SLOVENSKI STANDARD
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Kakovost vode - Spektrofotometrijsko določevanje klorofila-a po ekstrakciji z etanolom za rutinski monitoring kakovosti vode

Water quality - Spectrophotometric determination of chlorophyll-a content by ethanol extraction for the routine monitoring of water quality

Wasserbeschaffenheit - Spektrophotometrische Bestimmung des Chlorophyll-a-Gehalts durch Ethanolextraktion für das Routinemonitoring der Wasserqualität

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Water quality - Spectrophotometric determination of chlorophyll-a content by ethanol extraction for the routine monitoring of water quality

Wasserbeschaffenheit - Spektrophotometrische Bestimmung des Gehalts an Chlorophyll-a

This draft European Standard is submitted to CEN members for enquiry. It has been drawn up by the Technical Committee CEN/TC 230.

If this draft becomes a European Standard, CEN members are bound to comply with the CEN/CENELEC Internal Regulations which stipulate the conditions for giving this European Standard the status of a national standard without any alteration.

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EUROPEAN COMMITTEE FOR STANDARDIZATION
COMITÉ EUROPÉEN DE NORMALISATION
EUROPÄISCHES KOMITEE FÜR NORMUNG

CEN-CENELEC Management Centre: Rue de la Science 23, B-1040 Brussels

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European foreword

This document (prEN 17899:2022) has been prepared by the Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

Introduction

Chlorophyll-a is the most common essential photosynthetic pigment present in photoautotrophic plankton organisms. It is the main component of the dynamically regulated photosystem of these organisms, in which other accessory pigments are involved, some of which are chemically very similar to chlorophyll-a. Chlorophyll molecules isolated from the photosystem can be broken down by the influence of light and acids as well as chlorophyllases.

The chlorophyll-a content depends on the species composition of the phytoplankton, the time, the period, the place and the depth of sampling. It is also suitable for quantifying the change in the algal biomass (cell proliferation) in biological tests to check the toxicity or productivity of water or substances dissolved in water.

The chlorophyll concentration of a water sample can provide information about the trophic state of a water body. It is used as an easily determinable measure of the phytoplankton biomass and is a key variable in many trophy scoring systems. Even if this value cannot be used as an absolute measure for the phytoplankton biomass, the determination of the chlorophyll-a concentration together with other biomass and bioactivity parameters provides information about the quantitative occurrence and the potential metabolic performance of the phytoplankton in waters.

Due to the sensitivity of chlorophyll to light, acids and enzymes, there is currently no universally applicable routine analytical method that enables an accurate, artefact-free and at the same time simple determination of the chlorophyll-a content in water samples containing phytoplankton. The extractive, spectrophotometric method described in this document therefore provides an operationally defined value.

WARNING — Persons using this document should be familiar with usual laboratory practice. This document does not purport to address all of the safety problems, if any, associated with its use. It is the responsibility of the user to establish appropriate safety and health practices.

IMPORTANT — It is absolutely essential that tests conducted in accordance with this document be carried out by suitably qualified staff.

1 Scope

This document describes a spectrophotometric method for determining the chlorophyll-a content as a measure of the amount of phytoplankton for all types of water. Assuming a maximum sample volume of 2 l, chlorophyll-a content values of 5 µg/l or more can be determined. The determination limit can be calculated by each lab individually and it can be as low as 0,5 µg/l using 2 l of sample (or even more) and a 50 mm cuvette.

2 Normative references

The following documents are referred to in the text in such a way that some or all of their content constitutes requirements of this document. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

ISO 5667-4, *Water quality — Sampling — Part 4: Guidance on sampling from lakes, natural and man-made*

EN ISO 3696, *Water for analytical laboratory use - Specification and test methods (ISO 3696)*

EN ISO 5667-3, *Water quality - Sampling - Part 3: Preservation and handling of water samples (ISO 5667-3)*

EN ISO 5667-6, *Water quality - Sampling - Part 6: Guidance on sampling of rivers and streams (ISO 5667-6)*

3 Terms and definitions

For the purposes of this document, the following terms and definitions apply.

ISO and IEC maintain terminological databases for use in standardization at the following addresses:

— ISO Online browsing platform: available at https://www.iso.org/obp

— IEC Electropedia: available at http://www.electropedia.org/

3.1
chlorophyll-a
natural plant pigment that is present in phytoplankton, most important photosynthetic pigment of photoautotrophic organisms

Note 1 to entry: For the purposes of this document, the pigment whose concentration can be determined by measuring the absorption at 665 nm in an ethanolic solution (without phaeopigments).

3.2
phaeopigments
collective term for chlorophyll breakdown products, which do not contain any magnesium

EXAMPLE Phaeophytin is a phaeopigment.

3.3
phytoplankton
community of free-living, suspended, mainly photosynthetic organisms in aquatic systems comprising cyanobacteria and algae

3.4
accessory pigments
auxiliary pigments that support photosynthesis

EXAMPLES Carotinoids, phycobilins, chlorophyll-b and chlorophyll-c.

4 Principle

The determination of the chlorophyll-a concentration according to this document is based on the hot ethanolic extraction of a water sample's filter residue and the subsequent absorption measurement at 665 nm, whereby phaeopigments are also recorded. After the quantitative conversion of the chlorophyll-a into phaeopigments by means of acidification and renewed measurement at 665 nm, the original chlorophyll-a concentration of the water sample is calculated.

5 Interferences

Macroscopic parts of plants in the water sample (e.g. duckweed, drifted benthic filamentous algae, torn off macrophyte parts) as well as large zooplankton can falsify the measurement result. They shall be removed from the water sample, if they should not expressly be included.

In the case of water samples with a pH value of < 5, chlorophyll degradation by acid occurs in the filter residue. To prevent this, the pH value shall be neutralized by rinsing the filter with about 50 ml of a 0,1 molar ammonium acetate solution (see Reference [7]). This should be done shortly before the end of the filtration, when there are only a few millilitres of sample left in the filter funnel.

Chlorophyll is very sensitive to light, especially in extracted solution. If the extract is exposed to direct sunlight or bright artificial light, the pigment is destroyed photochemically.

Changes in the concentration of the extractant due to evaporation are to be avoided by working with firmly sealable extraction vessels.

During the extraction and homogenization of the filters, turbidities occurs which impairs the precision of the photometric measurement; it shall be removed by filtration (see 8.1) or centrifugation (see 8.1). The same applies to turbidities in the extract caused by other reasons (e.g. after acidification).

Pigments of (rarely occurring) autotrophic bacteria (e.g. chlorobium-chlorophyll, "bacterioviridin") may influence the determination of chlorophyll-a.

6 Reagents

Use reagents of an analytical pure quality and water according to class 2 of EN ISO 3696.

6.1 Ethanol, C_2H_5OH, volume fraction 96 %.

NOTE 1 Pure ethanol is preferred. Using ethanol 96 % with 5 % methanol added is allowed. Also, the denaturant methyl ethyl ketone (MEK) in ethanol does not interfere with the determination.

NOTE 2 Various other extractants (e.g. acetone or methanol) are described in the literature, but these can have lower extraction efficiency or are toxicologically problematic. This therefore prescribes ethanol as the extracting agent.

6.1.1 Ethanol, C_2H_5OH, volume fraction 90 %.

Add 60 ml of water to 900 ml of ethanol (6.1).

6.2 Hydrochloric acid, HCl, 12 mol/l, ρ = 1,19 g/ml.

6.2.1 Hydrochloric acid, HCl, 0,4 mol/l.

Add 10 ml of concentrated hydrochloric acid (6.2) to 290 ml of water.

6.3 Ammoniumacetate solution, $C_2H_7NO_2$, 0,1 mol/l.

7 Apparatus

Any work should preferably be done in a UV-free room. If this is not possible, the activities should be carried out using laboratory equipment and devices that do not let any UV light pass through.

7.1 Vacuum pump, preferably diaphragm vacuum pump with barometer, with suction bottle according to EN ISO 6556, nominal volume 2 l to 5 l, with silicone or rubber stopper.

7.2 Filtration apparatus for water samples with tightly closing funnel, preferably for filter diameters of 45 mm to 55 mm.

7.3 Filtration apparatus for extracts, e.g. Witt's pot to accommodate a volumetric flask, 200 mm high and internal width 150 mm, with side tube for hose attachment, flat ground lid with central tube, rough ground for rubber stopper connection, and filter attachment for round filters with low dead volume (e.g. perforated plate as filter support).

NOTE The filtration apparatus can be protected from excessive incidence of light by means of an opaque film or by painting it - with the exception of a viewing window.

7.4 Glass fibre filter, made of borosilicate glass, without binding agent, separation efficiency > 98 % for particles > 0,7 µm, recommended diameter 45 mm to 55 mm.

7.5 Filters for clearing of extracts, ash-free, slow-filtering paper filters with high separation efficiency or cellulose acetate membrane filters with a pore size of 0,45 µm.

NOTE Cellulose nitrate filters cannot be used because of losses of chlorophyl-a.

7.6 Extraction vessel, protected from light, tightly closable, e.g. wide neck bottles or vials made of (amber) glass with screw cap, nominal volume approx. 30 ml to 100 ml, preferably suitable for centrifugation.

7.7 Centrifuge, with an acceleration of 3 000 *g*, suitable for glass vials with tightly fitting screw cap centrifuge tubes with tightly fitting screw caps, nominal volume 15 ml to 50 ml; light protected (e.g. made of brown polypropylene), also transparent when working in darkened rooms.

7.8 Shaking water bath, set at (75 ± 1) °C.

7.9 Spectrophotometer, suitable for absorption measurements at 665 nm and 750 nm, with the following recommended performance characteristics: spectral bandwidth ≤ 2 nm, photometric accuracy ≤ ±0,005 at 1 abs. (decadic absorbance), wavelength accuracy ≤ ±1 nm.

NOTE 1 Suitable devices are in particular split-beam, dual-beam or reference beam path spectrophotometers.

Devices with an integrated self-test to check for their correct function are recommended.

7.10 Rectangular cuvette, with a path length of 10 mm to 50 mm (or even 100 mm can be used).

NOTE In most cases, the use of 50 mm cuvettes will be suitable.

7.11 Freezer, with a temperature lower than −18 °C.

7.12 Freezer, with a temperature lower than −80 °C.

7.13 pH meter with electrode, suitable for pH measurements in alcohol.

8 Procedure

8.1 General

Direct sunlight or bright artificial light shall be avoided in all steps of the procedure. The filtration of the water sample shall, the extraction and the analysis should take place in subdued light in the laboratory. Execution in a darkened room with low light is recommended. The illuminance should not exceed the level necessary for safe working with dark-adapted eyes. Execution in a room with UV free lamps is recommended.

8.2 Sample pre-treatment

Start with a sample taken according to EN ISO 5667-6 or ISO 5667-4 and preserved according to EN ISO 5667-3. The water samples are transported at (5 ± 3) °C in the dark. Plant material and/or water fleas can contribute to the chlorophyll content. If present, pre-filtering shall be carried out over plankton gauze of a mesh width of 1 mm. The water samples should be filtered as quickly as possible, at the latest 24 h after the sample was taken. Freezing of the water samples is not permitted.

8.3 Filtration

After the water sample has been sufficiently homogenized by shaking, a volume appropriate to the required extinction range at 665 nm between 0,01 and 0,90, is filtered through a glass fibre filter (7.4). With high concentrations, 100 ml will be sufficient, while in low concentrations the maximum possible sample volume up to the filter blockage will be required (e.g. 2 l to 5 l or even more).

Large amounts of non-chlorophyll-containing particles can quickly clog the glass fibre filters used. In this case, neither the filtration time should be greatly extended nor the filtration pressure increased, as this can cause losses through damage to the phytoplankton cells. Instead, a smaller sample volume should be used or the sample volume should be divided between several filters, if possible.

To minimize losses due to the destruction of algae cells, a vacuum of 30 kPa to 50 kPa shall be applied. The vacuum shall be switched off immediately when the water sample has been completely filtered. Avoid vacuuming the filters to drought!

After filtration, start the extraction according to 8.4. It is recommended to start the extraction immediately, however if this is not possible algae covered filters shall be stored in a freezer below −18 °C (7.11) for up to two weeks or stored in a freezer below −80 °C (7.12) for up to 1 month.

8.4 Extraction

Quantitatively transfer the algae covered filter to the extraction vessel (7.6). Add 25 ml (= V1) of ethanol 90 % (6.1.1). Seal the extraction vessel with the screw cap carefully and place it in a shaking water bath (7.8) at a temperature of (75 ± 1) °C. Then heat for 7 min (2 min of warming up and 5 min of extraction) at a shaking speed of 150 rpm to 200 rpm. Cool the extraction vessel with the extract at an accelerated pace until it is at room temperature by placing it in a reservoir filled with ice or holding it under cold running water.

In case of turbidity the extracts can be clarified by filtration or by centrifugation:

The volume of ethanol required for extraction depends on the intended nominal volume of the extract: if the extract is clarified by filtration, e.g. about 15 ml should be added to the extraction vessel (7.6) for a nominal volume of 25 ml, and about 30 ml for a nominal volume of 50 ml. If the extract is to be clarified by centrifugation the exact nominal volume shall be placed in suitable extraction vessels (7.6) at room temperature.

Clarification by filtration: the extract is filtered through a suitable filter (7.5). The extraction vessel and filter funnel are rinsed with about 5 ml to 10 ml of ethanol 90 % (6.1.1). Substance losses and carry-over in the filtration device are to be kept as low as possible. The clear filtrate is collected in a volumetric flask,

volume adjusted to the mark (nominal volume) with ethanol 90 % (6.1.1), then homogenized and kept protected from light.

Clarification by centrifugation: the filter is extracted and homogenized directly in a tightly sealable extraction vessel (7.6) and the extraction volume shall be set exactly at the beginning of the extraction. The extracts are centrifuged (7.7) at 3 000 *g* until the extracts are clear but not longer than 20 min. A measured partial volume of the clear supernatant is then transferred into a vessel.

The extract obtained can be transferred, if necessary into a volumetric flask or vessel, tightly sealed and shall be kept in the dark. Extracts can be kept for three days at a temperature of 1 °C to 5 °C.

8.5 Measurement

Fill the cuvette (7.10) with the (part of the) extract obtained and measure the absorption at 665 nm (Ex) and at 750 nm (E0) against an ethanol blank (6.1.1) using a spectrophotometer (7.9). It shall be ensured by selecting an appropriate sample volume or extract volume or cuvette pathlenght, that the measured absorption values are within the range suitable for photometric measurements (0,02 to 0,90).

If the extinction is more than 0,90, a shorter cuvette can be used or dilute the extract with a factor f with ethanol 90 % (6.1.1).

Acidify the extract (or a measured partial volume) with exactly 200 µl of hydrochloric acid (6.2.1) per 20 ml of extract volume and shake or stir it immediately. After a waiting time of 5 min, but at the latest after 30 min, measure again the absorption at 665 nm (E'x) and at 750 nm (E'0) against a blank of acidified ethanol.

NOTE Any residual turbidity that occurs before and after acidification can be corrected by subtracting the absorption value at 750 nm. Practically, a value of 0,002 per cm of cuvette thickness can be used. The extract can also be clarified again (but without readjusting the extract volume), especially if the difference between the absorption values at 665 nm and 750 nm is small.

9 Quality assurance

9.1 Blank values

It is advisable to determine blank values regularly, especially after opening a new batch of filters or ethanol used. Deionised water is used instead of the sample. The determined concentrations should be below the limit of quantification established internally in the laboratory.

9.2 pH after acidification

Check with a pH meter (7.13) that the pH of the acidified extract is 2,6 to 2,8.

9.3 Wavelength setting

Check at least once a year that the wavelength setting of the spectrophotometer is correct. See the instruction manual of the spectrophotometer.

9.4 Ratio E_n/E_z

The ratio E_n/E_z shall be in the range between 1,0 and 1,7. Otherwise it can be assumed that errors were made in the acidification or in the photometry, or that the result is below the limit of quantification. Results can only be reported if they are included with a disclaimer.