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Laboratories

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A. IMAP MONITORING GUIDELINES FOR CI13 AND CI14

A-1. Monitoring Guidelines/Protocols for Sampling and Determination of Hydrographic Physical and Chemical Parameters

Table of Contents

1 Guidelines for hydrographic physical parameters: sampling and measurement 3
1.1 Introduction 3
1.2 Technical note for measurement of temperature and salinity of seawater 3
1.2.1 Protocol for measurement of temperature and salinity using CTD 4
1.2.2 Protocols for the measurement of temperature using reversing thermometers 6
1.2.3 Protocol for sample preservation of seawater for the determination of salinity 7
1.2.4 Protocols for sample preparation and determination of salinity using bench salinometer 8
1.3 Technical note for measuring Secchi depth 9
1.3.1 Protocol for measuring Secchi depth 10
2 Guidelines for hydrographic chemical parameters: sampling and determination 11
2.1 Introduction 11
2.2 Technical note for measuring dissolved oxygen 11
2.2.1 Protocol for sample preparation and determination of dissolved oxygen in seawater by Winkler method 11
2.3 Technical note for measuring pH 16
2.3.1 Protocol for sample preparation and determination of pH using a potentiometric method 16
2.3.2 Protocol for sample preparation and determination of pH using spectrophotometric method 18
3 References 18

1 Guidelines for hydrographic physical parameters: sampling and measurement

1.1 Introduction

In this Guideline for the Determination of Hydrographic Physical Parameters, the supporting parameters temperature, salinity and transparency are presented. Temperature and salinity are essential in the basic calculation of other parameters as are dissolved oxygen and pH. On the other hand, they also serve as proxy for the definition of the water typology, an important tool in the water classification scheme on which the assessment of GES is based on, as presented in details in the IMAP Guidance Factsheets (UNEP/MAP, 2019)[1].

The IMAP Protocols elaborated within this Monitoring Guidelines for the Determination of Hydrographic Physical Parameters provide detail guidance on the necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the core hydrography physical supporting parameters, calculations, data transformation if necessary and identify weak points, including important specific notes and elaborated 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.

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 hydrographic chemical parameters, the needs of the measurements both in off-shore areas and in narrow coastal areas are addressed.

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. The Method identifiers are 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.

1.2 Technical note for measurement of temperature and salinity of seawater

Temperature is the property that regulates the transfer of thermal energy or heat between two bodies: the heat flow is directed from the warmer body to the colder one until thermal equilibrium is reached. Temperature measuring instruments are based on this basic principle. The temperature, together with salinity, is useful for identifying the mass of water sampled and for calculating, using an equation of state, the density and other derived quantities. This parameter also has effects on biological systems and in general on the chemical-physical balances in the marine environment, including the solubility of gases (e.g. oxygen solubility) and pH.

Prior to January 1, 1990, the temperature was expressed in the International Practical Temperature Scale of 1968 (IPTS-68). The 1990 International Temperature Scale (ITS-90) was subsequently adopted, which currently represents the best approximation of the thermodynamic temperature (T). In oceanography the convention is to measure the temperature on the Celsius (t) scale, whose unit is °C and with t = T – 273.15. The conversion between the old scale (t_{68}) and the new one (t_{90}) is given by the formula (Saunders, 1990)[5]:

$$t_{68} = 1.00024\ t_{90}$$

Salinity is a measure of the content of dissolved materials in sea water. Together with the temperature it is a parameter of fundamental importance for identifying the mass of water sampled and for calculating the density (using an equation of state) and other derived quantities. It affects other parameters such as dissolved oxygen and has effects on many biological and chemical processes and systems in the marine environment.

Absolute salinity (SA) is defined as the ratio between the total mass of materials dissolved in sea water and the total mass of water. SA is very difficult if not impossible to measure directly, because it would be necessary to fully know the composition of sea water. Therefore, in practice, an approximate definition is given, the measurement of which is more easily achievable.

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[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] Saunders, P., 1990. The International Temperature Scale of 1990, ITS-90. Woce Newsletter, 10, IOS, Wormley, UK.

The first practical definition of salinity is that given in 1899 by the International Commission for the study of the oceans led by Prof. Martin Knudsen which established that salinity is the residual mass of salt (measured in grams) per kilogram of sea water, when all the carbonates have been transformed into oxides, the bromides and iodides replaced by chlorides and all the organic substance has been oxidized (Forch et al., 1902)[6]. Since the various components contribute to salinity according to a practically constant ratio and the main component, chloride, is easy to accurately measure by a simple chemical analysis (titration), for a long time the salinity of seawater has been determined indirectly by measuring the mass of chlorides dissolved in the water and using empirical relationships (Forch et al., 1902; Wooster et al., 1969)[7].

The definition of salinity was revised when a technique was developed to be able to determine it from water conductivity measurements. In 1978, the JPOTS (UNESCO, 1981)[8] introduced the Practical Salinity Scale (PSS-78), which defines the practical salinity as a function of the ratio K_{15} between the electrical conductivity of a sample of sea water at temperature t_{68} = 15 °C (defined in the IPTS-68 temperature scale) and at the pressure of a standard atmosphere (101325 Pa in the SI, corresponding to 1013.15 millibar), and that of a solution of potassium chloride (KCl), in which the molar fraction of KCl is 0.0324356, under the same conditions of temperature and pressure. A $K_{15} = 1$ corresponds to a practical salinity of 35.

At 15 °C, the standard solution of KCl has an electrical conductivity which is equivalent to that of a North Atlantic seawater sample with chlorinity of 19.3740 at the same temperature. This fact guarantees:

- a certain continuity between the salinity measurements in the Practical Scale and the previous ones, which were largely based precisely on the measurement of chlorinity;
- the use of sea water with $K_{15} >> 1$ as a secondary standard for the routine calibration of instruments for measuring salinity.

Practical salinity is a dimensionless quantity, whose order of magnitude coincides with that of Knudsen's definition. Although it is an adopted practice, it is technically wrong to use the abbreviation "psu" (practical salinity units), and this practice should be absolutely avoided. This quantity enters into all the algorithms that are currently in use for calculating the thermodynamic properties of sea water (UNESCO, 1983)[9] and is also the one that is stored in databases.

Under this Technical Note, the Monitoring Guidelines for Determination of Hydrographic Physical Parameters elaborates the three following Protocols:

- Protocol for determination of temperature and salinity using CTD;
- Protocol for the determination of temperature using reversing thermometers;
- Protocol for the sample preservation of seawater for the determination of salinity
- Protocol for sample preparation and analysis of salinity using bench salinometer.

1.2.1 Protocol for measurement of temperature and salinity using CTD

The multiparameter probe, the only device with which the simultaneous and *in situ* measurement of temperature, salinity, pressure and any other bio-chemical parameters of interest are allowed. Multiparameter probes for oceanographic measurements have been in use since the middle of the last century. The central unit that incorporates and manages the sensors that measure the quantities of interest is their main part. The probes for the measurement of physical parameters at sea are commonly called CTD, an acronym that summarizes the three basic physical parameters measured (C = Conductivity, T = Temperature, D = Depth, i.e. conductivity, temperature and depth). In reality, CTD probes do not measure depth directly, but provide an indirect measure of it by detecting pressure. CTD probes have a dual use, as profilers, when they are dropped along the water column from the surface to the bottom or to a desired intermediate depth, or as fixed-point sampling instruments (as happens when they are installed on a buoy or on an instrumented anchor). In the first case, the measurement of a vertical profile of the parameters and in the second one a time series at a precise point in space are collected. The vertical resolution of the profile and the temporal resolution are related on the sampling frequency of the instrument.

To check the correct functioning of a CTD system during an oceanographic campaign, it is useful to make comparisons by collecting water samples with a sampler connected to the system, to be analysed with a salinometer and by carrying out temperature measurements using reversing thermometers mounted on the sampler. The pressure values measured by the sensor of the CTD system can be compared with those provided by an independent pressure sensor.

a. *Equipment*

CTD: preferably be equipped with dual sensors for salinity and temperature, to prevent loss of data and provide a first instance of quality control. For stratified waters CTDs should preferably have a sampling rate of 12 Hz or higher. A CTD equipped with a rosette for water samplers is preferred to individual sampling flasks

[6] Forch C., Knudsen M., Sorensen S.P.L. ,1902. Berichte über die Konstantenbestimmungen zur Aufstellung der hydrographischen Tabellen. Kgl. Danske Vidensk Selsk. Skrifter, 6 Raekke Naturvidensk, Mathem. Afd., 12: 1-151.

[7] Wooster W.S., Lee A.J., Dietrich G., 1969. Redefinition of salinity. Deep-Sea Res., 16: 321–322.

[8] UNESCO, 1981. The practical salinity scale 1978 and the international equation of seawater 1980. UNESCO Technical Papers in Marine Science, 36: 1-25.

[9] UNESCO, 1983. Algorithms for computation of fundamental properties of seawater. UNESCO Technical Papers in Marine Science, 44: 1-53.

clamped to a wire. It is recommended that the CTD is mounted vertically within the frame of the rosette frame to avoid fouling of sensors by debris or bubbles and promote free flow of water.

Reversing thermometers, mounted on the rosette frame or on samplers from which reference data for temperature is obtained.

Sampling bottles attached to the CTD-rosette or attached on a line from which reference samples for bench salinometers are obtained.

b. *Procedure*

Many protocols for CTD measurements (WOCE 1991[10], UNESCO 1994[11], UNESCO, 1988[12]) are available. Starting from what is suggested by these protocols and taking into account the field experience the following protocol is preferable:

The manufacturer's recommendations on preparations of the CTD and rosette sampler must be followed. If the CTD has not been used for a long time, e.g. the first cast of the cruise, problems with bottles leaking may occur since the O-rings for the bottle's caps are dehydrated. If this is known to happen, it can be prevented by rinsing and filling all bottles with freshwater for at least 1 hour before sampling.

When the CTD is on deck the system is started and the CTD pressure and temperature in the logbook noted.

The CTD must be lowered below the sea surface for at least 1 minute before starting the measurements. This gives time for all sensors to acclimatize and air bubbles have time to be flushed out by the pump.

The CTD is bring back to the surface and the measurement of the profile started. If the sea state is rough it is recommended to start the downcast from a few meters below the sea surface to prevent bubbles from breaking waves entering the sensors.

Care must be taken to keep the lowering speed as constant as possible, and around 0.5 m s^{-1}. If an Active Heave Compensation (AHC) system is available, a slower speed (0.3 m s^{-1}) can be used.

The CTD as close to the bottom as possible is lowered, though without risking bottom contact. The bottom depth and all the other information required by the CTD log or monitoring protocol are noted.

The rosette bottles should preferably be fired at selected standard depths during the up-cast in order to obtain an undisturbed CTD profile during the down-cast and undisturbed water samples on the way up. If the winch is maneuvered manually between each sampling depth, attention must be paid to approach the set depth as gentle as possible to reduce the disturbance of the water profile. This is especially important in stratified waters.

At each sampling depth the sampling bottles should have time to acclimatize and the effect of dragging water from deeper depth should be avoided. Wait at least 1 minute before the sampling bottles to be fired. If the CTD values still are not stable wait another 3 minutes before firing. If the bottles are equipped with reference sensors do not forget to wait the appropriate time for the sensors to measure after firing the bottle.

However, if the CTD and rosette is equipped and prepared for free-flow sampling bottles, it can be configured to fire water samples on predefined standard depths during the down-cast. Note that samples near the surface should be collected during up-cast to avoid trapping air bubbles mixed into the water by breaking waves and turbulence when the CTD is lowered.

When the CTD is back on deck, the pressure and temperature in the CTD log are noted. The pressure value must be approximately the same as that read before the cast; differences are due to thermal and mechanical hysteresis of the pressure sensor. Deck pressure as offsets to correct pressure is not used. Deck pressure should only be used as consistency check against laboratory measured historical drift.

If there is any leakage or malfunction to the CTD, water sampler or water bottles it must be reported. Questionable sensor readouts should also be noted. All events happened during the cast also must be noted. Manufacturer's instructions for cleaning the CTD after each cast must be followed.

Between casts and after the cruise; the CTD and rosette in a way to prevent contamination must be stored. All sensors should be treated and stored according to the manufacture's recommendations.

Reference data for temperature is obtained from reversing thermometers, mounted on the rosette frame.

CTD-rosette or line; It is recommended that reference samples are collected in triplicates.

c. *Symbol, units and precision*

For the parameters described in this protocol, the symbols and units 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:

Depth:

Symbol: z **Unit**: m

Precision: Pressure sensor: ± 0.01 m

Method identifier:

[10] WOCE, 1991.WOCE Operational Manual WHPO 91-1, WOCE Report No68/. (http://whpo.ucsd.edu/manuals.html).

[11] UNESCO, 1994. Protocols for Joint Global Flux Study (JGOFS) Core Measurements. Manual and Guide, 29: 1-181.

[12] UNESCO, 1988. The acquisition, calibration and analysis of CTD data. A report of SCOR Working Group 51. UNESCO Technical Papers in Marine Science, 54: 1-59.

SDN:P01:: **DEPHPR01**	Depth (spatial coordinate) relative to water surface in the water body by profiling pressure sensor and conversion to seawater depth using UNESCO algorithm

Temperature:

Symbol: *t* **Unit**: °C (degree Centigrade)

Precision: Temperature sensor: ± 0.01 °C

Method identifier:

SDN:P01:: **TEMPCC01**	Temperature of the water body by CTD and verification against independent measurements

Salinity:

Symbol: *S* **Unit**: -

Precision: Conductivity sensor: ± 0.01

Method identifier:

SDN:P01::**PSALCC01**	Practical salinity of the water body by CTD and computation using UNESCO 1983 algorithm and calibration against independent measurements

1.2.2 Protocols for the measurement of temperature using reversing thermometers

Digital electronic reversing thermometers

The digital reversing electronic thermometer, can perform the same functions as the mercury one, but with higher precision. Having the same dimensions of the mercury one it enters the housings provided for this type of thermometer. In this thermometer the temperature is measured by a platinum thermometer similar to the sensors used on the CTD probes. The advantages are that it does not use mercury, it covers a larger range of measurement, reading is easier because it is provided in digital form reducing the risk of loss of data, it is robust and easy to use.

a. <u>Procedure</u>

The thermometers are placed in the special thermometer holders with which the sampling bottles are equipped. In thermometer holders without a locking mechanism, the thermometers must be locked using para or neoprene rubber cylinders, usually supplied with thermometer holders, which cushion any mechanical shocks.

With the thermometer holder armed, a small magnet (supplied with the thermometer or common) is slid three times along the major axis of the thermometer, until on the display shows written "Samp"; in this way the thermometer is set in reversing mode. For information, the "Cont" mode is used to display the instantaneous temperature measured in real time by the thermometer, while the "Hold" mode displays the temperature data recorded after reversing the thermometer, while it was in "Samp" mode.

The bottles with the thermometers must be kept at the programmed depths for the time necessary to reach the balance with the surrounding medium. The time required for digital thermometers is about 30 seconds. Then the command to close is send the bottle and to reverse the thermometer and wait at least ten seconds after the closing confirmation signal before changing deep or to retrieve the bottle.

Once the bottles are brought back to the surface, sliding the long magnet once along the major axis of the thermometer, the temperature value will appear on the display for a few seconds recorded by the thermometer during the reversal.

The data are written in a form, reporting the serial number for each thermometer.

Mercury reversing thermometers

Mercury reversing thermometers consist of a main and a secondary thermometer, coupled in a glass container that protects them from mechanical changes induced by the water pressure. The main thermometer has a relatively large mercury tank that communicates, by means of a serpentine strangled in one point (called "break-off point") with a thin capillary ending with a small widening which constitutes a secondary tank. When the thermometer is in a straight position, at the desired depth, the mercury contained in the main tank changes volume according to the external temperature and occupies part of the thin capillary. When the thermometer overturn, due to the considerable surface tension, the mercury contained in the capillary breaks in the coil at the height of the choke and separates from the rest. The amount of mercury that separated, collected in the secondary tank and in part of the capillary, indicates the water temperature at the time of reversing. The auxiliary thermometer, mounted next to the reversing thermometer, is used to measure the ambient temperature, once the thermometer is brought back to the surface. **NOTE:** The use of mercury is prohibited except in exceptional cases.

a. <u>Procedure</u>

The thermometers are placed in the special thermometer holders with which the sampling bottles are equipped. In thermometer holders without a locking mechanism, the thermometers must be locked using para or neoprene rubber cylinders, usually supplied with thermometer holders, which cushion any mechanical shock.

The bottles with the thermometers are kept at the programmed depths for the time necessary to reach balance with the surrounding medium. The time required for mercury thermometers is 5-10 minutes. The reversing of the thermometer is then triggered.

The bottles are then returned to the surface and placed on a special support, making sure that are not directly affected by the sun, possibly covering them with a wet towel to avoid large fluctuations in temperature.

Once the balance between the temperature of the thermometers and the ambient temperature has been reached, using the appropriate eyepiece, the water temperature is read on the main thermometer (t’’) with accuracy to one hundredth of a degree or higher in relation to the characteristics of the scale.

Similarly, on the auxiliary thermometer, the air temperature (t'_a) is read with tenth degree precision. The eye must be in level with the upper part of the mercury column, to avoid errors due to refraction.

The data are written in a form, reporting the serial number for each thermometer.

b. Calculations

The reading provided by the thermometer, for the error caused by the capillary imperfections must be corrected proceeding as indicated in the calibration certificate that accompanies the thermometer. In the certificate, for temperature ranges of 5 °C, the correction to be made to the value read on the thermometer to obtain the real temperature value, or the value of the actual temperature at a given temperature value read, is reported. The temperature value read on the thermometer will probably not exactly match those indicated on the certificate, therefore first must be calculated the correction to be made to this value by applying a simple linear relationship between read values and real values. From the manufacturer's tables the values t''_{inf} and t''_{sup} within which the reading (t’’) is included and the corresponding correct values t'_{inf} and t'_{sup}, are read and the correct temperatures for the main thermometer t’ and for the auxiliary thermometer t'_a calculated from the following equations:

$$t' = t'_{inf} + (t'' - t''_{inf})\,(t'_{sup} - t'_{inf}) / (t''_{sup} - t''_{inf})$$

$$t'_a = t'_{a,inf} + (t''_a - t''_{a,inf})\,(t'_{a,sup} - t'_{a,inf}) / (t''_{a,sup} - t''_{a,inf})$$

t’and t'_a are inserted in the following equation:

$$c = (V_0 + t') / (t' - t'_a) / [K - ½\,(t' - t'_a) - (V_0 + t')]$$

where:

c = correction to be made,

K = inverse of the thermal expansion coefficient of the glass with which the thermometer is built.

V_0 = volume of mercury at 0 °C expressed as °C (=the degree volume).

The values of K and V_0 are obtained from the calibration certificate.

The water temperature *in situ* is calculated from the formula:

$$t_w = t' + c$$

a. Important notes related to both types of reversing thermometers

All reversing thermometers, regardless of type, must be calibrated with a reference thermometer at least once a year. The reference thermometers must in turn be properly calibrated. Calibration must take place in thermostated baths.

Mercury thermometers must be treated gently, avoiding sharp strokes as they can cause microfractures in the capillary compromising its functioning; digital electronic thermometers are less delicate, but like all electronic instruments, they must be treated with care.

For mercury thermometers, if mercury does not return to the tank, avoid the common practice of gently tap the thermometer, because this causes small damages to the capillary. The reunification must be produced by forcing the expansion of mercury with a heat source.

The main malfunction that mercury thermometers may face concerns the possibility that during the reversal the mercury contained in the capillary will break at a height other than the "break-off point". This can happen due to the presence of bubbles formed by residual gas remained inside the thermometer during construction. This gas should remain confined at the upper end of the thermometer, in the secondary tank, but it can happen that a bubble penetrates the column of mercury, causing it to break in the wrong place. To avoid the problem, the thermometers must be stored and transported in a vertical position (with the main tank down) and handled gently.

b. Symbol, units and precision:

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: *t* **Unit**: °C (degree Centigrade)

Precision: Mercury reversing thermometer: ± 0.01°C

Digital electronic thermometer: **Accuracy:** ±0.0001 °C

Method identifier:

SDN:P01::**TEMPRTNX**	Temperature of the water body by reversing thermometer
SDN:P01::**TEMPR601**	Temperature (IPTS-68) of the water body by reversing thermometer
SDN:P01::**TEMPR901**	Temperature (ITS-90) of the water body by reversing thermometer

1.2.3 Protocol for sample preservation of seawater for the determination of salinity

a. Equipment

The equipment for the sample preservation of seawater for the determination of salinity include:

i) Niskin bottles arranged on cable or on a multiple sampler (rosette);

ii) Glass bottles with perfect sealing caps from 120-250 ml (the necessary volume depends on the salinometer in use). To avoid leaks and evaporation, the use of glass bottles with cap and undercap is recommend.

b. *Procedure*

The sample bottle must be carefully rinsed (at least three times), using the same water as the sample.

The bottle must be filled up to the base of the neck, thus leaving enough space for the eventual thermal expansion of the water.

The cap, the screwing area and the neck of the bottle must be thoroughly rinsed and dried to avoid the formation of salt crystals that could precipitate and dissolve in the sample upon reopening in the laboratory.

The cap and undercap must be thoroughly tighten to avoid evaporation between the time of collection and analysis in the laboratory.

c. *Storage of samples*

For best results it is preferred to analyse the samples as soon as possible and only when their temperature is in equilibrium with that of the laboratory. Thermal equilibrium is typically achieved in 4-5 hours, but it can be accelerated by ensuring a good flow of air around the bottles or by immersing them in a water bath (Stalcup, 1991[13]). However, if kept at room temperature in bottles well capped, the samples remain unaltered for a few weeks, unless the variations of conductivity due to changes in pH, which can also cause changes in the salinity value to the second decimal place (Grasshoff, 1983)[14]. The tightness and chemical inertia of the bottles are factors determinants for a good conservation of the samples.

d. *Important notes*

It is advisable to write down the position number on the bottle that collects the sample Niskin bottle on the sampler. This will aid in the sampling phase and will minimize the possibility of collecting the sample on the wrong Niskin.

During the collection of the sample, to avoid contamination, attention must be paid to the water surface dripping from the external parts of the sampler. The same care should be taken in case of rain.

Undercaps should be changed every 2-3 years or when deformations occur.

1.2.4 Protocols for sample preparation and determination of salinity using bench salinometer

a. *Equipment*

The equipment for for sample preparation and analysis of salinity include: i) a laboratory salinometer; ii) IAPSO standard water bottles.

b. *General analytical procedure:*

b.1 Preparation

The salinometer must be turned on well in advance (at least two hours before the analysis), to stabilize the operation of its electrical parts and, when present, the temperature of the thermostatic bath.

Two bottles of standard water and the samples must be brought in the vicinity of the salinometer with which the measurement is carried out and allow a certain period of time to pass until they reach the same temperature.

b.2 Standardization

The measuring cell must be rinsed at least ten times with sea water with a salinity of about 35. There are bottles of water with these characteristics on the market, alternatively the standard water residues used in previous operations can be used.

The standard water bottles must be shacked carefully and gently to homogenize their contents, avoiding the formation of bubbles.

The standard water bottle must be opened and inserted into the salinometer sampling device.

The measuring cell must be rinsed at least four or five times with standard water.

The measuring cell is then filled with standard water and the salinometer standardized according to the procedure indicated by the manufacturer.

At least two or three measurements of the same standard water must be carried out, unloading and filling the cell each time and checking that the salinity value read after standardization coincides with the salinity value indicated on the standard bottle. If the value does not match, the standardization procedure with a new bottle of standard water must be repeated.

b.3 Measurement

With repeated overturning of the bottle the sample is homogenize avoiding too vigorous shaking not to allow the formation of air bubbles.

The measuring cell must be rinsed with the sample at least four or five times.

The measuring cell is filled with the sample and the outputs read.

[13] Stalcup M.C. ,1991. Salinity measurements. In: WOCE Operational Manual WHPO 91-1, WOCE Report No 68 (http://whpo.ucsd.edu/manuals.html).

[14] Grasshoff, K., 1983. Determination of salinity. In: Grasshoff K., Ehrhardt M., Kremling K. (eds), Methods of Seawater Analysis, Verlag Chemie; Weinheim: 31-60.

The measuring cell is unloaded and filled again with the sample for new reading.

The operation referred to in the previous point is repeated until the difference between two consecutive readings is not less than the level of precision declared by the manufacturer of the instrument.

c. Calculations:

Having determined the conductivity ratio, R_t, between the sample and the standard water at temperature t_{68} (expressed on the IPTS-68 scale), the practical salinity is calculated according to the following equation, valid in the interval for S [2.42] (UNESCO, 1983):

$$S = a_0 + a_1 R_t^{1/2} + a_2 R_t + a_3 R_t^{3/2} + a_4 R_t^2 + a_5 R_t^{5/2} + \Delta S$$

where

$$\Delta S = (b_0 + b_1 R_t^{1/2} + b_2 R_t + b_3 R_t^{3/2} + b_4 R_t^2 + b_5 R_t^{5/2}) \cdot (t_{68} - 15)/[1 + k(t_{68} - 15)]$$

t_{68} is expressed in °C. The temperature in the ITS-90 scale is converted in t_{68} with the equation,

$$t_{68} = 1.00024 \cdot t_{90}.$$

The values of constants are listed below:

a_0	=	0.0080	b_0	=	0.0005	k	=	0.0162
a_1	=	-0.1692	b_1	=	-0.0056			
a_2	=	25.3851	b_2	=	-0.0066			
a_3	=	14.0941	b_3	=	-0.0375			
a_4	=	-7.0261	b_4	=	0.0636			
a_5	=	2.7081	b_5	=	-0.0144			

d. Important notes

Depending on the salinometer in use for the measurement, the procedure indicated may require some modification. It is recommended to check it, following the instructions in the instrument's instruction manual.

The formation of air bubbles in the sample during the pouring or mixing of the sample itself must be avoided. If this happens, the problem can be solved emptying and refilling the cell.

In the presence of deposits and / or air bubbles on the internal components of the measuring cell during use, washing attempts by pumping soapy water or weakly acid solutions into the cell must be avoided, because in addition to the possibility of being ineffective in solving the problem, can have a negative effect on factory calibration and instrument standardization. In the case, the cell must be repeatedly rinsed with deionized water. If the problem persists, the cell can be removed carefully, disassembled and cleaned as indicated in the instrument's instruction manual.

It is recommended to repeat the standardization procedure at least once a day with which the stability of the measuring apparatus electronics is maintained. If variations in the standardization values is observed, it is advisable to check the quality of the standard water bottle in use by repeating the operation with a new bottle. If the variations persist, it may be that the salinometer needs maintenance and needs to be sent to the service company.

The exposure to the air of standard water must be minimized.

For each sample, the time taken to obtain a valid measurement must be limited to the minimum time necessary as the minimum number of readings, avoiding that the volume used falls below the minimum necessary for the analysis.

The use of standard water bottles from the same batch for the same campaign is recommended, otherwise it is necessary to take into account the differences between batches as described by Mantyla (1987)[15] to correct the final salinities. In addition, if the bottles are older than two or three years, it is recommended to compare them with fresher standards to highlight any changes in conductivity due to aging.

e. Symbol, units and precision

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 a Method identifier as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Salinity:

Symbol: S **Unit**: -

Precision: Conductivity sensor: ± 0.01

Method identifier:

SDN:P01::**PSALBSTX** Practical salinity of the water body by bench salinometer and computation using UNESCO 1983 algorithm

1.3 Technical note for measuring Secchi depth

Water transparency serves as an index for the trophic state of a water body. It reflects eutrophication through changes in the phytoplankton abundance; increase in the ambient nutrient status in the water leads to higher phytoplankton biomass that diminishes the propagation of light in the water. Water transparency is approached

[15] Mantyla, A.W., 1987. Standard seawater comparison. J. Phys. Oceanogr., 17: 543-548.

by Secchi depth (Cialdi and Secchi 1865[16], Whipple 1899[17]). Secchi depth is influenced by dissolved and/or colloidal inorganic and organic substances as well as total suspended solids and resident seston. It is thus affected by substances unrelated to eutrophication as well.

Secchi depth relates to primary production by being a proxy for the thickness of the euphotic zone wherein the large bulk of the gross production takes place. In principle, the euphotic depth is twice Secchi depth, but this relation varies largely in practice (French et al., 1982)[18].

Under this Technical Note, the Monitoring Guidelines for Determination of Hydrographic Physical Parameters elaborate the IMAP Protocol for measuring Secchi depth.

1.3.1 Protocol for measuring Secchi depth

The methodology is based on the ISO 7027-2:2019[19] standard.

a. Equipment:

Testing disk (Secchi disk). A white disk with a diameter of 30 cm. The disk should weigh at least 1.7 kg to descend quickly and not be affected by horizontal water movements. Should the disk be lighter, an additional weight can be fastened to the down-facing side of the disk. As the observed Secchi depth tends to increase with the diameter of the disk (Aas et al., 2014)[20], the disks of other sizes are not advised to be used.

Measuring tape/rope of non-elastic material. Depth[21] recognition:

- colour-coded marks at 10 cm intervals. The upper side of the disk equals 0 cm. Half and full meters should be marked to be easily distinguishable.
- depth indicator of a winch.

Weight for waters with currents, fixed in the middle of the down-facing side of the disk.

Optional devices for suppression of reflections, e.g., polarized glasses for the observer.

b. Measuring:

The observer should try to ensure that the measuring rope stays in an as upright position as possible. Deviations from the upright position stem from water currents and waves as well as ship's movement and thruster operation.

The Secchi depth is measured on the shaded side of the ship to avoid direct sunlight reflections from the water surface. However, the observer must consider the source of error in the shaded side that occurs whenever the Secchi depth stretches beyond the shade of the ship. In this case, the disk is suddenly lighted by the sun and a higher reading will be attained.

Enough time must be allowed (preferably 2 min) when looking at the disc near its extinction point for the eyes to completely adapt to the prevailing luminance level. The disc must be lowered further until it is no longer visible. The achieved depth is to be read and written down. After that, the disc is lowered by another 0.5 m. Then, during a slow elevation, the disc becomes visible as a greenish-bluish spot. The achieved depth is to be read and written down. It is recommended to repeat the test two times as a minimum. The Secchi depth is the arithmetic average of all readings.

The precision of a Secchi measurement depends on the turbidity of the water. In the waters of high turbidity, the precision can approach 0.1 m under calm seas. In clearer waters, the precision ranges from 0.2 to 0.5 m, depending on actual conditions (e.g., waving or sun glitter).

c. Important notes

Secchi depth determination is sensitive to weather conditions:

- *Waving:* Optimally, Secchi depth should be measured when the sea is relatively calm. Waving introduces a source of error in the Secchi measurement by worsening the overall visibility, and waves > 0.5 m in height obscure the identification of the actual surface. The length reading of the rope at the surface should be judged to be an average of the extreme values due to waving. The determination of Secchi depth is not meaningful in high seas.
- *Sunlight:* Secchi depth should be determined to avoid direct sunlight reflections from the water surface. Sun glitter decreases the Secchi depth estimation irrespective of optical properties of water; on the average by 12% (Aas et al., 2014).

[16] Cialdi, M. and Secchi, P. A., 1865., Sur la transparence de la mer. Comptes Rendu de l'Acadamie des Sciences 61: 100–104.

[17] Whipple, George C., 1899., The microscopy of drinking-water. New York: John Wiley & Sons. pp. 73-75.

[18] French RH, Cooper JJ, Vigg S., 1982., Secchi disc relationships. Water Resources Bulletin 18: 121-123.

[19] ISO 7027-2:2019 Water quality — Determination of turbidity — Part 2: Semi-quantitative methods for the assessment of transparency of waters.

[20] Aas, E., Høkedal, J., Sørensen. K., 2014., Secchi depth in the Oslofjord–Skagerrak area: theory, experiments and relationships to other quantities. Ocean Science 10: 177–199.

[21] Secchi depth measurement is dependent on the observer's eyesight, and any aids for vision tend to increase Secchi depth, which should be considered, e.g., in the context of long-term data series.

The length markings of the rope should be checked and made clearer annually. The rope should be changed whenever it stretches > 5%.

d. *Symbol, units and precision*

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 a Method identifier as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: z_{SD} **Unit:** m

Precision: 0.2-0.5 m

Method identifier:

SDN:P01::**SECCSDNX** Visibility in the water body by Secchi disk

2 Guidelines for hydrographic chemical parameters: sampling and determination

2.1 Introduction

In this Monitoring Guidelines for the determination of Hydrographic Chemical Parameters the supporting chemical parameters dissolved oxygen and pH are elaborated. Dissolved oxygen (DO) is an essential component which determines the water quality and trophodynamics of an aquatic system. On the other hand, the pH today is important mainly due to the acidification process: when CO_2 is absorbed by seawater, a series of chemical reactions occur resulting in the increased concentration of hydrogen ions (pH). This process has far reached implications for the ocean and the creatures that live there.

The IMAP Protocols elaborated within this Monitoring Guidelines for Determination of Hydrographic Chemical Parameters provide detail guidance on the necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the core hydrography chemical supporting parameters, calculations, data transformation if necessary and identify weak points, including important specific notes and elaborated 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.

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) and Data Quality Assurance schemes (UNEP/MAP, 2019b) 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), however providing detail procedures that are of relevance for IMAP implementation. With the details of the protocols for hydrographic chemical parameters, the needs of the measurements both in offshore areas and in narrow coastal area are addressed.

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.

2.2 Technical note for measuring dissolved oxygen

The concentration of dissolved oxygen (DO) present in sea water depends on physico-chemical factors that determine the solubility of the gas and on biological activities (photosynthesis and respiration). Knowing temperature and salinity of the water, it is possible to trace the concentration of the theoretical dissolved oxygen which does not consider the processes of organic production and consumption. The positive (over-saturation) or negative (under-saturation) imbalance between the experimentally obtained and theoretical oxygen concentration is often used as an estimate of the processes prevalent in the water column, i.e. the prevalence of oxygen production by photo-synthetic processes, or consumption by the processes of mineralization of organic debris. From a precise determination of the DO concentration, it is therefore possible to estimate the net production and respiration of the planktonic community.

Under this Technical Note, the Monitoring Guidelines for Determination of Hydrographic Chemical Parameters elaborate the Protocol for sample preparation and analysis of dissolved oxygen in seawater by Winkler method.

2.2.1 Protocol for sample preparation and determination of dissolved oxygen in seawater by Winkler method

The Winkler titration method for the determination is based on the method developed by Winkler in 1888 (Winkler 1888)[22]. The method has seen several modifications to encompass interferences, and the basic method today for the determination of oxygen

[22] Winkler, L.W., 1888. Die Bestimmung des in Wasser gelösten Sauerstoffen. Berichte der Deutschen Chemischen Gesellschaft, 21: 2843–2855.

concentration is the one prepared by Grasshoff (1983)[23]. It is an iodometric titration, in which the amount of oxygen in the sample is determined indirectly via iodine. It is the most precise and reliable titrimetric procedure for DO analysis.

Briefly: A divalent manganese solution is added followed by strong alkali to a water sample in a glass stoppered bottle. Any DO present in the sample rapidly oxidizes an equivalent amount of the dispersed divalent manganous hydroxide precipitate to hydroxides. The sample is then acidified with H_2SO_4. In the presence of iodide ions in an acidic solution, the oxidized manganese reverts to the divalent state, with the liberation of iodine equivalent to the original DO content. The iodine is then titrated with sodium thiosulfate and starch as an indicator. For the analysis of field samples, DO analysis is best done in the field, as there is less chance for the sample to be altered by atmospheric equilibration, changes in temperature and chance of escape of gasses.

a. <u>*Sample preparation*</u>

<u>*a1. Equipment*</u>

The equipment for sample preparation of dissolved oxygen in seawater by Winkler method include:

i) Transparent plastic tube (e.g. Tygon) to be connected to the sampling bottle taps;
ii) 60-90 ml pyrex bottles, BOD type, with ground flute beak or rounded truncated cone cap. Each bottle must have been pre-calibrated for its closed cap volume with an accuracy of ± 0.1ml;
iii) Laboratory glass-ware;
iv) Dispenser, automatic micropipettes or polyethylene syringes with notches every 0.5 mL;
v) Insulated container, shielded from light;
vi) 100 mL volumetric flasks;
vii) 6 bottles for the determination of the reagent blank. These bottles must be selected from those with a known volume used for the withdrawal of oxygen, preferably so that they are two by two of equal volume (± 0.1 mL), and with a difference in volume between one pair and the next of 1 ± 0.1 mL.

<u>*a.2. Chemicals*</u>

The following reagents and chemicals are needed:

i) manganese chloride [$MnCl_2 \cdot 4H_2O$] or manganese sulphate [$MnSO_4 \cdot H_2O$];

ii) sodium hydroxide [NaOH] or potassium hydroxide [KOH];

iii) potassium iodide [KI].

[23] Grasshoff, K., 1983. Determination of oxygen. In: Grasshoff, K., Ehrhardt M., Kremling K. (eds), Methods of Seawater Analysis, Verlag Chemie, Weinheim: 61-72.

<u>*a.3. Preparation of reagents*</u>

Solution of Mn_2^+ (R1)

40 g of $MnC1_2 \cdot 4H_2O$ or 35 g of $MnSO_4 \cdot H_2O$ is dissolved in 80 ml of reagent grade water and adjusted to volume in a 100 mL flask. The reagent, if stored in a closed bottle and not inadvertently contaminated with R2 containing iodide, is stable indefinitely.

Alkaline solution of ion I^- (R2)

20 g of sodium hydroxide or 30 g of potassium hydroxide is dissolved in 40 mL of reagent grade water. 60 g of potassium iodide is dissolved in 40 ml of reagent grade water. The two solutions are gradually mixed in a flask and adjusted to a final volume of 100 mL with H_2O. The solution should then be stored in a dark, well-capped plastic bottle. If it is not contaminated with R1 or with reducing or oxidizing agents, it is stable indefinitely.

<u>*a.4. Procedure*</u>

The sub-sampling of DO from the Niskin bottle, or similar, must be done quickly as the dissolved gas tends to balance itself with the atmosphere. This process will be further accelerated by the temperature difference existing between the sample and the environment.

For sub-sampling, the transparent plastic tube to the Niskin bottle, possibly with a diameter of no more than 5 mm and a length that can easily reach the bottom of the BOD bottles for sample collection is connected.

The bottles, previously cleaned from the residues of the previous samplings and analyzes, are rinsed with water from the sample to be analysed. To prevent the formation of a layer of supersaturated oxygen along the walls the bottles are not shacked vigorously.

The sample is allowed to flow into the bottle, checking that the filling tube is free of air bubbles and avoiding the bubbling of air in the sample. The sampling tube must touch the bottom of the bottle, which must be filled slowly by overflowing a quantity of water equal to at least half of its total volume.

The tube is slowly removed from the bottle, always letting the water flow, so that the bottle always remains full to the brim. Before adding the reagents, it is checked that no air bubbles are trapped in the bottle, otherwise the bottle is emptied, and the filling operation repeated.

In the case bottles for BOD of about 100 ml is used, 0.5 mL of R1 and 0.5 mL of R2 is added in rapid succession, using two automatic dispensers or two normal syringes equipped with a long and narrow needle in order to inject the reagents at least below the free surface of the sample, preferably at the bottom of the bottle. In the case bottles for BOD with a volume other than about 100 ml are used proportional volumes of R1 and R2 are added.

The cap is carefully inserted avoiding the formation of air bubbles between the cap and the liquid, letting excess

water escape. The well capped bottle is shacked inverting it several times for at least 30 seconds.

The bottles are placed in a dark place at a temperature similar to that of sampling. After the precipitate settle for 2/3 of the volume the bottles are shacked again. To further limit the possibility of gas exchange with the environment, it is suggested to keeps the cap firmly pressed on the neck of the bottle, using for example rubber bands, adhesive tape, etc.

Sampling for the determination of the reagent blank

Pre-selected bottles for blanks are sampled from the same sampling bottle, preferably not from the one relating to the surface level.

One dose of each reagent is added to the lowest volume pair of bottles, two doses to the volume greater than 1 mL and three doses to the volume greater than 2 mL. This operation must be carried out at least once during a sampling day.

a.5. Sample storage

The fixed samples should be stored in the dark and at a temperature as close as possible to that of sampling and analysed within the day of sampling.

Theoretically, the fixed samples could be kept for a longer time if there were no gaseous diffusion through the closures of the corks which unfortunately occurs, albeit to different degrees, in all bottles. In order to reduce this phenomenon, it is customary to keep the bottles, well closed, completely immersed in water of the same origin temperature as the sample.

b. *Analytical procedure*

b.1. Equipment in the laboratory

The equipment for analysis of dissolved oxygen in seawater by Winkler method includes:

i) 1 L class A volumetric flasks
ii) 1 mL or 5 mL glass or piston microburette
iii) 5 Pyrex bottles of the same type as those used for sampling
iv) 0.500 mL precision micropipette; 0.200 mL micropipette
v) Fluorescent lamp with opaque screen or diffuser
vi) Cold magnetic stirrer
vii) Magnetic stirrers
viii) 2 automatic dispensers or micropipettes or polyethylene syringes with notches every 0.5 mL (for oxygen reagents)
ix) 1 mL dispenser (for concentrated sulfuric acid).

An alternative to the micro burette is:

i) Potentiometric titrator;
ii) Combined redox platinum electrode, semi-micro.

b.2. Chemicals

The following reagents and chemicals are needed:

i) Sodium thiosulfate [$Na_2S2O_3 \cdot 5H_2O$];
ii) Potassium iodate [KIO_3], possibly ultrapure;
iii) Sodium chloride [NaCI];
iv) Chloroform [$CHC1_3$] or sodium-azide [NaN_3];
v) Soluble starch;
vi) Concentrated sulfuric acid, analytical grade [H_2SO_4].

b.3. Preparation of reagents

Thiosulfate solution ~ 0.1 mol L^{-1} (or ~ 0.1 M)

24.82 g of $Na_2S_2O_3 \cdot 5H_2O$ are dissolved in 800 ml of reagent grade H_2O in a 1 L volumetric flask and adjusted to the mark. Few drops of chloroform or sodium azide are added as a stabilizer.

The solution should be stored in a dark glass bottle. Since thiosulfate is involved in numerous redox reactions, the solution is relatively unstable and therefore must be standardized against the potassium iodate solution before and after use. It is possible to use pre-stabilized commercial vials of thiosulfate solution of known titre.

KIO_3 standard solution 0.01667 mol L^{-1} (or 0.01667 M)

About 5 g of iodate are dried in an oven at 110 °C for at least an hour and cooled in the dryer or taken directly from a stock that was once dried and stored cold in a dryer in the presence of a strong dehydrator. Exactly 3.567 g are weighted and then dissolved quantitatively in 800 mL of reagent grade water in a 1 L volumetric flask (class A). The solution is adjusted exactly to volume at a temperature around the flask calibration (usually 20-25 ° C). Commercial iodized standard vials are also available.

The solution must then be stored in tightly capped dark glass bottles, kept away from the sun and opened for the shortest time possible only for sampling. Under these conditions the standard solution is to be considered stable for at least one year.

Stabilized colloidal starch solution (starch weld)

A saturated solution of sodium chloride by dissolving approximately 350 g of it in 1 L of distilled water in a beaker is prepared. 10 g of soluble starch in the saturated sodium chloride solution is hot dissolved.

The solution should be kept in a dark bottle and can be used until it becomes cloudy and flocculates.

b.4. Preparation of standard solutions

5 BOD bottles are filled to 3/4 of the volume at least with sea water or with distilled water and 0.5 mL of concentrated sulphuric acid, 0.5 ml of R2 and 0.5 mL of reagent R1 are added to each one in succession using the same dispensers used to "fix" the samples. It is

preferable to carry out these operations under continuous stirring, allowing the complete mixing of each reagent before adding the next. The bottles can then be capped and stored in the dark until the iodate standard solution is added;

Exactly 10.00 mL of standard KIO_3 solution to each bottle using an automatic pipette are added;

The bottle is shacked a few seconds and placed in the dark for about 1 minute to allow the reaction of iodate dismutation to take place by producing molecular iodine. The standards are then titrated with the thiosulfate solution as indicated below for the samples.

b.5. Analysis of samples

Dissolution of the precipitate

The bottle number and its volume are recorded;

The cap is gently removed from the bottle containing the precipitate and placed on the switched off magnetic stirrer;

A magnetic stir bar is quickly inserted into the bottle trying to lift as little precipitate as possible, add 0.5 mL of concentrated sulphuric acid with a dispenser, the stirrer is started by adjusting its speed in order to avoid the formation of vortices and turbulence;

When the complete dissolution of the precipitate (the solution becomes a clear yellowish colour due to the presence of iodine) is achieved, as sun as possible the titration with sodium thiosulfate is performed.

Titration

The tip of the burette containing the thiosulfate solution is immersed in the bottle containing the sample or standard;

At the beginning the thiosulfate solution is added rapidly, and then the flow is slowed when the yellow colour of the sample clears and, importantly, and it is stopped before the total disappearance of the yellow colour.

When the solution is almost colourless, the lamp is turned on and about 0.2 mL of starch solution is added (an intense purple colour appears), the addition of thiosulfate is resumed slowly until the blue colour almost disappears.

After few seconds, when viewed transparently against diffuse fluorescent light, a faint dispersed colour like a cloud is displayed in the bottle. The titration is proceeded very slowly until the complete disappearance of the colour, the end point (EP) of the titration. The volume of added thiosulfate is recorded.

If an automatic titrator with combined redox / platinum electrode is used, the titration program must show a decrease in the titrant flow near the EP which will correspond to the inflection point of the titration curve.

c. Calculations

c.1. Standardization of thiosulfate (C_{tio})

The prepared KIO_3 standards are titrated with the ~ 0.1 M thiosulfate solution (see “Preparation of reagents”).

The molar titre C_{tio} of the thiosulfate solution is:

$c_{tio} = 6 * (V_{KIO3} * c_{KIO3}) / V_{tio}$

where:

c_{tio} = exact molar concentration (M) of the $Na_2S_2O_3 \cdot 5H_2O$ solution

V_{KIO3} = volume in mL of injected KIO_3 standard (see "Preparation of standard solutions")

c_{KIO3} = molar concentration (0.01667 M) of the KIO3 standard used

V_{tio} = volume in mL of thiosulfate required to titrate the standard

The mean and standard deviation of V_{tio} in the replicates must be calculated and any value that differ by more than two standard deviations from the mean discarded. The mean and standard deviation of V_{tio} which will be used in the calculation of c_{tio} must be recalculated with the new values.

c.2. Determination of the reagent blank

The 3 pairs of bottles dedicated to the determination of the blank must be titrated and the volume of thiosulfate used noted. The concentration of DO (see "Calculation of dissolved oxygen concentration") must be calculated as if the blank (c_{bl}) were zero. The slope of the correlation line between the concentrations of DO thus obtained and the volume of R1 + R2 added corresponds to the blank of the reagents (c_{bl}).

A simpler way would be to calculate the difference between the average values for each pair of bottles and the next, but given the considerable variability in the differences this method is to be used in the alternative. This procedure allows the determination of the reagent blank, not that of the sample blank, i.e. the presence in the sample of interfering chemical substances (e.g. iodate) capable of producing elemental iodine in the acidified solution. If the desired level of accuracy required it, the blank should also be measured for each sample, according to the procedure suggested by Tijssen and van Bennekom (1989)[24].

c.3. Calculation of the micro-molar concentration (M or μmol L^{-1}) of dissolved oxygen

The following equitation applies for calculation of the micro-molar concentration (M or μmol L^{-1}) of dissolved oxygen:

$c(O_2)/\mu mol\ L^{-1} = [(c_{tio} \cdot V_{tio}) / (4 \cdot (Y - y)) \cdot 10^6] - c_{bl}$

where:

[24] Tijssen S.B., Van Bennekom A.J., 1989. High precision determination of dissolved oxygen. ICES C.M. 1989/c:6, Annex C: 11-12.

c_{tio} = exact molar concentration of the $Na_2S_2O_3 \cdot 5H_2O$ solution, from the standardization procedure

V_{tio} = volume in mL of thiosulfate required to titrate the unknown sample

c_{bl} = reagent blank (see reagent blank determination)

Y = volume in mL of the specific BOD bottle used for each sample

y = total volume, in mL, of reagents R1 + R2 added to each sample bottle (in the case shown, 1 mL)

c.4. Transformations :

The transformation of units needs to follow below provided scheme:

The next numerical equation must be used

$c(O_2$, Unit A$) = c(O_2$, Unit B$) \cdot$ Conversion factor

Unit A	Unit B	Conversion factor
mg L^{-1}	mL L^{-1}	0.6997637
mg L^{-1}	µmol L^{-1}	0.0319988
mL L^{-1}	µmol L^{-1}	0,0223916

d. Dissolved oxygen expressed as a percentage of the saturation value

The calculation of the percentage of the saturation value can be made only by knowing the value of the oxygen solubility in the sea water sample that has been analysed. It is known that the solubility of a gas in a liquid depends not only on the properties of the solvent (composition and temperature), but also on the partial pressure exerted on the solution by the gas in question (Henry's law). The solubility value therefore corresponds to the amount of oxygen that would dissolve in water in conditions of equilibrium between the surface layer of the sea and the atmosphere above.

To determine it, reference is made to a sample in thermodynamic equilibrium with a gaseous mixture of composition equal to the standard atmosphere, at the pressure of a standard atmosphere (mole fraction of oxygen = 0.20946) and saturated with water vapour. Depending on whether the oxygen concentration is related to the unit of mass or volume of the solvent, two concentration values are obtained, called USAC (acronym for "Unit Standard Atmospheric Concentration). These values are represented by the symbols C_0^i and C_0^* according to the symbology introduced by Benson and Krause (1980[25], 1984[26]). These quantities have recently been recalculated on the basis of a more rigorous procedure introduced by the authors themselves and subsequently recommended by UNESCO (Millero, 1986)[27] to replace the values contained in the UNESCO oceanographic tables (1973)[28] which were based on the Weiss algorithms (1970)[29].

The equation is the product of numerical interpolations of data obtained from equations that more rigorously calculate the needed quantities. Furthermore, it should be noted that the equation is based on the practical temperature scale of 1968 (IPTS-68) and therefore, if values measured on the basis of the ITS-90 scale are used, the appropriate conversions must be applied.

d.1. Calculations

The following equitation applies for calculation of Dissolved oxygen expressed as a percentage of the saturation value:

$\varphi(O_2/O_2') = 100 \cdot c(O_2)/C_0$

where

$\ln C_0 = a_0 + a_1/T + a_2/T^2 + a_3T^3 + a_4/T^4 - S \cdot (b_0 + b_1/T + b_2/T^2)$

In the equation C_0 corresponds to the concentration of the theoretical DO C_0^i and C_0^* reported per unit of volume. The constants to be inserted in the equation are:

$a_0 =$	-135.90205
$a_1 =$	$1.575701 \cdot 10^5$
$a_2 =$	$-6.642308 \cdot 10^7$
$a_3 =$	$1.243800 \cdot 10^{10}$
$a_4 =$	$-8.621949 \cdot 10^{11}$
$b_0 =$	0.017674
$b_1 =$	-10.754
$b_2 =$	2140.7

e. Important notes

When fixing the samples contact between the reagents R1 and R2 must be avoided.

During manual titration, the same criterion for identifying the titration EP for both standards and samples must be used, best avoiding changing operator.

The titration must be performed quickly, decreasing the flow of thiosulfate only in the vicinity of the titration EP, in order to minimize errors due to the photo-oxidation of the iodide and the reduction of iodine by the starch.

[25] Benson B.B., Krause D. Jr., 1980. The concentration and isotopic fractionation of gases in freshwater in equilibrium with atmosphere. Limnol. Oceanogr., 25: 662-671.

[26] Benson B.B., D. Krause, Jr., 1984.The concentration and isotopic fractionation of oxygen dissolved in freshwater and seawater in equilibrium with atmosphere. Limnol. Oceanogr., 29: 620-632.

[27] Millero F.J., 1986. Solubility of oxygen in seawater. UNESCO Technical Papers in Marine Science, 50: 13- 17.

[28] UNESCO. 1973. International oceanographic tables, Vol. 2. NIO-UNESCO, Paris.

[29] Weiss, R.F., 1970. The solubility of nitrogen, oxygen and argon in water and seawater. Deep-Sea Research, 17: 721–735.

f. *Possible problems*

A problem that usually occurs is the formation of bubbles in the bottle containing the sample; to prevent this phenomenon, the bottles must be washed with detergents and rinsed thoroughly.

Sometimes an air bubble is formed under the cap of the bottle containing the sample already fixed; in this case, the possible existence of an error due to the excess, however not quantifiable, of the amount of dissolved oxygen must be considered and noted.

g. *Symbol, units and precision*

For the parameters described in this protocol, the symbols and units 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:

Concentration of dissolved oxygen

Symbol: $c(O_2)$ **Unit:** µmol L^{-1}

Precision: 0.1

Method identifier:

SDN:P01:: **DOXYWITX** Concentration of oxygen {O2 CAS 7782-44-7} per unit volume of the water body [dissolved plus reactive particulate phase] by Winkler titration

Saturation of Dissolved Oxygen

Symbol: $\varphi(O_2/O_2')$ **Unit:** % *(percent)*

Precision: 0.1

Method identifier:

SDN:P01:: **OXYSBW01** Saturation of oxygen {O2 CAS 7782-44-7} in the water body [dissolved plus reactive particulate phase] by Winkler titration and computation from concentration using Benson and Krause algorithm

2.3 Technical note for measuring pH

Since ocean acidification is a growing concern, monitoring of pH is necessary for studies of acidification and its effects on the carbonate buffer system. As many important biological processes are likely to be affected by rapid changes in pH, it is important to include accurate determination of pH among monitoring parameters.

pH is operationally defined, and several pH scales are used in environmental monitoring. The NBS (National Bureau of Standards) scale is suitable for waters of low ionic strength and used for freshwater monitoring. The total hydrogen ion scale is often used for pH determinations in oceanic waters.

pH is also used in marine environmental monitoring as a co-factor in measurements of primary production.

Two different principles for pH measurement are available, based on potentiometric and spectrophotometric detection. Potentiometric detection has the advantages of being fast and simple and requires no advanced or expensive equipment. Buffers used for calibration should ideally have an ionic strength matching that of the samples, which is challenging when an area with a large salinity gradient is monitored. Several pH meters, electrodes and buffers are commercially available.

Spectrophotometric detection is more accurate, has a higher precision, but requires expensive equipment. It is widely used in measurements under oceanic conditions, but less in estuarine waters. Since commercial applications for the spectrophotometric methods are not widely used; users must assemble instruments and software for data processing. Methods based on spectrophotometric detection are therefore not yet recommended for monitoring purposes.

Therefore, under this Technical Note, the Monitoring Guidelines for Determination of Hydrographic Chemical Parameters elaborates the two following Protocols: i) the Protocol for sample preparation and analysis of pH using a potentiometric method; and ii) the Protocol for sample preparation and analysis of pH using a spectrophotometric method.

2.3.1 Protocol for sample preparation and determination of pH using a potentiometric method

pH is measured using a glass/combined electrode. The total hydrogen ion scale should be used. Temperature is measured and recorded both during pH measurement and at sampling depth.

Subsamples for pH should be drawn from sampler bottles as early as possible (after samples for oxygen and hydrogen sulphide, but before samples for nutrients and salinity) to avoid gas exchange between water and air. Samples should be collected in gas-tight bottles. Bottles should be rinsed thoroughly with sample water before filling. Bottles are filled with a laminar flow of sample water, allowing 2-3 bottle volumes to overflow before capping. Bottles should be completely filled, leaving no headspace. Avoid trapping bubbles of air when capping bottles. Samples should preferably be analysed as soon as possible directly after sampling.

Determination of pH using a glass electrode is described in ISO 10523[30].

Temperature must be monitored and controlled during calibration of instrument and analysis, preferably by use of a tempered water bath. Make sure temperature of buffers and samples is constant (±1 °C) during the process. To maintain constant temperature, select a bath temperature slightly above ambient temperature (for normal room temperature, set bath temperature to 25 °C – in a cooler environment 20 °C may have to be used). pH analysis can also be made in + 15 °C in a cooling bath which has been shown to produce comparable results.

pH meter should be calibrated daily when in use. Manufacturer's instructions for a 2-point calibration (pH 7 and pH 9 are recommended) are followed. NBS buffers for calibration is used. Attention to expiry dates of buffers has to be paid.

Electrode and temperature probe must be rinsed with deionized water and wiped between buffers/samples.

Electrode must be allowed to equilibrate in sample water for 15 minutes before first measurement. The best is if equilibrium is reached for each sample before recording a reading.

Open-cell measurements allow gas exchange between sample and air during the time of measurements. Closed-cell measurements eliminate the interferences.

Manufacturer's instructions must be followed for handling and storage of electrodes. Electrodes may require cleaning and conditioning when exposed to samples from intense plankton blooms. Anoxic water containing high concentration of hydrogen sulphide may shorten the life of electrodes.

A correction for in situ pH (Gieskes, 1969)[31] is sometimes applied. A better option is to report measured pH, temperature from pH measurement and in situ temperature.

pH values from potentiometric detection should be reported with two decimals. Temperature from measurement and sampling depth should also be reported.

Information on which pH scale is used must be included in metadata.

A detailed analytical protocol for the analysis of pH using a potentiometric method (Dickson et al, 2007)[32] is presented in the Annex I. (Guide to best practices for ocean CO_2 measurements, Ch: 4. Recommended standard operating procedure, SOP6a: Determination of the pH of sea water using a glass/reference electrode cell).

a. <u>*Quality control*</u>

Laboratories carrying out analyses of pH should have established a quality management system according to ISO 17025[33].

Data for samples for estimation of measurement uncertainty (repeated measurements from a sample, multiple subsamples from different samplers closed at same depth).

An internal reference material (IRM) should be analysed daily.

It is strongly recommended that all laboratories participate in interlaboratory comparisons and proficiency testing programs, to provide external verification of laboratory performance. Proficiency testing for pH in environmental waters are provided by e.g. SYKE. More proficiency testing schemes are listed at www.eptis.bam.de.

Validation of the adopted method needs to be performed on the relevant matrix and concentration range e.g. by taking part regularly at intercomparison studies or proficiency testing schemes.

Measurement uncertainty should be estimated using ISO 11352[34].

Estimation should be based on within - laboratory reproducibility, IRM, and, if available, data from proficiency testing and CRM.

b. <u>*Symbol, units and precision*</u>

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 a Method identifier as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: *pH* **Unit:**

Precision: ± 0.003 **Accuracy:** -

Method identifier:

SDN:P01::**PHXXPR01** pH per unit volume of the water body by pH electrode

[30] ISO 10523: Water quality – Determination of pH.

[31] Gieskes, J. M., 1969. Effects of temperature on the pH of seawater. Limnology and Oceanography Vol 14 Issue 5, p 679-685.

[32] Dickson, A.G., Sabine, C.L. and Christian, J.R. (Eds.) 2007. Guide to Best Practices for Ocean CO2 Measurements. PICES Special Publication 3, 191 pp.

[33] ISO 17025: General requirements for the competence of testing and calibration laboratories.

[34] ISO 11352: Water quality – Estimation of measurement uncertainty based on validation and quality control data.

2.3.2 Protocol for sample preparation and determination of pH using spectrophotometric method

Recently, the spectrophotometric method of measuring the pH value of seawater has been proposed, which consists in measuring the visible absorption of a coloured pH indicator added to the seawater sample. The measurement is precise, sensitive, and theoretically free from the need for calibrations (of calibration lines), but the instrumentation is more expensive and the analysis speed lower than the potentiometric method (Dickson 1993)[35]. As précised before, this method is therefore not yet recommended for monitoring purposes. To compare the pH values obtained by this method with the potentiometric ones, it must be referred to the total hydrogen ion concentration pH scale.

A detailed analytical protocol for the analysis of pH using spectrophotometric method recommended by the International Scientific Community (IOC and SCOR) collected in Dickson et al., 2007 is presented in the Annex II. (Guide to best practices for ocean CO_2 measurements, Ch: 4. Recommended standard operating procedure, SOP6b: Determination of the pH of sea water using the indicator dye *m*-cresol purple).

a. Symbol, units and precision

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 a Method identifier as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: *pH* **Unit:**

Precision: 0.001 **Accuracy:** ± 0.005

Method identifier:

SDN:P01:: **PHXXSP01** pH per unit volume of the water body by spectrophotometry

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- ISO 17025: General requirements for the competence of testing and calibration laboratories.
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A-2. Monitoring Guidelines/Protocols for sampling and determination of key nutrients and chlorophyll a in seawater

Table of Contents

1 Guidelines for seawater sampling and sample preservation ... 22
1.1 Introduction ... 22
1.2 Technical note for sampling of seawater for the determination of key nutrients and chlorophyll *a*.. 22
1.2.1 Protocol for the use of a single water sampler attached to a line ... 23
1.2.2 Protocol for the use of a water sampler attached to a rosette ... 23
1.3 Technical note for the sample preservation of seawater for the determination of key nutrients and chlorophyll *a* ... 24
1.3.1 Protocol for the sample preservation of seawater for the determination of concentration of nutrients 25
1.3.2 Protocol for sample preservation of seawater for the determination of concentration of chlorophyll *a* 27
2 Guidelines for the determination of key nutrients in Seawater ... 28
2.1 Introduction ... 28
2.2 Technical note for determination of concentration of nitrite (NO_2^-) ... 29
2.2.1 Protocol for manual colorimetric determination of concentration of nitrite ... 29
2.2.2 Protocol for automated colorimetric determination of concentration of nitrite ... 31
2.3 Technical note for determination of concentration of nitrate (NO_3^-) ... 32
2.3.1 Protocol for manual colorimetric determination of concentration of nitrate ... 32
2.3.2 Protocol for automated colorimetric determination of concentration of nitrate ... 35
2.4 Technical note for determination of concentration of ammonium (NH_4^+) ... 37
2.4.1 Protocol for manual colorimetric determination of concentration of ammonium ... 37
2.4.2 Protocol for automated colorimetric determination of concentration of ammonium ... 39
2.5 Technical note for the determination of concentration of orthophosphate (PO_4^{3-}) ... 40
2.5.1 Protocol for manual colorimetric determination of concentration of orthophosphate ... 41
2.5.2 Protocol for automated colorimetric determination of concentration of orthophosphate ... 43
2.6 Technical note for the determination of concentration of orthosilicate (SiO_4^{4-}) ... 44
2.6.1 Protocol for manual colorimetric determination of concentration of orthosilicate ... 45
2.6.2 Protocol for automated colorimetric determination of concentration of orthosilicate ... 47
2.7 Technical note for the combined determination of concentration of total nitrogen and total phosphorous ... 48
2.7.1 Protocol for preparation of samples for a combined determination of concentration of total nitrogen and total phosphorus ... 49
2.7.2 Protocol for combined manual colorimetric determination of concentration of total nitrogen and total phosphorous ... 49
2.7.3 Protocol for the combined automated colorimetric determination of concentration of total nitrogen and total phosphorous ... 51

3 Guidelines for the determination of Chlorophyll a in Seawater 51
3.1 Introduction 51
3.2 Technical note for determination of concentration of chlorophyll *a* 52
3.2.1 Protocol for sample pre-treatment for determination of concentration of chlorophyll *a* 53
3.2.2 Protocol for spectrophotometric determination of concentration of chlorophyll *a* 53
3.2.3 Protocol for fluorometric determination of concentration of chlorophyll *a* 55
3.2.4 Protocol for High-Pressure Liquid Chromatography (HPLC) determination of concentration of chlorophyll *a* 57

1 Guidelines for seawater sampling and sample preservation

1.1 Introduction

In the Monitoring Guidelines for sampling and sample preservation of seawater for the analysis of CI13 and C14: concentration of key nutrients and chlorophyll *a*, the protocols for sampling and sample preservation for salinity, nutrients and chlorophyll *a* are elaborated. Sampling and sample preservation are an important step within the monitoring process of the marine environment. Through proper sampling and sample preservation assessment of GES regarding Ecological Objective 5 related to eutrophication as presented in details in the IMAP Guidance Factsheets (UNEP/MAP, 2019) [1] will be allowed and maintained.

The IMAP Protocols elaborated within this Monitoring Guidelines for sampling and sample preservation of seawater for the analysis of CI13 and C14 regarding concentration of key nutrients and chlorophyll *a* provides detail guidance on the necessary equipment, procedures 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.

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 sampling and sample preservation the needs of the measurements both in off-shore areas and in narrow coastal areas are addressed.

1.2 Technical note for sampling of seawater for the determination of key nutrients and chlorophyll *a*

Sampling is an important step within the monitoring process of the marine environment. Although significant efforts have been made in designing procedures for analytical measurements, very little attention has been given to the sampling. Historically, analytical scientists have primarily been concerned with measurements made in the laboratory, and the process of sampling has been conducted by different people, who often even work in different organizations. The analytical scientist's knowledge of the sampling process is therefore sometimes very limited.

Sampling could be defined as a process of selecting a portion of material small enough in volume to be transported conveniently and handled in the laboratory, while still accurately representing the part of the environment sampled. The main difficulties in sampling are representativeness and integrity. Many people think that the analysis starts when the sample arrives in the laboratory. However, sampling is an integral part of the analytical process and sampling is its starting point. Sampling is so important that, in some cases, it represents the main contribution to the error of the whole analytical process.

Sampling should always start by defining the purpose of the measurement (Stoeppler, 1997[5]). If the different stages are under the responsibility of different people, there needs to be good communication between all parties involved. Sampling planners and analytical scientists need to optimize the whole measurement procedure, including the sampling step. The sampling plan should be written as a protocol that includes the following aspects:

- when, where and how to collect samples;
- sampling equipment, including its maintenance and calibration;
- sample containers, including cleaning, addition of stabilizers and storage;
- sample-treatment procedures (e.g., handling prior to measurements);
- sub-sampling procedures; and
- sample record-keeping (e.g., labelling, recording information, auxiliary information, and chain-of custody requirements).

Sampling frequency is therefore an important factor in terms of representativeness. Low sampling frequency could underestimate the occasional presence of samples with high analyte concentration. Sampling frequency is subject to a number of factors, e.g., transportation, access to the sampling site, the availability of test organisms, and financial constraints.

Under this Technical Note, this Monitoring Guidelines provides the following IMAP Protocols for the sampling of seawater for the determination of hydrographic

[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] Stoeppler M. (Ed.), 1997. Sampling and Sample Preparation: Practical Guide for Analytical Chemists, Springer Verlag, Berlin, Germany.

parameter and the measurement of concentration of key nutrients and chlorophyll a:

- Protocol for the use of a single water sampler attached to a line;
- Protocol for the use of a water sampler attached to a rosette.

1.2.1 Protocol for the use of a single water sampler attached to a line

a. *Principle of work*

The measurement of salinity and oxygen, nutrients and chlorophyll *a* requires the collection of water samples from various depths. This essential task is achieved with "water bottles". The first water bottle was developed by Fritjof Nansen, the Nansen bottle. It consists of a metal cylinder with two rotating closing mechanisms at both ends. The bottle is attached to a wire. When the bottle is lowered to the desired depth it is open at both ends, so the water flows through it freely. At the depth where the water sample is to be taken the upper end of the bottle disconnects from the wire and the bottle is turned upside down. This closes the end valves and traps the sample, which can then be brought to the surface.

In an "oceanographic cast" several bottles are attached at intervals on a thin wire and lowered into the sea. When the bottles have reached the desired depth, a metal weight ("messenger") is dropped down the wire to trigger the turning mechanism of the uppermost bottle. The same mechanism releases a new messenger from the bottle; that messenger now travels down the wire to release the second bottle, and so on until the last bottle is reached.

The Nansen bottle has now widely been displaced by the Niskin bottle. Based on Nansen's idea, it incorporates two major modifications. Its cylinder is made from plastic, which eliminates chemical reaction between the bottle and the sample that may interfere with the measurement of tracers. Its closing mechanism no longer requires a turning over of the bottle; the top and bottom valves are held open by strings and closed by an elastic band. Because the Niskin bottle is fixed on the wire at two points instead of one (as is the case with the Nansen bottle) it makes it easier to increase its sample volume. Niskin bottles of different sizes are used for sample collection. Nansen and Niskin bottles are used on conjunction with reversing thermometers.

b. *Procedure*

When the oceanographic cast is lowered to the desired depth, enough time to adapt to the sampling environment must be provided. It is mainly related to the measurement of temperature as the thermometers have to equalize with the local temperature. For digital reversing thermometers 2 minutes is required and 10 minutes for the mercury ones.

After the cast is fired (messenger released) the necessary time to all bottle are closed must be waited.

After the recovery of the bottles, usually they must be put on a sampler holder that provide easy sampling of the content and are not exposed to the direct sunlight, to minimize the heat exchange.

If sampled, the first step is to read the temperature.

The next sub-sampling protocol is maintained:

i) Dissolved oxygen and pH samples using tygon tubing;

ii) Salinity;

iii) then in the order nitrite, other nutrients; and

iv) chlorophyll-*a*.

The contamination sources must be avoided:

i) Contamination from the sampling equipment, ship and on- board activities should be avoided while sampling is undertaken. Some details are provided with single parameter.

ii) Sampling bottles should be cleaned with dilute HCL acid and washed with pure water and always be kept closed when not in use.

1.2.2 Protocol for the use of a water sampler attached to a rosette

a. *Principle of work*

A rosette sampler is made of an assembly of 6 to 36 sampling bottles. Each bottle is a volume that range from a minimum value of 1.2 L to a maximum value of 30 L. All of them constitutes the rosette sampler and are clustered around a cylinder situated in the centre of the assembly where there is a sensing the CTD. The apparatus is attached to a wire rope. A winch on board of the boat unroll the rope during descent and roll up it during the ascent (i.e. at the end of the samples collection). During operations in the ocean, a rosette sampler can approach the seabed at a distance from 1 to 5 m, depending on the particular sea conditions. The opening of each sampling bottle can be automatic (by reaching a certain depth) or manual (by operator, remotely).

b. *Procedure*

The rosette and CTD is a unique instrument and as many protocols for CTD measurements (WOCE 1991[6],

[6] WOCE, 1991.WOCE Operational Manual WHPO 91-1, WOCE Report No68/. (http://whpo.ucsd.edu/manuals.html).

UNESCO 1994[7], UNESCO, 1988[8]) are available and starting from what is suggested by these protocols and taking into account the field experience the protocol as provided hereunder is preferable.

The manufacturer's recommendations on preparations of the CTD and rosette sampler must be followed. Details on the CTD operation is presented in Chapter A-1 of the IMAP Monitoring Guidelines (Monitoring Guidelines/Protocols for Sampling and Analysis of Hydrographic Physical and Chemical Parameters).

The rosette should be lowered as close to the bottom as possible, though without risking bottom contact. The bottom depth and all the other information required by the CTD log or monitoring protocol are noted.

The rosette bottles should preferably be fired at selected standard depths during the up-cast in order to obtain an undisturbed CTD profile during the down-cast and undisturbed water samples on the way up. If the winch is maneuvered manually between each sampling depth, attention must be paid to approach the set depth as gentle as possible to reduce the disturbance of the water profile. This is especially important in stratified waters.

At each sampling depth the sampling bottles should have time to acclimatize and the effect of dragging water from deeper depth should be avoided. Wait at least 1 minute before the sampling bottles to be fired. If the CTD values still are not stable wait another 3 minutes before firing. If the bottles are equipped with reference sensors do not forget to wait the appropriate time for the sensors to measure after firing the bottle.

However, if the CTD and rosette is equipped and prepared for free-flow sampling bottles, it can be configured to fire water samples on predefined standard depths during the down-cast. Note that samples near the surface should be collected during up-cast to avoid trapping air bubbles mixed into the water by breaking waves and turbulence when the rosette is lowered.

If there is any leakage or malfunction to the CTD, water sampler or water bottles it must be reported. Questionable sensor readouts should also be noted. All events happened during the cast also must be noted. Manufacturer's instructions for cleaning the CTD after each cast must be followed.

Between casts and after the cruise; the CTD and rosette in a way to prevent contamination must be stored.

c. *Procedure after CTD/ rosette recovery*

After the recovery, the CTD / rosette assembly must be put in a place not exposed to the direct sunlight or covered, to minimize the heat exchange.

The next sub-sampling protocol needs to be maintained:

i) Dissolved oxygen and pH samples using tygon tubing;

ii) Salinity, where sampled for control;

iii) Then in the order nitrite, other nutrients, and

iv) Chlorophyll-a.

The contamination sources must be avoided:

i) Contamination from the sampling equipment, ship and on- board activities should be avoided while sampling is undertaken. Some details are provided with single parameter.

ii) Sampling bottles should be cleaned with dilute HCL acid and washed with pure water and always be kept closed when not in use.

1.3 Technical note for the sample preservation of seawater for the determination of key nutrients and chlorophyll *a*

Apart from representativeness, one of the main difficulties in sampling is the preservation of the sample. The initial composition of the sample must be maintained from sampling through to analysis. If this is not the case, the final conclusions will not reflect the initial situation. For all of that, handling and storage of collected samples is of a great importance during sampling.

Proper preservation practices must be followed. Samples requiring preservation should be preserved as soon as possible after collection to maintain the integrity of the sample. Complete and certain preservation of samples, regardless of source, is a practical impossibility. Regardless of the sample nature, complete stability for every constituent can never be fully attained. At best, sample preservation only slows the biological and chemical changes that inevitably continue after the sample is collected. Methods of preservation are intended to retard biological action, retard hydrolysis of chemical compounds and complexes, and reduce volatility of constituents. Preservation methods are limited to pH control, chemical addition, amber or opaque bottles, filtration, refrigeration, and freezing.

Under this Technical Note, this Monitoring Guidelines provides the following IMAP Protocols for the sample preservation of seawater for the measurement of concentration of key nutrients and chlorophyll *a*:

- Protocol for the sample preservation of seawater for the determination of concentration of nutrients;

[7] UNESCO, 1994. Protocols for Joint Global Flux Study (JGOFS) Core Measurements. Manual and Guide, 29: 1-181.

[8] UNESCO, 1988. The acquisition, calibration and analysis of CTD data. A report of SCOR Working Group 51. UNESCO Technical Papers in Marine Science, 54: 1-59.

- Protocol for the sample preservation of seawater for the determination of concentration of chlorophyll *a*.

1.3.1 Protocol for the sample preservation of seawater for the determination of concentration of nutrients

The concentrations of nutrients and other bioactive elements are liable to change due to the activity of microorganisms naturally present in seawater. Therefore, as a rule, samples should not be exposed unnecessarily to light and analysed within a few hours after collection.

Nevertheless, it is sometimes necessary to postpone the analysis for some hours or days because of rough weather or shortage of personnel and laboratory space. There is ample literature on this subject (eg., Kirkwood, 1992[9], 1996[10]; Dore et al., 1996)[11] indicating that no single universal preservation regime will satisfy all requirements. For example, glass containers are not suitable if silicate is to be determined; in different seasons samples from the same location may contain microorganisms of different species and concentrations, so that a given preservation regime could be effective in spring but not in fall. The following two approaches to preservation are: refrigeration and poisoning.

Freezing (to -20 ºC) is the method of choice of many scientists if nutrient samples have to be stored for several weeks or even months (e.g., Macdonald and McLaughlin, 1982[12]; Macdonald et al., 1986[13]; Kremling and Wenck, 1986[14]; Chapman and Mostert, 1990[15]; Kirkwood, 1996). If the samples are visibly turbid, they should be filtered as soon as possible after sampling. Subsamples should be placed in carefully cleaned bottles and frozen, stored and thawed in an upright position. For storage, hard-glass bottles with Teflon-lined screw caps should be used or, preferably, high density polyethylene, polycarbonate or polypropylene bottles. For silicate samples only plastic bottles are recommended. The bottles should only be filled to 2/3 of their volume to prevent squeezing of the liquid through the screw caps during the freezing process. If possible, 'quick-freezing' in liquid nitrogen or in a dry ice-methane slurry (to -20°C within about 20 min) is recommended. The best practice suggests that nutrient samples can be stored for no longer than a month prior to analysis (ISO 5667-3:2012)[16]

The factors that affect the alteration of the samples can be mechanical, physical, chemical, biological and systematic. These drawbacks can be partially overcome by using the following measures:

i. the sample can be stored in disposable scintillation type vials, in high density polyethylene, with a cap suitable for ensuring perfect closure. Polyethylene has the advantage of being resistant to chemical agents and thermal variations, it has a greater mechanical resistance and, from experimental tests, it has been shown that it does not yield and does not absorb substances;

ii. the biological problem can be partially alleviated when the sample is filtered using syringes equipped with swinnex containing glass fiber filters with a pore size <1 µm previously rinsed with plenty of DDW and then, from time to time, with the water of the sample itself;

iii. a single vial is used to determine the concentration of the nutrient to be analysed;

iv. the containers must be washed with 10% HCl, then rinsed with DDW and finally with the sample itself;

v. the sample must be taken directly from the sampling bottle and stored in the dark at a temperature of +4 °C if it is analysed within 24 hours. If the sample is not analysed within this period, it must be frozen at a temperature of -20 °C, taking care to leave the vial upright;

vi. the vial should be filled no more than 3/4 of the volume.

This approach, with contained samples volumes, is more suited when samples are used with an automated analytical method.

Especially on small oceanographic vessels, in order to avoid contamination of the seawater sample with exhaust gas, it is advisable to sample directly from the spout of the water sampler using a 50 mL syringe. In this case, the syringe should be equipped with a Swinnex and two-way taps to facilitate washing of the syringe. The distribution of samples in scintillation vials for storage can be done in the ship's laboratories/environments not contaminated by exhaust gas.

The advantage of the scintillation vials, in addition to the practicality of organizing the vials themselves in

[9] Kirkwood, D.S., 1992. Mar. Chem., 38,151.

[10] Kirkwood, D.S., 1996. Nutrients: Practical notes on their determination in seawater. Copenhagen:

ICES Tech. Mar. Environ. Sci., 17,25 pp.

[11] Dore, J.E., Houlihan. T., Hebel, D.V., Tien, G., Tupas, L., Karl, D.M. (1996), Mar. Chem., 53, 173.

[12] 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.

[13] MacDonald R.W., McLaughlin F.A., Wong C.S. (1986) The storage of reactive silicate samples by freezing. Limnol. Oceanogr. 31, 1139-1142.

[14] Kremling, K., Wenck, A. (1986), Meeresforschung, 31,69.

[15] Chapman, P., Mostert, S. A. (1990), S. Afr J. Mar. Sci., 9,239.

[16] ISO 5667-3:2012 Water quality — Sampling. Part 3: Preservation and handling of water samples.

specially designed supports, is in the speed of freezing, which is still considered the best conservation procedure. Some operators have verified that the use of vials previously used reduces the possibility of contamination. Others rinse the vials with a diluted solution of HCl (0.1 M) and allow the vials to dry upside down. In summary, a reliable procedure is to use containers, even new ones, but previously protected from dust or other possible contamination, which must be washed several times with the sample and not completely filled in order to prevent the expansion of the liquid during freezing forcing the frost out of the container.

a. Specific details of sample collection and preservation

The specific details of sample collection preservation are taken from the Reference manual for sampling and analysis techniques for the eutrophication monitoring strategy of MEDPOL (UNEP/MAP/MED POL, 2005) with minor enhancement.

a.1. Orthophosphate - P

Water samples for phosphate analysis should be collected in stoppered glass or "aged" polyethylene bottles of 50 to 100 ml volume directly from the outlet tube of the in-line filter used to collect suspended particulates. The samples are stored in a cool dark place until the analysis can be performed. For phosphate, the analysis should be commenced as soon as possible, preferably within half an hour, certainly before 2 hours and only glass bottles should be used for intermediate storage of the samples. The samples should not be stored in new polyethylene or polyvinylchloride containers since phosphate has been shown to disappear rapidly in these containers. Therefore, aged high-density polyethylene bottles or other plastic e.g. polycarbonate may be satisfactory but all sample containers should be thoroughly tested before use. Once collected, samples should be stored out of the light in a refrigerator until required for the analysis.

The addition of acid to unfiltered samples cannot be recommended since this cause hydrolysis of any polyphosphates and release of phosphate from plankton and bacteria. The addition of all the reagents of the analytical procedure to the sample and postponement of the photometric measurement is also not possible, since arsenic and silicate will also react and cause erroneous phosphate readings.

Summarizing, the storage of samples for the analysis of dissolved phosphate for more than one hour should be avoided.

a.2. Ammonium - N

Samples for ammonium analysis should only be taken and stored in tightly sealed seawater-aged glass or high-density polyethylene bottles, which should only be used for the analysis of ammonia. Filtration of samples should also be avoided, if possible, because it is nearly impossible to obtain filters free of ammonium. Waters with high turbidity frequently contain high concentrations of ammonia and may therefore be diluted before the analysis (the residual turbidity may then be compensated by subtraction of the absorbance of the appropriately diluted sample without addition of reagents).

Ammonium is a nutrient compound, which rapidly undergoes biological conversion, i.e., oxidation into nitrite and nitrate and fixation as amino-bound nitrogen in organisms. The analysis of ammonia should be commenced without delay after sampling. Chemical methods for preservation have been proved unsatisfactory because of the fact that organisms rapidly release ammonia. It is therefore strongly recommended that the ammonia reagents be added within one hour after sampling.

a.3. Nitrite - N

Nitrite is an intermediate compound, which occurs if ammonia is oxidized or nitrate is reduced. The presence of higher amounts of nitrite (> 1.5 $\mu mol\ L^{-1}$) signifies the presence of high bacterial activity in the seawater sample. Storage of samples for nitrite analysis can therefore not be recommended. Chemical preservation (e.g. addition of chloroform) also seems to be unsatisfactory. In turbid waters a filtration step is necessary. Therefore, the sub-sample for nitrite determination directly from the outlet of the in-line filter described above in a 100 - 150 ml glass container must be collected. The nitrite reagents should, if possible, be added to the sample within one hour. Intermediate storage of the sample in glass bottles in a refrigerator for up to 3 hours causes, in most cases, no significant changes in the nitrite content, if the original ammonia level is low (< 0.07 $\mu mol\ L^{-1}$). Samples should be stored in tightly sealed glass or polyethylene bottles only. Sulphide ions have been reported to interfere with the determination of nitrite and, thus, when hydrogen sulphide is suspected to be present in a sample, the gas should be expelled with nitrogen after the addition of the acid sulphanilamide reagent (Grasshoff et al., 1983).

a.4. Nitrate - N

Nitrate is the final oxidation product of nitrogen compounds. Changes of the original nitrate content of a seawater sample can, therefore, only result from oxidation of ammonia and of nitrite or from adsorption of nitrate to the material of the sample container. Adsorption of nitrate into particles seems to be insignificant since the analytical procedure liberates any nitrate, which may be adsorbed. For reasons yet unknown, the nitrate content of a sample decreases rapidly if stored in polyethylene bottles, and at a level of 1.4 $\mu mol\ L^{-1}$ about half of the nitrate disappears within seven days after storage at room temperature. This indicates that only glass or "aged" high-density polyethylene bottles with tight screw caps (preferably with Teflon liners) should be used.

If larger plastic bottles are used for sub-sampling for all nutrient analysis, the amount needed for nitrate should be transferred into a glass or "aged" high-density polyethylene bottle within one hour after the sampling. The analysis should not be delayed for more than 5 hours. In this case the samples should be stored in a refrigerator. If longer storage is unavoidable, the sample should be quickly frozen to -20 °C after the addition of

the ammonium chloride buffer solution (Grasshoff et al., 1983).

a.5. Silicate - Si

It is obvious that glass bottles should not be used for storage and analysis of seawater samples for reactive silicate. The sub-sampling for silicate analysis should be performed with plastic bottles (made of polyethylene or polypropylene). A few days storage of the sample in the dark in a refrigerator does not lead to significant changes in the silicate content. However, during seasons of high productivity, do not store them for longer than a day. Polymerization of orthosilicate during storage of frozen samples has been reported from fresh water samples but does not occur in seawater. If kept frozen, it is recommended to thaw the sample gradually at room temperature for at least 24 hours. However, as with all nutrients immediate analysis of sample is the preferred option.

The best procedure for storage and preservation of fresh-water samples seems to be the acidification of the sample with sulfuric acid to a pH of 2.5 and storage in tightly sealed, seawater-aged, high density polyethylene bottles in the dark at about 4 °C. However, as with all nutrients immediate analysis of the sample is the preferred option.

1.3.2 Protocol for sample preservation of seawater for the determination of concentration of chlorophyll *a*

a. Equipment and reagents

The equipment for the sample preservation of seawater for the determination of concentration of chlorophyll *a* include:

i) Dark plastic bottles, 1 L (coastal waters) - 5 L (open sea)
ii) Plankton net with 250 µm mesh
iii) Plastic funnel suitable for bottles
iv) Filtration apparatus (for filters with 25 or 47 mm diameter)
v) Vacuum pump and trap
vi) 25 or 47 mm Whatman GF/F fiberglass filters (recommended)
vii) 10 ml calibrated centrifuge tubes
viii) Freezer or fridge
ix) Automatic sprayer or pipette for acetone
x) 1 L graduated cylinders
xi) Funnel for filtration and filter paper
xii) Acetone, ppa [$(CH_3)_2CO$]
xiii) Anhydrous sodium carbonate [Na_2CO_3]
xiv) Neutralized acetone: pure anhydrous sodium carbonate to the pure acetone (ppa) is added and shaken vigorously. After at least 24 hours, the acetone is filtered through paper and transferred to the hermetically sealed bottle or bottle.

b. Sampling

Water sample from the sampling bottles to the dark plastic bottles, through the net with 250 µm mesh, must be transferred and stored in a cool place, away from sunlight. The prefiltration of the sample is intended to retain zooplankton and fragments of macroalgae possibly present (Strickland and Parsons, 1968[17]; Lenz and Fritsche, 1980[18]).

c. Filtration procedure

Glass fibre filters, Whatman GF/F, are the most suitable for use and filtration must be carried out within a short time from the collection (max 1-2 hours), especially when the samples have been collected in eutrophic environments. The fiberglass filter is less prone to clogging and is cheaper than the synthetic membrane filter and are also used for their high retention capacity, ease of homogenization and versatility.

If the purpose is to estimate precise dimensional fractions of the particulate, filters based on synthetic membranes (polycarbonate) instead of glass fibre filters can be used. These filters, with an enucleation impression, have the advantage of having calibrated pores and therefore guarantee a very precise separation of the particles according to size. However, some disadvantages must be noted, such as the very low retention capacity, much slower filtration flow, making it necessary to distribute the sample on more filters if the sensitivity of the method want to be increased.

The filter is placed in the appropriate housing of the filtration apparatus, wetted and the vacuum pump with slight depression started, to allow it to uniformly adhere to the support.

Using a graduated cylinder, between 0.5 and 5 L of sample are poured into the funnel of the filtration system.

The vacuum pump is started, providing that the pressure difference between the lower and upper part of the filter does not exceed -25 KPa (about 150 mm Hg), to avoid breaking the plant cells with the consequent loss of pigments.

At the end of the filtration the filter the pump is kept running for a few seconds to avoid that a part of the material is lost. The amount of water to be filtered is related to the concentration of pigments (in the open sea where concentrations of chlorophyll *a* to about 1 µg L^{-1} are generally measured about 3 L of sea water must be

[17] Strickland Ld., Parsons T.R., 1968. A practical handbook of sea-water analysis. Bull. Fish. Res. Board Can., 167, 1-312.

[18] Lenz J., Fritsche P., 1980. The estimation of chlorophyll a in water samples: a comparative study on retention in a glass-fibre and membrane filter and on the reliability of two storage methods. Arch. Hydrobiol. Beih., 14: 46-51.

filtered while in coastal waters, 0.5 - 1 L may be sufficient).

As previously mentioned, algae suspensions must be filtered with a very small pressure difference between the two sides of the filter to minimize cell breakage.

The use of filtration supports that have a valve for the escape of air, not to limit the filtering surface due to the presence of air bubbles and that are tightened with the rotation of a ring nut independent of the upper face of the support, is advisable. Tearing the filter during assembly or disassembly of the support thus will be avoided.

d. *Storage of samples*

The conservation of the samples is a critical point of the analytical procedure, which can determine the onset of degradation processes of the chlorophylls that lead to their underestimation (Rai and Marker, 1982)[19]. Consequently, to perform the extraction and analysis of chlorophyll pigments immediately after filtering the sample is always preferable.

However, if this is not possible, immediately after filtration the filter is placed in a centrifuge tube with hermetic seal and a known volume of neutralized pure acetone is added to completely submerge the filter (approximately 5 ml). The test tube is then kept in the dark at -20 °C (or in any case at temperatures between -20 and +4 °C), paying particular attention to the tightness of the closure.

Storing the filtered material for a period of time that lasts from a few days to several weeks can have a negative effect, leading to the degradation of chlorophyll pigments (Yanagi and Koyama, 1971[20]; Blasco, 1973[21]; Neveux, 1979[22]; Lenz and Fritsche, 1980; Lazzara et al., 1990[23]; Mantoura et al., 2005[24]).

Alternative methods of storing the filters have been used, which however are not recommended, such as storing at -20 °C after freezing the damp or dried filters (Panella and Magazzù, 1978[25]) or the drying and freezing technique (freeze-drying) which does not give satisfactory conservation results according to Lenz and Fritsche (1980).

The conservation of chlorophyll *a* in microalgae samples collected on a filter has so far been the subject of a very limited number of studies, if the diffusion of the practice of conserving filters, even for prolonged periods (Mantoura et al., 2005) is to be considered. Almost all of the studies date back to before the 1980s, do not concern separate analyses of the extracted pigments and often present contrasting results (Marker et al., 1980)[26] so that no freezing practice can be recommended.

In conclusion, immediate measurements on the extracts is advisable to be carried out or, in the impossibility, the conservation of the filter immersed in pure acetone at -20 °C or better at -80 °C only for periods of less than a month, or of wet filter frozen in air (between -20 °C and -80 °C) but only for periods shorter than the week.

2 Guidelines for the determination of key nutrients in Seawater

2.1 Introduction

In the Monitoring Guidelines for Key nutrients in seawater, ~~the~~ protocols for manual and automated determination of the concentration Nitrogen, Phosphorus and Silica compounds 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), although in near future it will be complemented by those based on concentration of key nutrients in seawater.

The IMAP Protocols elaborated within these Monitoring Guidelines for Determination of Concentration of Key nutrients in Seawater provide detail guidance on the

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[19] Rai H., Marker A.F.M., 1982. The measurements of photosynthetic pigments in freshwaters and standardization of methods. Arch. Hydrobiol. Beih., 16: 1-130.

[20] Yanagi K, Koyama T., 1971. Thin layer chromatographic method for determining plant pigments in marine particulated matter, and ecologica1 significance of the results. Geochem. J., 5: 23-37.

[21] Blasco D., 1973. Estudio de las variaciones de la relacion fluorescencia in vivo chl a, y su aplicacion en ocea- nografia. Influencia de la limitacion de diferentes nutrientes, efecto del dia y noche y dependencia de la especie estudiada. Inv. Pesq., 37: 533-536.

[22] Neveux J., 1979. Pigments chlorophylliens. In: Jacques G. (ed), Phytoplancton, Biomasse, Production, Nu- meration et Culture. Edition du Castellet, Perpignan: 1-107.

[23] Lazzara L., Bianchi F., Falcucci M., Hull V., Modigh M., Ribera D'alcalà M., 1990. Pigmenti clorofilliani. In: Innamorati M., Ferrari I., Marino D., Ribera d'Alcalà M. (eds), Metodi nell'ecologia del plancton marino. Nova Thalassia, LINT, Trieste: 207-223.

[24] Mantoura R.F.C., Wright S.W., Barlow R.G., Cummings D.E., 2005 Filtration and storage of pigments from microalgae. In: Jeffery S.W., Mantoura R.F.C., Wright S.W. (eds), Phytoplankton pigments in ocea- nography: guidelines to modern methods. 2nd ed. SCOR UNESCO, Paris: 283-305.

[25] Panella S., Magazzù G., 1978. Analisi dei pigmenti fitoplanctonici. In: Magazzù G. (ed.), Metodi per lo studio del plancton e della produzione primaria. Edizioni GM: 19-33.

[26] Marker A.F.H., Nusch E.A., Rai H., Riemann B., 1980. The measurement of photosynthetic pigments in fresh waters and standardization of methods: conclusions and recommendations. Arch. Hydrobiol. Beih. Ergebn. Limnol., 14: 91–106.

necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the concentration of nutrient compounds 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.

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) and Data Quality Assurance schemes (UNEP/MAP, 2019b) 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), 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.

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.

Continuous flow methods

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)[27] and Grasshoff et al. (1999)[28] 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.

2.2 Technical note for determination of concentration of nitrite (NO_2^-)

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)[29], was subsequently modified by Shinn (1941)[30] and applied to the analysis of sea water by Bendschneider and Robinson (1952)[31].

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.

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.

2.2.1 Protocol for manual colorimetric determination of concentration of nitrite

a) *Equipment:*

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

1. graduated cylinders or 50 mL pipettes

[27] 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.

[28] Grasshoff, K., Kremling, K., Ehrhardt, M. (eds), 1999. Methods of Seawater Analysis 3rd Edition Wiley-VCH Weinheim, 634 pp.

[29] Ilosvay L. (1889) Determination of nitrite in saliva and exhaled air. Bull. Soc. Chim. Fr., 2, 388-391.

[30] Shinn M.B. (1941) A colorimetric method for the determination of nitrite. Ind. Eng. Chem Anal. Ed., 13, 33-35.

[31] Bendschneider K., Robinson R.J. (1952) A new spectrophotometric method for the determination of nitrite in sea water. J. Mar. Res., 11, 87-96.

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

b) <u>*Chemical products:*</u>

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

1. sulfocromic mixture
2. concentrated hydrochloric acid [HCl]
3. sulfanilamide [NH2SO2C6H4NH2]
4. N-(1-Naphthyl)ethylenediamine dihydrochloride [C10H7NHCH2CH2NH2 · 2HCl]
5. sodium nitrite [NaNO3]
6. chloroform [CHCl3]

c. <u>*Preparation of stock solutions:*</u>

Sulfanilamide reagent

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

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}

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. <u>*Preparation of specific equipment for analysis:*</u>

d.1. Maintenance of reaction vessels

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.

e. <u>*Analytical procedure:*</u>

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

Preparation of standard solutions

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

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;

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

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.

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

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

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.

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.

f. <u>*Calculations*</u>

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

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.

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

$c(NO_2^-)$ / µmol 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

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.

g. *Important notes:*

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

h. *Possible problems:*

The suggested method is trouble- and interference-free. However, any hydrogen sulfide present in the sample must be removed before analysis (Grasshoff, 1983[32]; Airey et al., 1984[33]).

2.2.2 Protocol for automated colorimetric determination of concentration of nitrite

a. *Reagents*

Sulfanilamide reagent

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

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*

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.

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*

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.

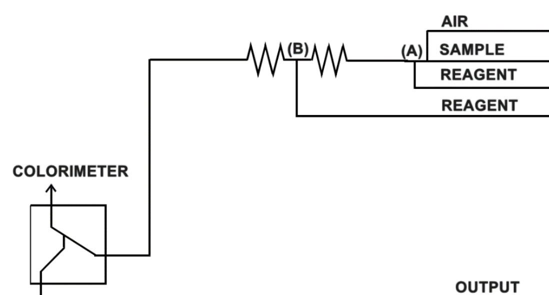

Figure 1. Manifold for nitrite measurement.

d. *Important notes:*

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

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.

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

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

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.

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*

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

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

[33] 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.

BODC Parameter Usage Vocabulary are provided as follows:

Symbol: $c(NO_2^-)$ **Unit**: $\mu mol\ L^{-1}$

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 auto-analysis

2.3 Technical note for determination of concentration of nitrate (NO_3^-)

The method was introduced by Morris and Riley (1963)[34], but only later was the dynamics of the involved reactions studied in depth (Nydhal, 1976[35]; Grasshoff, 1983[36]). 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.

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 $\mu mol\ L^{-1}$, calibration curves for a low and high range must be established.

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).

[34] Morris A.W., Riley J.P. (1963) The determination of nitrate in sea water. Analytica Chim. Acta, 29, 272-279.

[35] Nydhal F. (1976) On the optimum conditions for the reduction of nitrate to nitrite by cadmium. Talanta, 23, 349-357.

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.

2.3.1 Protocol for manual colorimetric determination of concentration of nitrate

a. *Equipment:*

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:*

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]

[36] Grasshoff K. (1983) Determination of nitrate. In: “Methods of Seawater Analysis”, Grasshoff K., M. Ehrhardt, K. Kremling Eds, Verlag Chemie, Weinheim, 143-150.

9. granular cadmium for reactors [Cd]
10. copper sulphate pentahydrate [$CuSO_4 \cdot 5H_20$]
chloroform [$CHCl_3$]

c. *Preparation of stock solutions:*

Sulfanilamide reagent

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

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

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}

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

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}

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}

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

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

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.

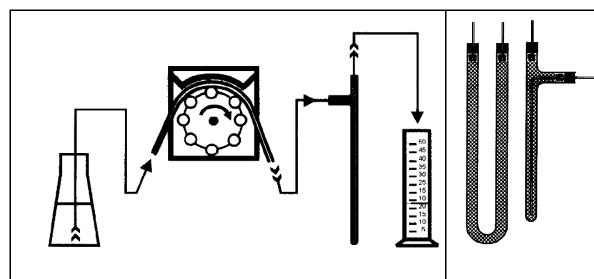

Figure 2. Reduction column for the analysis of nitrate and type of columns.

d.3. Preparation of the reduction column

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.

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.

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.

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.

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.

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%).

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

e. <u>*Analytical procedure:*</u>

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

Preparation of standard solutions

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.

Three 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

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;

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

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

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

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.

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

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

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

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.

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.

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

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

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.

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.

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. <u>*Calculations*</u>

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

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.

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%.

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

$c(NO_3^-)$ / µmol 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

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. <u>*Important notes*</u>

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.

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.

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

h. <u>*Possible problems*</u>

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).

The efficiency of the column can be reduced if concentrations of phosphates higher than 2 µmol L^{-1} are present (Olsen, 1980)[37].

2.3.2 Protocol for automated colorimetric determination of concentration of nitrate

a. <u>*Reagents*</u>

Buffer

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

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

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. <u>*Standard*</u>

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.

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. <u>*Reducer*</u>

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

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

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.

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. <u>*Manifold*</u>

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

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

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.

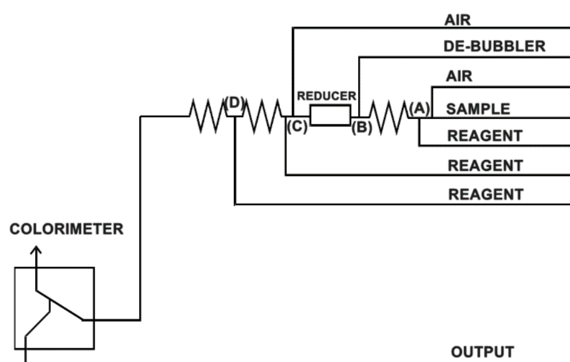

Figure 3. Manifold for nitrate measurement.

e. Important notes

Air passage through the reducer is not allowed.

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.

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.

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.

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.

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.

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

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.

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.

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

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.

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.

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.

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

2.4 Technical note for determination of concentration of ammonium (NH_4^+)

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)[38].

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)[39].

Finally, given the importance of pH control in the development of the reaction (Sasaki and Sawada, 1980)[40], the significant salt effect (different yield of the reaction in fresh or salt water) that occurs in this method (Koroleff, 1983)[41] 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)[42].

The procedure outlined here, mainly follows the methods described by Grasshoff and Johansen (1973)[43] and by Koroleff (1983) as described by Hansen and Koroleff (1999)[44] and is adapted to this manual from the previous one (UNEP/MAP/MED POL, 2005).

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.

2.4.1 Protocol for manual colorimetric determination of concentration of ammonium

a. *Equipment:*

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, 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_4Cl]
11. ammonium hydroxide [NH_4OH]
12. chloroform [$CHCl_3$]

c. *Preparation of stock solutions:*

"Ammonia-free" water

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.

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[38] Berthelot, M.P. (1859) Repertoire de Chemie Appliquée, pp. 284.

[39] Ivancic I., Degobbis D. (1984) An optimal manual procedure for ammonia analysis in natural waters by indophenol blue method. Water Res., 18, 1143-1147.

[40] Sasaki K., Sawada Y. (1980) Determination of ammonia in estuary. Bull. Jap. Soc. Sci. Fish., 46, 319-321.

[41] Koroleff F. (1983) Determination of ammonia. In: "Methods of seawater analysis", Grasshoff K., M. Ehrhardt, K. Kremfing Eds, Verlag Chemie, Weinheirn, 150-175.

[42] 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.

[43] 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.

[44] 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.

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

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

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

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)

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)

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

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.

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

Seven (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

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

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

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

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

One 100 mL flask is filled with 50 mL and one with 47.5 mL of distilled water or "open sea surface water"

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

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.

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:*

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

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}.

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

$c(NH_4)$ / µmol 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

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:

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

where

S_s = salinity of the sample.

g. *Important notes:*

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.

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:*

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.

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

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

2.4.2 Protocol for automated colorimetric determination of concentration of ammonium

a. *Reagents*

Buffer

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

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

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*

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.

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*

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

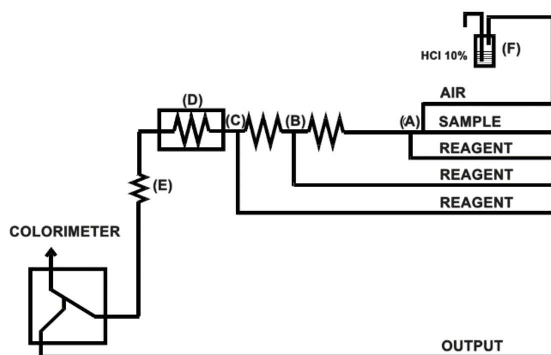

Figure 4. Manifold for ammonium measurement.

a. *Important notes*

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:

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

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

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

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;

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;

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

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

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;

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;

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

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. *Symbol, units and precision*

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

2.5 Technical note for the determination of concentration of orthophosphate (PO_4^{3-})

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)[45].

[45] Deniges M.G. (1920) Reaction de coloration extrêmement sensible des phosphates et des arseniates. Ses applications. C. R. Acad. Sci., Paris, 171, 802-804.

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[46]). 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.

Murphy and Riley (1962)[47] 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[48], 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)[49].

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

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

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

2.5.1 Protocol for manual colorimetric determination of concentration of orthophosphate

a. *Equipment:*

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:*

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

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

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

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}

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[46] 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.

[47] 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.

[48] 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.

[49] Koroleff F. (1983) Determination of phosphorus. In: "Methods of Seawater Analysis", Grasshoff K., M. Ehrhardt, K. Kremling Eds, Verlag Chemie, Weinheim, 125-139.

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

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

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

In a glass container are mixed: 100 mL of ammonium molybdate solution, 250 mL of 5 N sulphuric 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

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 $\mu mol\ L^{-1}$ plus the orthophosphate content of oligotrophic seawater.

e.2. Analytical treatment

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

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

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.

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

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

e.3. Preparation of reagent blanks

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

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

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

e.4. Spectrophotometric measurements

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.

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:

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.

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.

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 $\mu mol\ L^{-1}$.

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

For a cell with a 50 mm optical path, the colorimetric factor is equal to about 9.9 $\mu mol\ L^{-1}$, i.e. a difference in concentration of 1 $\mu mol\ L^{-1}$ (for example between standard solution 3 and 5) should be the difference in absorbance of about 0.1.

g. Important notes and possible problems:

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.

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.

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

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

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

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)[50]. In this case the sulphides from the sample should be removed (Airey et al., 1984).

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).

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)[51] to be applied.

2.5.2 Protocol for automated colorimetric determination of concentration of orthophosphate

a. *Reagents:*

Ammonium molybdate

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

Antimony potassium tartrate (KAT)

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

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.

Ascorbic acid

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

c. *Standards:*

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}.

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*

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.

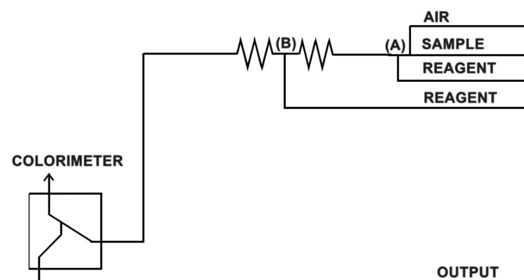


Figure 1. Manifold for orthophosphate measurement.

e. *Calculations*

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

f. *Important notes and possible problems*

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.

If during the analysis there is an evident increase in the baseline, the colorimeter reading cell is immediately

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

[51] 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.

cleaned by injecting 50% hydrochloric acid directly into the cell without stopping the circuit.

DDW is deionized if possible, in the water container of the instrument. If change of components of the circuit (injectors, bubblers) is necessary, the circuit by changing the flow rates of the pipes must be rebalanced.

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.

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.

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

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

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

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

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

2.6 Technical note for the determination of concentration of orthosilicate (SiO_4^{4-})

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.

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.

Silicomolybdic acid exists in at least two isomers α and β (Strickland, 1952[52]; Morrison and Wilson, 1963[53]; Truesdale and Smith, 1975[54]), 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)[55]. Also, in this process it is important to control the pH to avoid a direct reduction of excess molybdenum by metol.

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).

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.

[52] 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.

[53] 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.

[54] Truesdale V.W., Smith C.J. (1975) The formation of molybdosilicic acids from mixed solutions of molybdate and silicate. Analyst, 100, 203-212.

[55] 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.

2.6.1 Protocol for manual colorimetric determination of concentration of orthosilicate

a. Equipment

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

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

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

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

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

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) sulphuric acid solution

250 mL of concentrated sulphuric 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})

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.

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

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

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

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

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.

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.

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.

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

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.

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

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.

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*

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

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.

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

$c(SiO_4)$ / µmol L^{-1} = (ABS - bl - $bl_{c,\,i}$) · 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

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*

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[56]; MacDonald and McLaughlin, 1982; MacDonald et al., 1986).

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

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.

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

[56] Burton J.D., Leatherland T.M. (1970) The reactivity of dissolved silicon in some natural waters. Limnol. Oceanogr., 15, 473-476.

around 35 a value of about 0.85 is observed (Bien, 1958[57]; Fanning and Pilson, 1973[58]; Koroleff, 1983).

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.

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).

2.6.2 Protocol for automated colorimetric determination of concentration of orthosilicate

a. Reagents

Stannous chloride.

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.

Tartaric acid

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

Ammonium molybdate

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

b. Solutions for use

Molybdate

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

Stannous chloride

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

c. Standard

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}.

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.

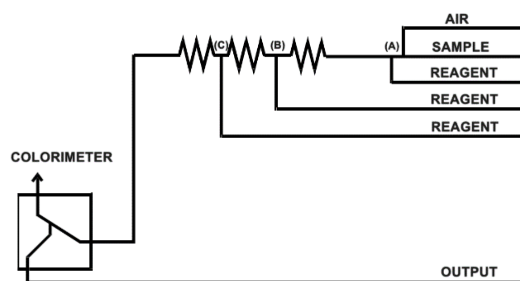


Figure 2. Manifold for orthosilicate measurement.

e. Calculations

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

f. Important notes and possible problems

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.

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.

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

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

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.

[57] Bien G.S. (1958) Salt effect correction in determining soluble silica in sea water silicomolybdic acid method. Anal. Chem., 30, 1525-1526.

[58] Fanning K.A., Pilson M.E.Q. (1973) On the spectrophotometric determination of dissolved silica in natural waters. Anal. Chem., 45, 136-140.

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.

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

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

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

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

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.

<u>*Symbol, units and precision*</u>

Symbol: $c(SiO_4^{4-})$ **Unit:** µmol L^{-1}

Precision: ± 0.05 **Accuracy:**±0.05 **LOD:** 0.10

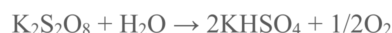
Method identifier:

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

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

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.

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



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

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.

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)[59]. Therefore, adding OH^- to the reaction mixture or shaking the reaction vessels is suggested. Koroleff (1968[60]; 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[61]) and Valderrama (1981)[62] believe that a unique method for the determination of nitrogen and total phosphorus in seawater are equally reliable.

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).

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:

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[59] Nydahl F. (1978) On the peroxidisulphate oxidation of total nitrogen in waters to nitrate. Talanta, 12, 1123-1130.

[60] Koroleff F. (1968) Determination of total phosphorus in natural waters by means of persulphate oxidation. ICES C.M./C, 33, 209-212.

[61] Koroleff, F. (1983b) Total and organic nitrogen. In: "Methods of Seawater Analysis", Grasshoff K., M. Ehrhardt, K. Kremlin Eds, Verlag Chemie, Weinheim, 162-173.

[62] Valderrama J.C. (1981) The simultaneous analysis of total nitrogen and total phosphorus in natural waters. Mar. Chem., 10, 109-122.

- 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.

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

a. *Equipment*

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*

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

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*

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.

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

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*

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.

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

a. *Equipment*

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*

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*

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})

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.

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

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

250 mL of concentrated sulphuric 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

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

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

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

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

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

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):

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;

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):

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.

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

d.5. Analytical treatment

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.

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.

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.

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

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.

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

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

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

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

e. Calculations

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.

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.

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).

The concentrations are calculated according to the equations:

$c(TN)$ / µmol L^{-1} = $(ABS_N - bl_N - bl_{c,i,N}) \cdot f_N$

$c(TP)$ / µmol L^{-1} = $(ABS_P - bl_P - bl_{c,i,P}) \cdot f_P$

where:

ABS_N	=	absorbance of sample at 553 nm
ABS_P	=	absorbance of sample at 882 nm
$bl_{c,i,N}$	=	blank of i-th cell at 553 nm
$bl_{c,i,P}$	=	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

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}.

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.

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.

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

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

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

3 Guidelines for the determination of Chlorophyll a in Seawater

3.1 Introduction

In the Monitoring Guidelines for Determination of Chlorophyll a in Seawater, the four protocols for determination of the concentration of chlorophyll *a* are elaborated. The concentration of in the sea is an important indicator for the presence of algae and other plant-like organisms that carry out photosynthesis. As such, phytoplankton, which contains the chlorophyll, is an essential element of the food chain in the seas as it provides the food for numerous animals. Variations and changes in the chlorophyll levels are also relevant for the study of the ecology of the sea. 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 the IMAP Guidance Factsheets (UNEP/MAP, 2019).

The IMAP Protocols elaborated within this Monitoring Guidelines for Determination of Chlorophyll *a* in Seawater provide detail guidance on the necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the concentration of chlorophyll *a* in sea water,

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.

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) and Data Quality Assurance schemes (UNEP/MAP, 2019b) 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), however providing detail procedures that are of relevance for IMAP implementation. With the details of the protocols for determination of chlorophyll *a*, the needs of the measurements both in off-shore areas and in narrow coastal areas are addressed.

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.

3.2 Technical note for determination of concentration of chlorophyll *a*

In this note the photometric, fluorometric and HPLC methods are presented, that are based on a characteristic common to all autotrophic organisms, i.e., the presence of pigments that allow to capture the light and transfer it to the reaction centres where photosynthesis begins. In the marine environment, except for a small fraction of very ancient bacteria (Kolber et al., 2001)[63], all phototrophic organisms, i.e. those that use light to live, have either chlorophyll *a*, or a very similar pigment, divinyl chlorophyll *a*, while accessory pigments, mostly carotenoids, can change from group to group.

The methods are based on the evidence that the amount of pigments present in a planktonic organism are related with its total biomass. It should also be added that the methods used for the collection of phytoplanktonic biomass (essentially represented by filtration) do not allow to separate the phytoplanktonic carbon from the non-phytoplanktonic carbon (organic debris), simultaneously present in marine water.

In summary, although the carbon measurements are the most correct for an estimate of the phytoplankton biomass, those based on chlorophyll *a* are still the most used, both for historical and practical reasons. In fact, the former, despite the recent technical progress, are more expensive and complicated than those of the pigments proposed here.

These methods, both photometric and fluorometric, are optimal when a limited economic and time commitment is expected, while the recent chromatographic separation techniques of the pigment mixture (essentially by HPLC: Robinson, 1979[64]) are costly and time demanding and not always sustainable for all laboratories.

The wide diffusion of the spectrophotometric method is also motivated by the fact that the instrument used is almost always present in an analytical laboratory, for the many determinations based on the measurement of the absorbance of coloured substances. The contraindication of the spectrophotometric method is its reduced sensitivity, compared to methods based on fluorescence. This entails either the use of cells with a higher optical path (10 cm) (which in any case generate the problem of having a larger volume of solvent for extraction and waiting for the reading to stabilize), or for the filtration of large volumes of water. This is not always possible given that in the open sea and in periods other than those of intense blooms, 4-5 L are the minimum quantity necessary to obtain reliable results. Filtering large volumes of water always presents many difficulties, both for sampling, and for increasing the filtration time and for the need to use larger filters and / or ad hoc filtration systems.

On the contrary, the fluorometric method allows to obtain reliable data by filtering smaller quantities of water, using filters of smaller diameter and obtaining lower extract volumes. All these aspects make the measurement of fluorescence in overall more practical and economical, excluding the initial acquisition of a fluorimeter, both with filters and with monochromator, even if recently instruments at affordable costs have been placed on the market. However, it is good to consider that these analytical tools are suitable for fewer applications for environmental analysis. Finally, it should be remembered that all methods of measuring pigment concentrations, including HPLC and fluorometric techniques, are based on calibrations that necessarily use optical density measurements, which makes the use of the spectrophotometer irreplaceable.

The differences between the concentrations obtained by spectrophotometry-UV, fluorimetry, and

[63] Kolber Z.S., Gerald Plumley F., Lang A.S., Beatty T.J., Blankenship R.E., Vandover C.L., Vetriani C., Koblizek M., Rathgeber C., Falkowski P.G., 2001. Contribution of Aerobic Photoheterotrophic Bacteria to the Carbon Cycle in the Ocean. Science, 292: 2492-2495.

[64] Robinson, A.L., 1979. HPLC: the new king of analytical chemistry. Science, 203: 1329-1332.

spectrophotometry is visible; after the chromatography, can be significant if degradation products are present (Dos Santos et al. 2003[65]). The simultaneous use of the methods in the same programs is not encouraged.

Under this Technical Note, the Monitoring Guidelines for Determination of Chlorophyll *a* in Seawater elaborates the four following Protocols:

- Protocol for sample pretreatment for determination of concentration of chlorophyll *a;*
- Protocol for spectrophotometric determination of concentration of chlorophyll *a;*
- Protocol for fluorometric determination of concentration of chlorophyll *a;*
- Protocol for HPLC determination of concentration of chlorophyll *a*.

3.2.1 Protocol for sample pre-treatment for determination of concentration of chlorophyll *a*

After the suspended particulates containing fat-soluble pigments have been concentrated on a glass fibre filter by means of filtration the chlorophyll pigments are extracted from the cells, shredding and by homogenizing the filters, immersed in a mixture of acetone and water.

a. *Specific equipment*

The equipment for sample pre-treatment includes the following pieces:

i) Centrifuge for 12 mm diameter tubes, capable of reaching 4000 rpm, preferably refrigerated.

ii) Homogenizer (potter) with ground glass or Teflon pestle.

b. *Chemical products and reagents*

For sample pre-treatment for determination of concentration of chlorophyll *a*, the following products and reagents are needed:

i) Acetone, p.a. [$(CH_3)2CO$]

ii) Sodium carbonate [$NaCO_3$]

iii) Hydrochloric acid [HCl]

iv) 90% v/v neutral acetone: 100 mL of reagent grade water and 900 mL of neutral acetone (see above) separately measured are mixed. The solution is always kept away from light and in the presence of sodium carbonate.

v) Hydrochloric acid 0.66 mol L^{-1}: 55 mL of concentrated hydrochloric acid (HCl 37% v/v) is slowly poured (under stirring) in 950 mL of reagent grade water.

c. *Procedure*

After filtration, filters are either frozen in liquid nitrogen (after being folded and placed into cryotubes) and transferred at -80°C until analyses or directly placed in the freezer at -80°C. For pigment extraction, frozen filters are directly placed in 90% acetone and triturated and homogenized for a maximum of 2 min by carefully rinsing the pestle of the homogenizer several times.

This operation must be carried out using a volume of acetone equal to that of the pure acetone used to store the filter. Since the final extract must be in 90% acetone and considering that the filter retains water (for a 47 mm GF/F filter about 0.7 mL), generally 5 mL of 90% acetone are added to the 5 ml of pure acetone.

If the sample is analysed immediately after filtration, the shredding and homogenization operations must be carried out directly with 90% acetone. The homogenization of the filter by potter causes a gradual heating of the extraction liquid, with possible partial degradation of the pigments. This inconvenience can be limited by using cold acetone (4 °C) or by placing the test tube in a beaker with ice, in any case containing the operation within a maximum time of 2 minutes.

The sample can also be homogenized by manual shredding with a glass rod, directly inside the test tube used for storage; in this case it is appropriate to estimate quantitatively what the possible decrease in efficiency is, compared to the instrument shredding.

Note, that the use of ultrasound does not seem to give good results (Nusch, 1980)[66] as it produces excessive heating of the extract and is therefore not recommended.

The test tube carefully capped with the obtained suspension (10 mL of 90% acetone) must be kept at 4 °C in the dark for 24 hours to complete the extraction. The closed tubes are centrifugated for 10 minutes at 4000 rpm (or 3500 for 12 minutes, if not refrigerated).

3.2.2 Protocol for spectrophotometric determination of concentration of chlorophyll *a*

The spectrophotometer to be used should preferably be equipped with an interference grid and a bandwidth of 1-2 nm, with cells of at least 50 mm (preferably 100 mm) with optical path and reduced volume (max 7 mL). It is important that the wavelength is carefully adjusted, frequent checks must be carried out following the instructions of the manufacturer of the equipment. For spectrophotometers with a hydrogen or mercury lamp, the respective lines (hydrogen - 656 nm; mercury - 546 nm) must be checked.

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[65] Dos Santos, A.C.A., Calijuri, M.C., Moraes, E.M., Adorno, M.A.T., Falco, P.B.,Carvalho, D.P., Deberdt, G.L.B., Benassi, S.F. 2003. Comparison of three methods for Chlorophyll determination: Spectrophotometry and Fluorimetry in samples containing pigment mixtures and spectrophotometry in samples with separate pigments through High Performance Liquid Chromatography. Acta Limnol .Bras., 15(3):7- 18.

[66] Nusch, E., 1980. Comparison of different methods for chlorophyll and phaeopigment determination. Arch. Hydrobiol. Beih., 14: 14-35.

With the new generations of diode lattice spectrophotometers these tasks are easier to be performed. They are even connected to a PC that allows data to be stored in digital format and therefore immediately usable for the calculations necessary for estimating concentrations.

a. *Reading and calculations*

After the final centrifugation of the extract, the supernatant, using a pipette or syringe, is transferred to the cell.

Three different methods for estimating photosynthetic pigments are available:

i) method for estimating chlorophyll *a* with phaeopigments;

ii) method for the separate estimation of chlorophylls *a*, *b* and *c*;

iii) method for the separate estimation of chlorophyll *a* and phaeopigments.

The first method reported involves an error of variable magnitude, due to the presence of both accessory pigments (chlorophylls *b* and *c*) which have an absorption maximum even at 664 nm, both for pheophytins and pheophorbides, the main degradation products of chlorophylls. However, this method is preferable when you want to lower the sensitivity threshold of the estimate (e.g. for concentrations lower than 0.4 $\mu g\ L^{-1}$), as it allows a more "robust" and reliable estimate of the pigment biomass.

The other two methods allow to obtain a more precise estimate of chlorophyll *a* alone in the presence of significant quantities of chlorophyll *b* and *c*, using readings at multiple wavelengths (Jeffrey and Humphrey, 1975[67]; Lorenzen and Jeffrey, 1980[68]) or in the presence of significant quantities of its degradation products, having treated the extract with hydrochloric acid (Lorenzen, 1967)[69].

b. *Method 1. Concentration of chlorophyll a*

This method is based on the assumption that the maximum absorption peak of chlorophyll *a* is at 664 nm with a specific absorption coefficient of 87.67 $cm^{-1}\ g^{-1}$ L (Jeffrey and Humphrey, 1975) and the phaeopigments are not present in high quantity.

Absorbance of the sample is read at 664 and 750 nm against a blank of 90%-acetone (not neutralized).

The concentration (*c*) of chlorophyll *a* (Chl *a*) is calculated applying the following formula:

$$c(\text{Chl } a)/\mu g\ L^{-1} = \{[A(s,664)-A(b,664)]-[A(s,750)-A(b,750)]\}\ v\ 10^6/(a^*\ op\ V)$$

where:

A (s, 664) = Absorbance of the sample at 664 nm;

A (s, 750) = Absorbance of the sample at 750 nm;

A (b, 664) = Absorbance of white at 664 nm;

A (b, 750) = Absorbance of the blank at 750 nm;

a* = specific absorption coefficient of chlorophyll *a* in 90% acetone at 664 nm (87.67 $cm^{-1}g^{-1}$ L)

op = optical path of the cell (cm);

v = volume of the extract (mL); and

V = volume of filtered sample (mL).

c. *Method 2. Concentrations of chlorophylls a, b and c*

The method should be used to provide accurate estimates of chlorophylls *a*, *b* and $c_1 + c_2$ on phytoplanktonic samples of mixed populations, when no significant quantities of their degradation products are present (Jeffrey and Welschmeyer, 2005[70]; Humphrey and Jeffrey, 2005[71]).

Absorbance at wavelengths of 630, 647, 664 and 750 nm are to be read, to estimate the incidence of the concentration of the chlorophylls *b* and *c* on the concentration of chlorophyll *a* (Lorenzen and Jeffrey, 1980). By applying this method, it is also necessary to read the blanks at respective wavelengths.

Determine the net absorbance of the extract at each wavelength [A (l)] according to the equation:

$$A(l) = [A(s, l)-A(b, l)]-[A(s, 750)-A(b, 750)]$$

where:

A (b, l) = Absorbance of the blank at l nm;

A (s, l) = Absorbance of the sample at l nm;

A (b, 750) and A (s, 750) are defined as above.

Calculate the concentrations of chlorophylls (Chl *a*, *b* and *c*) by applying the following equations:

[67] Jeffrey S.W., Humphrey G.F., 1975. New spectrophotometric equations for determining chlorophylls *a, b, c1* and *c2* in higher plants, algae and natural phytoplankton. Biochem. Physiol. Pfanzen., 167: 191-194.

[68] Lorenzen C.J., Jeffrey S.W., 1980. Determination of chlorophyll in sea water. UNESCO Tech. Pap. Mar. Sci., 35: 1-20.

[69] Lorenzen C.J., 1967. Determination of chlorophyll and phaeopigments spectrophotometric equations. Limnol. Oceanogr., 12: 343-346.

[70] Jeffrey S.W., Welschmeyer N.A., 2005. Spectrophotometric and fluorometric equations in common use in oceanography. In: Jeffrey S.W., Mantoura R.F.C., Wright S.W. (eds), Phytoplankton pigments in oceanography: guidelines to modern methods. 2nd ed. SCOR UNESCO, Paris: 597-615.

[71] Humphrey G.F., Jeffrey S.W., 2005. Test of accuracy of spectro-photometric equations for the simultaneous determination of chlorophylls *a*, *b*, *c1* and *c2*. In: Jeffery S.W., Mantoura R.F.C., Wright S.W. (eds), Phytoplankton pigments in oceanography: guidelines to modern methods. 2nd ed. SCOR UNESCO, Paris: 616-621.

c(Chl a)/µg L^{-1} = [11.85 A(664)-1.54 A(647)-0.08 A(630)] v 10^3/(op V)

c(Chl b)/µg L^{-1} = [-5.43 A(664)+21.03 A(647)- 2.66 A(630)] v 10^3/(op V)

c(Chl c_1+c_2)/µg L^{-1} = [-1.67 A(664)-7.60 A(647)+24.52 A(630)] v 10^3/(op V)

where:

A (l), op, v and V have the meaning already expressed above.

The values of the concentrations of chlorophylls b and c can be negative when these pigments are present in very low concentrations and cannot be determined with this method, or if there are many phaeopigments that disturb the readings.

d. *Method 3. Concentrations of chlorophyll a and phaeopigments*

The method allows to determine the concentrations of chlorophyll *a* and phaeopigments (pheophytins, pheophorbides, chlorophyllides) assuming that the ratio between their specific absorption coefficients is equal to that between chlorophyll *a* and pheophytin *a* (Lorenzen, 1967).

The analytical procedure involves the addition of 50 µL (one drop) of HCl (0.66 mol L^{-1}) for every 5 mL of extract directly into the spectrophotometer cell immediately after the readings at 665 and 750 nm. The cell must be shaken repeatedly and it is necessary to wait 30 to 60 seconds before repeating the readings at the same wavelengths. In this way, all the chlorophyll *a* present in the extract is converted into pheophytin *a*. It is important to keep in mind that the final acid concentration in the extract must not greatly exceed the value of 3 10^{-3} mol L^{-1} (30 µL of HCl 0.66 mol L^{-1} for each ml of extract), to avoid that the carotenoids present are transformed into a compound that absorbs in the red, thus altering the value of the reading of the phaeopigments (Riemann, 1978)[72].

Determine the net absorbance of the extract before acidification [A (665o)] and after acidification [A (665a)] according to the equation:

A(665α) = [A(s, 665α) -A(b, 665α)] - [A(s, 750α) -A(b, 750α)]

where:

A(b, 665) = Absorbance of the blank at 665 nm;

A(b, 750) = Absorbance of the blank at 750 nm;

A(s, 665α) = Absorbance of the sample at 665 nm before (α = o) or after acidification (α = a);

A(s, 750 α) = Absorbance of the sample at 750 nm before (α = o) or after acidification (α = a).

The concentrations of chlorophyll *a* (Chl *a*) and phaeopigments are calculated applying the following equations:

c(Chl a)/µg L^{-1} = 26.73 [A(665o)-A(665a)] v 10^3/(op V)

c(Phaeopigments)/µg L^{-1} = 26.73 [1.7 A(665a)-A(665o)] v 10^3/(op V)

where:

A(665o) = net optical density of the sample at 665 nm before acidification;

A(665a) = net optical density of the sample at 665 nm after acidification;

op, v and V have the meaning already expressed above.

e. *Important notes*

Instruments with interferential lattice has an optimal reading range, with respect to the measurement error, between 0.2 and 0.8 absorbance units (Strickland and Parsons, 1968). The minimum concentration of chlorophyll a at which, using 100 mm cells of optical path in the extract is 228 µg L^{-1}, which is equivalent to an *in situ* concentration of 0.46 µg L^{-1}, in the case 5 L of sample have been filtered. However, if the optical conditions of the measurement and the accuracy are satisfactory (± 0.002 A), readings are also valid with absorbances, at 664 nm, of 0.050 (Neveux, 1979)[73] corresponding to an *in situ* value of 0.11 µg L^{-1}.

If the absorbance of the blank exceeds 0.008 it is necessary to carefully clean the outside of the cells and if the readings value is still high, it is necessary to immerse the cells in sulphochromic mixture for 10 minutes and then rinse them abundantly with water before to repeat the reading. If the absorbance does not decrease, check that the disturbance is not due to impurities present in the acetone and if necessary, filter it carefully.

The reading at 750 nm gives an estimate of the turbidity of the sample and must not exceed the value of 0.010 of absorbance (i.e. 0.002 for each cm of optical path); otherwise it is necessary to repeat the centrifugation or filter the sample with a syringe equipped with a "Swinnex" support in which a 13 mm diameter Teflon filter with a porosity of 0.2 µm is inserted.

3.2.3 Protocol for fluorometric determination of concentration of chlorophyll *a*

The estimation of the concentration of chlorophyll *a* and phaeopigments with the fluorometric method is based on the measurement of the fluorescence of the pigments in acetone extract, before and after acidification with hydrochloric acid. The photosynthetically active (chlorophyll *a*) and inactive (phaeopigments) fractions of the chlorophyll pigments present (Yentsch and

[72] Riemann, B., 1978. Carotenoid interference in the spectrophotometric determination of chlorophyll degradation products from natura1 population of phytoplankton. Limnol. Oceanogr., 23: 1059-1066.

[73] Neveux, J., 1979. Pigments chlorophylliens. In: Jacques G. (ed), Phytoplancton, Biomasse, Production, Numeration et Culture. Edition du Castellet, Perpignan: 1-107.

Menzel, 1963[74]; Holm-Hansen et al., 1965[75]) are measured. Compared to spectrophotometric ones, fluorometric methods are more sensitive, precise and rapid, however the use is recommended only when the concentration of the pigments is low, since, for high values, the relationship between fluorescence and concentration is no longer linear. The upper limit within which the relationship remains such is approx. 750 µg L^{-1} in the acetone extract (Neveux, 1979) and approx. 1.5 µg L^{-1} in sea water (Bianchi, 1986)[76]. In any case, this linearity interval must be verified for each instrument. Furthermore, the validity of these methods is strongly conditioned by the heterogeneity of the pigment mixture, in particular by the concentration of chlorophyll *b* in the acetone extract (Yentsch, 1965[77]; Loftus and Carpenter, 1971[78]; Gibbs, 1979[79]). In fact, the pheophytin *b* produced by the degradation of this pigment shows an emission peak at 651 nm which, inversely to that of pheophytins *a* and *c*, shows a strong increase compared to the corresponding chlorophyll, thus causing, if present, an overestimation of the phage pigments.

Finally, the presence in the samples of other compounds that fluorescence in red should not be underestimated, since they can lead to erroneous estimates of chlorophyll *a* and phaeopigments.

a. *Equipment*

The equipment for fluorometric determination of concentration of chlorophyll *a* include: i) Spectrophotometer, see considerations in the previous paragraphs; and ii) Filter fluorometer or spectrofluorometer.

If a filter fluorometer is used, it is recommended to use an F474-BL lamp as the light source and a Corning CS.5-60 or Kodak Wratten 47B as excitation filter and a Corning CS.2-64 as emission filter. The instrument must be equipped with a photomultiplier with sensitivity extended to the 800 nm band (e.g. Hamamatsu R446). Even if measurements are taken with a spectrofluorometer, it is necessary to use a photomultiplier with extended sensitivity in the red region. Furthermore, it is necessary to calibrate/check the wavelengths of the monochromators; the simplest calibration consists in the emission scan of a sample of deionized water, placing the excitation monochromator at 350 nm: the maximum peak (called "Raman water peak") must be at 397 ± 2 nm. As a bandwidth, the recommended setting is 4-5 nm in excitation and 10 nm in emission.

b. *Procedure*

b.1. Fluorometric measurements

After the extract become clear the extracts are transferred to the fluorometric cuvettes.

Fluorometric readings (excitation – exc; emission – ems) are taken at the maximum wavelengths of chlorophyll *a*, (l_{exc} = 430 nm, l_{ems} = 665 nm) if a spectrofluorometer is used;

Two fluorometric readings for each sample are taken: i) F_o: the sample as it is; ii) F_a: the sample after adding 1 drop of a 1N HCl solution (after 1 minute); and the fluorescence range is noted in which all measured samples are included.

b.2. Preparation of the initial standard

A standard solution of pure commercial chlorophyll *a* (stock solution) is prepared by dissolving the standard, supplied in crystalline form, in a 90% (v / v) acetone solution;

The optical density of this solution is read with a spectrophotometer (in general an absorbance at 664 nm equal to about 0.09 units with a 10 mm cell is obtained);

The concentration of the stock solution (in mg L-1) is calculated using the following equation:

$c(\text{Chl } a)/\mu g\ L^{-1} = [A(664) - A(750)].\ (a^*\ op)^{-1}\ 10^6$

where

A(664) = Absorbance at 664 nm;

A(750) = Absorbance at 750 nm;

a* = specific absorption coefficient of chlorophyll *a* in 90%-acetone at 664 nm (87.67 $cm^{-1}g^{-1}$);

op = optical path of the cuvette, in cm.

The spectra (SPT) are scaned before (SPT_o) and after (SPT_a) acidification of the mother solution with a drop of 1N HCl; saved and the maximum excitation and emission noted.

The spectra have to be compared with those of chlorophyll *a* and pheophytin a reported in the literature, these scans must be repeated frequently to verify the possible existence of degradation processes in progress in the standard solution.

The linearity of the instrumental response must be verified: a series of substandards are prepared for a range of three orders of magnitude, using automatic

[74] Yentsch, C.S., Menzel, D.W., 1963. A method for the determination of phytoplankton chlorophyll and phaeophytine by fluorescence. Deep Sea Res., 10: 221-231.

[75] Holm-Hansen O., Lorenzen C.J., Holmes R.W., Strickland J.D.H., 1965. Fluorimetric determination of chlorophyll. J. Cons. Int. Explor. Mer., 30: 3-15.

[76] Bianchi, F., 1986. Relazioni fra misure di clorofilla in Adriatico settentrionale. Arch. Oceanogr. Limnol., 20: 287-292.

[77] Yentsch, C.S., 1965. Distribution of chlorophyll and phaeophytine in the open ocean. Deep Sea Res., 12: 653- 666.

[78] Loftus M.E., Carpenter J.H., 1971. A fluorometric method for determining chlorophylls *a, b* and *c*. J. Mar. Res., 29: 319-338.

[79] Gibbs, C.F., 1979. Chlorophyll b interference in the fluorometric determination of chlorophyll *a* and phaeopigments. Aust. J. Mar. Freshwater Res., 30: 597-606.

pipettes or calibrated glassware, with 1:2 dilutions in succession.

Following the same methods of reading the samples (l_{exc} = 430 nm, l_{ems} = 665 nm), each substandard must be read before (F_o) and after (Fa) acidification.

A table containing the dilutions carried out, the concentrations obtained and the fluorescence read before (F_o) and after (F_a) acidification must be prepared.

After bringing the pairs of concentration / fluorescence values on an x-y graph; a linear relationship at low values and a loss of linearity at higher values, caused by self-quenching phenomena present in molecules of fluorescent compounds, such as chlorophylls (Lakowicz, 2006)[80] will be noticed.

It is necessary for each operator to write down the limit beyond which linearity is lost for their instrument.

If the discrete samples show fluorescence values beyond this value, the sample must be diluted to bring it back into the linearity range of the instrumental response

b.3. Routine standardization after fluorometric measurement of samples

After each batch of analysis, starting from the stock solution, a series of 3-5 substandards must be prepared by dilutions, which fall within the fluorescence range obtained from the readings of the samples; and

For each substandard a reading before (F_o) and after (F_a) acidification with HCl must be performed.

c. Calculations of the concentrations of the samples:

The factor C is calculated as the average of the ratios between the 3-5 concentrations of each substandard ($C_{Chl\ a}$) and the relative fluorescence values before acidification (F_o)

The R factor is calculated as the average of the ratios between F_o and F_a for each of the 3-5 measured substandard;

The concentrations of chlorophyll *a* and phaeopigments are calculated from the sample values using the following equations proposed by Holm-Hansen et al. (1965):

$$c(\text{Chl } a)/\mu g\ L^{-1} = R\ (R-1)^{-1}\ C\ (F_o - F_a)\ v\ V^{-1}$$

$$c(\text{Phaeopigments})/\mu g\ L^{-1} = R\ (R-1)^{-1}\ C\ [(R\ F_a) - F_o)]\ v\ V^{-1}$$

where:

R = F_o / F_a average;

C = C (Chl a) / average Fo;

F_o = fluorescence of the sample before acidification;

F_a = fluorescence of the sample after acidification;

v = volume of the extract (mL);

V = volume of filtered sample (mL).

3.2.4 Protocol for High-Pressure Liquid Chromatography (HPLC) determination of concentration of chlorophyll *a*

The separation of pigments is possible thanks to their difference in polarity which determines the affinity between a mobile phase (elution solvents) and a stationary (column). In practice, it is determined by their different speed of crossing the column (composed of a support consisting of silicon and molecules of C18 or C8) which represents the stationary phase, while the mixture of solvents and pigments, which runs through the column, forms the mobile phase. The stationary phase is less polar than the mobile phase and, therefore, a reverse phase HPLC is implied. The polarity of the mobile phase varies over time, thus the pigments adsorbed on the stationary phase are eluted and therefore sequentially separated from the phase mobile according to their polarity gradient. Typically, an elution gradient is used that allows to decrease the retention time of the less polar compounds and, consequently, to increase the sensitivity of the method.

Once separated, the pigments are detected and quantified according to spectrophotometric methodologies and / or fluorometric. The result of the analysis is a chromatogram (spectrophotometric and/or fluorometric), in which the position of the peaks on the time axis allows to identify the different pigments present in the sample, while from the peak areas it is possible to quantify them. With the chromatogram obtained with a spectrophotometric detector the identification and quantification of both chlorophylls and carotenoids is allowed, while from the fluorescence chromatogram the identification of only the chlorophylls and their degradation products are possible.

Currently, the most accurate spectrophotometric detectors are diode ones (Diode Array Detector: DAD) which allow the determination of the absorption spectrum of each pigment; this allows, not only to quantitatively determine the chlorophylls and carotenoids, but also to evaluate their purity. In the absence of a DAD spectrophotometric detector it is advisable to use methods with analysis times longer that limit the overlap of the peaks. The solvent gradient, flow and run time (20-40 min) are characteristic of the selected method. However, it is advisable to seek optimization of the method to minimize time and amount of solvents and maximize the resolution of the pigments.

While these chromatographic separation techniques of the pigment mixture are costly and time demanding and not always sustainable for all laboratories routine work. If the choice of implementing this method for chlorophyll *a* analysis is selected, due to the complexity

[80] Lakowicz J. R., 2006. Principles of Fluorescence Spectroscopy. 3rd ed. Springer, Berlin: 954 pp.

in choosing the various components in order to optimize the cost /effectiveness of the apparatus that build the method it is advisable to start with consulting chapters 9 and 11 of the “Monograph on Oceanographic methodology (UNESCO Publishing, Publishers: Jeffrey SW, Mantoura RFC and Wright SW, 1997[81]) (Wright et al., 1997[82] and Mantoura et al., 1997[83], respectively). The most complete, up-to-date information about the analysis of pigments, particularly for the use of aquatic scientists, can be found in a recent book edited by Roy, Llewellyn, Egeland and Johnsen (2011)[84].This book follows the 1997 monograph edited by Jeffrey, Mantoura and Wright and together, these two books cover sample collection, methods for pigment extraction and analysis, with emphasis on HPLC methods, comparisons with non-chromatographic methods, preparation of pigment standards and a key for identification of the various algal pigments.

a. Symbol, units and precision

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 a Method identifier as provided in the Library P01 of BODC Parameter Usage Vocabulary are provided as follows:

Symbol: c(Chl a) **Unit:** $\mu g\ L^{-1}$

Precision: 0.01 **Accuracy:** ± 0.05

Method identifier:

SDN:P01:: **CPHLSXP1** Concentration of chlorophyll-a {chl-a CAS 479-61-8} per unit volume of the water body [particulate >GF/F phase] by filtration, acetone extraction and spectrophotometry

SDN:P01:: **CPHLFLP1** Concentration of chlorophyll-a {chl-a CAS 479-61-8} per unit volume of the water body [particulate >GF/F phase] by filtration, acetone extraction and fluorometry

SDN:P01::**CPHLHPP5** Concentration of chlorophyll-a {chl-a CAS 479-61-8} per unit volume of the water body [particulate >0.2um phase] by filtration, acetone extraction and high performance liquid chromatography (HPLC)

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[84] Roy, S.; Llewellyn, C. A.; Egeland, E. S.; Johnsen, G., 2011.Phytoplankton Pigments – Characterization, Chemotaxonomy and Applications in Oceanography. Cambridge University Press: Cambridge, 843 pp.

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