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Agenda item 12: Harmonization and standardization of IMAP Pollution Cluster Monitoring

Monitoring Guidelines/Protocols for Determination of Concentration of Key nutrients in Seawater – Nitrogen Compounds

UNEP/MAP
Athens, 2021

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Annexes:

Note by the Secretariat

In line with the Programme of Work 2020-2021 adopted by COP21 the MED POL Programme has prepared the Monitoring Guidelines related to IMAP Common Indicators 13, 14, 17 and 20 for consideration of the Integrated Meeting of the Ecosystem Approach Correspondence Groups on Monitoring (December 2020), whilst the Monitoring Guidelines for Common Indicator 18, along with the Monitoring Guidelines related to data quality assurance and reporting are under finalization for consideration of the Meeting on CorMon on Pollution Monitoring planned to be held in April 2021.

These Monitoring Guidelines present coherent manuals to guide technical personnel of IMAP competent laboratories of the Contracting Parties for the implementation of the standardized and harmonized monitoring practices related to a specific IMAP Common Indicator (i.e. sampling, sample preservation and transportation, sample preparation and analysis, along with quality assurance and reporting of monitoring data). For the first time, these guidelines present a summary of the best available known practices employed in marine monitoring by bringing integrated comprehensive analytical practices that can be applied in order to ensure the representativeness and accuracy of the analytical results needed for generation of quality assured monitoring data.

The Monitoring Guidelines/Protocols build upon the knowledge and practices obtained over 40 years of MED POL monitoring implementation and recent publications, highlighting the current practices of the Contracting Parties' marine laboratories, as well as other Regional Seas Conventions and the EU. A thorough analysis of presently available practices of UNEP/MAP, UNEP and IAEA, as well the HELCOM, OSPAR and European Commission Joint Research Centre was undertaken to assist an innovative approach for preparation of the IMAP Monitoring Guidelines/Protocols.

In order to support national efforts, this Monitoring Guidelines for Determination of Concentration of Key nutrients in Seawater – Nitrogen Compounds provide the six protocols gathered under tree Technical Notes for determination of concentration of nitrite, nitrate and ammonium in seawater, as follows: a) Technical note for determination of concentration of nitrite that includes i) Protocol for manual colorimetric determination of concentration of nitrite and ii) Protocol for automated colorimetric determination of concentration of nitrite; b) Technical note for determination of concentration of nitrate that includes i) Protocol for manual colorimetric determination of concentration of nitrate and ii) Protocol for automated colorimetric determination of concentration of nitrate; and c) Technical note for determination of concentration of ammonium that includes i) Protocol for manual colorimetric determination of concentration of ammonium and ii) Protocol for automated colorimetric determination of concentration of ammonium.

The Monitoring Guidelines/Protocols for IMAP Common Indicators 13 and 14, including the one related to Key nutrients in Seawater, establish a sound ground for further regular update of monitoring practice for a purpose of successful IMAP implementation.

In accordance with the Conclusions and Recommendations of the Integrated Meetings of the Ecosystem Approach Correspondence Groups on IMAP Implementation (CORMONs) (Videoconference, 1-3 Dec. 2020), and in particular paragraph 22, this Meeting requested the Secretariat to amend the Monitoring Guidelines by addressing agreed technical proposals that were described in the Report of the Meeting in line with its agreement to proceed with submission of these documents to the Meeting of MEDPOL Focal Points. Given the Integrated Meetings of CORMONs did not provide any request for further changes in this Monitoring Guideline, it is submitted for consideration of the present Meeting of MEDPOL Focal Points in the format as it has been discussed and agreed by the Integrated Meetings of CORMONs.

List of Abbreviations / Acronyms

ASTM	American Society for Testing and Materials
BDH	British Drug Houses, a big chemical company that was merged with Merck KGaA
BODC	British Oceanographic Data Centre
CAS	CAS Registry Number, is a unique numerical identifier assigned by the Chemical Abstracts Service (CAS)
CI	Common Indicator
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
DDW	Double-distilled water
EcAp	Ecosystem Approach
EO	Ecological Objective
EPA	United States Environmental Protection Agency
EU	European Union
GES	Good Environmental Status
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
HPLC	High Performance Liquid Chromatography
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
ISO	International Standard Organization
JGOFS	Joint Global Ocean Flux Study
LOD	Limit of Detection
MAP	Mediterranean Action Plan
MEDPOL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MSFD	Marine Strategy Framework Directive
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
OSW	Oligotrophic Sea Water
SI	International System of Units (SI, abbreviated from the French Système international (d'unités))
SCOR	Scientific Committee on Oceanic Research
SFA	Segmented Flow Autoanalyser
UNESCO	United Nation Educational Scientific and Cultural Organization
WOCE	World Ocean Circulation Experiment

1. Introduction

1. In the Monitoring Guidelines for Key nutrients – Nitrogen compounds in Seawater, the protocols for manual and automated determination of the concentration nitrite, nitrate and ammonium are elaborated. Probably the most important property of seawater in terms of its effect on life in the marine environment is the concentration of dissolved nutrients. The most critical of these nutrients are nitrogen and phosphorus because they play a major role in stimulating primary production by plankton. These elements are known as limiting because plants cannot grow without them. At the moment, the water classification scheme on which the assessment of GES regarding Ecological Objective 5 related to eutrophication is based on chlorophyll *a* concentration as presented in details in the IMAP Guidance Factsheets (UNEP/MAP, 2019) [1], although in near future it will be complemented by those based on concentration of key nutrients in seawater.

2. The IMAP Protocols elaborated within this Monitoring Guidelines for Determination of Concentration of Key nutrients in Seawater – Nitrogen Compounds provide detail guidance on the necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the concentration of nitrite, nitrate and ammonium in seawater, calculations, data transformation if necessary and identify weak points all endorsed through important notes and possible problems. However, they are not intended to be analytical training manuals, but guidelines for Mediterranean laboratories, which should be tested and accordingly modified, if need be, in order to validate their final results.

3. This Monitoring Guidelines builds upon the UNEP/MAP Integrated Monitoring and Assessment Programme (IMAP) respectively IMAP Guidance Fact Sheets for IMAP Common Indicators 13 and 14 (UNEP/MAP, 2019); standardized protocols (UNEP/MAP, 2019a)[2] and Data Quality Assurance schemes (UNEP/MAP, 2019b)[3] in order to allow the comparability of the data and build of regional assessment schemes. They also take into account previous Sampling and Analysis Techniques for the Eutrophication Monitoring Strategy of MED POL (UNEP/MAP/MED POL, 2005)[4], however providing detail procedures that are of relevance for IMAP implementation. With the details of the protocols for determination of Key nutrients, the needs of the measurements both in offshore areas and in narrow coastal areas are addressed.

4. In the Subchapters "Symbol, units and precision" at the end of each Protocol, for all parameters described in it, the symbol and unit suggested by the International System of Units (SI) are presented. The expected accuracy, precision and where possible the Limit of Detection (LOD) are also presented. A Method identifier is also presented as it is provided in the Library P01 of the British Oceanographic Data Centre (BODC) Parameter Usage Vocabulary respectively included in Data Dictionaries and Data Standards for eutrophication built in IMAP Pilot Info System.

5. The below flow diagram informs on the category of this Monitoring Guidelines related to determination of concentration of key nutrients in seawater respectively concentration of nitrogen compounds in seawater within the structure of all Monitoring guidelines prepared for IMAP Common Indicators 13, 14, 17, 18 and 20.

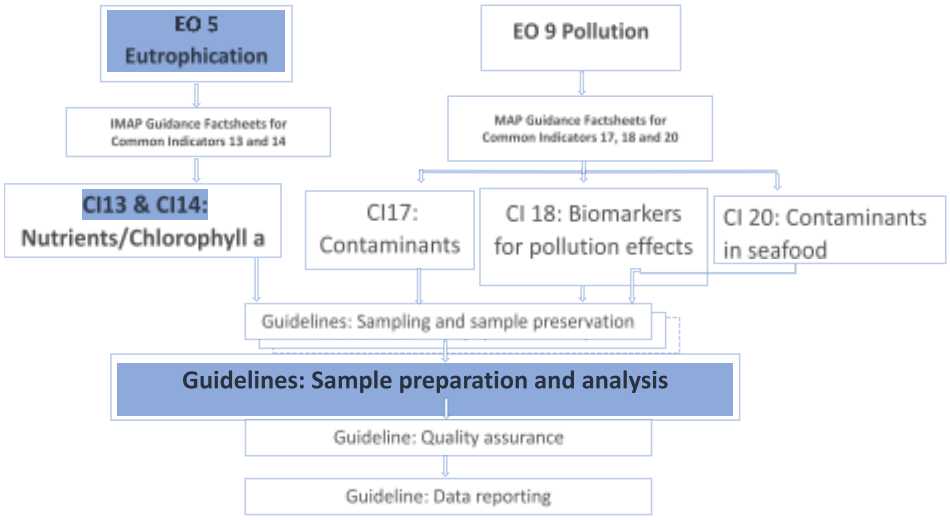
a. Continuous flow methods

6. The principle used by the continuous segmented-flow auto-analysers (SFA) is recognized as the most reliable and accurate method for determination of nutrients. Different systems are available and can be configured to meet the standard methods such as ISO, EPA, ASTM, etc… Wherever possible it is strongly recommended that such analysers are used because of the considerable increase in precision and sample throughput that they offer. Ideally such analysers can be used in laboratories on board a research vessel allowing problems of sample deterioration during storage to be circumvented.

7. The multiplicity of methods reported in the literature is more related to the optimization of methods for different environments that a significant difference in the reactions used. In the Protocols dedicated to the individual methods, some specific aspects will be mentioned. On the general principles of SFA systems, in addition to the documentation provided by the manufacturers to the classic textbooks of Strickland and Parsons (1965)[5] and Grasshoff et al. (1999)[6] can be referred. Equally numerous are the technical reports of the various laboratories produced to homogenize the methods within the programs international like JGOFS or WOCE. In

[1] (UNEP/MAP, 2019), UNEP/MED WG.467/5. IMAP Guidance Factsheets: Update for Common Indicators 13, 14, 17, 18, 20 and 21: New proposal for candidate indicators 26 and 27.
[2] (UNEP/MAP, 2019a), UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution.
[3] (UNEP/MAP, 2019b), UNEP/MED WG.46710. Schemes for Quality Assurance and Control of Data related to Pollution
[4] (UNEP/MAP/MED POL), 2005. Sampling and Analysis Techniques for the Eutrophication Monitoring Strategy of MED POL. MAP Technical Reports Series No. 163. UNEP/MAP, Athens, 46 pp.
[5] Strickland J.J., Parsons T., 1965. A manual of sea water analysis: with special reference to the more common micronutrients and to particulate organic material. Fisheries Research Board of Canada, 311 pp.
[6] Grasshoff, K., Kremling, K., Ehrhardt, M. (eds), 1999. Methods of Seawater Analysis 3rd Edition Wiley-VCH Weinheim, 634 pp.

the Protocols only the most essential indication on the most frequently used method will be provided. Important notes on the critical parts of the methods, for it successful performance will also be indicated.



Flow Diagram: Monitoring Guidelines for IMAP Ecological Objective 5 and 9.

2. Technical note for determination of concentration of nitrite

8. This technical note elaborates the method for determination of concentration of nitrite that is based on a series of reactions that lead to the formation of a coloured diazo compound and measured colorimetrically. This procedure, one of the most sensitive among direct colorimetric analyses, is specific for nitrites and does not show any variation in efficiency in relation to the ionic strength of the solution. The original method, proposed by Griess-Ilosvay (Ilosvay, 1889)[7], was subsequently modified by Shinn (1941)[8] and applied to the analysis of sea water by Bendschneider and Robinson (1952)[9].

9. The analytical procedure is based on the formation, in an environment with a pH lower than 2 and a temperature not higher than 40 °C, of a diazonium salt (diazosulfanilamide chloride) which subsequently reacts with naphthylethylenediamine to generate a diazo dye.

10. Under this Technical Note, this Monitoring Guidelines provides the following IMAP Protocols for the colorimetric determination of concentration of nitrite:

- Protocol for manual colorimetric determination of concentration of nitrite;
- Protocol for automated colorimetric determination of concentration of nitrite.

2.1. Protocol for manual colorimetric determination of concentration of nitrite

b. Equipment:

11. The equipment for manual colorimetric determination of concentration of nitrite include:

1. graduated cylinders or 50 mL pipettes
2. 100 mL borosilicate glass containers (beaker)
3. laboratory glassware for chemical preparations
4. 1 mL automatic dispenser
5. 500 mL volumetric flasks
6. volumetric flasks of 100 mL class A
7. 1 L class A volumetric flask
8. precision micropipettes to measure volumes in the range of 10-100 µL
9. analytical scale
10. stove
11. microwave oven
12. dryer
13. spectrophotometer or colorimeter sensitive to 543 nm equipped with cells of at least 50 mm optical path

c. Chemical products:

[7] Ilosvay L. (1889) Determination of nitrite in saliva and exhaled air. Bull. Soc. Chim. Fr., 2, 388-391.
[8] Shinn M.B. (1941) A colorimetric method for the determination of nitrite. Ind. Eng. Chem Anal. Ed., 13, 33-35.
[9] Bendschneider K., Robinson R.J. (1952) A new spectrophotometric method for the determination of nitrite in sea water. J. Mar. Res., 11, 87-96.

12. The chemical products for manual colorimetric determination of concentration of nitrite include:

1. sulfocromic mixture
2. concentrated hydrochloric acid [HCl]
3. sulfanilamide [$NH_2SO_2C_6H_4NH_2$]
4. *N*-(1-Naphthyl)ethylenediamine dihydrochloride [$C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$]
5. sodium nitrite [$NaNO_3$]
6. chloroform [$CHCl_3$]

d. Preparation of stock solutions:

Sulfanilamide reagent

13. 50 mL of concentrated hydrochloric acid is poured into a beaker of at least 600 mL, containing 400 mL of reagent grade water, and stirred until completely mixed. 5 g of sulfanilamide in this solution are dissolved. The volume with reagent grade water is adjusted to 500 ml. The solution is stable for many months if stored in plastic or glass containers, in the refrigerator.

NNEDDC reagent

14. 500 mg of *N*-(1-Naphthyl) ethylenediamine dihydrochloride in 450 mL of reagent grade water is dissolved and adjusted to volume with reagent grade water in a 500 mL flask. The solution stored in the refrigerator, in dark bottles, is stable for 1-2 months and must be discarded if brown colour is developed.

Standard solution of sodium nitrite - 2 mmol L^{-1}

15. Few grams of sodium nitrite in an oven at 110 °C are dried and cooled in a silica gel dryer. 138 mg are weighted on an analytical balance, and in 800 mL of reagent grade water in a 1 L (class A) flask dissolved and adjusted to volume. The solution should be kept refrigerated, in a dark bottle, adding a few drops of chloroform and is stable for about a month.

e. Preparation of specific equipment for analysis:

d.1. Maintenance of reaction vessels

16. The reaction flasks with boiling sulfochromic mixture are periodically washed, rinsed abundantly with reagent grade water and them dried. For ordinary maintenance, after use, are rinsed with reagent grade water and placed upside down on filter paper.

f. Analytical procedure:

e.1.Reagents to be prepared at the time of use

Preparation of standard solutions

17. 5 standards of known nitrite concentration are prepared: by diluting, in 100 mL flasks (class A), respectively 10, 25, 50, 75, 100 µL of standard solution of sodium nitrite (measured with a precision pipette) with oligotrophic seawater. The concentrations of nitrite are thus between 0.2 and 2 µmol L^{-1} plus the nitrite content of oligotrophic seawater.

e.2. Analytical treatment

18. At the time of analysis, if the sample had been frozen, possibly using a 37 °C bath or in a microwave oven is quickly thawed;

19. The beakers with 50 mL of sample or each of the standards (measured with a graduated cylinder) are filled.

20. 1 mL of sulfanilamide reagent to each sample or standard with a dispenser are added and the reaction allowed to take place for 5 minutes.

21. 1 mL of NNEDDC reagent to each sample or standard with a dispenser are added and the reaction allowed to take place for additional 10 minutes.

e.3. Preparation of reagent blanks

22. At least two replicates of reagent blanks in the same type of 100 mL borosilicate glass container, using 50 mL of reagent grade water are prepared applying the same procedure as for samples and standards.

e.4. Spectrophotometric measurements

23. The absorbance of the blank ($bl_{c,i}$) of each cell of the spectrophotometer or colorimeter, used for reading against the reference cell, at 543 nm is measured, both filled with water without regents. The operation is superfluous if only one cell is used.

24. For each flask, the number of the cell used, the identification of the contents of the flask (sample, standard solution, blank) are noted in a form. The cell is rinsed with part of its contents, filled and the absorbance at 543 nm read, recording the reading on the same form.

g. Calculations

25. The reagent blank (bl) as the average of the two blank readings is calculated.

26. The correlation between the absorbance values of the 5 standards and the assumed concentrations, using the Ordinary Least-Squares Regression is calculated. The colorimetric factor (f) is represented by the slope.

27. The concentration of nitrite in the samples is calculated with the following equation:

$$c(NO_2^-) / \mu mol\ L^{-1} = (ABS - bl - bl_{c,\,i})\ f$$

where

$c(NO_2^-)$ = concentration of orthophosphates

ABS = absorbance of the sample

bl = blank of the reagents

$bl_{c,\,i}$ = blank of the i-th cell used

f = colorimetric factor

28. For a cell with a 50 mm optical path, the colorimetric factor is equal to about 4.0 µmol L^{-1}, i.e. a difference in concentration of 1 µmol L^{-1} (for example between standard solution 3 and 5) should be the difference in absorbance of about 0.25.

h. Important notes:

29. The standard stock solution is renewed frequently (at least once a month).

i. Possible problems:

30. The suggested method is trouble- and interference-free. However, any hydrogen sulfide present in the sample must be removed before analysis (Grasshoff, 1983[10]; Airey et al., 1984[11]).

2.2. Protocol for automated colorimetric determination of concentration of nitrite

a. Reagents

Sulfanilamide reagent

31. 10 g of sulfanilamide in 100 ml of concentrated HCl is dissolved and adjusted to one liter with DDW. The solution should be stored in a dark glass bottle and is stable at least 1 month.

NNEDDC reagent

32. 1 g of *N*-(1-Naphthyl) ethylenediamine dihydrochloride in 950 mL of reagent grade water is dissolved and adjusted to volume with DDW in a 1000 mL flask. The solution stored in the refrigerator, in dark bottles, is stable for 1-2 months and must be discarded if brown colour is developed.

b. Standard

33. About 2 g of $NaNO_2$ is dried in an oven at 100 °C, checking the weight of the salt remain constant over time. The salt is placed in a silica gel dryer for additional 24 hours. 138 mg are weighted on an analytical balance, and in 800 mL of DDW in a 1 L (class A) flask dissolved and adjusted to volume. A final concentration of 2 mmol L^{-1} is obtained. The solution should be kept refrigerated, in a dark bottle, adding a few drops of chloroform and is stable for about a month.

34. This standard is used in the daily procedure for the preparation of 5 standards. The concentration of the standards is chosen based on the amount of NO_2 salt expected to be found covering the entire range of expected concentrations. From the 5 standards the multiplication factor necessary to calculate the concentrations is obtained.

c. Manifold

[10] Grasshoff, K. (1983) Determination of nitrite. In: “Methods of Seawater Analysis”, Grasshoff K., M. Ehrhardt, K. Kremling Eds, Verlag Chemie, Weinheim, 139-142.

[11] Airey D., Dal Pont G., Sandars G. (1984) A method of determining and removing sulphide to allow the determination of sulphate, phosphate, nitrite and ammonia by conventional methods in small volumes of anoxic waters. Analytica Chim. Acta, 166, 79-92.

35. The manifold (Fig. 1) is composed of two injectors and four coils of 10 turns each. The first injector (A) is equipped with 3 inputs: the first is for the sample, the second is for the air and the third input provided for the introduction of the first reagent. Immediately after there are 4 composite coils with 10 coils each: in the first 2 the first reagent is mixed, in the other 2 the second reagent is introduced at point (B), by means of the second injector.

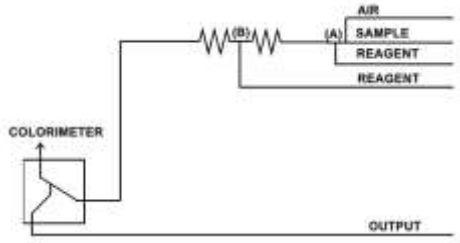


Figure 1. Manifold for nitrite measurement.

d. Important notes:

36. If an unstable baseline appears when the device is turned on in the absence of reagents, wash the circuit with 10% HCl.

37. If during the analysis there is an evident increase in the baseline, clean immediately the colorimeter reading cell by injecting 50% hydrochloric acid directly into the cell without stopping the circuit.

38. Use DDW water by deionizing it directly in the sample container of the instrument.

39. If it is necessary to change components of the circuit (injectors, bubblers), rebalance the circuit by changing the flow rates of the pipes.

40. Use suitable containers for the different reagents to be used. The container cap must be provided of small holes in which to insert capillaries (needles, etc.) for the withdrawal of the reagent.

41. Nutrient-poor water, i.e. oligotrophic water (OSW), as wash water between samples is used. OSW of salinity similar to the samples must be used.

e. Symbol, units and precision

42. For the parameter described in this protocol, the symbol and unit suggested by the International System of Units (SI), as well as the expected accuracy, along with the Method identifiers as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: $c(NO_2^-)$	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.03
Method identifier:	SDN:P01::**NTRIMATX**	Concentration of nitrite {NO2- CAS 14797-65-0} per unit volume of the water body [dissolved plus reactive particulate phase] by manual colorimetric analysis
	SDN:P01::**NTRIAAZX**	Concentration of nitrite {NO2- CAS 14797-65-0} per unit volume of the water body [unknown phase] by colorimetric autoanalysis

3. Technical note for determination of concentration of nitrate

43. The method was introduced by Morris and Riley (1963)[12], but only later was the dynamics of the involved reactions studied in depth (Nydhal, 1976[13]; Grasshoff, 1983[14]). The method for the determination of nitrate (NO_3^-) is based on its reduction to nitrite, which is then determined colorimetrically via the formation of an azo dye. It had proved to be reliable and useful for work at sea and is widely free from interferences in nearshore and oceanic waters.

[12] Morris A.W., Riley J.P. (1963) The determination of nitrate in sea water. Analytica Chim. Acta, 29, 272-279.
[13] Nydhal F. (1976) On the optimum conditions for the reduction of nitrate to nitrite by cadmium. Talanta, 23, 349-357.
[14] Grasshoff K. (1983) Determination of nitrate. In: "Methods of Seawater Analysis", Grasshoff K., M. Ehrhardt, K. Kremling Eds, Verlag Chemie, Weinheim, 143-150.

44. The method determines the sum of nitrite and nitrate; therefore, a separate determination of nitrite must be conducted, and concentration subtracted from that obtained with this method. At concentration levels higher than about 20 μmol L^{-1}, calibration curves for a low and high range must be established.

45. Nitrate is reduced to nitrite in a reduction column filled with copper-coated cadmium granules. The yield of the reduction depends on the pH of the solution and on the activity of the metal surface. The conditions of the reduction described in the method are adjusted to a pH of about 8.5, so that nitrate is converted to nitrite almost quantitatively (90-95 %) and not reduced further. Ammonium chloride buffer is used to control the pH and to complex the liberated cadmium ions. The nitrite formed is then determined colorimetrically (at 540 nm). The proposed method is substantially that illustrated by Grasshoff (1983).

46. Under this Technical Note related to determination of concentration of nitrate, this Monitoring Guidelines provides the following IMAP Protocols for the colorimetric determination of concentration of nitrate:

- Protocol for manual colorimetric determination of concentration of nitrate;
- Protocol for automated colorimetric determination of concentration of nitrate.

3.1. Protocol for manual colorimetric determination of concentration of nitrate

a. Equipment:

47. The equipment for manual colorimetric determination of concentration of nitrate include:

1. graduated cylinders or 50 mL pipettes
2. 100 mL borosilicate glass containers (beaker)
3. laboratory glassware for chemical preparations
4. 1 mL automatic dispenser
5. 1000 mL and 500 mL volumetric flasks
6. volumetric flasks of 100 mL class A
7. 1 L class A volumetric flask
8. precision micropipettes to measure volumes in the range of 10-100 μL
9. analytical scale
10. stove
11. microwave oven
12. dryer
13. spectrophotometer or colorimeter sensitive to 543 nm equipped with cells of at least 50 mm optical path
14. peristaltic pump with one or more channels
15. reduction columns
16. 4-4.5 mm internal diameter tygon tube
17. glass wool
18. pH meter
19. 0.25- and 0.42-mm mesh sieves for particle size (60 and 40 mesh)

b. Chemical products:

48. The chemical products for manual colorimetric determination of concentration of nitrate include:

1. sulfocromic mixture
2. concentrated hydrochloric acid [HCl]
3. sulfanilamide [$NH_2SO_2C_6H_4NH_2$]
4. *N*-(1-Naphthyl) ethylenediamine dihydrochloride [$C_{10}H_7NHCH_2CH_2NH_2 \cdot 2HCl$]
5. potassium nitrate 99.999% [KNO_3]
6. sodium nitrate [$NaNO_2$]
7. ammonium chloride [NH_4CI]
8. ammonium hydroxide [NH_4OH]
9. granular cadmium for reactors [Cd]
10. copper sulphate pentahydrate [$CuSO_4 \cdot 5H_20$]
11. chloroform [$CHCl_3$]

c. Preparation of stock solutions:

Sulfanilamide reagent

49. 50 mL of concentrated hydrochloric acid is poured into a beaker of at least 600 mL, containing 400 mL of reagent grade water, and stirred until completely mixed. 5 g of sulfanilamide in this solution are dissolved. The volume with reagent grade water is adjusted to 500 ml. The solution is stable for many months if stored in plastic or glass containers, in the refrigerator.

NNEDDC reagent

50. 500 mg of *N*-(1-Naphthyl)ethylenediamine dihydrochloride in 450 mL of reagent grade water is dissolved and adjusted to volume with reagent grade water in a 500 mL flask. The solution stored in the refrigerator, in dark bottles, is stable for 1-2 months and must be discarded if brown colour is developed.

Copper sulphate solution

51. 20 g of copper sulphate pentahydrate in reagent grade water in a 1 L volumetric flask are dissolved and stored in a dark bottle. The solution is stable indefinitely.

Hydrochloric acid about 0.2 mol L^{-1}

52. 100 mL of concentrated hydrochloric acid and 500 mL of reagent grade water are mixed in a beaker while stirring. The solution is stable indefinitely stored in a glass bottle.

Ammonium-ammonium chloride buffer

53. 10 g of ammonium chloride for analysis in 1 L of reagent grade water in a beaker are dissolved. The pH of the solution is adjusted to 8.5 adding, drop by drop while stirring and checking the pH with a pH meter, a small quantity of ammonium hydroxide solution (about 1.5 mL should be sufficient). The buffer solution must be stored in a dark bottle and is stable for many months.

Standard solution of potassium nitrate 5 mmol L^{-1}

54. Few grams of potassium nitrate in an oven at 110 °C are dried and cooled in a silica gel dryer. 505.6 mg are weighted on an analytical balance, and in 800 mL of reagent grade water in a 1 L (class A) flask dissolved and adjusted to volume. The solution should be kept refrigerated, in a dark bottle, adding a few drops of chloroform and is stable for about a month.

Standard solution of sodium nitrite e 2 mmol L^{-1}

55. Few grams of sodium nitrite in an oven at 110 °C are dried and cooled in a silica gel dryer. 138 mg are weighted on an analytical balance, and in 800 mL of reagent grade water in a 1 L (class A) flask dissolved and adjusted to volume. The solution should be kept refrigerated, in a dark bottle, adding a few drops of chloroform and is stable for about a month.

d. Preparation of specific equipment for analysis:

d.1. Maintenance of reaction vessels

56. The reaction flasks with boiling sulfochromic mixture are periodically washed, rinsed abundantly with reagent grade water and them dried. For ordinary maintenance, after use, are rinsed with reagent grade water and placed upside down on filter paper.

d.2. Reduction column

57. The major part of the reduction column is made of a U-shaped glass tube with a total length of about 10-25 cm and an inner diameter of 3 mm. Connections to the 100 ml sample bottle and the 25 ml (marked) Erlenmeyer flask are made from flexible capillary tubing (Tygon). The sample is drawn through the column by a small peristaltic pump. For practical purpose, the whole set-up can be mounted in a box. Suitable flow rates should be determined by experimentation.

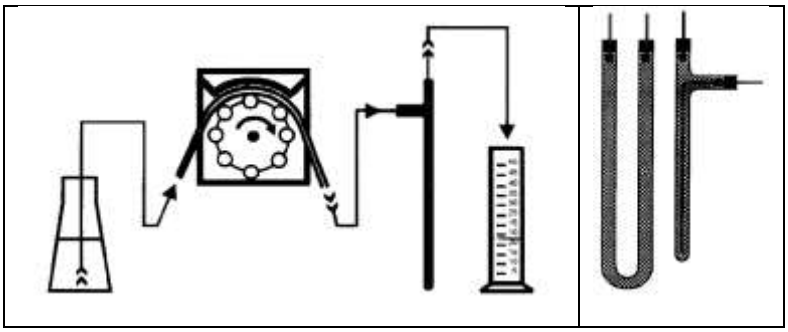


Figure 2. Reduction column for the analysis of nitrate and type of columns.

d.3. Preparation of the reduction column

58. Commercially available granulated cadmium (e.g. coarse powder for reductors grade - BDH) is sieved and the fraction between 40 and 60 mesh (i.e. around 0.25 and 0.42 mm) is retained and used.

59. The sieved cadmium granules are freed from oxides by washing them in 0.2 mol L^{-1} hydrochloric acid. The granules in a 200 mL beaker vigorously (for about 3 minutes) with 100 mL of the copper sulphate solution are shaken. Afterwards, the copperized cadmium granules under gentle shaking are rinsed, the water decanted and washed again until the water is free from finely dispersed copper.

60. Cadmium is poisonous. It should, therefore, be handled with great care. The dust is never inhaled and all operations on the dry metal are perform in a fume hood.

61. The copperized granules are poured into the reduction column (with the aid of distilled water and a funnel). The effective packing is encouraged by gently tapping the column with a pencil. When one arm is filled, the funnel is connected to the other arm and the procedure repeated. Some space in both side arms is leaved in order to pack in some glass wool.

62. The Cd is activated by passing through about 250 mL of buffer solution (ammonium chloride) containing about 100 µmol L^{-1} nitrate and rinsed thoroughly with buffer solution before the reducer is used for analysis.

63. The reduction efficiency of the reduction column is checked by analysing a nitrate standard solution of suitable concentration (e.g. equimolar). The determined absorbance is compared with that of a nitrite solution of the same concentration (e.g. if $A_{NO3} = 0.200$, $A_{NO2} = 0.210$, the reduction efficiency would be (0.200 x 100) / 0.210 = 95.2%).

64. The column is ready for use and is stable for a few months.

e. Analytical procedure:

e.1. Reagents to be prepared at the time of use

Preparation of standard solutions

65. 5 standards of known nitrate concentration are prepared: by diluting, in 100 mL flasks (class A), respectively 10, 25, 50, 75, 100 µL of standard solution of potassium nitrate (measured with a precision pipette) with oligotrophic seawater or reagent grade water. The concentrations of nitrate are thus between 0.5 and 5 µmol L^{-1} plus the nitrate content of oligotrophic seawater, if used for dilution.

66. 3 standards of known nitrite concentration are prepared: by diluting, in 100 mL flasks (class A), respectively 50, 75, 100 µL of standard solution of sodium nitrite (measured with a precision pipette) with oligotrophic seawater or reagent grade water. The concentrations of nitrate are thus between 1 and 2 µmol L^{-1} plus the nitrite content of oligotrophic seawater, if used for dilution.

e.2. Analytical treatment

67. At the time of analysis, if the sample had been frozen, possibly using a 37 °C bath or in a microwave oven is quickly thawed;

68. The 100 mL beakers with 50 mL of sample or each of the standards (measured with a graduated cylinder) are filled.

69. 50 mL of ammonium buffer (measured with a graduated cylinder) are added and mixed well.

70. The end of the capillary tube is inserted in the beaker containing the first sample to be analyzed.

71. The peristaltic pump is adjusted in such a way to ensure a flow rate between 2.5- and 3-mL min^{-1} and started allowing the sample to pass through the reduction column. The first 25 mL of sample is discarded.

72. The next 25 mL is collected and transferred in a 50 mL flask or beaker.

73. The other samples to be analysed, the nitrate standards and the nitrite standards is passed through the system, interrupting the operation of the peristaltic pump between each operation

74. After passing the last sample, the reduction column is washed with 50 mL of ammonium buffer and always kept completely full of buffer.

75. With a graduated cylinder for of each of the nitrite standards a substandard is prepared: 12.5 mL of standard and 12.5 mL of ammonium buffer is added to a beaker and properly mixed. The preparation of these standards, which have not passed through the reduction column, is necessary to verify the degree of transformation of nitrite to compounds with a lower oxidation number, independently of the degree of efficiency of the column, except for impurities of nitrate present in the nitrite standard.

76. 1 mL of sulfanilamide reagent to each flask containing the samples and the three series of standards (nitrates, nitrites and nitrites not passed through the reduction column) with a dispenser are added and the reaction allowed to take place for 5 minutes.

77. 1 mL of NNEDDC reagent to each sample or standard with a dispenser are added and the reaction allowed to take place for 10 minutes.

e.3. Preparation of reagent blanks

78. At least two replicates of reagent blanks in the same type of 100 mL borosilicate glass container, using 50 mL of reagent grade water are prepared applying the same procedure as for samples and standards, including the passage through the reduction column.

e.4. Spectrophotometric measurements

79. The absorbance of the blank ($bl_{c,i}$) of each cell of the spectrophotometer or colorimeter, used for reading against the reference cell, at 543 nm is measured, both filled with water without regents. The operation is superfluous if only one cell is used.

80. For each flask, the number of the cell used, the identification of the contents of the flask (sample, standard solution, blank) are noted in a form. The cell is rinsed with part of its contents, filled and the absorbance at 543 nm read, recording the reading on the same form.

81. The reading of the blanks is affected by a small error due to the different matrix used, but it is usually negligible as it is related only to the nitrate impurities in the ammonium buffer.

f. Calculations

82. The reagent blank (bl) as the average of the two blank readings is calculated.

83. The correlation between the absorbance values of the three series of standards and the assumed concentrations, using the Ordinary Least-Squares Regression is calculated. The colorimetric factor for the nitrates (f_1), for the nitrites (f_2) and for the nitrites not passed on the reduction column (f_3) is represented by the slopes.

84. The efficiency of the column for the reduction of nitrate and for the preservation of the nitrite present in the sample is indicated by the ratios f_1/f_2 and f_2/f_3. If the reduction efficiency is unsatisfactory (<90%), the length of the column must be increased, while this must be decreased if the nitrite yield is less than 95%.

85. The concentration of nitrate in the samples is calculated with the following equation:

$$c(NO_3^-) / \mu mol\ L^{-1} = (ABS - bl - bl_{c,i}) - c(NO_2^-) / f_2) \cdot f_1$$

where

$c(NO_3^-)$ = concentration of nitrate

$c(NO_2^-)$ = concentration of nitrite in the sample (independently derived)

ABS = absorbance of the sample

bl = blank of the reagents

$bl_{c,i}$ = blank of the i-th cell used

f1 = colorimetric factor of nitrate

f2 = colorimetric factor of nitrite

86. For a cell with a 50 mm optical path, the colorimetric factor of nitrate is equal to about 8.0 µmol L^{-1}, i.e. a difference in concentration of 2 µmol L^{-1} (for example between standard solution 1 and 3) should be the difference in absorbance of about 0.25.

g. Important notes

87. Before carrying out the analysis, the characteristics of the column must be carefully checked. If air bubbles enter the column, it is preferable to empty them and repack, as the retention time becomes variable following the progressive expulsion of air. Alternatively, the buffer solution can be allowed to pass through the column for about 20-30 minutes, to expel most of the air. In both cases it is necessary to pass through the column at least a series of standards to verify any variations in the yield of the reduction.

88. The determination of the factor f_2 is superfluous when the nitrite concentrations turn out to be of an order of magnitude lower than those of nitrates. In this case it is enough to calculate the colorimetric factor f_1 and subtract the nitrite concentration from the values obtained.

89. If a large number of samples are to be analysed, the efficiency of the reduction column during the analysis must be checked periodically.

h. Possible problems

90. The suggested method is trouble- and interference-free. However, Hydrogen sulphide, hardly present in samples containing nitrate, can be precipitated as copper or cadmium sulphide (Grasshoff, 1983).

91. The efficiency of the column can be reduced if concentrations of phosphates higher than 2 µmol L^{-1} are present (Olsen, 1980)[15].

3.2. Protocol for automated colorimetric determination of concentration of nitrate

[15] Olsen R.J. (1980) Phosphate interference in the cadmium reduction analysis of nitrate. Limnol. Oceanogr., 25, 758-760.

a. Reagents

Buffer

92. 10 g of ammonium chloride dissolved in 700 mL of DDW, and then brought to the volume of one liter must be prepared. To the solution must be added 1 mL of Brij and sodium hydroxide in a percentage such as to bring the pH of the solution to a value of 8.5. The solution is very stable.

Sulfanilamide

93. 10 g of sulfanilamide in 100 mL of concentrated HCl are dissolved and adjusted to 1 L with DDW. The solution should be stored in a brown glass bottle and is stable for at least one month.

Ethylenediamine dihydrochloride

94. 1 g of Ethylenediamine dihydrochloride is dissolved in 1 L of DDW. The solution should be stored in a dark glass bottle and is stable for at least one month.

b. Standard

51. Few grams of potassium nitrate in an oven at 110 °C are dried and cooled in a silica gel dryer. 505.6 mg are weighted on an analytical balance, and in 800 mL of DDW in a 1 L (class A) flask dissolved and adjusted to volume. The solution should be kept refrigerated, in a dark bottle, adding a few drops of chloroform and is stable for about a month. This standard is used in the daily procedure for the preparation of 5 lower concentration standards.

95. The concentration of the minor standards is chosen based on the amount of NO_3^- salts expected to be found, so that the set of sub-standards covers the entire range of expected concentrations. From the 5 standards a multiplication factor is obtained which is necessary to calculate the concentrations.

c. Reducer

96. The reducer is composed of a 20 cm long Pyrex glass tube with an internal diameter of 2 mm and U-bent.

97. Cadmium granules previously prepared according to the procedure described below are inserted into the tube.

98. Some granular cadmium are sieved to obtain a fraction of granules between 0.42 and 0.60 mm, then washed with 10% HCl and with DDW at the end. 2 g of copper sulphate are dissolved in 100 mL of DDW. The cadmium is immersed in the solution and shaken until the colour disappears. The cadmium is washed until the total elimination of colloidal copper bound to cadmium, silvery colour of the grains. The glass tube is filed with DDW and the granules inserted from the flask with a Pasteur pipette. Once the reducer has been filled, glass wool at the ends is inserted, to prevent cadmium to escape.

99. There are alternatives to the use of granular cadmium such as the use of cadmium coils or with internal walls covered with cadmium or the use of polyethylene coils with a cadmium wire inside. In all cases, the activation of cadmium with the copper sulphate solution is necessary in some procedures, copper sulphate is added continuously with the buffer.

d. Manifold

The manifold (Figure 3) is built of three injectors and five coils, one with 5 turns and four with 10 turns, and a reducer. The first injector (A) is equipped with three inputs: the first is for the sample, the second is for air bubbles and with the third input the first reagent is introduced. Immediately after, a coil made up of 5 turns in which the liquid is mixed with the buffer, is located. At the end of the coil a de-bubbler, which has the function of eliminating the bubble from the circuit to prevent air from entering the reducer that is connected to the bubbler itself at point (B), is present. At point (C) after the reducer, the second injector equipped with three inputs can be found: The first for the sample to be reduced from NO_3^- to NO_2^-, the second to restore the air bubbles and with the third the second reagent is introduced. Immediately after 4 coils made up of 10 coils each can be found: in the first 2 the mixing with the second reagent takes place, in the other 2 is where the third reagent at point (D) is injected.

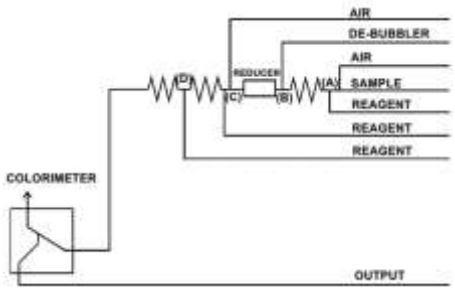


Figure 3. Manifold for nitrate measurement.

e. Important notes

100. Air passage through the reducer is not allowed.

101. The efficiency of the reducer is checked by comparing the nitrate standard with that of the nitrites according to the next methodology: 1) Two nitrate standards are prepared: one at a concentration of 5 µmol L^{-1}, the other at 10 µmol L^{-1}. The doubling of the concentration must correspond to an effective doubling of the reading. 2) Two nitrate standards of the same concentration of 5 µmol L^{-1} are prepared. The two standards are run in the same circuit prepared for nitrates and that they give the same reading value must be checked; to ensure that there has not been a reduction in the concentration of nitrites in the cadmium column.

102. The air bubbles point of the circuit must be adjusted each time the reducer is replaced by acting on the flow rates of the pipes.

103. The reducer by passing a standard of NO_3^- with a concentration of 25 µmol dm-3 through the circuit each time it is replaced must be activated.

104. If an unstable baseline is observed when the appliance is turned on in the absence of reagents, the circuit with 10% HCl must be washed.

105. If during the analysis an evident increase in the baseline is observed, the colorimeter reading cell by injecting 50% hydrochloric acid directly into the cell without stopping the circuit must be immediately cleaned.

106. The DDW is deionized if possible, directly in the water container of the instrument.

107. If a change of components of the circuit (injectors, bubblers) is necessary, the circuit by changing the flow rates of the pipes must be rebalanced.

108. Suitable containers for the different reagents must be used. The cap of the container must be provided with small holes in which to insert capillaries (needles, etc.) for the withdrawal of the reagent.

109. When mixed standards are used, NO_3^- standards with either NH_4^+ or NO_2^- standards never must be combined.

110. Water poor in nutrients, or oligotrophic water (OSW), as washing water between one sample and another must be used. OSW with salinity values similar to the sample to be analysed must be used.

111. The NO_2^- standard, passed through the nitrate reduction column must have the same reading value as the NO_2^- standard analysed in the nitrite circuit.

112. Since the concentration of nitrates is determined after their reduction to nitrites: The copper cadmium does not have a reduction efficiency of 100% and in certain conditions it also reduces nitrite. Therefore, if it were necessary to discriminate the two ions, the efficiency of the reducer should be accurately determined both for nitrite with a solution with a concentration of nitrite only and for nitrate with a solution with a known concentration of nitrate only.

113. For the parameter described in this protocol, the symbol and unit suggested by the International System of Units (SI), as well as the expected accuracy, along with the Method identifiers as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: $c(NO_3^-)$	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.03
Method identifier:	SDN:P01::**NTRAMADZ**	Concentration of nitrate {NO3- CAS 14797-55-8} per unit volume of the water body [dissolved plus reactive particulate <unknown phase] by filtration and manual colorimetric analysis and correction for nitrite

SDN:P01::**CHEMM012**	Concentration of nitrate {NO3- CAS 14797-55-8} per unit volume of the water body [dissolved plus reactive particulate phase] by colorimetric autoanalysis and correction for nitrite

4. Technical note for determination of concentration of ammonium

114. The determination of concentration of ammonium is based on a series of photochemically catalysed reactions that lead to the formation of indophenol blue. The concentration of the compound is then measured colorimetrically. The first analytical application of the formation of indophenol from phenol and hypochlorite was performed by Berthelot (1859)[16].

115. The formation of monochloramine predominates, compared to that of di- and trichloramine, for pH values higher than 7.5. The next stage of the reaction consists in the attack of monochloramine on the benzene ring of the phenol to form, probably, chloraminoquinone. Finally, quinone, or in any case the intermediate formed, produces indophenol by copulation with another phenol. This stage is strictly pH dependent, as OH^- enters directly into the reaction. For this reason, all methods that use phenol and hypochlorite require an environment with a pH of around 10.5 (Ivancic and Degobbis, 1984)[17].

116. Finally, given the importance of pH control in the development of the reaction (Sasaki and Sawada, 1980)[18], the significant salt effect (different yield of the reaction in fresh or salt water) that occurs in this method (Koroleff, 1983)[19] largely depends on the buffer capacity of the sample matrix. For this reason, the method can be applied to samples collected in estuarine environments, where the variations in alkalinity are strong, by adequately buffering the solution (Mantoura and Woodward, 1983)[20].

117. The procedure outlined here, mainly follows the methods described by Grasshoff and Johansen (1973)[21] and by Koroleff (1983) as described by Hansen and Koroleff (1999)[22] and is adapted to this manual from the previous one (UNEP/MAP/MED POL, 2005).

118. Under this Technical Note for determination of concentration of ammonium, this Monitoring Guidelines provides the following IMAP Protocols for the colorimetric determination of concentration of ammonium:

- Protocol for manual colorimetric determination of concentration of ammonium;
- Protocol for automated colorimetric determination of concentration of ammonium.

4.1. Protocol for manual colorimetric determination of concentration of ammonium

a. Equipment:

119. The equipment for manual colorimetric determination of concentration of nitrate include:

1. graduated cylinders or 50 mL pipettes
2. 100 mL borosilicate glass containers (beaker)
3. laboratory glassware for chemical preparations
4. 1 mL automatic dispenser
5. 1000 mL and 500 mL volumetric flasks
6. volumetric flasks of 100 mL class A
7. 1 L class A volumetric flask
8. precision micropipettes to measure volumes in the range of 10-100 µL
9. analytical scale
10. stove
11. microwave oven
12. dryer

[16] Berthelot, M.P. (1859) Repertoire de Chemie Appliquée, pp. 284.
[17] Ivancic I., Degobbis D. (1984) An optimal manual procedure for ammonia analysis in natural waters by indophenol blue method. Water Res., 18, 1143-1147.
[18] Sasaki K., Sawada Y. (1980) Determination of ammonia in estuary. Bull. Jap. Soc. Sci. Fish., 46, 319-321.
[19] Koroleff F. (1983) Determination of ammonia. In: "Methods of seawater analysis", Grasshoff K., M. Ehrhardt, K. Kremfing Eds, Verlag Chemie, Weinheirn, 150-175.
[20] Mantoura R.F.C., Woodward E.M.S. (1983) Optimization of the indophenol blue method for the automated determination of ammonia in estuarine water. Eustar. Coast. Shelf, Sci., 17, 219-229.
[21] Grasshoff K., Johannsen H. (1972) A new automatic and direct method for the automatic determination of ammonia in sea water. J. Cons. Int. Explor. Mer, 34, 516-521.
[22] Hansen H.P., Koroleff, F. (1999) Determination of nutrients. In Methods of Seawater Analysis. K. Grasshoff, K. Kremling and M. Ehrhardt (eds) 3rd Edition Wiley-VCH Weinheim pp159-228.

13. spectrophotometer or colorimeter sensitive to 543 nm equipped with cells of at least 50 mm optical path, preferable 100 mm

b. Chemical products:

1. The chemical products for manual colorimetric determination of concentration of nitrate include:sulfocromic mixture
2. concentrated hydrochloric acid [HCl]
3. Sodium hydroxide [NaOH]
4. Potassium persulfate [$K_2S_2O_8$]
5. phenol [C_6H_5OH]
6. disodium EDTA [$C_{10}H_{14}N_2Na_2O_8$]
7. sodium dichloroisocyanuric acid [$C_3HCl_2N_3NaO_3$]
8. sodium nitroprusside dihydrate [$Na_2Fe(CN)_5NO{\cdot}2H_2O$]
9. trisodium citrate dihydrate [$C_6H_5Na_3O_7{\cdot}2H_2O$]
10. ammonium chloride [NH_4CI]
11. ammonium hydroxide [NH_4OH]
12. chloroform [$CHCl_3$]

c. Preparation of stock solutions:

"Ammonia-free" water

120. There is no standard procedure for the preparation of water with very low ammonia content. De-ionized water may sometimes be used without subsequent distillation, but it must be noticed that ion exchange resins potentially bleed out organic substances and ammonia. In case the ammonia blank concentrations are higher than 0.3 µmol L^{-1}., the water should be subjected to subsequent distillation. In this second step, 0.3 g NaOH and 1 g $K_2S_2O_8$ are added to 1000 mL of water (in a 2 L flask). The solution should be boiled for 10 minutes to remove ammonia (without the condenser) and then distilled until a residue of about 150 mL. The distilled water should be stored in a tightly sealed container, preferably made of glass. The method of preparation of ammonia-free water should be regularly checked and appropriate blanks must be analysed with every batch of samples. As an alternative, "open sea surface water" can be used as "ammonia-free" water.

Buffer solution

121. 240 g trisodium citrate dihydrate ($C_6H_5Na_3O_7{\cdot}2H_2O$), 20 g of disodium EDTA ($C_{10}H_{14}N_2Na_2O_8$) and 0.4 g NaOH in about 600 ml distilled water are dissolved. The solution is boiled (to remove ammonia) until the volume is below 500 mL. It is then cooled and diluted to 500 mL with "ammonia-free" water. The solution is stable and should be stored in a well-stoppered polyethylene bottle.

Phenol reagent

122. 80 g colourless phenol (C_6H_5OH) is dissolved in 300 mL of ethanol, added 600 mL of distilled water and 600 mg of sodium nitroprusside dihydrate ($Na_2Fe(CN)_5NO{\cdot}2H_2O$) in "ammonia-free" water and diluted to 1000 ml. When stored in a tightly closed dark bottle and in a refrigerator, the solution should be stable for several months. **Phenol is a particularly toxic compound and safety glasses and gloves should be worn and all handling conducted in a fume cupboard.**

Hypochlorite reagent

123. 1 g of sodium dichloroisocyanuric acid ($C_3HCl_2N_3NaO_3$; dichloro-s-triazine-2, 3, 6 (1H, 3H, 5H)-trione) and 8 g NaOH in "ammonia-free" water are dissolved and diluted to 500 mL. The sodium dichloroisocyanuric acid is employed as a hypochlorite donor (in comparison to generally used commercial hypochlorite solutions) has the advantage of being a stable solid, and that it is easy to prepare. The solution should be stored in a dark bottle in a refrigerator and is stable for at least a week.

Ammonia stock solution (A) (10 mmol L^{-1} NH3)

124. Ammonium chloride (NH_4Cl) is dried at 100 ºC to constant weight. Then dissolve 0.0535 g NH4Cl in "ammonia-free" water and dilute to 100 mL in a volumetric flask. When kept in a glass bottle (protected from sunlight) and in a refrigerator, the solution should be stable for at least several weeks.

Ammonia working solution (B) (100 µmol L^{-1} NH3)

125. Exactly 10.0 ml of the stock solution is diluted with "ammonia-free" water to a final volume of 1000 ml in a volumetric flask made of glass.

d. Preparation of specific equipment for analysis:

d.1. Treatment of reaction vessels

126. All flasks and tubes to be used should be cleaned with hot HCl, rinsed well with "ammonia-free" water and kept closed between analyses. The analysis should be performed in a well-ventilated room with no ammoniacal solutions stored (**Note:** this should include any cleaning agents containing ammonia and used by laboratory cleaning staff during or outside normal working hours). This includes the NH_4Cl reagent used for nitrate analysis. **Smoking should be forbidden.**

127. Alternative: Before use, all flasks should be treated by performing the reaction in them with the addition of chemical reagents to the "ammonia-free water" or "open sea surface water". The reaction should proceed at least for 6 hours and the flasks should be shaken time to time during the reaction period. Later, the flask should be rinsed with ammonia-free water and kept stoppered when not in use. The flasks should not be washed between the analysis of different sets of samples/standards, but just rinsed with "ammonia-free" water and kept closed.

e. *Analytical procedure:*

e.1. Reagents to be prepared at the time of use

Preparation of standard solutions

128. 7 standards of known ammonia concentration are prepared: by diluting, in 100 mL flasks (class A), respectively 0.5, 1, 2, 3, 5, 7 and 10 mL of Ammonia working solution (B) (measured with a precision pipette) with "ammonia-free" water or "open sea surface water" and filled to the 100 mL mark. The concentrations of ammonia are thus between 0.5 and 10 µmol L^{-1}. In this instance, it is probably best not to use low nutrient seawater unless it is known to have a suitably low ammonia concentration.

e.2.Analytical treatment

129. At the time of analysis, if the sample had been frozen, possibly using a 37 °C bath or a microwave oven is quickly thawed.

130. The flasks with an aliquot of samples or standard solutions of different concentrations are pre-rinsed.

131. The flasks with 50 mL of sample or each of the standard (measured with a graduated cylinder) are filled.

132. 2 ml phenol reagent, 1 ml buffer solution and 2 ml hypochlorite reagent are added. The solution is mixed by swirling between the additions. The reaction bottles are closed properly and kept in a dark place during the reaction time which is at least 6 hours at room temperature, but which is reduced to 30 minutes if the samples are incubated in a water bath at 37 ºC ± 1 ºC. Note that standards and samples of the same series must be treated simultaneously, and in the same way.

e.3. Preparation of reagent blanks

133. One 100 mL flask is filled with 50 mL and one with 47,5 mL of distilled water or "open sea surface water"

134. To the first 2 ml phenol reagent, 1 ml buffer solution and 2 ml hypochlorite reagent are added and to the other 3 ml phenol reagent, 1.5 ml buffer solution and 3 ml hypochlorite reagent. The solutions are mixed by swirling between the additions. The reaction bottles are closed properly and kept in a dark place during the reaction time as for samples and standards.

e.4. Spectrophotometric measurements

135. The absorbance of the blank ($bl_{c,\,i}$) of each cell of the spectrophotometer or colorimeter, used for reading against the reference cell, at 630 nm is measured, both filled with water without regents. The operation is superfluous if only one cell is used.

136. For each flask, the number of the cell used, the identification of the contents of the flask (sample, standard, blank) are noted in a form. The cell is rinsed with part of its contents, filled and the absorbance at 630 nm read, recording the reading on the same form.

f. *Calculations:*

137. The reagent blank (bl) as the average difference between the values of the two blanks is calculated.

138. The correlation between the absorbance values of the 7 standards and the assumed concentrations, using the Ordinary Least-Squares Regression is calculated. The colorimetric factor (f) is represented by the slope, covering a concentration range 0.5 to 10 µmol L^{-1}.

139. The concentration of ammonia in the samples is calculated with the following equation:

$$c(NH_4) / \mu mol\ L^{-1} = (ABS - bl - bl_{c,\,i})\ f$$

where

$c(NH_4)$ = concentration of ammonia

ABS = absorbance of the sample

bl = blank of the reagents

$bl_{c,\,i}$ = blank of the i-th cell used

f = colorimetric factor

140. As already mentioned, for any given concentration of ammonium the blue color produced in seawater is less intensive than in distilled water. Thus, for each sample a correction must be made with respect to its salinity and the resulting pH. In many circumstances a simple correction (Hansen and Koroleff , 1999) may be used where the correction is given by:

$$c(NH_4)_{corr} / \mu mol\ L^{-1} = [1 + 0.0073\ S_s]\ c(NH_4)_{uncorr}$$

where

S_s = salinity of the sample.

g. Important notes:

141. The method is very sensitive to the effects of a possible contamination of the glassware or reagents; therefore, it is recommended to strictly follow the instructions given for washing the glassware and to use the recommended chemical products.

142. It is essential to ensure that the work environment is smoke-free and that there are no reactants in the vicinity that can release ammonia.

h. Possible problems:

143. Interferences from amino acids and urea (at seawater levels) can be neglected but may be significant in estuarine or brackish waters, especially where these are contaminated with urban waste.

144. Hydrogen sulphide can be tolerated up to about 60 µmol L^{-1}. Samples with higher H_2S concentrations should be diluted.

145. The blue colour of the indophenol, however, is influenced by salinity, which must be compensated by the application of a salt factor (see above).

4.2. Protocol for automated colorimetric determination of concentration of ammonium

a. Reagents

Buffer

146. The buffer is composed of 120 g of trisodiocitrate, dissolved in 500 mL DDW, and adjusted to 1L. Sodium hydroxide must be added to this solution in a percentage such as to bring the pH of the solution to a value of 11. This reagent must be stored in a glass bottle and is very stable.

Phenol reagent

147. 35 g of phenol and 0.40 g of sodium dichloroisocyanuric acid are dissolve in 800 mL of DDW and adjusted to 1000 mL. This reagent is stable for 24 hours.

Hypochlorite reagent

148. 5 g of sodium hydroxide and 1 g of dichloroisocyanurate are dissolved in 400 mL of DDW and adjusted to 500 mL. This reagent must be stored in a glass bottle at a temperature of + 4 °C and is stable for a week.

b. Standards

149. About 2 g of ammonium chloride is dried in an oven at a temperature of 100 °C to constant weight and then placed in a silica gel dryer for another 24 hours. The ammonium chloride is dissolved in DDW in such a proportion as to obtain a concentration of 2 mmol L^{-1}. This standard is used in the daily procedure for the preparation of 5 lower concentration standards.

150. The concentration of the minor standards is chosen based on the amount of NH_4^+ salts that are expected to be found, so that the set of sub-standards covers the entire range of expected concentrations. From the 5 standards a multiplication factor is obtained which is necessary to calculate the concentrations.

c. Manifold

151. The manifold (Fig. 4) is built of three injectors, three coils of 10 turns each, a thermostatic bath and a trap containing 10% hydrochloric acid. The first injector (A) is equipped with 3 inputs: the first is for the sample, the second for air bubbles, by which the liquid is divided into many equal segments and with the third input the first reagent is introduced. Immediately after, there are 2 coils made up of 10 coils each: in the first the liquid is mixed with the buffer; in the second at point (B) the second reagent is injected. At point (C) the third reagent is injected. To accelerate the blue production of indophenol, the solution is passed through a coil immersed in a thermostated bath (D) at a temperature of 75 °C. At the exit of the bath, at point (E) the solution is cooled passing through the last coil. The air for producing the air bubbles is introduced into the circuit through a trap (F) containing 10% HCl. This measure must be adopted to ensure that any ammonia vapours contained in the laboratory air are eliminated

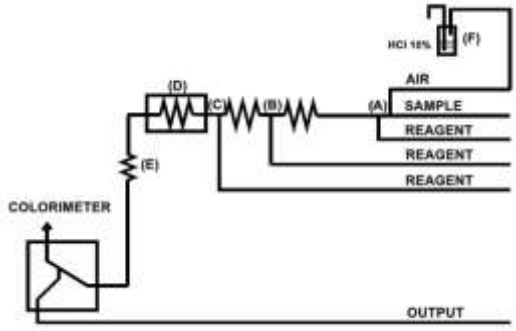


Figure 4. Manifold for ammonium measurement.

a. <u>Important notes</u>

152. The base line must be stable and if any fluctuations as if even small variations are noted it would mean that flocculate has formed in the sample caused by the phenol that is no longer stable:

153. The reagents one at a time, in strict order from the first to the third mast be inserted;

154. The circuit must be washed with the progressive elimination of the reagents from the third to the first;

155. If precipitate is observed to form near the hypochlorite injector the circuit is probably dirty or the buffer inefficient;

156. If an unstable baseline is observed when the appliance is turned on in the absence of reagents, the circuit with 10% HCl must be washed;

157. If during the analysis an evident increase in the baseline is observed, the colorimeter reading cell by injecting 50% hydrochloric acid directly into the cell without stopping the circuit must be immediately cleaned;

158. The DDW is deionized if possible, directly in the water container of the instrument;

159. If the ambient temperature is higher than + 20 °C a heat sink to the cooling coil must be installed;

160. If a change of components of the circuit (injectors, bubblers) is necessary, the circuit by changing the flow rates of the pipes must be rebalanced;

161. Suitable containers for the different reagents must be used. The cap of the container must be provided with small holes in which to insert capillaries (needles, etc.) for the withdrawal of the reagent;

162. When mixed standards are used, NO_3^- standards with either NH_4^+ or NO_2^- standards never must be combined;

163. Water poor in nutrients, or oligotrophic water (OSW), as washing water between one sample and another must be used. OSW with salinity values similar to the sample to be analysed must be used.

a. <u>Symbol, units and precision</u>

164. For the parameter described in this protocol, the symbol and unit suggested by the International System of Units (SI), as well as the expected accuracy, along with the Method identifiers as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: $c(NH_4^+)$	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.03
Method identifier:	SDN:P01::**AMONMATX**	Concentration of ammonium {NH4+ CAS 14798-03-9} per unit volume of the water body [dissolved plus reactive particulate phase] by manual colorimetric analysis
	SDN:P01::**AMONAADZ**	Concentration of ammonium {NH4+ CAS 14798-03-9} per unit volume of the water body [dissolved plus reactive particulate <unknown phase] by filtration and colorimetric autoanalysis

Annex I

References

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