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Monitoring Guidelines/Protocols for Determination of Chlorophyll *a* in Seawater

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Note by the Secretariat

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

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

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

The Monitoring Guidelines for Determination of Chlorophyll *a* in Seawater provide the four protocols gathered under Technical Note for measurement of chlorophyll *a* in seawater, for consideration of Integrated Meeting of the Ecosystem Approach Correspondence Groups on Monitoring (CORMON) Biodiversity and Fisheries, Pollution and Marine Litter, and Coast and Hydrograph, as follows:

- Protocol for sample pre-treatment 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*.

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

List of Abbreviations / Acronyms

CAS	CAS Registry Number, is a unique numerical identifier assigned by the Chemical Abstracts Service (CAS)
CI	Common Indicator
COP	Conference of the Parties
CORMON	Correspondence Group on Monitoring
DAD	Diode Array Detector
EcAp	Ecosystem Approach
EO	Ecological Objective
EU	European Union
GES	Good Environmental Status
HELCOM	Baltic Marine Environment Protection Commission - Helsinki Commission
HPLC	High Performance Liquid Chromatography
IMAP	Integrated Monitoring and Assessment Programme of the Mediterranean Sea and Coast and Related Assessment Criteria
INFO/RAC	Information and Communication Centre of the Barcelona Convention
ISO	International Standard Organization
MAP	Mediterranean Action Plan
MEDPOL	Programme for the Assessment and Control of Marine Pollution in the Mediterranean Sea
MSFD	Marine Strategy Framework Directive
OSPAR	Convention for the Protection of the Marine Environment for the North-East Atlantic
SI	International System of Units (SI, abbreviated from the French Système international (d'unités))
SCOR	Scientific Committee on Oceanic Research
UNESCO	United Nation Educational Scientific and Cultural Organization

1. Introduction

1. 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) [1].

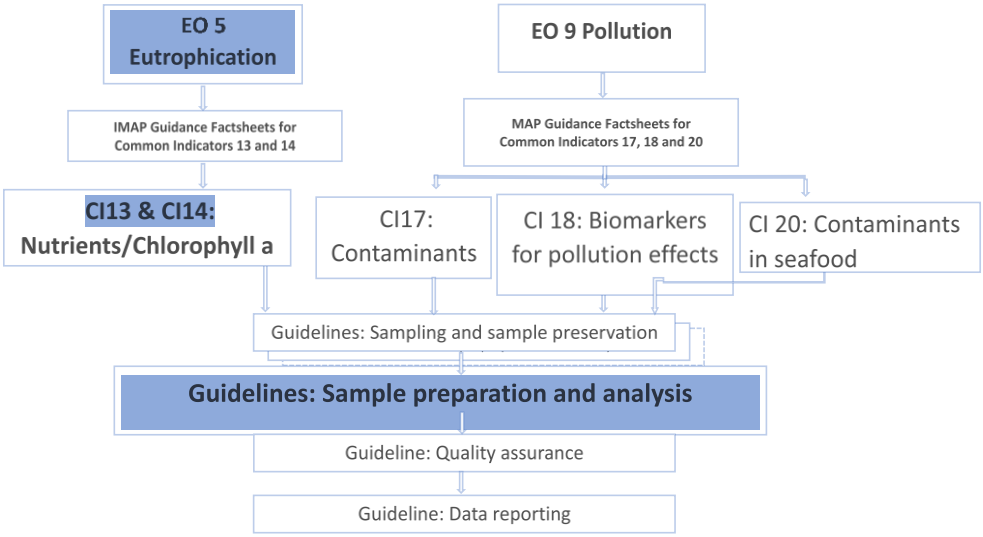
2. The IMAP Protocols elaborated within this Monitoring Guidelines for Determination of Chlorophyll *a* in Seawater provide detail guidance on the necessary equipment, chemical reagents, analytical procedures along with appropriate methodologies for measurement of the concentration of chlorophyll *a*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

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

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

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

[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.



Flow Diagram: Monitoring Guidelines for IMAP Ecological Objectives

2. Technical note for determination of concentration of chlorophyll *a*

6. 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)[5], 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.

7. 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.

8. 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.

9. 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[6]) are costly and time demanding and not always sustainable for all laboratories.

10. 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

[5] 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.
[6] Robinson, A.L., 1979. HPLC: the new king of analytical chemistry. Science, 203: 1329-1332.

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.

11. 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.

12. 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*.

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

13. 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

14. 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

15. 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

16. The filter, stored in pure acetone, must be triturated and homogenized for a maximum of 2 min by carefully rinsing the pestle of the homogenizer several times.

17. 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.

18. 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.

19. 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.

20. Note, that the use of ultrasound does not seem to give good results (Nusch, 1980)[7] as it produces excessive heating of the extract and is therefore not recommended.

21. 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).

2.2. Protocol for spectrophotometric determination of concentration of chlorophyll *a*

22. 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.

23. 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

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

25. 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*;

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

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

26. 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.

27. 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[8]; Lorenzen and Jeffrey, 1980[9]) or in the presence of significant quantities of its degradation products, having treated the extract with hydrochloric acid (Lorenzen, 1967)[10].

b. Method 1. Concentration of chlorophyll a

28. 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.

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

30. 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).

[8] 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.
[9] Lorenzen C.J., Jeffrey S.W., 1980. Determination of chlorophyll in sea water. UNESCO Tech. Pap. Mar. Sci., 35: 1-20.
[10] Lorenzen C.J., 1967. Determination of chlorophyll and phaeopigments spectrophotometric equations. Limnol. Oceanogr., 12: 343-346.

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

31. 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[11]; Humphrey and Jeffrey, 2005[12]).

32. 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.

33. 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.

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

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

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

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

where:

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

35. 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

36. 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).

37. 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

[11] 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.

[12] 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.

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)[13].

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

$$A(665\alpha) = [A(s, 665\alpha) - A(b, 665\alpha)] - [A(s, 750\alpha) - A(b, 750\alpha)]$$

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).

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

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

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

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. <u>Important notes</u>

40. 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)[14]. 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)[15] corresponding to an *in situ* value of 0.11 µg L^{-1}.

41. 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

[13] Riemann, B., 1978. Carotenoid interference in the spectrophotometric determination of chlorophyll degradation products from natura1 population of phytoplankton. Limnol. Oceanogr., 23: 1059-1066.
[14] Strickland, J.D.H., Parsons T.R., 1968. A Practical Handbook of Seawater Analysis. Bull. Fish. Res. Bd. Can., 167: 1-310.
[15] Neveux, J., 1979. Pigments chlorophylliens. In: Jacques G. (ed), Phytoplancton, Biomasse, Production, Numeration et Culture. Edition du Castellet, Perpignan: 1-107.

absorbance does not decrease, check that the disturbance is not due to impurities present in the acetone and if necessary, filter it carefully.

42. 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.

2.3. Protocol for fluorometric determination of concentration of chlorophyll *a*

43. 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 Menzel, 1963[16]; Holm-Hansen et al., 1965[17]) 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 $\mu g\ L^{-1}$ in the acetone extract (Neveux, 1979) and approx. 1.5 $\mu g\ L^{-1}$ in sea water (Bianchi, 1986)[18]. 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[19]; Loftus and Carpenter, 1971[20]; Gibbs, 1979[21]). 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.

44. 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

45. 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.

46. 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.

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[16] 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.

[17] 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.

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

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

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

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

b. Procedure

b.1. Fluorometric measurements

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

48. 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;

49. 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

50. 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;

51. 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);

52. 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.

53. 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.

54. 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.

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

56. 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.

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

58. 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)[22] will be noticed.

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

60. 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

61. 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

62. 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:

63. 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)

64. 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;

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

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

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

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).

2.4. Protocol for HPLC determination of concentration of chlorophyll *a*

66. 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

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

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.

67. 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.

68. 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.

69. 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 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[23]) (Wright et al., 1997[24] and Mantoura et al., 1997[25], 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)[26].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

70. 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

[23] Jeffrey, S. W.; Mantoura, R. F. C.; Wright, S. W., 1997. Phytoplankton Pigments in Oceanography: Guidelines to Modern Methods. UNESCO Publishing: Paris, 691 pp.
[24] Wright, S.W.; Jeffrey, S.W.; Mantoura, R.F.C., 1997. Evaluation of methods and solvents for pigment extraction, in: Jeffrey, S.W. et al. Phytoplankton pigments in oceanography: guidelines to modern methods. Monographs on Oceanographic Methodology, 10: pp. 261-282.
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[26] 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.

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)

Annex I
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