SLOVENSKI STANDARD

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Kakovost vode - Smerni standard za štetje fitoplanktona z invertno mikroskopijo (postopek po Utermöhlu)

Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)

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Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)

Qualité de l'eau - Norme guide pour l'analyse de routine de l'abondance et de la composition du phytoplancton par microscopie inversée (méthode d'Utermöhl)

Wasserbeschaffenheit - Anleitung für die Zählung von Phytoplankton mittels der Umkehrmikroskopie (Utermöhl-Technik)

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Foreword

This document (EN 15204:2006) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This European Standard shall be given the status of a national standard, either by publication of an identical text or by endorsement, at the latest by February 2007, and conflicting national standards shall be withdrawn at the latest by February 2007.

According to the CEN/CENELEC Internal Regulations, the national standards organizations of the following countries are bound to implement this European Standard : Austria, Belgium, Cyprus, Czech Republic, Denmark, Estonia, Finland, France, Germany, Greece, Hungary, Iceland, Ireland, Italy, Latvia, Lithuania, Luxembourg, Malta, Netherlands, Norway, Poland, Portugal, Romania, Slovakia, Slovenia, Spain, Sweden, Switzerland and United Kingdom.

Introduction

The European Water Framework Directive (2000/60/EC) has created a need for a uniform procedure to assess ecological quality of surface waters using phytoplankton abundance and composition. This European Standard will meet this need and will help laboratories improve the quality of their analytical results.

A single standard procedure for the assessment of phytoplankton composition and abundance cannot be given as the questions which drive monitoring programmes are diverse in character and therefore require specific protocols. This European Standard, therefore, aims to provide guidance on basic aspects of microscopic algal analyses and to provide statistical procedures for the design, optimization and validation of methods and protocols. Though mentioned in Annex C, a method for the estimation of biovolume is not included.

WARNING — Persons using this European Standard should be familiar with normal laboratory practice. Long periods of microscopic phytoplankton analysis can cause physical fatigue and affect eyesight. Attention should be given to the ergonomics of the microscope and advice from a health and safety practitioner should be sought to ensure that risks are minimized. The use of chemical products mentioned in this European Standard can be hazardous and users should follow guidelines provided by the manufacturers and take necessary specialist advice.

This European Standard 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 health and safety practices and to ensure compliance with any national regulatory guidelines.

1 Scope

The procedure described in this European Standard is based on the standard settling technique as defined by Utermöhl in 1958 [31]. It describes a general procedure for the estimation of abundance and taxonomic composition of marine and freshwater phytoplankton by using inverted light microscopy and sedimentation chambers, including the preceding steps of preservation and storage. Emphasis is placed on optimizing the procedure for the preparation of the microscopic sample. Many of the general principles of the approach described may also be applied to other techniques of enumerating algae (or other entities) using a (conventional) microscope, some of which are described in Annex E. This guidance standard does not cover field collection of samples or the analysis of picoplankton, quantitative analysis of free-floating mats of Cyanobacteria or specific preparation techniques for diatoms.

2 Normative references

Not applicable.

3 Terms and definitions

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

3.1
accuracy
closeness of agreement between a test result or measurement result and the true value

3.2
algal object
unit/cluster of one or more algal cells encountered during the phytoplankton analysis that is discrete from (liable to settle independently of) other particles in the sample

3.3
detection limit
minimum number and/or size of a specific taxon or group of organisms in a sample at which its presence can be detected with a specified probability

NOTE This definition is analogous to the definition used in chemistry (smallest true value of the measurand which is detectable by the measuring method).

3.4
error
difference between an individual result and the true value

3.5
fixation
protection from disintegration of the morphological structure of organisms

3.6
microscope counting field
delimited area (e.g. a square or grid) in the microscope field of view, used for enumeration

3.7
nanoplankton
small algae between 2 µm and 20 µm in size

3.8
numeric aperture (NA)
difference in refraction index of the medium between objective and object multiplied by the sine of half the angle of incident light

3.9
performance characteristic
characteristics of a specific analysis protocol which encompass qualitative and quantitative aspects for data precision, bias, method sensitivity and range of conditions over which a method yields satisfactory data

3.10
phytoplankton
community of free-living, suspended, mainly photosynthetic organisms in aquatic systems comprising Cyanobacteria and algae

3.11
picoplankton
very small algae between 0,2 µm and 2 µm in size

3.12
precision
closeness of agreement between independent test/measurement results obtained under stipulated conditions

3.13
preservation
process that protects organic substances from decay

3.14
(analysis) protocol
specific analytical procedure concerning (sub)sample volume, magnification, number of cells to count, taxonomic level of identification etc.

3.15
repeatability
precision under repeatability conditions

3.16
repeatability conditions
conditions where independent test/measurement results are obtained with the same method on identical test/measurement items in the same test or measuring facility by the same operator using the same equipment within short intervals of time

NOTE This definition should be interpreted as the error occurring between replicate sub-samples from the same sample, counted using the same counting chamber, performed by one analyst using one microscope in a continuous run on one day.

3.17
reproducibility
precision under reproducibility conditions

3.18
reproducibility conditions
conditions where independent test/measurement results are obtained with the same method on identical test/measurement items in different test or measurement facilities with different operators using different equipment

3.19
uncertainty
parameter associated with the result of a counting that characterizes the dispersion of values that could reasonably be attributed to the measurand

3.20
validation
confirmation by examination and the provision of effective evidence that the particular requirements for a specific intended use are fulfilled

4 Principle

After preservation and storage, if applicable, the sample is homogenized and a sub sample is placed in a sedimentation chamber. When the algae have settled to the bottom of the chamber, they are identified and counted using an inverted microscope.

5 Equipment and preservatives

5.1 Sampling bottles

A sampling bottle should meet the following requirements (the relevance of some of these may depend on the duration of storage of the sample):

— the bottle should be clean and easily be cleaned. It should not be permeable to, or react with, the preservative used;

— the bottle should be transparent (so that the state of preservation and the presence of aggregates can be examined easily), but stored in the dark.;

— the combination of bottle and screw cap should ensure a closure that is watertight (to facilitate homogenisation) and almost gastight (to minimize evaporation) to allow long periods of storage;

— the neck of the bottle should be wide enough for filling the counting chamber. The bottle should not be too large for easy handling and filling of the counting chamber: generally, a volume of some 100 ml to 200 ml is satisfactory;

— to facilitate homogenisation, bottles should not be filled completely with sampling water (preferably fill to around 80 %).

5.2 Sedimentation chamber

Sedimentation chambers consist of a vertical column, with a base through which the contents can be observed with an inverted microscope. The column is filled with a sample and the particles in the sample are allowed to settle on the bottom of the chamber. By using a relatively small cross-sectional area in comparison with column height, the sample can be concentrated effectively. A common type of chamber has 2 pieces: a top-piece column that is placed above a well in a base-piece, the top-piece being slid aside and replaced with a cover glass once the algae have settled on the bottom. Sedimentation chambers may be square or circular. The thickness of the base plate should not exceed 0,17 mm as this directly affects image quality. Counting chambers should be calibrated so that the volume of sub-sample contained can be determined.

Counting chambers should be cleaned and dried between uses. For best results, cleaning should include washing with detergent using a soft paintbrush or small scrubbing brush; afterwards, the chamber should be rinsed in distilled water. Other agents that can be used, depending on the chamber material, are methanol, ethanol (90 %), commercial ‘denatured’ alcohol or isopropanol.

5.3 Inverted microscope

The use of an inverted microscope allows the algae, settled on the bottom of the chamber, to be brought into clear focus (see Annex A). The optical properties of the microscope determine the discriminating potential and hence the identification possibilities. For phytoplankton counting, an inverted microscope should be equipped with a condenser with a NA of at least 0,5 and plan objectives with a NA of 0,9 or more (see Annex A). Phase-contrast and/or Normarski interference-contrast is usually used in marine phytoplankton analysis. It can assist greatly in the identification of certain taxa, including flagellates, diatoms and delicate forms such as chrysophytes. Ideally, the microscope should be equipped with a (digital) camera.

The microscope should have binocular, wide-field × 10 or × 12,5 eyepieces. One eyepiece should be equipped with a calibrated ocular micrometer. The other eyepiece should be equipped for counting by use of an appropriate calibrated counting-graticule:

a) for counting of randomly-selected microscope fields, the graticule should have a square field or grid (available commercially, e.g. a Whipple disc), or the equivalent using 4 crossing threads, or

b) for counting transects or the whole chamber, 2 parallel threads within the eyepiece forming a transect, preferably with a third vertical thread crossing the other two in the centre.

The ocular micrometer and counting-graticule shall be calibrated for each magnification being used, and for each microscope. To do this, a stage micrometer slide composed of 100 µm × 10 µm divisions is viewed and focused through the ocular micrometer/counting-graticule and used to measure the scale of the ocular micrometer and the dimensions (to permit calculation of area) of the counting-field.

Though inverted microscopy is the recommended method for enumeration of phytoplankton, conventional (non-inverted) compound light microscopes may also be used for enumerating phytoplankton under some conditions (see Annex E).

5.4 Preservatives

5.4.1 Acid Lugol's iodine [35]

Dissolve 100 g of KI (potassium iodide) in 1 l of distilled or demineralised water; then add 50 g of iodine (crystalline), shake until it is dissolved and add 100 g of glacial acetic acid. As the solution is near saturation, any possible precipitate should be removed by decanting the solution before use. Lugol's solution can be stored in a dark bottle at room temperature for at least 1 year.

5.4.2 Alkaline Lugol's iodine (modified after [37])

Dissolve 100 g of KI (potassium iodide) in 1 l of distilled or demineralised water; then add 50 g of iodine (crystalline), shake until it is dissolved and add 100 g of sodium acetate (CH_3COO-Na). As the solution is near saturation, any possible precipitate should be removed by decanting the solution before use.

The use of 5 ml of Lugol's solution per litre of sample is standard. However, this is dependent on the algal density: for meso- and especially oligotrophic waters more than 2 ml might already cause over-saturation rendering the algae difficult to identify, in which case a lower volume of Lugol's should be used. In general enough Lugol should be added to turn the sample to a cognac or straw colour.

5.4.3 Formaldehyde 37 % volume fraction

For long term storage, formaldehyde should be added to give a final concentration of 4 %. This should only be done if no reanalysis of the sample is planned, since naked small flagellates will be destroyed. Another risk is a quick decoloration of the sample.

Most preservatives are commercially available. The reader is referred to Annex B for more details on the use of different preservatives.

6 Sample processing

6.1 General

Samples should be divided into two with one part being preserved and stored at low temperature for later analyses whilst the other sub-sample is kept unpreserved to allow examination of live material (6.1 and 6.2). Before a sub-sample is taken for analysis acclimatize the sample to the appropriate temperature and homogenise (6.3 to 6.5). Thereafter transfer a sub-sample directly to a calibrated counting chamber (6.5 to 6.6). Then count the number of algal objects in a known area of the chamber with the aid of eyepiece graticules, and from this determine the concentration of algal units (Clause 7).

The precise counting protocol used will vary depending on, for example, the purpose and objectives of the study, the nature of the samples being analysed, and the resources/equipment available. The error associated with each protocol will differ and so it is important to validate each protocol before it is used (Clause 8 and Annex F).

The detailed analyses of certain groups of organisms may require special treatment. For (benthic) diatoms, guidance can be found in EN 13946 and EN 14407.

6.2 Preservation of samples

Samples should be preserved as soon as possible after they have been taken, with one of the specified preservatives. Living samples should also be retained for preliminary analysis of the algal flora (6.3.1).

6.3 Storage

6.3.1 Living samples

Living samples for preliminary analysis (7.2) should be kept in the dark at a temperature between 4 °C and 10 °C. Samples taken from ambient water at a higher temperature may need to be cooled gradually in order to avoid damage to phytoplankton cells. A maximum storage time of 36 h should not be exceeded, prior to analysis.

NOTE In samples with a very high density of organisms, blooms or surface scums, depletion of oxygen (and, hence, degradation) should be prevented by diluting the sample with filtered (0,45 µm) water from its origin.

6.3.2 Preserved samples

Samples preserved with Lugol's solution (or formaldehyde) should be stored in the dark and cooled to 1 °C to 5 °C, unless they are analysed within three weeks, in which case they can be stored in the dark at room temperature. The level of the sample in the bottle should be marked on the bottle prior to storage.

Storage at low temperature will slow down the rate of physical and chemical processes thus leading to a reduction in sample quality. Storage in the dark is always necessary to prevent photo-oxidation. The maximum storage time for Lugol preserved samples in the dark and between 1 °C and 5 °C is 12 months. Preservation and storage for longer periods is possible only after addition of formaldehyde (Annex B).

New samples should be checked after a couple of days for oxidation of the Lugol's iodine. The sample should have a Cognac or straw colour. If not, Lugol's solution should be added until the sample has regained this colour. When properly sealed sample bottles are stored at a temperature between 1 °C to 5 °C no significant evaporation should occur.

6.4 Acclimatization

In order to promote a random distribution of plankton in the sedimentation chamber, the sample and all equipment used should be of a similar temperature. Usually, an acclimatization period to room temperature of some 12 h is adequate but this depends upon actual ambient temperatures and the sample volume.

NOTE Temperature differences between sedimentation chamber and medium may produce convection currents that have different effects on the settling of phytoplankton species, depending on their physical properties. Furthermore, bubbles may develop in relatively cold samples as the solubility of gases declines with the gradual rise of the temperature of the sample. Therefore, acclimatization is an important step in a controlled procedure for phytoplankton analyses.

6.5 Sample homogenisation

The first critical step in preparing a sample for microscopic analysis is homogenisation of the sample. During sample storage, suspended particles settle out and (small) algae become indiscernible by incorporation in detritus aggregates or by adhesion to other large algal cells. Re-suspension and separation of particles can be achieved by shaking the sample as gently as possible. This may be performed manually or preferably by the use of an appropriate shaking device. Devices (tumbler mixers) based on a three-dimensional motion (figure-of-eight movement with rotation: Paul-Schatz principle) are preferred over standard orbital shakers, which induce a vortex movement of the water sample leading to incomplete mixing.

The method used for manual shaking should be described clearly in order to minimise differences between operators. A combination of alternating horizontally rolling and vertical turning upside down of the sample bottle for a specific number of times provides better mixing than straightforward shaking.

NOTE 1 Vigorous shaking may lead to the disintegration of fragile colonies, which is a problem if colony size has to be determined. When a lot of small bubbles are produced (which will affect sedimentation adversely), then allow one hour before taking the sub-sample, gently re-shaking the bottle before doing so.

NOTE 2 Disintegration of colonies to facilitate cell counts can be promoted by exposure to ultrasonic vibration taking care not to damage cells by over exposure [25], [3] or by hydrolysis of the colony mucus [2], after which the sample may need to be homogenised by gentle shaking. Some colonies (e.g. *Phaeocystis*, certain chlorophytes and colonial chrysophytes as *Synura* and *Uroglena* will partly or fully disintegrate shortly after preservation in acid Lugol´s solution.

6.6 Sub-sample preparation

After homogenisation, a known volume of sample should be used to fill the counting chamber. The filling of the counting chamber is crucial, as it affects the final distribution of settled particles. Random distribution allows for simple and uniform counting strategies and statistical procedures to assess measurement uncertainty.

The chamber should be filled directly from the sample bottle. The exact volume depends on the phytoplankton density, the volume of the counting chamber and its surface to volume ratio. Larger sub-sample volumes (up to 100 ml) will be required from oligotrophic waters (see also B.3). At high phytoplankton biomass a dilution step may be necessary to ensure that the concentration of particles is sufficiently low to prevent clogging of particles by adhesion and to optimise the counting process (6.6). Dilution should be performed with relatively large volumes (e.g. using a graduated measuring cylinder). For marine samples filtered (0,45 µm) sea water should be used for dilution.

NOTE The height of sedimentation tubes should not be higher than 5 times of chamber diameter [19]. This means for instance that 100 ml-tubes with a diameter of 26 mm are not suitable for counting of nanoplankton and picoplankton. If more than 50 ml sedimentation volume is required a pre-sedimentation is indicated (see Annex B.3).

When using small volume pipettes (1 ml to 5 ml) with removable tips, the end of the tip should be cut off to widen the opening to a diameter between 3 mm and 4 mm, to ensure that large taxa such as *Ceratium* are not excluded. Since cutting the tip will affect the accuracy of the pipette, calibrate each individually.

The following points should be noted for optimal filling of sedimentation chambers:

— ensure that all equipment (including filling tips etc.) is allowed to equilibrate to the ambient temperature of the room where the analyses are to be performed. The ambient temperature should be as constant as possible;

— place the chamber on a horizontal flat surface that is a poor heat conductor (e.g. a thin acrylic plate). Wait until all materials have reached an equal temperature;

— take enough sample (diluted if necessary), to completely fill the chamber in a single addition (with no air spaces at the top);

— if it becomes obvious that many algal cells are obscured by adhesion to detritus, the quality of the sub-sample can be improved by prolonged and/or more intensive shaking of the sample and appropriate dilution;

— close the chamber with a cover glass; avoid trapping air bubbles in the process;

— the sedimentation should take place in the dark at a constant ambient temperature that is similar to the temperature of the sub-sample; avoid vibrations;

— for freshwater samples preserved with Lugol's iodine a settling time of at least 4 h per cm is recommended [19], [20] and for seawater samples preserved with formaldehyde a settling time of at least 16 h per cm [9]. For Lugol preserved seawater samples the following settling times are advised [12];

Table 1 — Settling times for Lugol preserved seawater samples [12]

Volume of chamber ml	Height of chamber cm	Settling time h
2	1	3
10	2	8
25	5	12
50	10	24
100	20	48

— after sedimentation, slide the chamber column aside and place a cover glass on the counting chamber to close it; avoid enclosing air bubbles; these can be eliminated by topping up with water using a small dropper pipette whilst sliding the coverslip back;

— gentle moving the counting chamber to the microscope will not affect the settled particles when the chamber is filled completely and a cover glass is used. However, settled algae in an open sedimentation chamber are easily disturbed even when moved carefully. Open chambers should not be used for this reason;

— an intense light source (including that from the microscope) might cause settled algae to float again even in a closed counting chamber. It should be checked for each microscopic configuration and procedure whether this phenomenon will indeed affect the analysis.

When incomplete sedimentation has been observed or when there are specific sampling or historical indications that buoyant algae like many Cyanobacteria or for instance the lipid containing green alga *Botryococcus* may be present, the fluid in the upper column of the chamber should be centrifuged at an appropriate speed to estimate whether the amount of buoyant algae is significant. Furthermore, some small Cyanobacteria remain in suspension just above the bottom glass under some circumstances. This can be checked by focusing above the bottom prior to counting. If necessary, gas vesicles of Cyanobacteria can be collapsed, after which a new sub-sample may be prepared. Gas vesicles can be collapsed by putting a sample in a large plastic syringe from which the needle has been removed, leaving an opening of 1 mm or 2 mm in diameter. If the needle end is hit firmly against a wall whilst holding the piston, the sudden increase of pressure inside the syringe will collapse the gas vesicles. Another option is to put a rubber stopper in the opening of a sample bottle and then hit it carefully with a hammer. Collapsing of gas vesicles may sometimes be achieved using acidified Lugol with a slightly higher concentration of glacial acetic acid.