

Guidelines for monitoring of chlorophyll *a*

1. Background

1.1 Introduction

Increase in phytoplankton biomass is a direct consequence of advancing eutrophication. For monitoring purposes, phytoplankton biomass is estimated by chlorophyll *a* (Chl *a*) concentration.

The Chl *a* concentration of a water sample provides information about the trophic state of a water body. Although this parameter is not a direct index for phytoplankton biomass because of a highly variable ratio of cellular carbon to Chl *a* in phytoplankton (Geider 1987; [Jakobsen & Markager 2016](#)) it is easily-determinable providing a wealth of data and thus a key variable in trophic evaluation of waters.

The scope of this guideline is the determination of Chl *a* concentration; measured from water samples using wet analytics and/or estimated from *in vivo* Chl *a* fluorescence recordings.

1.2 Purpose and aims

Monitoring of Chl *a* provides information that is used for assessing direct effects of eutrophication. The aim is to provide spatiotemporal information for detection of short-term status and long-term trends and to ensure that the data is compatible for the HELCOM core indicator 'Chlorophyll *a*'. The indicator description, including its monitoring requirements, is given in the HELCOM core indicator web site: [<http://helcom.fi/baltic-sea-trends/indicators/chlorophyll-a>].

2. Monitoring methods

2.1 Monitoring features

Chl *a* is an optically active pigment, and hence, its concentration within a sample can be determined optically by spectrophotometry (light absorption), fluorometry (fluorescence emission), and high-performance liquid chromatography (HPLC).

The *in vivo* fluorescence yield is considerably lower for cyanobacteria than for eukaryotic groups using the traditional *in vivo* Chl *a* fluorescence method (Lorenzen et al. 1966), and hence, this method does not reliably detect cyanobacterial biomasses (Seppälä et al. 2007).

2.2 Time and area

2.2.1 Station-based determination of Chl-*a*

Chl *a* concentration patterns vary substantially both in space and time, including inter-annual variation, obscuring the determination of temporal trends. For this reason, the station-based determination of Chl *a* using wet analytics should be carried out throughout the growth season, offering the possibility of assessing mean values for the spring, ~~summer and~~ autumn season, and for the entire growth season. As a minimum, this should be done during the summer months (depending on the Baltic region).

Chl *a* monitoring is carried out by all HELCOM Contracting Parties, and the monitored area covers the entire Baltic Sea area, both the open sea and coastal areas.

2.2.2. Ship-of-opportunity

In the ship-of-opportunity (SOOP) approach, Chl *a* is estimated along the routine operating merchant routes using both wet analytics and *in vivo* Chl *a* fluorescence emission recorded by Ferryboxes, enabling the description of spatial distribution and surface coverage of phytoplankton blooms along the ferry route.

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2.3 Monitoring procedure

2.3.1 Monitoring strategy

To reach sufficiently high confidence criteria of the core indicator, the joint monitoring should produce annually at least 15 measurements, collected within the station network of each assessment unit, i.e., for open sea sub-basins. The measurements should be as evenly spatially distributed as possible.

2.3.2 Sampling method(s) and equipment

2.3.2.1 Station/ship based monitoring

To describe the distribution of Chl *a* concentration, horizontal and vertical sampling at an appropriate number of stations is required. The sampling strategy for sea-truth measurements follows the general HELCOM strategy of shared ship cruise measurements on agreed stations in the Baltic Sea.

Chl *a* samples are collected in the ship-based monitoring as follows:

- For the open sea, the standard sampling depths in the upper water column: 1, 5, 10, 15 and 20 m (the same as for nutrients). Additional sample(s) should be obtained from Chl *a* maxima present at other depths
- In coastal monitoring, an individual sample at 1 m or an integrated sample (1 to 10 m) can be collected
- For helicopter sampling, an individual sample from the mixed surface layer (optimally at 1 m depth) can be taken
- Additionally, Chl *a* should be analyzed as a subsample from the sample used for phytoplankton taxonomy and/or primary production analyses

Sampling equipment

- Discrete sampling bottles
- CTD-rosette bottles. A deployable fluorometer typically installed into the CTD-system offers exact information on the vertical phytoplankton distribution

2.3.2.2. Ship-of-opportunity

In Ferryboxes, single samples are collected underway typically at 3–5 m depth, and stored by a refrigerated automatic sampler. Due to the turbulent effect created by the hull of the ferry, samples describe best the surface mixed layer, and cannot be linked to any certain depth.

2.3.3 Sample handling

2.3.3.1 Station/ship based monitoring

Due to seasonal variations in phytoplankton biomass the sample volume has to be optimised to fit the volume of the extraction solvent and the cuvette length. The sensitivity of the fluorometer is about ten times higher than that of the spectrophotometer, and hence, the required sample volume for the fluorometer could be one tenth of that for the spectrophotometer.

The sample water for Chl *a* measurement should be filtered immediately after sampling through GF/F filters, and extracted with an appropriate amount of $\geq 96\%$ ethanol without any drying period. The samples shall be filtered in subdued light. Suction pressure must not exceed 3×10^4 Pa. Filtration time must not exceed 30 min for the volumes needed for the spectrophotometric method and 3 min for the fluorometric method.

Filters should not be stored dry, but extracted as soon as possible (Wasmund et al. 2006); only short time storage [of filters is allowed, ideally shock-frozen in liquid nitrogen at -80 °C \(up to 3 months\), or if this equipment is not available, frozen at -20 °C for max. 4 weeks](#). Recommended extraction time is 6–24 hours at room temperature. Extracts can be stored in an ultracold freezer (–80°C), or otherwise at –20°C, up to three months prior to the measurement (Wasmund et al. 2006).

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2.3.3.2. Ship-of-opportunity

The samples cannot be filtered immediately after sampling in the SOOP approach, so the sample bottles should be shaken well prior to filtration. Otherwise, the same sample treatment applies as for station/ship based monitoring samples.

2.3.4. Sample analysis

All work with the chlorophyll extracts shall be carried out in subdued or green light.

2.3.4.1. Spectrophotometric method

The Chl *a* concentration is measured from individual extracts using light absorption peak at 663–665 nm with 2 nm band-width (absorption at 750 nm should be subtracted to prevent falsification of the measured values due to turbidity). Clear filtration with a cellulose acetate membrane filter (pore size of 0.45 µm) or centrifugation for 10–20 minutes at about 10,000 m/s² is necessary in order to reduce the blank reading to < 0.002 per cm path length of the cuvette. The method is based on ISO 10260, and Arvola (1981), except that here the Chl *a* determination is not corrected by phaeopigment content operated by an acidification step. It is to note that the exclusion of phaeopigment correction can lead to an overestimation of the true Chl *a* content.

Calculation:

$$\text{Chl } a \text{ (mg/m}^3\text{)} = 1000 \times e \times A \times (83 \times V \times I)^{-1}$$

where e = volume of ethanol (cm³), A = absorbance at 750 nm subtracted from absorbance at 665 nm after correction by the cell-to-cell blank, 83 = absorption coefficient in 96% ethanol, V = water volume filtered (dm³), and I = length of cuvette (cm).

The sample volume, ethanol volume and the length of cuvette must be chosen so as to deliver absorbance at a range of 0.05–0.8 at 663–665 nm, i.e., within the optimum range of the instrument.

2.3.4.2. Fluorometric method

It is necessary to make sure that the concentration of the extraction is at the optimum range of the instrument used. Synthetic detergents should be avoided when cleaning the cuvettes, as they may interfere with the fluorescence

The Chl *a* concentration is measured from individual extracts against the instrument calibration curve. The calibration factor is determined from the spectrophotometric reading based on known concentrations of pure Chl *a*. The wavelength combination for the determination is 425–430 nm / 663–672 nm (excitation / emission, depends on the instrument). The method is based on Holm-Hansen et al. (1965), Lorenzen (1966), and Jespersen and Christoffersen (1987).

Calculation:

$$\text{Chl } a \text{ (mg/m}^3\text{)} = R \times f \times s \times e \times V^{-1}$$

where R = fluorescence reading, f = calibration factor, s = slit correction, e = volume of ethanol (cm³), and V = sample volume (dm³).

The calibration factor is determined as follows:

$$f = KR^{-1} \times Ve^{-1}$$

where K = concentration of Chl *a* (mg/m³) determined spectrophotometrically as described by Arvola (1981).

For the measurement of phaeopigments, an additional fluorescence reading is recorded after the acidification of the extract with 1 M HCl (0.06 cm³ to 5 cm³ of extract) after the first reading. A new reading should be taken 0.5-3 minute after the acidification. Phaeopigments are determined as:

$$\text{Phaeopigment (mg/m}^3\text{)} = f_a \times ((r \times R_a) - R) \times s \times e \times V^{-1}$$

where R_a = fluorescence reading after acidification and r = ratio of R to R_a obtained from an extract free from phaeopigments. Calibration factor f_a :

$$f_a = f \times r \times (r^{-1}).$$

2.3.4.3. High pressure liquid chromatography

High pressure liquid chromatography (HPLC) techniques allow simultaneous determination of the concentrations of a wide range of carotenoids, chlorophylls and their degradation products. For pigment separation, most of HPLC methods employ reversed-phase conditions and columns packed with stationary phases having an aliphatic chain length of C_8 , C_{18} or C_{30} (Bidigare et al. 2005, Carrido et al. 2011). Separated pigments peaks are identified by a comparison of retention times with those of standards. Quantification of pigments is typically based on their absorption or fluorescence signals.

2.4 Data analysis:

2.4.1. Ship-of-opportunity

For high-frequency Chl a mapping with Ferryboxes, flow-through based *in vivo* Chl a fluorescence data is converted to Chl a concentration by validating a fluorescence emission – recorded at the time of sampling – with a parallel wet Chl a measurement from the water sample. A statistical relation is derived from the collected transect data and the resulting conversion factor is extrapolated with certain reliability to flow-through data between the sampling points. This conversion factor varies regionally and seasonally due to variations in the phytoplankton physiology and community structure, and has to be treated as transient; the factor should be re-estimated every time Chl a data is being collected.

This basic modelling provides adequate results during the times of season when algal biomasses are high or eukaryotic groups dominate. The relation weakens when the algal biomasses are low or cyanobacteria comprise a significant part of the phytoplankton community. In that case, auxiliary parameters, such as phycocyanin fluorescence (indicating the presence of cyanobacteria) and turbidity correct the relation substantially (Seppälä et al. 2007).

Ferrybox system probes the phytoplankton inhabiting the upper layers of the water column. There, phytoplankton exhibit non-photochemical fluorescence quenching under excessive light levels, which lowers *in vivo* Chl a fluorescence emission relative to Chl a concentration. This diel variation in *in vivo* Chl a fluorescence needs to be taken into account when using samples collected in the daytime to convert fluorescence data recorded in the nighttime, and vice versa (Babin 2008). Therefore, the calibration factors should be determined for day and night separately.

3. Data reporting and storage

The wet analytics data is included in the station data along with depth-dependent variables, stored by the contracting parties, and reported annually to the COMBINE database hosted by ICES.

4. Quality control

4.1 Quality control of methods

Laboratories carrying out Chl a analyses should have established a quality management system according to EN ISO/IEC 17025 standard.

Replicate samples (at least one duplicate sample should be included in every batch of samples), producing data for X-bar charts, are included in the analysis in order to clarify the magnitude of random error for Chl a results introduced by sampling and handling of the samples.

The limit of quantification should be determined annually. Since there is no stable standard (reference material) for Chl *a*, the blank value method should be used. For this purpose, a practically analyte free sample whose matrix largely corresponds to that of monitoring samples (e.g. the filtrate of a real sample) is used for a 10-fold determination.

In order to clarify the magnitude of systematic error in the analytical chain, laboratories should participate in ring-tests and interlaboratory proficiency tests.

Contracting parties should follow the HELCOM monitoring guideline but minor deviations from this are acceptable if the method achieves comparable results. The deviations have to be documented. Validation of the adopted method needs to be performed on the relevant matrix and concentration range e.g. by taking part regularly at inter-comparison studies or proficiency testing schemes.

4.2 Quality control of data and reporting

Measurement uncertainty should be estimated using ISO 11352 standard. Estimation should be based on within-laboratory reproducibility, data from proficiency testings, and internal / commercial reference material.

Data must be flagged if normal QA routines or recommended storage conditions cannot be followed.

See HELCOM (2015).

5. Contacts and references

5.1 Contact persons

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* For undated references, the latest edition of the referenced document (including any amendments) applies