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Integrated Meetings of the Ecosystem Approach Correspondence Groups on IMAP Implementation (CORMONs)

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Agenda item 5: Parallel CORMON Sessions for Pollution, including Marine Litter and Biodiversity

Monitoring Guidelines/Protocols for Determination of Concentration of Key Nutrients in Seawater – Phosphorous and Silica Compounds

UNEP/MAP
Athens, 2020

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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 – Phosphorous and Silica Compounds provide the seven protocols gathered under tree Technical Notes for determination of concentration nitrite, nitrate and ammonium in seawater, as follows: a) Technical note for the determination of concentration of orthophosphate which includes: Protocol for manual colorimetric determination of concentration of orthophosphate; Protocol for automated colorimetric determination of concentration of orthophosphate; b)Technical note for the determination of concentration of orthosilicate which includes: Protocol for manual colorimetric determination of concentration of orthosilicate; Protocol for automated colorimetric determination of concentration of orthosilicate; and c) Technical note for a combined determination of concentration of total nitrogen and total phosphorus which includes: Protocol for preparation of samples for a combined determination of concentration of total nitrogen and total phosphorus; Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous; Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous, for consideration of Integrated Meeting of the Ecosystem Approach Correspondence Groups on Monitoring (CORMON) Biodiversity and Fisheries, Pollution and Marine Litter, and Coast and Hydrography.

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

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
TPX	Polymethylpentene
UNESCO	United Nation Educational Scientific and Cultural Organization
WOCE	World Ocean Circulation Experiment

1. Introduction

1. In the Monitoring Guidelines for Key nutrients – Phosphorous and Silica compounds in Seawater, the protocols for manual and automated determination of the concentration of orthophosphate, orthosilicate, and total phosphorous and total nitrogen 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 – Phosphorous and Silica Compounds provides 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 build 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 off-shore 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.

The below flow diagram informs on the category of this Monitoring Guidelines related to determination of chlorophyll *a* in seawater within the structure of all Monitoring guidelines prepared for IMAP Common Indicators 13, 14, 17, 18 and 20.

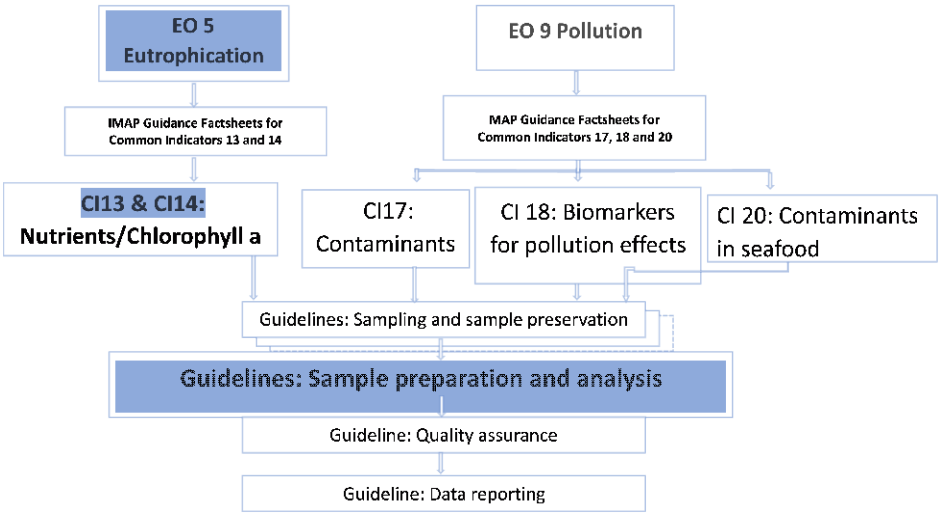
a. *Continuous flow methods*

5. 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.

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

[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.

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 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 the determination of concentration of orthophosphate

6. The method is based on the formation of a blue phosphomolybdic complex (from the molybdenum blue group) whose concentration is measured by colorimetry (spectrophotometer or colorimeter) (Deniges, 1920)[7]. The aspects relevant to the development of the phosphomolybdic complex are summarized as follow: The molybdate ion and its polymers form, in an acid environment, stable heteropoly acids with elements of the IV and V groups (Boltz and Mellon 1947[8]). Phosphomolybdic acid is a yellow complex. The reduction of molybdate from Mo (VI) to Mo (V) in this complex produces a blue coloured heteropoly acid. The maximum absorbance peak varies according to the type of reducing agent used, probably in relation to the variation of the ratio between Mo (VI) and Mo (V) as a whole and to the type of aggregation of the basic units in the solution.

7. Murphy and Riley (1962)[9] introduced, in the procedure for the determination of phosphates in seawater, the use of a trivalent antimony salt, which enters the heteropoly acid in a ratio of about 1:1 with phosphorus. This modification induces a shift of the maximum absorbance towards the infrared, with an increase in the molar extinction coefficient and a drastic increase in the rate of formation. The subsequent reduction occurs by ascorbic acid, thus eliminating dependencies on ionic strength (saline effect) and on temperature (Murphy and Riley, 1958[10], 1962). To minimize the interference of other ions that react in a similar way with molybdates, it is necessary to keep the pH of the final solution below 1, a condition in which the formation of hetero-polyacids with Si and As is decidedly disadvantaged (Koroleff , 1983)[11].

8. The methodology of Murphy and Riley (1962) as reported by Strickland and Parsons (1968) is described in this note.

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

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[6] Grasshoff, K., Kremling, K., Ehrhardt, M. (eds), 1999. Methods of Seawater Analysis 3rd Edition Wiley-VCH Weinheim, 634 pp.
[7] Deniges M.G. (1920) Reaction de coloration extrêmement sensible des phosphate et des arseniates. Ses applications. C. R. Acad. Sci., Paris, 171, 802-804.
[8] Boltz D.F., Mellon M.G. (1947) Determination of phosphorus, germanium, silicon, and arsenic by the heteropolyblue method. Ind. Eng. Chem. Anal. Ed., 19, 873-877.
[9] Murphy J., Riley J.P. (1962) A modified single solution method for the determination of phosphate in natural waters. Analytica Chim. Acta, 27, 31-36.
[10] Murphy J., Riley J.P. (1958) A single-solution method for the determination of soluble phosphate in sea water. J. Mar. Biol. Ass. U.K., 37, 9-14.
[11] Koroleff F. (1983) Determination of phosphorus. In: “Methods of Seawater Analysis”, Grasshoff K., M. Ehrhardt, K. Kremling Eds, Verlag Chemie, Weinheim, 125-139.

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

2.1. Protocol for manual colorimetric determination of concentration of orthophosphate

a. Equipment:

10. The equipment for manual colorimetric determination of concentration of orthophosphate include:

1. graduated cylinders or 50 mL pipettes
2. 100 mL borosilicate glass containers (preferably flasks with cap)
3. laboratory glassware for chemical preparations
4. 5 mL automatic dispenser
5. 50, 250 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 880 nm (as a fullback 705 nm) equipped with cells of at least 50 mm optical path

b. Chemical products:

11. The chemical products for manual colorimetric determination of concentration of orthophosphate include:

1. sulfocromic mixture
2. concentrated sulfuric acid [H_2SO_4]
3. ammonium heptamolybdate tetrahydrate [$(NH_4)\ 6Mo_7O_{24}\cdot 4H_2O$]
4. potassium antimony tartrate [$K(SbO)C_6H_4O_6$]
5. ascorbic acid [$C_6H_8O_6$]
6. potassium dihydrogen phosphate [KH_2PO_4]
7. chloroform [$CHCl_3$]

c. Preparation of stock solutions

5 N sulfuric acid

12. 140 mL of concentrated sulfuric acid are poured slowly into a beaker containing about 800 mL of reagent grade water. Allow to cool and the volume is adjusted to 1 L. The solution, stored in a dark glass bottle, is stable indefinitely.

Molybdate ammonium solution

13. 15 g of crystalline ammonium heptamolibdate tetrahydrate are dissolved in 450 mL of reagent grade water in a 500 mL flask and adjusted to volume. The solution, stored in a plastic or borosilicated glass bottle, away from direct light, is usable until a white precipitate is formed.

Solution of potassium antimony tartrate

14. 0.34 g of potassium antimony tartrate are dissolved in 250 mL of reagent grade water in a 250 mL flask. The solution, stored in a glass or plastic bottle, is stable for many months, unless a white flocculate is formed.

Standard solution of potassium dihydrogen phosphate 2 mmol L^{-1}

15. Few grams of potassium dihydrogen phosphate in an oven at 110 °C are dried. 272.18 mg on an analytical balance are weighted and in 900 mL of reagent grade water in a 1 L (class A) flask dissolved. Up to volume is adjusted and a few drops of chloroform as a preservative added. The solution, stored in a borosilicate glass bottle, is stable for a few months.

d. Preparation of specific equipment for analysis

d.1. Treatment of reaction vessels

16. The reaction flasks with boiling sulfochromic mixture are periodically washed. Keep them tightly capped, filled with reagent grade water and mixed reagent (if necessary, the residue of the analysed sample can be left in the flask).

e. Analytical procedure

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

Ascorbic acid solution

17. 2.7 g of ascorbic acid is dissolved in 45 mL of reagent grade water in a 50 mL flask and to volume adjusted. The solution, stored in a plastic or glass bottle, is stable for 24 hours.

Mixed reagent

18. In a glass container are mixed: 100 mL of ammonium molybdate solution, 250 mL of 5 N sulfuric acid, 100 mL of ascorbic acid solution and 50 mL of potassium antimony tartrate solution. The solution is sufficient for about 100 samples but deteriorates within a few hours, and must be replaced when its color changes from light yellow to very dark yellow.

Preparation of standard solutions

19. 5 standards of known phosphate concentration are prepared: by diluting, in 100 mL flasks (class A), respectively 10, 25, 50, 75, 100 µL of standard solution of potassium dihydrogen phosphate (measured with a precision pipette) with oligotrophic seawater. The concentrations of phosphate are thus between 0.2 and 2 µmol L^{-1} plus the orthophosphate content of oligotrophic seawater.

e.2. Analytical treatment

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

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

22. The flasks with 50 mL of sample or each of the standard solutions (measured with a graduated cylinder) are filled. Given the remarkably low concentrations of phosphates and the relative sensitivity of the analytical method, it is advisable to carry out at least two determinations for each sample to be analysed.

23. 5 mL of mixed reagent to each sample or standard solution with a dispenser are added and shaken.

24. For the reaction to take place is necessary at least 5 minutes and no more than 2 hours.

e.3. Preparation of reagent blanks

25. Four 100 mL flasks are filled with 50 mL of oligotrophic seawater, low in phosphates, after rinsed with the same water.

26. 5 mL of mixed reagent are added to two flasks and double the amount in the other two.

27. The time the reaction to take place is necessary as for samples and standard solutions.

e.4. Spectrophotometric measurements

28. The absorbance of the blank ($bl_{c,\,i}$) of each cell of the spectrophotometer or colorimeter, used for reading against the reference cell, at 882 nm is measured, both filled with water without regents. The operation is superfluous if only one cell is used.

29. 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 882 nm read, recording the reading on the same form. Alternatively, with a loss of sensitivity of about 30%, the absorbance can be read at 705 nm.

f. Calculations:

30. The reagent blank (bl) as the average difference between the values of the blanks containing 10 mL and those containing 5 mL of mixed reagent is calculated.

31. 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.

32. A standard with zero concentration of orthophosphates represented by the sample of oligotrophic seawater to which a single dose of mixed reagent has been added is considered. In this way, a total of 6 standards are obtained, covering a concentration range of 2.0 µmol L^{-1}.

33. The concentration of orthophosphate in the samples is calculated with the following equation:

$$c(PO_4) / \mu mol\ L^{-1} = (ABS - bl - bl_{c,\,i})\ f$$

where

$c(PO_4)$ = 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

34. For a cell with a 50 mm optical path, the colorimetric factor is equal to about 9.9 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 0.1.

g. Important notes and possible problems:

35. The cells of the spectrophotometer (or colorimeter) should be washed periodically with a 5% solution of soda or hydrofluoric acid because the phosphomolybdic complex tends to stick to the walls, giving them a slight blue colour.

36. The samples should not be lived in plastic containers at room temperature for a long time. Both due to the bacterial activity that develops on the walls of the bottle and to adsorption phenomena the concentration of phosphates tends to decrease.

37. After thawing the samples, the analysis must be complete in a short time to avoid phenomena of hydrolysis of organic phosphates or polyphosphates.

38. If the standard solution has been stored in the refrigerator, it must be brought to laboratory temperature before starting the standardization procedure.

39. Spectrophotometer measurement mast be performed within two hours of adding the reagent to avoid the slow formation of silicomolybdic heteropoly acids.

40. Sulphides can interfere with the reaction, if present in concentrations higher than 50 µmol L^{-1} of S^{2-}, as the extinction coefficient and the maximum absorbance are altered (De Jonge and Villerius, 1980)[12]. In this case the sulphides from the sample should be removed (Airey et al., 1984)[13].

41. Silicates interfere if present at concentrations higher than 150 µmol L^{-1} as a complex that absorbs in the same band is developed (Koroleff, 1983).

42. The reagent blank, if prepared using distilled water, may have a higher optical density than the samples to be analysed. This can derive from different causes, it is advised the procedure indicated in the paragraph "Preparation of reagent blanks" to strictly be followed or the method suggested by Novoselov et al. (1976)[14] to be applied.

2.2. Protocol for automated colorimetric determination of concentration of orthophosphate

a. Reagents:

Ammonium molybdate

43. 10 g of molybdate are dissolved in 800 mL of DDW. The solution is stable for at least one month.

Antimony potassium tartrate (KAT)

44. 2.5 g of KAT is dissolved in 800 mL of DDW and adjusted to 1 L. The solution is stored in a glass bottle and is stable for at least one month.

b. Solutions for use

Mixed reagent

45. In a 250 mL graduated glass cylinder and shaking after each addition are mixed: 100 mL of molybdate stock + 25 mL of KAT + 30 mL of H_2SO_4 conc. + 1 mL of SLS (Sodium-Laurel-Sulphate) and adjusted to 250 ml. The reagent is very stable and should be stored in a glass bottle.

[12] De Jonge V.N., Villerius L.A. (1980) Interference of sulphide in inorganic phosphate determination in natural waters. Mar. Chem., 9, 191-197.

[13] 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.

[14] Novoselov A.A., Sheremet'Yeva A.I., Danilenko A.F. (1976) Method for simultaneous obtaining silicon-free and phosphate-free sea water aboard ship. Oceanology, 16, 358-359.

Ascorbic acid

46. 1.8 g of ascorbic acid is dissolved in 100 mL of DDW.

c. Standards:

47. About 2 g of KH_2PO_4 are dried in an oven at a temperature of 110 °C, checking for constant weight of the salt over time. The salts are then placed in a silica gel dryer for another 24 hours. It is then dissolved in reagent grade water in such a proportion to obtain a concentration of 2 mmol L^{-1}.

48. This standard is used in the daily procedure for the preparation of 5 lower concentration standards. The concentration of the minor standards is chosen based on the amount of PO_4^{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.

d. Manifold

49. 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 3 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.

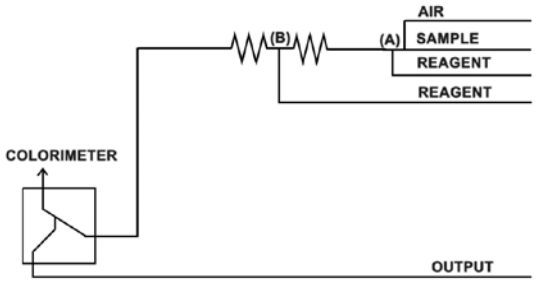

Figure 1. Manifold for orthophosphate measurement.

e. Calculations

50. The calculations are performed as is generally indicated in the Annex I: Automated methods for determination of concentration of key nutrients in seawater – Calculation of the concentration.

f. Important notes and possible problems

51. If an unstable base line occurs when the appliance is turned on in the absence of reagents, wash the circuit with NaOH and then with 10% HCl.

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

53. DDW is deionized if possible, in the water container of the instrument.

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

55. 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.

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

57. At temperatures below 10 °C a thermostated bath at a temperature of 40 °C must be added to the manifold.

58. If precipitate forms in the molybdate the reagent must be discarded.

59. In case of preparation of mixed standards of PO_4 and SiO_4 the standards must never be prepared in the same flask.

60. A colorimeter with a very narrow entrance of the reading cell to avoid refractive disturbances must be used.

61. High sensitivity reading phototubes for 880 nm readings must be used.

a. Symbol, units and precision

Symbol: $c(PO_4^{3-})$	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.03
Method identifier:	SDN:P01::**PHOSMAZX**	Concentration of phosphate {PO43- CAS 14265-44-2} per unit volume of the water body [unknown phase] by manual colorimetric analysis
	SDN:P01::**PHOSAAZX**	Concentration of phosphate {PO43- CAS 14265-44-2} per unit volume of the water body [unknown phase] by colorimetric autoanalysis

3. Technical note for the determination of concentration of orthosilicate

62. The determination of the dissolved silicates is carried out by inducing the formation of a silicomolibdic polyacid which is subsequently reduced to molybdenum blue. The final compound has a maximum absorbance at 810 nm, and is measured by colorimetry.

63. The chain of reactions is strongly influenced by even minimal variations in the reaction conditions due to the multiplicity of intermediate products and their instability. Silicomolybdic acid is formed with different speed in relation to the degree of polymerization of the silicate.

64. Silicomolybdic acid exists in at least two isomers α and β (Strickland, 1952[15]; Morrison and Wilson, 1963[16]; Truesdale and Smith, 1975[17]), of which the former is thermodynamically more stable but kinetically disadvantaged at pH values below 2. The two isomers α and β of silicomolybdic acid have a peak of maximum absorbance in the blue part of the spectrum, but with quite different extinction coefficients, none of which are particularly high. Furthermore, for the reasons mentioned above, they do not guarantee sufficient stability over time. The subsequent reduction of isomer β by p-methylaminophenol (metol) sulphate in an acid environment and in the presence of sulphite produces a stable molybdenum blue for at least 2 hours from the completion of the reaction (Mullin and Riley, 1955)[18]. Also in this process it is important to control the pH to avoid a direct reduction of excess molybdenum by metol.

65. All the reactions outlined above depend both on the ionic strength of the solution and on the presence of specific ions, especially the divalent ones; therefore, the concentration of the final product and perhaps also its molar extinction depend on the salt concentration of the reaction mixture and, consequently, of the sample. The formation of polyacids with molybdate, in fact, is also characteristic of other ions, in particular phosphate and arsenate (Boltz and Mellon, 1947); to avoid the interference of the phosphomolybdates, these can be eliminated with oxalic acid (Strickland and Parsons, 1968).

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

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

3.1. Protocol for manual colorimetric determination of concentration of orthosilicate

a. Equipment

67. The equipment for manual colorimetric determination of concentration of orthosilicate include:

[15] Strickland J.D.H. (1952) The preparation and properties of silicomolybdic acid. II. The preparation and properties of alpha silicomolybdic acid. J. Amer. Chem. Soc., 74, 868-871.
[16] Morrison I.R., Wilson A.L. (1963) The absorptiometric determination of silicon in water. Part I. Formation, stability and reduction of [β- and α-molybdosilicic acids. Analyst, 88, 88-99.
[17] Truesdale V.W., Smith C.J. (1975) The formation of molybdosilicic acids from mixed solutions of molybdate and silicate. Analyst, 100, 203-212.
[18] Mullin J.B., Riley J.P. (1955) The colorimetric determination of silicate with special reference to sea and natural waters. Analytica Chim. Acta, 12, 162-176.

1. 25 mL cylinder or pipette, preferably in plastic
2. 50 mL plastic containers (preferably flasks or bottles with polyethylene or polymethylpentene cap)
3. laboratory glassware for chemical preparations
4. automatic dispensers or pipettes of 10 and 15 mL
5. Whatman paper filters n. 1
6. 500 mL volumetric flasks
7. volumetric flasks of 100 mL class A
8. 1 L class A volumetric flask
9. precision micropipettes to measure volumes in the range of 10 - 100 µL
10. spectrophotometer or colorimeter sensitive to 810 nm, which has cells of at least 50 mm optical path
11. platinum crucible
12. agitator
13. analytical scale
14. stove
15. dryer

b. Chemical products

68. The chemical products for manual colorimetric determination of concentration of orthosilicate include:

1. sulfocromic mixture
2. concentrated sulfuric acid [H_2SO_4]
3. concentrated hydrochloric acid [HCl]
4. ammonium heptamolybdate tetrahydrate [$(NH_4)_6Mo_7O_{24}\cdot 4H_2O$]
5. oxalic acid [$C_2H_2O_4\cdot 2H_2O$]
6. 4-methylaminophenol sulfate (metol) [$(CH_3NHC_6H_4OH)\cdot 2H_2SO_4$]
7. anhydrous sodium sulphite [$Na2SO_3$]
8. powdered silica [SiO_2] and anhydrous sodium carbonate [Na_2CO_3] (alternatively, sodium hexafluorosilicate [Na_2SiF_6])

c. Preparation of stock solutions

Molybdate reagent

69. 4.0 g of ammonium heptamolybdate tetrahydrate (preferably crystalline) are dissolved in about 300 mL of reagent grade water. 12 mL of concentrated hydrochloric acid are diluted in 100 -150 mL of reagent grade water and mixed well. While stirring, the molybdate solution is added in that of hydrochloric acid and adjusted to 500 mL with reagent grade water. The solution, stored in a polyethylene bottle, away from direct light, is usable until a white precipitate is formed or turned blue.

Solution of metol and sulphite

70. 6 g of anhydrous sodium sulphite are dissolved in 400 mL of reagent grade water and 10 g of metol added, while stirring until it is completely dissolved. The solution is filtered, on a Whatman No. 1 filter, previously rinsed with reagent grade water, and and adjusted to 500 mL. The solution is stored in a tightly closed borosilicate glass bottle and therefore should not be stored for more than a month.

Oxalic acid solution

71. A saturated solution of oxalic acid by dissolving 50 g of acid in 400 mL of reagent grade water is prepared. The solution is decanted separating it from the residual crystals and adjusted to the volume of 500 mL. The solution is stored in a polyethylene bottle and stable indefinitely.

50% (v / v) sulfuric acid solution

72. 250 mL of concentrated sulfuric acid are poured into 250 mL of reagent grade water while stirring. Cool to room temperature and make up to volume with reagent grade water in a 500 cm3 mat. The solution, stored in a container of dark plastic, is stable indefinitely.

Standard solution of silicate (10 mmol L^{-1})

73. The pure silica is heated to 1000 °C, cooled in a desiccator and checked for constant weight with repeated weighing. 601.0 mg of silica (the theoretical amount corresponding to 10 mmol of Si) are weighted in a platinum crucible and 1.5 g of anhydrous sodium carbonate added. Everything is mixed with a metal spatula and melted, until completely homogenized, at a temperature of 1000 °C. The melted product is kept at 1000 °C until clear. Then is cooled and in several portions of very hot water dissolved and transferred after cooling to a 1 L flask (class A). Adjusted to volume with reagent grade water and quickly transferred to a high-density polyethylene bottle. The solution is stable for a few months.

74. Alternatively, sodium hexafluorosilicate can be used. It must be dried in an oven at 105 °C for one hour in a metallic melting pot. In this case, given the low solubility, it is preferable to prepare solutions with concentrations not exceeding 2 mmol L^{-1}, therefore the dilutions must be corrected proportionally. Since the product is not yet supplied in analytical purity, the quantity to be weighed must be calculated based on the purity indications of the supplier. The sodium hexafluorosilicate is dissolved in 700 mL of reagent grade water in a

plastic container, under gentle heating, and the solution into a 1 L (class A) flask transferred. The dissolution time is correlated to the crystalline form of the product and few hours may be necessary. The volume is adjusted to 1 L and quickly transferred to a plastic bottle to prevent the fluoride from removing silicon from the glass. The solution is stable for few months.

d. Preparation of specific equipment for analysis:

d.1. Treatment of reaction vessels

75. Wash the 50 cm3 polyethylene or polymethylpentene containers with sulphochromic mixture, rinse them thoroughly with reagent grade water and dry them. For routine maintenance it is sufficient, after use, to rinse them with reagent grade water and place them upside down on filter paper.

e. Analytical procedure:

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

Reducing reagent

76. 100 mL of metol and sulphite solution and 60 mL of oxalic acid solution are mixed. Slowly 60 mL of 50% sulfuric acid are added and adjusted to 300 mL in a cylinder with reagent grade water. This reagent must be prepared immediately before use.

e.2. Preparation of standard solutions

77. 5 solutions of known concentration of silicate by diluting, in 100 mL flasks (class A), respectively 10, 25, 50, 75, 100 µL of standard silicate solution (measured with a precision micropipette) with oligotrophic sea water are prepared, resulting in concentrations between 1 and 10 µmol L^{-1} of silicate, plus the silicate content of the oligotrophic water.

e.3. Analytical treatment

78. At the time of analysis, the sample, if had been frozen, is thawed slowly keeping it away from light. The analysis must be performed after 12 hours, to allow the polymeric forms of silicates to depolymerize.

79. 10 mL of molybdic reagent is poured (using a dispenser) into the container and while stirring 25 mL of sample or of each of the standard solutions (measured with a graduated cylinder) added.

80. Respecting the same times for all samples and calibration standards the reaction is allowed to take place for at least 15 minutes but not more than 30 minutes.

81. Using a dispenser, 15 mL of reducing reagent are added and allowed the reaction to take place for at least 1 hour. For the whole group of samples the same reaction times must be respected.

e.4. Preparation of reagent blanks

82. At least two replicates of reagent blanks in 50 mL polyethylene containers, using 25 mL of oligotrophic seawater must be prepared and treated with the same analytical procedure applied to the samples and standards.

83. In some cases, due to a high concentration of silicates in the oligotrophic sea water too high blank value may be observed. In that case it is advisable to remove them (Novoselov et al., 1976) or the blanks must be prepared with reagent grade water.

e.5. Spectrophotometric measurement

84. The absorbance of the blank ($bl_{c,\,i}$) of each cell of the spectrophotometer or colorimeter, used for reading against the reference cell, at 810 nm is measured, both filled with water without regents. The operation is superfluous if only one cell is used.

85. 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 810 nm read, recording the reading on the same form.

f. Calculations

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

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

88. The concentration of orthosilicate in the samples is calculated with the following equation:

$$c(SiO_4) / \mu mol\ L^{-1} = (ABS - bl - bl_{c,\,i}) \cdot f$$

where

$c(SiO_4)$ = concentration of orthosilicates
ABS = absorbance of the sample
bl = blank of the reagents
$bl_{c,\,i}$ = blank of the i-th cell used
f = colorimetric factor

89. For a cell with a 50 mm optical path, the colorimetric factor is equal to about 19 µmol L^{-1}, i.e. a difference in concentration of 10 µmol L^{-1} (such as for example between the water used to dilute the standard solutions and the standard 5) should be approximately 0.52 in the case of samples with salinity of 37.

g. Important notes and possible problems

90. As already mentioned, after thawing, the sample must be kept in dark for at least 12 hours at room temperature to favour the depolymerization of the silicates. In fact, polymerization is promoted by freezing and an underestimation of the concentration of reactive silicates will be observed (Burton and Leatherland, 1970[19]; MacDonald and McLaughlin, 1982[20]; MacDonald et al., 1986[21]).

91. The sample must be added to the molybdic reagent, and not vice versa, in order to guarantee a correct pH value.

92. During the analysis, all the samples must be kept at the same temperature, possibly around 20 °C, to avoid a variability depending on the thermal coefficient of the reaction.

93. Calibration standards using seawater with salinity equal to that of the samples must be prepared. If working in an estuarial environment, a set of standards that cover the range of salinity values found in the samples must be prepared. The saline coefficient (ratio between the colorimetric factor value in reagent grade water and in salt water) is quite variable: for water with salinity around 35 a value of about 0.85 is observed (Bien, 1958[22]; Fanning and Pilson, 1973[23]; Koroleff, 1983).

94. An abnormal yield of the reaction is related almost always to pH values different from 2 or on bad handling. The pH in the final mix must be between 1.8 and 2.2. Sometimes a bad mixing of the reaction mixture, as well as an incorrect pH value is responsible for the formation of a blue colour due to the direct reduction of the molybdate and not to that of the polyacids.

95. The suggested method is generally free from interference for sea water. However, interference of cations such as copper, iron, cobalt and nickel with the colour of their ions may be observed. In this case the absorbance of the sample at the same wavelength without adding the reagents is necessary to be measured and the value of this reading must be added to the reagent blank. If iron ions are present, which form ferric molybdate during the reaction, a hydroxylamine hydrochloride solution (Mullin and Riley, 1955) must also be added to the samples before analysis. The development of colour is not observed if the sulphides are present in a concentration below 5 mg L^{-1}, otherwise they must be oxidized with bromine water (Koroleff, 1983).

3.2. Protocol for automated colorimetric determination of concentration of orthosilicate

a. Reagents

Stannous chloride.

96. 20 g of stannous chloride are dissolved in 12.5 mL of concentrated HCl + 27.5 mL of DDW. The reagent is dissolved at a temperature of 70 °C.

[19] Burton J.D., Leatherland T.M. (1970) The reactivity of dissolved silicon in some natural waters. Limnol. Oceanogr., 15, 473-476.
[20] MacDonald R.W., McLaughlin F.A. (1982) The effect of storage by freezing on dissolved inorganic phosphate, nitrate and reactive silicate for samples from coastal and estuarine waters. Water Res., 16, 95-104.
[21] MacDonald R.W., McLaughlin F.A., Wong C.S. (1986) The storage of reactive silicate samples by freezing. Limnol. Oceanogr., 31, 1139-1142.
[22] Bien G.S. (1958) Salt effect correction in determining soluble silica in sea water silicomolybdic acid method. Anal. Chem., 30, 1525-1526.
[23] Fanning K.A., Pilson M.E.Q. (1973) On the spectrophotometric determination of dissolved silica in natural waters. Anal. Chem., 45, 136-140.

Tartaric acid

97. 100 g of tartaric acid are dissolved in 1 L of DDW.

Ammonium molybdate

98. 40 g of molybdate are dissolved in 800 mL of DDW and then adjusted to l L.

b. Solutions for use

Molybdate

99. 50 mL of 10% HCl + 40 mL of molybdate + 15 mL of DDW are mixed.

Stannous chloride

100. 2.5 mL of stannous chloride + 48 mL of 10% HCl + 50 mL of DDW are mixed.

c. Standard

101. About 2 g of Na_2SiF_6 are dried in an oven at a temperature of 105 °C until a constant weight over time is reached. The salt is placed in a silica gel desiccator for another 24 hours. Then the salt is dissolved in reagent grade water in such a proportion to obtain a concentration of 10 mmol L^{-1}.

102. This standard is used in the daily procedure for the preparation of 5 lower concentration standards. The concentration of the sub-standards is chosen based on the amount of SiO_4 that is expected to be found, in a way the entire range of expected concentrations are covered. The multiplication factor for the calculation of concentrations is obtained from the 5 standards.

d. Manifold

The manifold (Fig. 2) is composed of three injectors and six coils of 10 turns each. The first injector (A) is equipped with 3 inputs: The first for the sample, the second for air bubbles and the third where the first reagent is introduced. Immediately after 6 coils made up of 10 coils each can be found: in the first two the first reagent is mixed, in the second two where at point (B) the second reagent is injected, and finally in the last two where at point (C) the third reagent is injected.

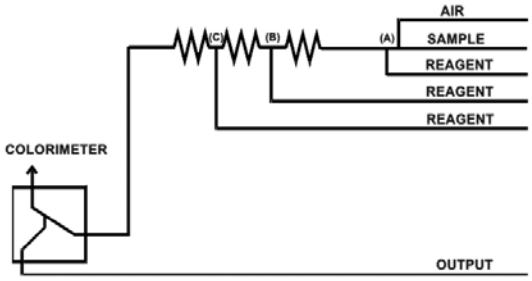

Figure 2. Manifold for orthosilicate measurement.

e. Calculations

103. The calculations are performed as is generally indicated in the Annex I: Automated methods for determination of concentration of key nutrients in seawater – Calculation of the concentration.

f. Important notes and possible problems

104. If an unstable base line occurs when the appliance is turned on in the absence of reagents, wash the circuit with NaOH and then with 10% HCl.

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

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

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

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

110. If precipitate forms in the molybdate the reagent must be discarded.

111. In case of preparation of mixed standards of PO_4 and SiO_4 the standards must never be prepared in the same flask.

112. A colorimeter with a very narrow entrance of the reading cell to avoid refractive disturbances must be used.

113. High sensitivity reading phototubes for 820 nm readings must be used.

114. If, when inserting the reagents, a blue colour is noticed in the sample, at the exit from the second series of coils, this would indicate that the tartaric acid is to be discarded.

Symbol, units and precision

Symbol: $c(SiO_4^{4-})$	**Unit:** µmol L^{-1}	
Precision: ±0.05	**Accuracy:** ± 0.05	**LOD:** 0.10
Method identifier:	SDN:P01::**SLCAMAZX**	Concentration of silicate {SiO44- CAS 17181-37-2} per unit volume of the water body [unknown phase] by manual colorimetric analysis
	SDN:P01::**SLCAAAZX**	Concentration of silicate {SiO44- CAS 17181-37-2} per unit volume of the water body [unknown phase] by colorimetric autoanalysis

4. Technical note for the combined determination of concentration of total nitrogen and total phosphorous

115. The concentration of total nitrogen or phosphorus in a water sample is represented as the sum of the moles of the element in question present in the form of organic and inorganic, dissolved and particulate species. In this analytical procedure both elements are determined after oxidation and hydrolysis of most of the compounds initially present in the sample in the same reaction mixture, with the production of nitrate and orthophosphate respectively. The procedure for the common mineralization of the two elements are presented.

116. The oxidizing agent used is potassium persulfate $K_2S_2O_8$, which decomposes when hot according to the reaction:

$$K_2S_2O_8 + H_2O \rightarrow 2KHSO_4 + 1/2O_2$$

117. During the oxidation reaction, H + is produced which determines a pH variation. The behaviour of the various nitrogen compounds in the oxidation reaction is different. Those containing N-N bonds are oxidized more difficultly while those with N = N bonds are rather refractory to nitrate oxidation. Furthermore, a time of at least 30 minutes is necessary to ensure the complete disappearance of the persulfate from the oxidation solution, thus preventing possible interference in the subsequent phases of the analytical assay, especially for the determination of nitrate.

118. The appearance in the reaction mixture, even in an alkaline environment, of Cl_2 which would interfere with the subsequent reduction of nitrates by cadmium is due to the subtraction of OH- by magnesium in the form of a precipitate, which does not readily neutralize the H^+ ion produced by the reaction (Nydhal, 1978)[24]. Therefore, adding OH^- to the reaction mixture or shaking the reaction vessels is suggested. Koroleff (1968[25]; 1983) on the other hand argued that in an alkaline environment, while the complete hydrolysis of the bound phosphorus into organic compounds is achieved, a yield of polyphosphates decomposition around 60% is observed. However, the concentration of the latter is generally of secondary importance compared to bound phosphorus in organic compounds, for which Koroleff (1968; 1983b[26]) and Valderrama (1981)[27] believe that a unique method for the determination of nitrogen and total phosphorus in seawater are equally reliable.

[24] Nydahl F. (1978) On the peroxidisulphate oxidation of total nitrogen in waters to nitrate. Talanta, 12, 1123-1130.
[25] Koroleff F. (1968) Determination of total phosphorus in natural waters by means of persulphate oxidation. ICES C.M./C, 33, 209-212.
[26] Koroleff, F. (1983b) Total and organic nitrogen. In: “Methods of Seawater Analysis”, Grasshoff K., M. Ehrhardt, K. Kremlin Eds, Verlag Chemie, Weinheim, 162-173.
[27] Valderrama J.C. (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem., 10, 109-122.

119. In the method reported by Valderrama (1981), thanks to the use of a buffer based on the boric acid-borate couple and based on the reactions involved, the pH of the mixture starts from about 9.7 and reaches the end of the process at about 4-5, thus creating the appropriate conditions for the oxidation-hydrolysis of both nitrogen and phosphorus and the necessary decomposition of excess persulphate. The method presented is the method of Valderrama (1981) in the version of Koroleff (1983a, b).

120. Under this Technical Note, this Monitoring Guidelines provides the following IMAP Protocols for the combined colorimetric determination of concentration of total nitrogen and total phosphorous:

- Protocol for preparation of samples for a combined determination of concentration of total nitrogen and total phosphorus;
- Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous;
- Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous.

4.1. Protocol for preparation of samples for a combined determination of concentration of total nitrogen and total phosphorus

a. Equipment

121. The equipment for preparation of samples for determination of concentration of total nitrogen and total phosphorous include:

1. graduated cylinders of 50 mL
2. 100 mL borosilicate glass, polypropylene, TPX or Teflon containers with hermetically sealed screw cap fitted with flange or Teflon gasket. It is recommended to use polyethylene bottles if the samples will be frozen.

b. Chemical products

122. The chemical products for preparation of samples for determination of concentration of total nitrogen and total phosphorous include:

1. potassium persulfate [K_2S_2O8] (nitrogen content <0.001%)
2. sodium hydroxide [NaOH] (nitrogen content <0.001%)

c. Preparation of reagents:

Oxidizing solution

123. 50 g of potassium persulfate (low N content) and 30 g of boric acid are dissolved in 1 L of sodium hydroxide 0.375 mol L^{-1} (15 g of NaOH are dissolved and diluted to 1 L with distilled water and stored in a polyethylene bottle). The reagent, if stored in a well capped polyethylene bottle and wrapped in aluminium foil, is stable for at least one week.

d. Sampling procedure

124. Using a 50 mL cylinder, rinsed at least twice with the sample, 50 mL of water for each sub-sample are poured directly from the sampling bottle into the reaction containers, which have also been previously rinsed with the sample.

125. If the sample is particularly turbid (frequent occurrence in coastal waters), a duplicate sampling is necessary to determine the turbidity.

126. As in all determinations that include particulate matter, sub-samples must be taken after having carefully shaken the sampling bottle or within very short times that prevent significant sedimentation of the particulate.

e. Sample storage

127. As regards conservation, one of the three methods indicated below can be used, which ensure acceptable results:

1. The samples are kept, at the time of collection, in the hermetically sealed reaction containers. The analysis can also be performed after a long period of time. In fact, following the oxidation reaction, the nitrates and phosphates produced remain constant.
2. Immediately after sampling, 5 mL of oxidizing solution are added and the sample containers hermetically sealed. Under these conditions the samples are stable for at least 48 hours. If the oxidation

reaction takes place within this time, the nitrates and phosphates produced remain constant even for 2 ÷ 3 months (Nydhal, 1978).
3. The samples, in a polyethylene bottle, are quickly frozen, without filtering.

4.2. Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous

a. Equipment

128. The equipment for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous include:

1. all that indicated for nitrate and orthophosphate determination
2. autoclave or normal pressure cooker (in the latter case it may be more practical to use, as sample containers, test tubes of about 50 mL with screw caps and Teflon seals and use a small volume)

b. Chemical product

129. The chemical products for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous include:

1. all that indicated for nitrate and orthophosphate determination
2. disodium EDTA [$C_{10}H_{14}N_2Na_2O_8$]

c. Preparation of stock solutions

130. For the ***determination of total nitrogen***, the stock solutions indicated in the Protocol for determination of nitrates and an organic nitrogen solution must be prepared.

Organic nitrogen solution (10 mmol L^{-1})

131. 186.2 mg of disodium-EDTA are dissolved in 90 mL of reagent grade water, adjusted to100 mL in a volumetric flask (100 mL, class A) and stored in the refrigerator in a dark glass bottle. The solution is stable for a few months.

132. For the ***determination of total phosphorus***, the solutions listed below must be prepared:

Sulfuric acid (4.5 mol L^{-1})

133. 250 mL of concentrated sulfuric acid to 750 mL of reagent grade water are carefully added, allowed to cool and adjusted to 1 L. Stored in a reagent bottle, the solution is stable indefinitely.

Mixed reagent

134. 12.5 g of crystalline ammonium heptamolybdate tetrahydrate are dissolved in 125 mL of reagent grade water. 0.5 g of potassium antimony tartrate in 20 mL of reagent grade water are dissolved separately. The molybdate solution, while stirring, is added to 350 mL of 4.5 mol L^{-1} sulfuric acid, then the potassium antimony tartrate solution is added and mixed. The solution is preserved in a dark glass bottle and stable for several months.

d. Analytical procedure

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

Acidified solution of ascorbic acid

135. 10 g of ascorbic acid are dissolved in 50 mL of reagent grade water and 50 mL of 4.5 mol L-1 sulfuric acid added. The solution is stored in a dark glass bottle in the refrigerator. The solution can be used as long as it remains colorless (about a week), but is preferable to be prepared at the time of use.

d.2. Preparation of standard solutions

136. The Protocol for determination of concentrations of nitrate and orthophosphate must be followed.

d.3. Preparation of the solution for checking the efficiency of the oxidizing reagent

10 µmol L^{-1} solution of nitrogen

137. In a 100 mL flask (class A) 100 µL (measured with a precision pipette) of organic nitrogen stock solution is diluted with reagent grade water. The solution is divided into two 50 mL subsamples and 5 mL of oxidizing reagent added. The entire amount of nitrogen present in the solution (10 µmol L^{-1}) must be determined. If this does not happen, the oxidizing solution must be prepared again.

d.4. Preparation of reagent blanks

138. 50 mL of reagent grade water are transferred into 3 reaction vessels and each inoculated with 5 mL of oxidizing reagent.

139. The prepared blanks are autoclaved following the procedure indicated for the analytical treatment of the samples.

To prepare the blanks of the reagents related to the analysis of total nitrogen (blN):

140. 5 mL from each of the 3 containers are sampled with an automatic pipette and transferred into 100 mL beakers; 45 mL of reagent grade water are added to each;

141. To the blanks of the nitrogen reagents the same procedure applied to the samples and illustrated in detail in the Protocol for manual colorimetric determination of nitrate (ammonium buffer, reduction column, sulphanilamide, NNEDDC, spectrophotometric assay) is applied.

To prepare the blanks of the reagents related to the analysis of total phosphorus (blP):

142. To each of the 50 mL left in the 3 containers as indicated for the analytical treatment of the samples the reagents (acidified solution of ascorbic acid and mixed reagent) are added.

143. The spectrophotometric measurement as indicated in the Protocol for manual colorimetric determination of orthophosphate are performed.

d.5. Analytical treatment

144. The containers with the samples and the solutions to be analysed are put in an autoclave or pressure cooker and autoclaved/cooked for at least 30 minutes at 120 °C.

145. The containers are brought to room temperature and checked that the sample volume has remained unchanged. If necessary, the volume is adjusted back to 55 mL with reagent grade water, but the change in volume which may have led to a parallel contamination of the sample recorded.

146. At the end of the oxidation stage, all the nitrogen in the sample should have been converted to nitrate and all the phosphorus to phosphate.

147. The procedures as indicated in the Protocols for the determination of nitrate and phosphate are followed, considering the following additions and modifications.

For nitrogen analysis

148. 5 mL from each of the samples and of the two control samples with EDTA are sampled with an automatic pipette and transferred into 100 mL beakers; 45 mL of reagent grade water are added to each.

149. The same procedure illustrated in detail in the Protocol for manual colorimetric determination of nitrate (ammonium buffer, reduction column, sulphanilamide, NNEDDC, spectrophotometric assay) is applied.

For phosphorus analysis

150. The remaining 50 mL of sample are used and 1 mL of acidified solution of ascorbic acid with is added with a dispenser;

151. The solution is shaken and after about 30 seconds 1 mL of mixed reagent (measured with a dispenser) is added while shaking;

152. The same procedure illustrated in detail in the Protocol for manual colorimetric determination of orthophosphate is applied for the spectrophotometric measurement.

e. Calculations

153. The blanks of the of the cells ($bl_{c,i,N}$ and $bl_{c,i,P}$) are calculated as indicated in the Protocols for manual colorimetric determination of nitrate and orthophosphates.

154. The blank of the reagents (oxidizing reagent plus colour development reagent), both for phosphate (bl_P) and for nitrate (bl_N) are calculated as the average of the absorbance values of the three solutions with reagent grade water.

155. The colorimetric factor for the two components are calculated according to the procedure indicated in the Protocols for manual colorimetric determination of nitrate (f_N) and orthophosphate (f_P).

156. The concentrations are calculated according to the equations:

$$c(\text{TN}) / \mu\text{mol L}^{-1} = (\text{ABS}_N - \text{bl}_N - \text{bl}_{c,i,N}) \cdot f_N$$

$$c(\text{TP}) / \mu\text{mol L}^{-1} = (\text{ABS}_P - \text{bl}_P - \text{bl}_{c,i,P}) \cdot f_P$$

where:

ABS_N = absorbance of sample at 553 nm
ABS_P = absorbance of sample at 882 nm
$\text{bl}_{c,i,N}$ = blank of i-th cell at 553 nm
$\text{bl}_{c,i,P}$ = blank of i-th cell at 882 nm
bl_N = blank of nitrogen reagents
bl_P = blank of phosphorus reagents
f_N = colorimetric factor for nitrate
f_P = colorimetric factor for phosphate

f. Important notes and possible problems

157. The containers should be rinsed with reagent grade water for at least a couple of times. Between the determinations the containers should be kept filled with an HCl solution of approximately 0.1 mol L^{-1}.

158. The test solution should not be used to correct the reaction yield, but only as a rough check of the oxidation efficiency, since a reduced oxidizing power can occur in relation to the different type of nitrogen compounds involved in the reaction.

159. About the determination of nitrogen, it should be noted that some problems may be due to impurities of the reactants. It is important to use low nitrogen persulfate or to follow the recrystallization technique reported in Nydhal (1978). It is also important to always check the quality of the reagent grade water being used. In fact, ammonia is almost always present in closed environments and dissolves easily in water, water as soon as it comes out of the purifier must be used, even if this does not eliminate the risk of substances released by the exchange resins.

4.3. Protocol for the combined automated colorimetric determination of concentration of total nitrogen and total phosphorous

160. This Protocol do not differ in the analytical treatment of the samples from the Protocol for combined manual colorimetric determination of total nitrogen and total phosphorous (4.2) as is based on the identical methodology. The same equipment and chemical reagents must be prepared. The samples prepared and treated in the identical way together with the prepared reagent blanks and standards. At the time when the determination of the nitrate and orthophosphate are necessary the Protocol for automated colorimetric determination of nitrate and orthophosphate must be used for each of the analysed compound. The same problems identified for the automated methods for determination of nitrate and orthophosphate may arise and must be handled as indicated. The calculations are performed as is generally indicated in the Annex I: Automated methods for determination of concentration of key nutrients in seawater – Calculation of the concentration.

a. Symbol, units and precision

Symbol: *c*(TN)	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.03
Method identifier:	SDN:P01::**NTOTWCTX**	Concentration of total nitrogen {total_N} per unit volume of the water body [dissolved plus reactive particulate phase] by oxidation and colorimetric autoanalysis
Symbol: *c*(TP)	**Unit:** µmol L^{-1}	
Precision: ±0.02	**Accuracy:** ± 0.02	**LOD:** 0.05
Method identifier:	SDN:P01::**TPHSPP01**	Concentration of total phosphorus {total_P CAS 7723-14-0} per unit volume of the water body [dissolved plus reactive particulate phase] by oxidation and colorimetric analysis

Annex I
Automated methods for determination of concentration of key nutrients in seawater
Calculation of the concentration

Many automatic nutrient analysis systems are equipped with software that allows, with more or less sophisticated algorithms, to determine the height of the peak and to provide the concentration of the individual samples having previously determined the calibration and the reagent blank value. Although the software may be sophisticated, it is not always able to manage anomalous events, so it is advisable to continuously monitor the operation of the instrument.

Considering the current availability of calculation programs such as spreadsheets, the best solution is to obtain from the analyser the values of the three components necessary to calculate the concentrations, i.e. the blank value, that of the baseline near the samples and that of the individual samples. The procedure suggested below is certainly not the only one possible and, once again, it will be up to the operator to decide which paths to follow.

Operationally: The instrument is stabilized with the reagents and ultrapure water (DDW) for 15-20 minutes. It is verified that the hydraulics are stable (regular bubbles and stable baseline). The refractive index is then determined by continuously sampling first the ultrapure water (DDW) and then the washing water (OSW), having replaced one of the reagents, usually the one with lower flow rate, with ultrapure water. The difference in reading is recorded which corresponds to the false absorption due to refraction. The replaced reagent is reinserted and the baseline is re-stabilized with DDW. For the analysis of the samples the DDW is replaced with OSW (the drawing needle is moved from one container to another) and waited for the baseline to re-stabilize. Then the sampler is activated by arranging the samples in groups (usually one or more stations) and taking care to combine the groups with an OSW reading. In this way each group of samples is sandwiched between two OSW readings, which allow a good control of baseline drift. It is also good practice to periodically analyze a series of solutions of known concentration (standards) which must always be prepared daily. Usually, at least one series of standards with increasing concentration is inserted at the beginning of the series of samples and one at the end. If the series of samples is very long, further series of intermediate standards can be inserted. Standards must be in increasing concentrations so that the difference between the lowest and highest includes the range of concentrations expected for the samples to be analysed. This procedure allows both to determine the linearity of response to the Lambert-Beer law (i.e. to determine the slope of the extinction / concentration line of the increasing standards which, in the absence of blank, should pass through the origin of the axes) in the expected concentration range, and to determine any variations in the efficiency of the method (gain) which would be highlighted by significant variations of this slope with the progress of the analysis. In fact, the slope of the initial standardization line, ie its angular coefficient and its reciprocal value F, will almost never coincide with those of the final and / or intermediate standards. The simplest solution to this problem is to take the average between initial, final and / or intermediate F and use this average F_m in the calculation. The most correct, but more complicated, procedure consists in determining the sequential reading "gain" sample by sample, similar to what is done for the calculation of the baseline drift, and multiplying the reading value of each sample by its own F so determined. Once the samples have been analysed, it is good practice to re-measure the DDW reading and wash the circuit without the reagents.

To calculate the concentrations, the following quantities (the values are in the unit in use, cm if you read on the trace of a recorder or digital counts if you work on the outputs of the A / D converter) must be obtained:

- V_{DDW} = value of the DDW reading at the time of blank determination
- V_{OSW0} = value of the OSW reading after the blank (DDW)
- R = OSW baseline variation in mm by refractive index
- V_{OSWi} = value of the OSW reading that precedes the first of the samples of the group
- N_{OSWi} = sequential number of the OSW reading that precedes the group of samples
- V_{OSWn} = value of the OSW reading that follows the last of the group samples
- N_{OSWn} = sequential number of the OSW reading after the last sample of the group
- V_s = value of the sample reading
- N_s = sequential number of the sample reading
- D = drift
- F_m = average factor obtained from the standard curves (the reciprocal of the slope or angular coefficient of the straight line reading-concentration of the calibration samples)
- *c* = concentration of the sample

the concentration of the sample is given by the equation:

$$c(\text{Nut.})/\ \mu\text{mol L}^{-1} = [V_s - D\ (N_s - N_{OSWi}) - V_{OSWi} + (VOSW0 - V_{DDW}) - R]\ F_m$$

where the drift (D) is given by:

$$D = (V_{OSWn} - V_{OSWi}) / (N_{OSWn} - N_{OSWi})$$

The refractive index refers to the shift of the baseline in the absence of reactants due to the difference in salinity between deionized distilled water (DDW) and oligotrophic water (OSW).

Annex II
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