UNITED NATIONS

EP

UNEP/MED WG.509/15

30 August 2021
Original: English

Meeting of the MED POL Focal Points

Teleconference, 27-28 May and 6-7 October 2021

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

Monitoring Guidelines/Protocols for sampling and sample preservation of seawater for the analysis of CI13 and C14: concentration of key nutrients and chlorophyll a

UNEP/MAP
Athens, 2021

Table of Contents

Annexes:

Note by the Secretariat

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

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

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

The Monitoring Guidelines for sampling and sample preservation of seawater for the analysis of CI13 and C14 regarding concentration of key nutrients and chlorophyll *a* provide the five protocols gathered under two Technical Notes, as follows: a) Technical note for the sampling of seawater for the determination of hydrographic parameter and the measurement of concentration of key nutrients and chlorophyll a that includes the two following Protocols: i) Protocol for the use of a single water sampler attached to a line and ii) Protocol for the use of a water sampler attached to a rosette; and b) Technical note for the sample preservation of seawater for the determination of hydrographic parameter and the measurement of concentration of key nutrients and chlorophyll *a* that includes the three following Protocols: i) Protocol for the sample preservation of seawater for the determination of salinity ii) Protocol for the sample preservation of seawater for the determination of concentration of nutrients iii) Protocol for the sample preservation of seawater for the determination of concentration of chlorophyll *a*.

The Monitoring Guidelines/Protocols for IMAP Common Indicators 13 and 14, including this one related to sampling and sample preservation of seawater for the analysis of CI13 and C14 regarding concentration of key nutrients and chlorophyll *a*, establish a sound ground for further regular update of monitoring practice for a purpose of successful IMAP implementation.

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

List of Abbreviations / Acronyms

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

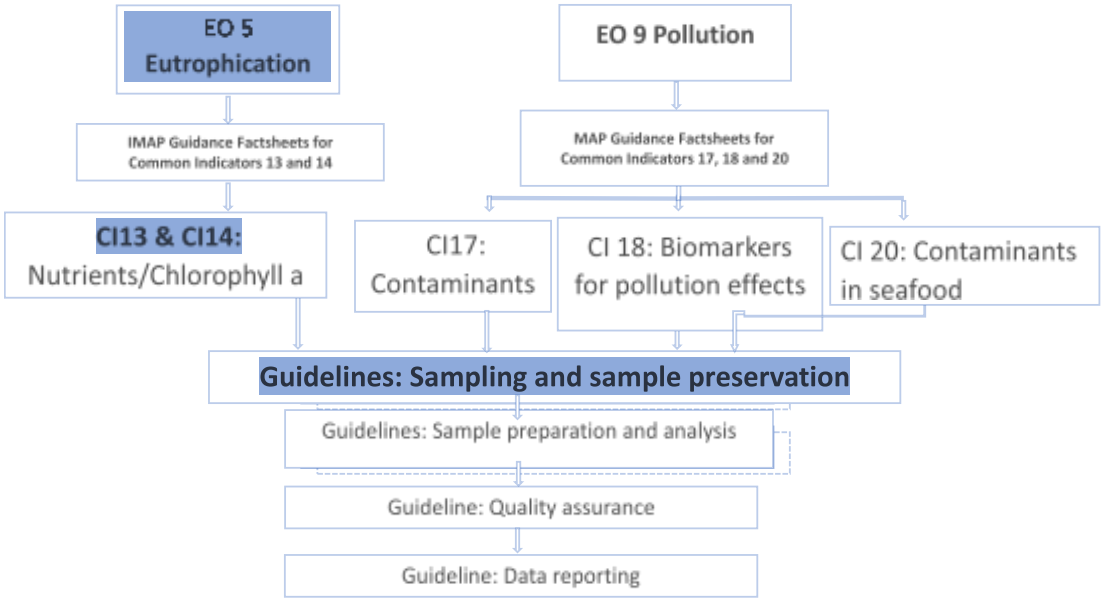
1. Introduction

1. In the Monitoring Guidelines for 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.

2. 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.

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

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



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

[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.

2. Technical note for the sampling of seawater for the determination of hydrographic parameter and the measurement of concentration of key nutrients and chlorophyll *a*

5. 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.

6. 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 it's starting point. Sampling is so important that, in some cases, it represents the main contribution to the error of the whole analytical process.

7. 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).

8. 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.

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

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

a. Principle of work

10. 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.

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

11. 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.

12. 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

13. 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.

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

15. 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.

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

17. 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*.

18. 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.

2.2. Protocol for the use of a water sampler attached to a rosette

a. Principle of work

19. 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

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

21. 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.

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

23. 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.

24. The CTD is bring back to the surface and the measurement of the profile is 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.

25. 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.

26. 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.

27. 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.

28. 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.

29. 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.

30. 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.

31. 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.

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

32. 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

33. 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.

34. 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.

35. 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.

3. Technical note for the sample preservation of seawater for the determination of hydrographic parameter and the measurement of concentration of key nutrients and chlorophyll *a*

36. 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.

37. 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.

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

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

3.1. Protocol for the sample preservation of seawater for the determination of salinity

a. Equipment

38. 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

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

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

41. 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.

42. 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

43. 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[9]). 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)[10]. The tightness and chemical inertia of the bottles are factors determinants for a good conservation of the samples.

d. Important notes

44. 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.

45. 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.

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

3.2. Protocol for the sample preservation of seawater for the determination of concentration of nutrients

47. 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.

48. 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[11], 1996[12]; Dore et al., 1996)[13] 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

[9] Stalcup M.C. ,1991. Salinity measurements. In: WOCE Operational Manual WHPO 91-1, WOCE Report No 68 (http://whpo.ucsd.edu/manuals.html).
[10] Grasshoff, K., 1983. Determination of salinity. In: Grasshoff K., Ehrhardt M., Kremling K. (eds), Methods of Seawater Analysis, Verlag Chemie; Weinheim: 31-60.
[11] Kirkwood, D.S., 1992. Mar. Chem., 38,151.
[12] Kirkwood, D.S., 1996. Nutrients: Practical notes on their determination in seawater. Copenhagen: ICES Tech. Mar. Environ. Sci., 17,25 pp.
[13] Dore, J.E., Houlihan. T., Hebel, D.V., Tien, G., Tupas, L., Karl, D.M. (1996), Mar. Chem., 53, 173.

effective in spring but not in fall. The following two approaches to preservation are: refrigeration and poisoning.

49. Freezing (to -20 "C) 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[14]; Macdonald et al., 1986[15]; Kremling and Wenck, 1986[16]; Chapman and Mostert, 1990[17]; 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)[18]

50. 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.

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

52. 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.

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

[14] Macdonald, R.W., McLaughlin, F.A. (1982), Water Res, 16,95.
[15] Macdonald, R.W., McLaughlin, EA., Wong, C.S. (1986), Limnol. Oceanogr., 31, 1139.
[16] Kremling, K., Wenck, A. (1986), Meeresforschung, 31,69.
[17] Chapman, P., Mostert, S. A. (1990), S. Afr J. Mar. Sci., 9,239.
[18] ISO 5667-3:2012 Water quality — Sampling. Part 3: Preservation and handling of water samples.

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

54. 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

55. 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.

56. 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.

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

a.2. Ammonium - N

58. 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).

59. 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

60. Nitrite is an intermediate compound, which occurs if ammonia is oxidized or nitrate is reduced. The presence of higher amounts of nitrite (> 1.5 µmol 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 µmol 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

61. 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 µmol 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.

62. 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

63. 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.

64. 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.

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

a. <u>Equipment and reagents</u>

65. 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

66. 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[19]; Lenz and Fritsche, 1980[20]).

c. Filtration procedure

67. 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.

68. 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.

69. 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.

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

71. 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.

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

73. 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.

74. 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

75. 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)[21]. Consequently, to perform the extraction and analysis of chlorophyll pigments immediately after filtering the sample is always preferable.

76. 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

[19] Strickland Ld., Parsons T.R., 1968. A practical handbook of sea-water analysis. Bull. Fish. Res. Board Can., 167, 1-312.
[20] 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.
[21] Rai H., Marker A.F.M., 1982. The measurements of photosynthetic pigments in freshwaters and standardization of methods. Arch. Hydrobiol. Beih., 16: 1-130.

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.

77. 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[22]; Blasco, 1973[23]; Neveux, 1979[24]; Lenz and Fritsche, 1980; Lazzara et al., 1990[25]; Mantoura et al., 2005[26]).

78. 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[27]) or the drying and freezing technique (freeze-drying) which does not give satisfactory conservation results according to Lenz and Fritsche (1980).

79. 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)[28] so that no freezing practice can be recommended.

80. 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.

[22] 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.

[23] 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.

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

[25] 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.

[26] 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.

[27] 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.

[28] 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.

Annex I: References

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.

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.

UNEP/MAP, 2019a. UNEP/MED WG.463/6. Monitoring Protocols for IMAP Common Indicators related to pollution.

UNEP/MAP, 2019b. UNEP/MED WG.463/10. Schemes for Quality Assurance and Control of Data related to Pollution.

Salinity

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

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

Key nutrients

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

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

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

Kirkwood, D.S., 1992. Mar. Chem., 38,151.

Kirkwood, D.S., 1996. Nutrients: Practical notes on their determination in seawater. Copenhagen: ICES Tech. Mar. Environ. Sci., 17,25 pp.

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

Macdonald, R.W., McLaughlin, F.A. (1982), Water Res, 16,95.

Macdonald, R.W., McLaughlin, EA., Wong, C.S. (1986), Limnol. Oceanogr., 31, 1139.

Chlorophyll a

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.

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.

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.

Mantoura R.F.C., Wright S.W., Barlow R.G., Cummings D.E., 2005 Filtration and storage of pig- ments 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.

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.

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

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.

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

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

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.