline solution precipitates and coagulates the Millipore membrane material which should collect at the interface of the two liquids. All pigments except chlorophyll *c* are removed from the lower layer (*see* Note *d*).

(*b*) If care is taken to avoid particles of precipitated membrane material, 10.0 ml of clear solution should result. Centrifugation at this stage should rarely be necessary but the 15-ml centrifuge tubes make convenient measuring vessels.

(*c*) The use of this technique of converting chlorophyll *c* to phaeophytin *c* and measuring the corresponding *decrease* in extinction at 4500 Å gives a method having less sensitivity than could be obtained if the extinction at 4500 Å alone were measured but the procedure is more specific for chlorophyll *c* and removes the possibility of interference from traces of carotenoids, etc. It should be noted that the spectrophotometer cell must be completely free from the acid used in one determination before the non-acidified reading of the next determination is at-

tempted. Cells should be cleaned by a generous washing with 100% acetone between determinations.

(d) There is no blank determination, as such, in this method. The method is difficult to calibrate in the absence of a pure source of chlorophyll *c* and the factor given here, obtained by taking known weights of chlorophyll *c* throughout the whole procedure, is probably applicable directly with all correctly aligned spectrophotometers. The only compounds known to give interference are chlorophyllides *a* and *b*, as some of these would remain with the chlorophyll *c* in the acetone layer and be converted on acidification to the corresponding phaeophorbides, with a spectral shift. The present technique minimizes the interference from such compounds and fortunately it does not appear likely that they will often be present in significant amounts in samples from the open sea.

IV.3.IV. FLUOROMETRIC DETERMINATION OF CHLOROPHYLLS

INTRODUCTION

The method described here is based on the use of the Turner fluorometer as suggested by C.S. Yentsch and D.W. Menzel (*Deep-Sea Res.*, 10: 221, 1963) and subsequently investigated by Holm-Hansen et al. (*J. Conseil, Conseil Perm. Intern. Exploration Mer*, 30: 3, 1965). The method is not so accurate as the spectrophotometric approach but has the convenience of speed and the requirement of much smaller sample volumes for a given sensitivity. Only chlorophyll *a* is determined.

METHOD

A. Capabilities

The limit of detection will depend upon the volume of water filtered and the sensitivity of the fluorometer. With a 2-liter sample about 0.01 mg chlorophyll a/m^3 should be detectable with surety. The precision is very much dependent on the amount of pigment being determined but P (*see* NOTE ON STATISTICAL LIMITS) is better than 8% of any value of chlorophyll *a* exceeding 0.5 mg/m^3.

B. Outline of Method

Extracts obtained as described in IV.3.I are measured fluorometrically with the Turner fluorometer.

C. Special Apparatus and Equipment

See IV.3.I,C but only glass filters should be used. The Turner fluorometer is fitted with the "high sensitivity" door, F.4T4-BL lamp, Wratten 47B or Corning CS.5-60 filter for the excitation light and Corning CS.2-64 filter for the emitted light.

D. Sampling Procedure and Sample Storage

See IV.3.I,D.

E. Special Reagents

See IV.3.I,E.

F. Experimental

The extracts from 0.25–2 liters, obtained exactly as described in IV.3.I,F.1–5, but using *only 10.0 ml* of 90% acetone, are measured in a Turner fluorometer with the scale "zeroed" for each door opening against a tube of 90% acetone. Provided that phaeo-pigments are absent:

$$\text{mg chlorophyll } a/m^3 = F_D \times R$$

where R is the reading of the fluorometer and F_D is a factor for each door (*see* Section H *below*). Do not use solutions which necessitate the use of door 1 or which give readings much greater than 50 on door 3. With such solutions the concentrations of chlorophyll are too great for there to be linearity between fluorescence and concentration. If solutions are too concentrated dilute 3 ml (pipette) of extract with 3 ml of 90% acetone in a second clean, dry tube.

G. Determination of Blank

As the conditions used in the fluorometer are specific for chlorophyll and this is not introduced as a contaminant in reagents we have never found a blank, *per se*, with this method. The output (probably from scatter) of a tube of 90% acetone is not negligible on door 10 and the instrument should be zeroed against a tube of 90% acetone with all doors immediately prior to use.

H. Calibration

This must be done on extracts from marine phytoplankton as pure chlorophyll *a* is difficult to obtain. With the conditions recommended in this method the instrument responds almost exclusively to chlorophyll *a* but there is a slight and variable response to other chlorophylls. For this reason factors vary a little from species to species. We recommend that a healthy culture of *Skeletonema costatum* or, even more, a mixture of about equal amounts (by pigment) of *Skeletonema costatum*, *Coccolithus huxleyii*, and *Peridinium trochoidium* be used as a source of chlorophyll. If such cultures are not available natural populations can be used but there is then always uncertainty as to the presence of phaeo-pigments. Take samples from near the surface in eutrophic waters under early "bloom" conditions.

Extract sufficient culture or natural population to give 50 ml of extract having a reading of about 50 on door 3 of the fluorometer (R_3). Determine the amount, C_e, as described in IV.3.I,F, having ensured that the wavelength alignment of the spectrophotometer is carefully adjusted. Determine F_3 for door 3 from the formula:

$$F_3 = \frac{C_e}{R_3}$$

Dilute a known volume of the extract with a known volume of 90% acetone so that readings greater than 50 are obtained for doors 10 and 30 with known new values of C_e. Calculate F_{10} and F_{30} from expressions analogous to the above.

Note: Generally it is so little extra trouble to determine both chlorophyll and phaeo-pigments together that the approach given in the following addendum is recommended for work with samples other than phytoplankton cultures.

ADDENDUM TO IV.3.IV. FLUOROMETRIC DETERMINATION OF PHAEO-PIGMENTS

METHOD

This procedure is taken from Holm-Hansen, et al. (*J. Conseil, Conseil Perm. Intern. Exploration Mer*, 30: 3, 1965) and is essentially the same as IV.3.IV except that after the first reading, R_B, is taken on the fluorometer, the tube is removed, and 2 drops of 5% v/v hydrochloric acid are added. The contents of the tube are mixed by inverting once or twice, and a second reading, R_A, is taken between 30 and 60 sec later, after a stable value is reached.

Note: The tube must be washed out well with 90% acetone between determinations to make sure that no acid remains.

The precision varies from sample to sample and is not so high as the precision obtainable when only measurements on unacidified solutions are made. Quantities of phaeo-pigments less than 10% of the total pigment should be interpreted with caution.

$$\text{mg chlorophyll } a/\text{m}^3 = F_D \frac{\tau}{\tau - 1} (R_B - R_A) \qquad \frac{\text{Volume of extract}}{\text{Volume extracted}}$$

$$\text{mg phaeo-pigment}/\text{m}^3 = F_D \frac{\tau}{\tau - 1} (\tau R_A - R_B)$$

$F_3 = .111$

$F_{30} = .0152$

where F_D is the door factor and τ is a ratio obtained as described below. There is no blank *per se* with this method.

$\tau = 1.36$

CALIBRATION

The door factors, F_D, are identical with those obtained in IV.3.IV,H and should be obtained in the same way, ideally from mixed cultures. The ratio, τ, is the ratio $\frac{R_B}{R_A}$ obtained on any extract free from phaeo-pigments but, preferably, an extract used for standardization. This ratio, which should be near to 2.2 with the equipment specified here, must be obtained by making all measurements on one door, the R_B value being as near to 100 as practicable. The value of τ is not quite constant, especially if a great deal of chlorophyll *c* is present in some extracts, so slightly negative values for phaeo-pigments may sometimes be obtained. A single formula is good enough, however, for most field work and will estimate the fraction of phaeo-pigments present in samples sufficiently well for most ecological studies. For work of the highest precision use the method given in the Addendum to IV.3.I.

IV.3.V. AUTOMATED ESTIMATION OF CHLOROPHYLL PIGMENTS BY FLUORESCENCE

INTRODUCTION

This method derives from a report by C. J. Lorenzen (*Deep-Sea Res.*, 13: 223, 1966) with some further work reported by one of us (Strickland, *Deep-Sea Res.*, In Press). Sea water is pumped through a cuvette in a specially sensitive Turner fluorometer and the fluorescence of the living plankton cells measured directly. The method is an invaluable semiquantitative tool for measuring the surface concentration of phytoplankton in a ship underway but requires very frequent standardization if it is to be used as a precise technique in eutrophic waters. No generally applicable precision can be quoted. The method will measure between about 0.05 and 20 mg chlorophyll a/m^3. Greater quantities (such as found in red-tide blooms) can be handled if a specially constructed door 1 is made with a very small hole.

METHOD

A. Special Apparatus and Equipment

A Turner fluorometer, Model III with a flow-through door and cuvette (¼-inch orifices). Use a high intensity F4T.5. blue lamp with a blue Corning filter CS.5-60 for the excitation light and a red Corning filter CS.2-64 for the emitted light. The standard photomultiplier *must* be replaced by a red sensitive one (R136).

Sea water or distilled water should be drawn through the cuvette using a small pump and a three-way tap. Depending on the source of the sample some form of debubbling device will generally be needed (*see* Section IV.13,C).

Using a heat exchanger (Section IV.13) may prevent condensation of cold water on the cuvette when the sample is below room temperature or wiping the faces of the cuvette once a day with a strong solution of organic detergent may suffice. (The detergent causes very little increase of blank as a red fluorescence is being measured.)

A 10-mv recorder for the output of the fluorometer with the output adjusted as described in the Turner instrument manual is required.

B. Experimental

PROCEDURE

1. Draw distilled water through the cuvette until it has been thoroughly flushed and filled. Turn off the pump to conserve water and adjust the zero with door 1 so that the blank is near zero. Switch successively to doors 3, 10, and 30; record blanks for each door. Repeat this operation once or twice a day.

2. Suck sea water and measure the output of the fluorometer using a suitable door. Change doors if the output exceeds 9 mv or decreases below 1 mv. Calculate the chlorophyll *a* content of the water using the expression

$$\text{mg chlorophyll } a/m^3 = F_D \times R \qquad -1$$

The values for F_D for doors 1, 3, 10, and 30 must be determined as described in Section C.

C. CALIBRATION

Very frequent standardization is necessary if this method is to be anything but a semiquantitative technique for detecting major changes of phytoplankton concentration. The output for a given concentration of chlorophyll (in suspension) depends on species in an (as yet) unpredictable fashion. The value for F_D can vary at least threefold for samples taken within a few miles of each other. The factor is nearly always greater near the surface than deeper in the euphotic zone and should be determined at several depths for the best work. Although the fluorescence is proportional to concentration in most circumstances (equation 1), if the concentration of chlorophyll *a* exceeds *about* 5 mg/m³ or if cells are suspended in filtered sea water (e.g. phytoplankton cultures) a power function may be found in some instruments and thus should be carefully checked.

Every time a door is changed or, for the best work with the ship underway, every 30 min, take a liter of effluent from the fluorometer and at the same time mark the chart. Analyze the effluent for chlorophyll *a* as described in IV.3.IV. Calculate the door factor F_D from the expression

$$F_D = \frac{\text{mg chlorophyll } a/\text{m}^3}{R}$$

where R is the reading (corrected for blank) at the moment the sample is taken.

The method does not distinguish between chlorophyll and phaeo-pigments and is best calibrated in chlorophyll equivalents even though some degradation products may be present in the water. The method is, essentially, an exploratory tool and discrete samples should always be taken and analyzed fully by methods IV.3.I or IV.3.IV whenever precise data are needed.