BRITISH STANDARD

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

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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of an identical text or by endorsement, at the latest by February 2007, and conflicting national standards shall be withdrawn at the latest by February 2007.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.

When all particles are settled, the overall distribution pattern should be judged using a stereo-zoom microscope to confirm a random distribution. At a low magnification, large-scale patterns can be observed easily, though the smallest particles might be overlooked. When temperature is not controlled, the most regularly observed pattern is a concentric pattern in which the larger and heavier particles tend to concentrate towards the chamber wall and the smallest particles more towards the centre of the chamber. This pattern arises when the temperature of the chamber is higher than that of the sub-sample. If the sub-sample has a higher temperature, the reverse pattern arises with the smallest particles near the chamber wall. A random distribution of particles is recognized by its irregular pattern, and, depending on the particle density, lots of 'open' spaces (Figure 1). When a new protocol/equipment is used, the presence of a random distribution should be demonstrated by validation (Clause 8), otherwise a visual inspection will suffice.

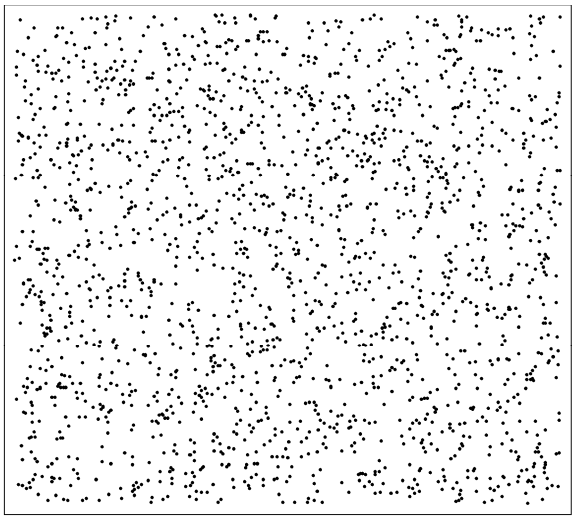


Figure 1 — Random distribution of particles (note the open spaces)

6.7 Algal density

For optimum accuracy in identification and counting, the density of settled algae in the counting chamber should be considered and the volume of sub-sample (or dilution) adjusted accordingly. Although the optimum range is subjective, some general guidelines can be given. Firstly, the number of particles should allow for the independent settling of particles. If there are too many particles, agglutination and piling-up of particles (including algae) may occur, resulting in non-random distribution. Secondly, if the number of algae per field is too low, there will be a large random error when counting random fields or transects and hence inefficient counting (many fields or grids to count), but detection levels will be high (algae are not easily overlooked). Conversely, high densities of settled algae will reduce the error and number of fields to count but will reduce the detection accuracy. A chamber/field with a high particle density may result in analyst fatigue, with algae then being overlooked. The ideal density depends on the relative size of the algae (visibility) and the number of non-algae particles (detritus etc.) in combination with the skills of the analyst. Evaluation may be carried out by counting a relevant sample at different densities.

7 Counting procedure

7.1 General

The choice of counting strategy depends on the information needed. As microscopic phytoplankton analyses are time consuming and therefore costly, it is important to optimize this strategy (see also Annex C).

7.2 Preliminary (qualitative) study

A taxon list should be compiled before the quantitative analysis of the sample starts. The main objective of this qualitative analysis is to provide an overview of phytoplankton composition prior to detailed quantitative analysis. This may determine the counting strategy and also helps subsequent identification of the preserved sample. Additional information may also be apparent that is of relevance to the condition of the sample (e.g. it may be useful to make a note of colony size in a fresh sample, if the colonies disintegrate upon preservation). Several approaches are suitable for the compilation of this list. Whilst quantification is not necessary, an expression of relative abundance may be valuable for future comparisons.

If possible, a preliminary study of living samples should be made as preservation often obscures diagnostic identification characteristics. Living samples permit the use of auto-fluorescence of chlorophyll-*a* for the distinction between small Cyanobacteria and Eubacteria and are also valuable to distinguish between Xanthophyceae and Chlorophyceae and to identify delicate flagellates (e.g. Haptophyceae and Chrysophyceae). Living flagellates may be concentrated by their positive phototaxis. If the sample bottle is wrapped in aluminium foil leaving the bottleneck exposed to the ambient light, the flagellates can be sampled from the bottleneck after approximately one hour. The 'larger' and less common species can be collected and concentrated by taking net samples using an appropriate mesh size. Generally, many species may be identified more easily in the final preserved sample after they have been observed alive.

A preliminary taxon list should be available for consultation during the final analysis of the preserved sample. Any observed differences should be investigated. Any taxa that are uncommon in the waterbody/region should be highlighted and expert confirmation sought. Once differences have been resolved, the numbers of algae present in the quantitative sample should be counted. Taxa found in the preliminary analysis but having been absent in the quantitative sample should be recorded as such.

NOTE As many living algae do not or only slowly sediment, fresh samples should be studied in a very shallow sedimentation chamber or simply as a wet mount under a coverslip on a slide. The sample may be concentrated by gentle vacuum filtration using polycarbonate membrane filters. By leaving a small amount of fluid above the filter, the concentrate can be pipetted easily to a glass or small chamber without damaging the algae. Gentle centrifugation may also be used to concentrate fresh samples but it should be ensured that the centrifuge is not provided with an automatic brake.

7.3 Quantitative analysis

7.3.1 General

The procedure for quantitative analysis involves recording the taxa observed and the number of algal objects for each taxon, in a known area of the counting chamber. If the area and volume of the whole chamber is known, the concentration of each individual taxon may be calculated.

The taxa to be counted depend on the objective(s) of the study. Although this will often be to analyse the composition of the phytoplankton (in terms of the abundance of species or genera or groups present), it might also be to determine the abundance of a single species or a particular group of phytoplankton (e.g. Cyanobacteria), or the total abundance of phytoplankton (i.e. the total number of algal objects irrespective of their identity).

The chamber area to be counted will also depend on the objective of the study as well as on the composition and density of the phytoplankton sample itself. There are three alternative counting strategies when using sedimentation chambers:

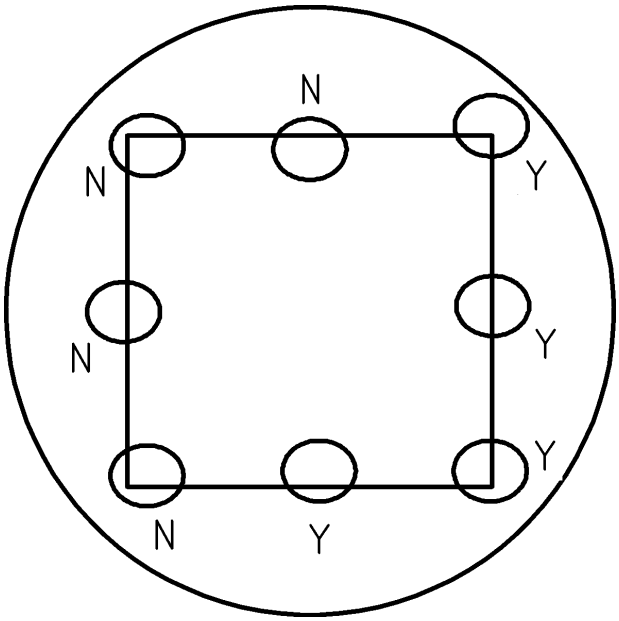
a) counting a number of randomly-selected fields,

b) counting transects, or

c) counting the whole chamber.

The latter may be appropriate for low densities of phytoplankton (or detecting rare species), or for counting large species whose distribution in the chamber may not be random.

For randomly selected fields, use a square or grid eyepiece graticule to delimit the counting field and a tally is kept of the number of fields counted. Fields can be selected either by the operator in a pseudo-random fashion (which can be tested for randomness during validation of the protocol), or using an electronic stage with built-in random-position controller. When using a microscope counting field, it is important to ensure that a consistent approach is taken to decide whether algal objects traversing the grid lines are recorded as being in or out of the field. A simple rule should be established, such as that depicted in Figure 2 (see 7.3.2.4 for its application to coenobia, colonies or filaments).



Key

Y counted
N not counted

Figure 2 — Example of rule for counting cells on the edge of the field. Algae objects crossing both the top and left hand side of grid are not counted whilst those crossing the bottom and right hand side of grid are counted

Counting a whole chamber is achieved by traversing back and forwards across the chamber, from top to bottom (or *vice versa*). An eyepiece graticule is used comprising 2 horizontal parallel lines which delimit the transect, preferably with a third vertical line crossing these. Algal objects between the transect lines are counted as they pass the vertical line. Those crossing the top transect line are included, but not those crossing the bottom line as these will be counted on the next transect (or vice versa). The same type of eyepiece can be used for counting transects, taking care to ensure (by validation) that a random distribution of settled algal objects exists and that there are no 'edge effects' (more algae settling towards the periphery).

NOTE Counting a random number of fields is claimed by some authors to give the most accurate data (smaller coefficient of variation) and to be less time consuming than counting transects (e.g. [25]). When algae are sedimented in more or less concentric-like patterns, as often observed, the algae in the central area of the counting chamber are over-estimated and algae closer to the chamber wall are under-estimated when counting transects. This problem might be anticipated by counting random fields, segments of the chamber or by treating the central and peripheral areas as two or more distinct 'populations'. In the case of random fields the probability of counting a specific region is proportional to the surface area of that region relative to the total surface area of the counting chamber.

7.3.2 How many fields or algal objects to count?

7.3.2.1 General

The number of fields or algal objects to be counted can be decided according to the level of precision required, as the precision depends on the number of algal objects/fields. The precision (*D*) of a count may be expressed either as the standard error or as a proportion of the mean, or as the 95 % confidence limits as a proportion of the mean. In most routine counts, the precision should usually be pre-set. However, for some purposes (e.g. biodiversity studies) it may be more appropriate to pre-set the detection limit.

7.3.2.2 Precision

The easiest way to set the precision (*D*) for estimating the number of objects in a chamber is according to:

$$D = \frac{\text{standard error}}{\text{arithmetic mean}} = \frac{1}{\bar{x}}\sqrt{\frac{s^2}{n}} = \frac{1}{\bar{x}}\sqrt{\frac{\bar{x}}{n}} = \frac{1}{\sqrt{\sum x}} \quad (1)$$

where

n is the number of fields counted;

$\bar{x}$ is the mean number of objects per field and $\sum x$ is the total number of algal objects counted.

If a precision of 5 % in the estimation of the mean number of objects per field is required, then:

$$\sum x = \left(\frac{1}{0{,}05}\right)^2 = 400$$

objects should be counted. It makes no difference whether 10 fields are counted with 40 objects per field or 80 fields with just 5 objects.

If precision is considered in terms of percentage confidence limits, multiply the above equation by t^2 (Student's t), which yields:

$$\sum x = \frac{t^2}{D^2}$$

for n-1 degrees of freedom. In this case, a count of 400 objects will result in 95 % confidence limits that lie within 10 % of the mean ($t \approx 2$). The estimate of *D* is only valid when algal objects can be considered to be distributed randomly in the counting chamber. Otherwise it should only be used as a rough approximation.

NOTE The precision relates to the type of algal objects counted. If all taxa should be counted and the precision is set for the total number of algal objects, then the precision applies only to the total number of algae, not to individual groups/species. If a single taxon should be counted, the precision should be set for that taxon.

7.3.2.3 Quantitative detection limit

The detection limit is an important performance characteristic in phytoplankton surveys. For a single taxon (assuming a random distribution), the detection limit may be determined by Poisson statistics according to:

$$n_{\text{det}} = -\ln(\alpha) \cdot f_{\text{total}} \,/(V \,.\, f_{\text{counted}}) \quad (2)$$

where

α is the level of significance;

n_{det} is the detection limit;

f_{total} is the total number of microscope fields in the chamber;

$f_{counted}$ is the number of fields counted;

V is the volume of the sub-sample in the chamber.

In multi-taxon samples, correct α for the number of taxa. The chance of finding each taxon in a sample is determined by the product of the probabilities of each independent taxon. This implies that if ten taxa have each a concentration of n_{det}, the probability that they will all be detected in the same analysis at α = 0,01 is only $100 \times (1\text{-}0{,}01)^{10}$ = 90 %. Any knowledge of taxon richness of a sample prior to analysis can be used to correct α and determine a proper detection limit or the number of fields to be counted. In some studies, an α of 0,01 will be sufficient whereas in other studies an α of 0,001 or even less is necessary.

NOTE 1 By contrast to estimates of precision, the detection limit depends on the number of fields (actually the absolute volume of sample) counted, rather than the number of algal objects. If the number of algal objects to be counted is fixed, then a variation in the detection limit caused by variation in the total numbers of algae per unit of volume may occur within the same sample series. This variation may render comparisons difficult.

NOTE 2 The detection limit also applies to the size of algal objects. At a magnification of 400 × to 600 ×, the smallest countable particles have a size of approximately 2 µm to 4 µm. In this size range, many identification characteristics are difficult to observe and need experience to detect. Direct identification even to the genus level is often impossible (Annex D). The limit of detection does not take account of the (identification) skills of the analyst. The limit of detection, from an identification point of view, corresponds with the laboratory species list (7.4).

7.3.2.4 Counting coenobia, colonies and filaments

A coenobium should be treated as a single unit and not counted as individual cells. If part of the coenobium lies within the counting field/transect lines (and applying rules such as depicted in Figure 2), then the whole coenobium is counted.

Counts of whole colonies and filaments can be made by applying a similar rule. Generally this requires a lower magnification (200 ×) than when counting cells. When intact colonies/ filaments are surveyed and are large with respect to the field of view, an objective basis should be agreed for determining whether it should be counted in a particular grid or not. For instance, colonies are counted when the extreme tip at the right or left side lies inside the field and filaments when the centre cell lies inside the field.

If the total number of cells is required for colonial/filamentous algae, there are three alternative approaches:

1) *Direct counts of cells* – Cells of colonies and filaments are counted as if they are individual cells, counting only cells within the counting field/transect lines. However, unless it can be shown that the cells are distributed randomly within the chamber (i.e. the variance is not significantly different from the mean), then the data will need to be transformed, for example by log transformation or (if there are sufficient data to plot variance against mean) by application of Taylor's power law (as described in standard statistical textbooks, e.g. [4]).

2) *Making separate counts of the number of colonies/filaments and the mean number of cells per colony/filament* – The number of algal objects (whole colonies/filaments and individually occurring cells of the same taxon) is counted as normal, with a separate count made of the number of cells in at least 30 objects (see Annex F). The mean number of cells per object multiplied by the number of objects gives the total number of cells in the count. This method can only be used when the number of cells per object does not vary widely within the sample (e.g. it would not be applied to a cyanobacterium ranging in size from 10 to 10 000 cells per colony). The frequency distribution for total cells is the product of the distribution of objects (assuming these to be random) and an unknown and variable distribution of cells per object. However, as the error for the estimate of cells per object can be low compared to the error in the estimate of the number of objects, the confidence limits in

terms of cell numbers can be calculated by multiplying the confidence limits for the count of objects (colonies/filaments) by the mean number of cells per object [15]. When counting species whose cells are very small or difficult to distinguish (e.g. some species of *Oscillatoria*), the number of cells per unit length or area can be counted at higher power, then multiplied up using the mean length/area of 30 or more colonies/filaments.

3) *Disintegration of colonies into their constituent cells* – This may be achieved, for example, by ultrasonic vibration or hydrolysis of the colony mucus (see Note in 6.4).

When cells of filamentous algae are difficult to discern (e.g. *Oscillatoria*), estimated length of filaments can be used instead of the number of cells. This can be measured directly using a micrometer, with the mean length per filament then multiplied by the number of filaments to give the total length or by counting filaments in units of 100 µm. When the number of cells per colony or filament is important, care should be taken to ensure that sampling, transport and homogenisation do not alter the colony or filament size.

7.3.3 Calculation of phytoplankton concentration

The number of algal objects counted is converted to give a concentration per unit volume of sample according to:

$$N = X \frac{Ad}{av} \tag{3}$$

where

N is the number per unit volume;

X is the mean number per field (or the total count for the whole chamber);

A is the total effective area of the chamber;

v is the volume of the sub-sample in the chamber;

a is the area of a counting field or grid;

d is the dilution factor (diluted by 100 %, d = 2; concentrated by half, d = 0,5) if applicable.

Generally, for freshwater the unit of measurement is cells · ml^{-1} whereas for marine waters cells · l^{-1} is used.

7.4 Identification

Proper identification should be controlled by setting up one or more taxon lists depicting all taxa identified by the analysts in a laboratory. This should specify the taxonomic level to which identification is required. Reference should be made to keys, guides and Floras relating to the relevant geographical area and preferably in the working language of the analyst and more general and well-accepted Floras (e.g. *Süsswasserflora von Mitteleuropa*) with detailed keys. Care should be taken to check descriptions of species and not just to match illustrations.

NOTE The availability of validated historical data from a specific sampling location and, if available, the results of a preliminary study of the living sample (7.1) will facilitate the analysis and reduce misidentifications.

The list of all taxa identified by analysts within the laboratory should include identification characteristics and relevant references in the literature for each taxon. Drawings or digital photographs of taxa observed should be made and retained as a reference collection. Taxa should never be identified beyond the level at which the analyst is confident.

Inter- and intra-laboratory comparison tests should be performed on a regular basis in order to avoid/minimize

identification difficulties between analysts. A representative natural sample should be selected and analysed. In principle, two methods may be followed. The easiest way is to analyse field by field, or transect by transect, by switching the microscope from one analyst to the other. After a field or transect has been analysed by all participants, the individual results should be compared directly and evaluated. A more advanced method is to use a computer-controlled XY-stage, which can reproduce identical series of random fields. In this way different analysts may carry out a standard survey one after the other. After the last analyst has finished the analysis, each counted field can be reviewed together.

8 Quantitative validation

8.1 General

Quantitative validation of microscopic phytoplankton analyses should focus on the provision of effective evidence of the accuracy of sub-sampling, of the distribution pattern of the settled algae in the sedimentation chamber and of the specific counting protocol.

Validation should be carried out before a specific protocol comes into operation and relevant parts should be repeated when a change in procedure, instrument, personnel etc. is likely to affect the analytical result. During operation, quality control steps should be taken to secure that the performance characteristics assessed by the validation are maintained.

Validation should be carried out on a range of natural samples representative of the range of waters to be analysed, on samples spiked with a laboratory-cultured alga, or artificial samples composed entirely of cultured algae. It is important that at least one very small species ($\varnothing < 10$ µm) with a low sedimentation rate should be included in the validation, and at least one large species ($\varnothing \geq 25$ µm) with a high sedimentation rate.

It is important that the algal objects used in validation relate to those that are to be analysed. For example, a protocol only validated for small unicellular taxa may not be accurate for larger, filamentous or colonial forms and, similarly, evidence of a random distribution of the total number of algal objects in a sedimentation chamber does not necessarily mean that the distribution of individual taxa is also random. Ideally, therefore, validation should include assessment of the whole phytoplankton community in the representative samples, i.e. the abundance of all taxa present and of the total number of algal objects. However, if a monitoring programme focuses solely on selected taxa, then validation based on those taxa alone is sufficient.

8.2 Validation of homogenisation

The objective of this validation step is to assess the minimum shaking time and intensity (see 6.4) necessary to produce reproducible results. Replicate counts are made from one or several representative samples. The counts are best performed with a Sedgwick-Rafter counting chamber or similar as these chambers with a low sedimentation depth give the smallest counting errors (Annex E). Alternatively, electronic particle counters such as flow cytometers and Coulter counters may be used. Counts can be compared by standard parametric tests if the central limit theorem is applicable (F.4).

8.3 Validation of sub-sample preparation

8.3.1 General

Apart from the accuracy of the method used, the process of sub-sampling will, itself, introduce an error. This error can be estimated by Poisson statistics, in which the variance is equal to the mean number of objects. For example, a 1 ml sub-sample with a mean of 10 000 objects ml^{-1} or more in the sample will have a coefficient of variation of less than 1 % and will be negligible. However, for individual species with a very low abundance, the sub-sample error might become significant.

When it appears from direct observation (6.5) that the algal objects are randomly distributed with neither significant clumping nor a centrifugal or centripetal pattern, the assumption of random distribution is tested in two steps. Collect data for this analysis by counting two perpendicular transects or diagonals of microscopic

fields. If more than 60 objects were counted per transect the deviation should not be greater than 25 % of the mean value. If this is not the case a third and fourth transect has to be counted [42].Record the number of algal objects for each taxon observed for each field in succession.

8.3.2 Step 1 random distribution

The first test should be to assess whether or not the algal objects (or a single taxon, if only a single taxon is being validated) can be considered to be randomly distributed across the area of the chamber counted. The easiest way is to determine the variance to mean ratio (F.1). If the algae are not distributed according a Poisson series, the filling process should be evaluated and improved when possible (6.5). When no random distribution can be realized the actual variation between different counts should be assessed, at the very least.

8.3.3 Step 2 random order of occurrence

Where more than one taxon is being counted in the analysis, a second step in the validation procedure is to test whether successive observations are independent (the probability of counting a specific taxon is independent of the preceding counted taxon). This so-called serial randomness can be tested with a Run-test (F.3). The same data collected for assessing the overall distribution of algal objects can be used.

NOTE It is possible that the distribution of algal objects is random and that the run test reveals no clustering (objects are independent) whereas a diagonal gradient in number of algae is still observed. It might be possible that towards the chamber wall, irrespective of the taxa, more or less algae have sedimented. The only concern then will be to count random fields inside the chamber. If this is omitted, a systematic error may result (also see Note at the end of 7.2).

8.4 Repeatability and reproducibility

For the proper interpretation of repeatability and reproducibility, a record should be made of the general condition of the sample, e.g. whether the sample is rich or poor in algae, detritus etc.

Quantitative results should be assessed on at least two of the most numerous species or, less preferably, at the level of chlorophytes, diatoms, Cyanobacteria and other major taxonomic groups. The measured error should be compared with the expected random error based on Poisson statistics in order to gain an insight into the performance of the procedure and analyst.

Repeatability can be assessed by an analyst recounting several sub-samples (counting chambers) from the same sample in one series. Inter-laboratory reproducibility can be assessed by recounting a representative set of samples under different relevant conditions and by different analysts. Counts can be compared by standard parametric tests, provided the central limit theorem is applicable (F.4).

Besides errors related to the absolute number of algae, repeatability and reproducibility investigations should also evaluate whether different counts result in the same proportion of taxa (phytoplankton composition). To test whether different sub-samples and counts are homogeneous with respect to the proportion of the taxa, multinomial statistics can be applied (F.4). Different counts will be homogenous (they can be judged to derive from the same population with regard to their taxonomic composition) if no additional source of significant error is introduced during the preparation and counting of the sub-samples/counting chambers.

9 Measurement uncertainty

9.1 General

An abundance or composition estimate is of little value without some knowledge of its uncertainty. Uncertainty associated with the result of a microscopic analysis encompasses the uncertainties of the whole measurement process: sampling (not covered in this European Standard), storage, sub-sampling, homogeneity, identification, and quantification. A clear distinction should be made between quantitative and qualitative uncertainty.

9.2 Qualitative uncertainty

Qualitative uncertainty refers to mis- and non-identification of taxa. It can be expressed in the form of a general statement based on inter- and intra-laboratory comparisons. This statement can be in the form of a maximum percentage of misidentification.

9.3 Quantitative uncertainty

When it has been demonstrated by validation that the procedure of homogenization, sub-sampling, sedimentation and counting has been brought into a state of statistical control (reproducible within certain limits), a meaningful uncertainty statement can be developed. Measurement uncertainty can be thought of as the sum of the intra-laboratory reproducibility and the deviation from the true value. The true exact quantitative composition of a sample is impossible to assess. Nevertheless, a relative uncertainty value may be derived from the deviation of the mean score in inter-laboratory studies (when available). Another approach can be obtained by using some other method, for example a Sedgwick-Rafter chamber (Annex E) or an electronic particle counter, as a reference method. The applicability of these methods as suitable reference methods has to be checked before use.

NOTE 1 Quantitative uncertainty depends on the abundance of each taxon in the sample. This means that, in the same sample, the uncertainty of the abundance estimate of one taxon might be different to that of another taxon. In general, the uncertainty will increase with a decrease in abundance. Consequently, uncertainty cannot be expressed as a single value for each analysis.

NOTE 2 For the time being, it is not possible to combine qualitative and quantitative uncertainty estimates into one meaningful overall uncertainty estimate. However, when the final assessment is expressed as an index value, both qualitative and quantitative uncertainty may be combined at the level of the index by judging the variation in index value obtained among identical samples by different analysts and under different conditions etc. This should be part of a sensitivity analysis in which the discriminative power of the index is assessed.

Annex A
(informative)

Optical characteristics of inverted microscopes

A.1 Illumination

The microscope is inverted in the sense that the light source and condenser illuminate the sedimentation chamber from above and the objectives view the specimens from below through a thin base plate. A powerful light source (50 W to 100 W halogen) is needed, particularly for phase contrast and photomicrography, and may be powered by a regulating transformer (rheostat). Analysis is made by bright-field illumination, phase-contrast or Nomarski interference-contrast illumination. The use of phase-contrast and especially Nomarski interference-contrast illumination is advantageous for the examination of colourless and low-contrast structures in some organisms or species, which may be hardly visible under bright-field illumination, for instance details of weakly silicified diatoms, and small (chrysophyte) flagellates. In contrast, bright-field gives colour information (Cyanobacteria versus chlorophytes) and will generally be satisfactory for most freshwater analysis. A bright-field illumination system using high quality objectives that allows an easy switch to interference-contrast illumination is the ideal configuration. A disadvantage of phase-contrast can be the appearance of haloes around objects.

In special cases, for instance the identification of thecate dinoflagellates or situations where it is necessary to discriminate between very small Cyanobacteria and other bacteria, fluorescence microscopy is very useful.

A.2 Condenser

A condenser with a NA of at least 0,5 is recommended. This allows a working distance of about 3 cm. Ultra long-working distance condensers allow a larger working space which may be useful when micromanipulating organisms in the chamber, but such condensers often have a NA of less than 0,5 (for instance 0,3) making them less suitable for identifying and counting phytoplankton. Long-working distance condensers, which have a high NA, can produce optical artefacts.

A.3 Stage

The microscope should be equipped with a good-quality movable stage. Coaxial controls for the stage suspended by a shaft lessen the strain on the arm of the investigator and leave the other hand free for the focusing controls. The focusing controls should be calibrated to offer the possibility for measuring the vertical dimensions of the object. Computer controlled XY-stages are now available which can reproduce identical series of (random) fields. This kind of control also allows for smooth joystick operation of the stage, which effectively reduces the risk of repetitive strain injuries.

A.4 Objectives and oculars

A × 10 and/or × 20 (phase) objective(s) should be available for counting the larger organisms, which are easy to distinguish and to identify. A × 20 objective of at least NA 0,5 (for instance a plan-apo objective with a resolution of at least 1 µm) may be very useful for difficult identifications. A × 40 phase and a × 60 or × 100 plan-apochromatic (oil immersion) objective (NA > 0,9 and a resolution of about 0,3 µm) should be present for smaller algae.

A.5 Sharpness (resolution)

A.5.1 General

The resolution of the image is determined by a number of factors:

A.5.2 NA of the objective

With a NA of 0,9 or more immersion oil shall be used. The NA has a large influence on the resolution of the objective and is directly coupled to the quality of the objective. The best lenses, which also correct for spherical aberration, are the so-called plan-lenses: plan-achromatic, plan-apochromatic and planfluorite, of which the plan-apochromatic lenses produce the clearest view. Normally these lenses have, at the appropriate magnification of 40 × to 60 ×, a NA of 0,90 or more.

A.5.3 Numeric aperture of the condenser

In general, the condenser is adjusted to the NA of the objective and used in such a way that the NA of the condenser diaphragm is 0,7 to 1 times the NA of the objective. A smaller NA results in a loss of resolution and increased contrast. For inverted microscopes, a NA of the condenser diaphragm of about 0,5 is standard. Condensers with a high NA are not applicable with sedimentation chambers with a height of 4 mm or more.

A.5.4 Thickness of the glass bottom of the chamber

The thickness should not exceed 0,17 mm. With some objectives it is possible to correct for the (greater) thickness of the chamber's bottom, but the resolution of these combinations is far below that necessary for counting phytoplankton.

A.5.5 Other aspects of image resolution

Only completely settled organisms can be studied under optimal conditions. When free floating or resting on setae, for example, the observation of organisms suffers from poor resolution and a limited ability to focus. Although the resolution of the objective is the most important factor determining image quality, the difference in contrast of the details of the organism against the background is also important. The only details of transparent and non-coloured objects being visible will be those with a refraction index different to that of the surrounding medium. The materials of the object will influence the image quality, resulting in light being scattered or absorbed. Higher scattering of light (e.g. by denaturised proteins of protoplasm) will result in a darker image. Absorption may result in colour, which gives the object more contrast.

Annex B
(informative)

Sample treatment

B.1 Sample bottles

These may be made from glass, polyethene (PE) or polyvinyl chloride (PVC). PE has disadvantages. Soft PE, in particular, absorbs iodine, which can diffuse slowly through the bottle wall, leaving the sample in an unpreserved state. In addition, bottles of soft PE are relatively difficult to close tightly. Bacteria may be introduced to the sample by fluid via the screw thread, leading to disintegration of the sample when preservative concentrations are too low.

A disadvantage of glass and hard PVC is their higher fragility, especially during sampling tours. For storage of samples, however, both kinds of material are suitable. For the collection and transport of samples, soft PE bottles may be used. On arrival in the laboratory, the sample should be transferred to glass bottles. In order to minimize the loss of fluid by evaporation during prolonged storage, the stopper may be sealed by dipping the inverted bottles to the neck in melted beeswax [29].

B.2 Preservation

B.2.1 General

Preservatives are toxic by definition. Addition leads to death of living organisms, preceded by a strong chemical irritation. Consequently, delicate organisms without strong cell walls may collapse before preservation is complete. It is important that the preservative agent enters the cell quickly in order to minimize this effect. The entrance rate may be enhanced by the addition of dimethyl sulfoxide [17]. Some preservatives may eventually lead to the loss of some groups of organisms.

Preservatives should meet the following requirements:

— the effect of the agent on the loss of organisms by chemical shock or otherwise shall be known beforehand;

— the preservative shall prevent the microbial degradation of organic matter, at least during the storage period of the samples;

— the preservative shall guarantee good recognition of taxa, at least during the storage period of the samples.

The most frequently used preservatives in phytoplankton research are Lugol's solution and a solution of formaldehyde. For specific organism groups or advanced research methods, several other preservatives may be used. These include glutaraldehyde [17], [28], osmium tetroxide [14] or, for delicate flagellates, a mixture of glutaraldehyde, acroleïc acid and tannic acid [32]. The latter preservatives are not considered in this document because of their high toxicity and their rare use during routine monitoring.

B.2.2 Lugol's solution

Lugol's solution is a mixture of iodine (I_2) and potassium iodide (KI). It is oxidized in the air and by light. Therefore the solution shall be stored in the dark, or in brown glass bottles, with a small head-space volume. Samples preserved with Lugol's solution shall also be stored in the dark. Lugol's solution does not fix organic biomass, leading to the risk of the inner cell structure disintegrating after 'long' storage times. In order to

prevent this, formaldehyde should be added as quickly as possible but preferably after counting. Samples preserved with Lugol's solution may be cleared completely (e.g. to show cell wall ornamentation in *Cosmarium*) by reducing the iodine with sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$).

Advantages of Lugol's solution:

— it enters the cell more quickly than formaldehyde, leaving shock-sensitive organisms better preserved in the sample;

— the specific weight of organisms is increased by iodine, resulting in faster settling times;

— detection of phytoplankton cells is made easier by the enhanced contrast between organisms and the surrounding fluid;

— it stains starch, aiding recognition of those groups of algae (e.g. Chlorophyta) which use this as a storage compound.

Disadvantages of Lugol's solution:

— the intense staining may obscure certain cellular structures that need to be observed for proper identification (e.g. surface structures, eye spots) – overstaining can be overcome by adding sodium thiosulfate ($Na_2S_2O_3 \cdot 5H_2O$) to reduce the iodine;

— occasionally some cell structures may disappear within one year (e.g. spines in some chlorophytes and coccoliths and aerotopes in Cyanobacteria;

— disintegration of certain colonies (e.g. *Microcystis*);

— organic matter is not fixed and soft materials may lose their characteristic structure during storage of the sample – this alteration can be prevented by the addition of formaldehyde;

— auto-fluorescence of chlorophyll-*a* is not detectable after preservation with Lugol's solution;

— iodine is oxidized with time, therefore samples that are stored for a long time and samples that contain a large amount of organic matter need attention to prevent them from decay.

Without any addition, Lugol's will become acidic with time. The solution of iodine in potassium iodide is commonly used in two varieties:

a) acid Lugol's: Lugol's solution to which acetic acid has been added. Acid Lugol's is recommended for the preservation of seawater samples with delicate flagellates [29]. Additional disadvantages of acid Lugol's are the frequently observed deformation of cell membranes in certain algal groups (Cryptophyceae), the dissolution of coccolithophorids and (in the long term) dissolution of silica in diatoms leading to the loss of colony structure, and the volatility and irritating effect of acetic acid. If coccolithophores need to be preserved with the coccoliths intact, a parallel sub-sample should be fixed with alkaline Lugol's solution or formaldehyde;

b) alkaline Lugol's: Lugol's solution with sodium acetate added (the solution reacts slightly alkaline). According to general experience this preservative yields better results than acid Lugol's for freshwater samples. With time (several years) silica structures of diatoms may become less prominent, probably by dissolution.

In general, the use of Lugol's solution is recommended for quantitative analyses. Only if the sample is to be stored for more than 12 months or contains large amounts of organic matter, post-fixation with formaldehyde is recommended.

B.2.3 Formaldehyde

Formaldehyde (HCHO) acts as a fixative by forming hydrogen bridges between protein molecules. As a result, organic tissues and cellular structures are preserved in a form that can be stored for long periods of time. Formaldehyde, however, slowly transforms into formic acid and methanol ('Cannizzaro reaction'), which negatively affects fixation and preservation. For this reason, formaldehyde should not be kept in stock for too long and should be buffered to a pH 8 to pH 9. A suitable buffer is hexamethylene tetramine. The transformation into formic acid and methanol can be counteracted if the solution is prepared from paraformaldehyde, a polymer of formaldehyde [24]. If the concentration of formaldehyde exceeds 20 % volume fraction, there is a risk of precipitation [29].

Advantages of formaldehyde:

— good fixing and preserving agent for those algae with a more rigid cell wall;

— cell wall structures and other characteristics such as eye spots remain visible;

— when stored properly in appropriate bottles, samples will stay in good condition for many years without attention;

— auto-fluorescence of chlorophyll-*a*, though decaying, remains intact for several days at least if the samples are stored in continuous dark.

Disadvantages of formaldehyde:

— formaldehyde may trigger allergies or cancers and should be handled with care and appropriate precautions;

— some algal species (e.g. many naked flagellates) can be distorted or cannot be recovered at all (organisms with trichcysts like *Gonyostomum* disintegrate totally);

— organisms may shrink resulting in lower cell volumes and calculated biomasses.

Formaldehyde can be oxidized by adding hydrogen peroxide (H_2O_2).

B.3 Concentration and dilution

The concentration of phytoplankton samples is time consuming and contributes to the overall measuring error. This step should be avoided if possible, but it is necessary for samples with an extremely low phytoplankton abundance. Several methods are available for concentrating phytoplankton samples, based on sedimentation, filtration and centrifugation [27]. Sedimentation is the most frequently-used method. Sedimentation can proceed in the original sample bottle if it is made of transparent material. Otherwise, measuring cylinders (1 l or 2 l) equipped with stoppers and measuring bars of known precision (Class A), should be used. The cylinders or sample bottles should be placed in the dark, at a constant ambient temperature and in the absence of vibrations, preferably in the immediate vicinity of the place where decantation is to be carried out. Some organisms may not settle at all but keep floating or adhere to the cylinder wall. To loosen organisms adhered to the wall, the cylinders/bottles should be rotated quickly by a quarter turn along their axis while leaving them standing on the table. This should be performed once a week at least. The supernatent can be decanted at low pressure using a water pump. A Pasteur pipette can be used as the mouth of the tube. Filtration using plankton gauzes with mesh sizes of 10 µm or 25 µm is not appropriate for concentration of phytoplankton in general, but can be used to concentrate organisms whose minimum dimensions are greater than these mesh sizes.

It is important to moisten the filter before filtration and to prevent it from drying out during the filtration process. When samples need to be concentrated, validation shall be carried out to assess the recovery of the different algal taxa in accordance with the objectives of the analysis.

Dilution of phytoplankton samples is sometimes necessary for samples of highly eutrophic waters. To some extent, the number of observations per microscope field can be adjusted by taking a smaller sub-volume to settle out, but there is a lower limit to this (see 6.5). For the dilution of sub-samples, filtered tapwater or seawater can be used. A stock solution that can be used for dilution can be made by the addition of Lugol's solution in a similar concentration to that added to the sample.

B.4 Behaviour of algae during sedimentation

It is a general phenomenon that, after settling, the distribution of particles on the bottom of a chamber is very variable. In one chamber particles may be random, whereas in another a concentric or asymmetric distribution may arise. These patterns can be observed in both round (tubular) and square (van Heusden) sedimentation chambers.

After the chamber is filled and closed with a glass cover, fluid flow within it will cease rapidly due to surface and internal resistance (viscosity). Usually, this process will take less than a minute and will not cause any irregular distribution of algae upon sedimentation. Most algae have a low Reynolds number and so random sedimentation should occur unless there is an additional flow-inducing force. In the absence of a precise temperature control system, however, a temperature difference nearly always develops. Close to the chamber wall an upward or downward flow will develop, depending on the heating or cooling of the fluid. In the upward flow, small particles will sediment less than larger and heavier particles, whereas in the downward flow the opposite will happen. Temperature differences of less than one degree are sufficient to induce a convection flow and even the heat from fingers picking up or holding a chamber can induce these flows. The heat currents stop when the temperature differences are neutralized. The sedimentation pattern produced is the result of the sedimentation rate of the particles and the length of time over which the current operates. Asymmetrical and non-concentric distributions have been seen to arise when temperature differences are induced at one side by holding the chamber for some time.

Convection flows giving rise to non-random distributions are generally associated with chambers with a sedimentation depth of > 5 mm. In contrast, convection flows in shallow counting chambers (e.g. 1 mm, Sedgwick-Rafter) are not significant due to the combination of a low depth and a relatively large surface area. Shallow chambers thus perform well in producing a random distribution of settled algae.

Annex C
(informative)

Phytoplankton analysis strategies

C.1 General

Phytoplankton analyses are carried out for various reasons. The analysis may start from a clear hypothesis, with the analytical approach directed to particular species, variables and confidence limits, yielding the necessary answers in an efficient way. Many analyses, however, are carried out for environmental monitoring programmes that are characterized by roughly-formulated questions. The aim of such work may be the assessment of the ecological quality of surface waters, or a description of the effects of water management measures. The analytical approach may result from the requirements of the system used for the biological assessment of water quality. Sometimes, new questions are formulated afterwards that cannot be answered with the data collected and require additional analyses with different variables, a different level of identification or a higher counting precision. The following questions are commonly involved:

— Which species occur in the sample?

— What is the species composition in terms of number of algal units (cells, filaments etc) per ml or litre?

— What is the species composition in terms of biovolume per litre?

— What is the structural composition of the phytoplankton community in terms of biovolume per litre?

The term (species) composition refers to the phytoplankton community. For practical reasons a representative description of the whole community is hard or impossible, to achieve. Those parts of the community that will be included is not always clearly defined *a priori*, which may result in serious discrepancies between data sets. The structural composition of phytoplankton communities (in terms of organism size) encompasses a range of particle sizes from 0,3 µm (the smallest picoplankton) to several 1 000 µm (large filaments of diatoms or chlorophytes or large colonies of Cyanobacteria). As a rule of thumb, it can be assumed that the abundance of phytoplankton organisms decreases with increasing size of cells or colonies. The density of picoplankton may be in the order of 10^5 cells to 10^6 cells per ml, while the abundance of large dinoflagellates or diatoms may be in the order of 10^0 per ml to 10^2 per ml. In marine waters these figures may be considerably lower. In phytoplankton analyses, the lower size limit of the community may be set by the optical resolution of the microscope whilst the upper size limit may depend on the counting strategy and the species composition of the sample. A counting strategy that enumerates the most abundant species tends to overestimate the biomass contribution of smaller algae and underestimate the biomass contribution of larger ones [21].

In biological monitoring programmes for water quality assessment these sources of discrepancies and statistical inaccuracies may be avoided by selecting a well-defined set of indicator species to quantify. In selecting such a set, useful criteria could be identified, average abundance during the monitoring season and indicative value in accordance with the ecosystem properties and monitoring goals.

C.2 Approach

C.2.1 General

The underlying questions determine the choice of preservatives, the method of microscopic analysis, the variables to be measured and the counting strategy. Common to each question is the importance of a homogenised plankton sample. The common methods for a quantitative phytoplankton analysis are derived from the Utermöhl method [31] and involve the use of sedimentation chambers and an inverted microscope.

This approach is generally accepted as the standard, except for picoplankton, as there is evidence that this group of organisms does not always settle effectively [23]. An advantage of the inverted-microscope method over methods using conventional microscopes, is that samples do not have to be concentrated in a separate step, unless the algal density is very low (e.g. in samples of ocean waters). This saves time and eliminates possible errors from decantation or centrifugation. Another and probably more important advantage is the easier predictability of the distribution of particles in sedimentation chambers compared with the distribution in microscope slides or in low volume (<<1 ml) counting chambers (haemacytometers).

C.2.2 Species composition in terms of number of cells per unit volume

Estimations of the species composition in cells per millilitre or litre, are commonly carried out in biological monitoring programmes and aquatic research. This is a quantitative analysis where quality assurance measures should guarantee the reliability of the identifications and provide an appropriate figure of the precision of the density estimations. The estimation of relative (percentage) abundance only should be avoided to facilitate time-series analysis.

C.2.3 Species composition in terms of bio-volume per unit volume

A description of the species composition in terms of bio-volume per millilitre or litre is carried out in scientific research and many monitoring programmes. Since the measurement of the chlorophyll-*a* content, as a measure of total phytoplankton biomass, is common practice in monitoring surveys, a description of the taxonomic composition of the phytoplankton community expressed in a biomass-related variable allows better interpretation of the phytoplankton biomass than a description in terms of cells per litre. However, calculation of bio-volume is time consuming and also introduces extra sources of error which may cancel out these benefits. A counting strategy is required resulting in a reliable estimation of the bio-volume of the taxa that contribute most to the total phytoplankton bio-volume. Quality assurance should be directed to the reliability of the identification, and to estimations of the density and bio-volume of each taxon.

C.2.4 Structural composition of the phytoplankton community in terms of bio-volume per litre

A description of the structural composition of the phytoplankton community is useful in research on the grazing of phytoplankton by zooplankton. Measured bio-volume can be converted into a biomass size spectrum to establish carbon flows or predator:prey ratios. A counting strategy is required yielding a reliable estimation of the bio-volume of all size classes represented in the phytoplankton community. Quality assurance should provide an estimation of the reliability of estimates of both the density and the bio-volume per size-class.

C.3 Counting strategy

C.3.1 General

For quantitative analysis there are two main types of counting strategies:

1) counting strategy directed to the most numerous organisms;

2) counting strategy directed to the organisms contributing most to total bio-volume.

Counting strategy 1 is followed in many monitoring programmes and in research where a lower level of precision is accepted to save time, the so-called ‘short-cut’ counting methods [33]. However, as the chlorophyll-*a* content is determined as a measure of total phytoplankton biomass in most monitoring surveys, counting strategy 2 is recommended over strategy 1 if the important question is how the biomass is shared amongst taxa.

C.3.2 Counting strategy directed to the most numerous organisms

In this approach the algae in the sample are identified and counted at a constant magnification until the total number of observations has reached a certain value (e.g. 100, 200 or 300), depending on the level of precision required. The precision of the total density of observations per millilitre is defined, but the precision of the estimation of constituent taxa depends on species richness and the relative abundance of each taxon. In samples with few species, 90 % of the total number of observations can refer to just one species. A characteristic of this strategy is a failure to detect less abundant species, whether large or small.

C.3.3 Counting strategy directed to the organisms contributing most to total bio-volume

This strategy does not necessarily need to be combined with an estimation of bio-volumes. It should, however, result in a description characterized by a precise estimation of the density of large species that contribute significantly to the total phytoplankton bio-volume but that may be relatively low in abundance. Enumeration is built-up of succeeding steps, using different magnifications and sub-sample volumes. Enumeration should begin with a small sub-sample volume at relatively high magnification. This step is finished as soon as sufficient observations of the most numerous organisms have been collected. Taxa with insufficient observations are taken to the next enumeration step: the examination of a larger sub-volume at a similar magnification. This procedure can be repeated several times before changing to a lower magnification for the examination of relatively large sub-volumes. In this step, only those species are enumerated which can be detected and counted precisely at low magnification. This step can also be repeated, stopping to count organisms with sufficient observations and taking others with too few observations along to the next step. This strategy is characterized by a failure to detect small, less abundant species. Modifications of this strategy might include varying the area of the chamber counted rather than the sub-sample volume (e.g. counting the whole chamber for large taxa and random fields for smaller taxa).

Annex D
(informative)

Identification

There are two methods of identifying organisms: by matching (pattern recognition) and by elimination (typically through the use of keys). Matching depends on the availability of a comprehensive set of specimens or illustration and keen observation. Elimination depends on knowing what the possibilities are and how to eliminate them on the basis of identification characteristics [1]. The success of the matching method comes largely from the relative ease with which humans can often detect subtle differences in the range of visual characteristics displayed by two or more organisms. However, identification based on illustrations alone should be avoided. If the accompanying text is clear to the analyst, it will include more information on variation and salient morphological features of a given species. Moreover, most authors will discuss similar species and clarify how they are distinguished [10]. The primary attraction of the elimination methods lies in the possible precision and objectivity once the technicalities of using the key have been mastered [1]. However, many traditional dichotomous keys can be difficult to use. The application of computer-based multi-access keys may make it easier for non-specialists to use the elimination method accurately.

The name given to a species upon identification links the organism with the original ‘type specimen’ of that species. This is often one or more individuals of the species, preserved in a herbarium, described according to the rules of the International Code of Botanical Nomenclature [6] using text, measurements and illustrations. In practice, however, it is rarely necessary to consult the original description, and instead most algal identification is based upon comprehensive books and Floras, in which the species characteristic of a region are described. However, users need to take care, when using such Floras, to ensure consistency. In particular:

— many algal species are named on the basis of morphological criteria alone and there are many examples of taxa with variable morphology described under several different names. The chlorophycean genus *Scenedesmus* offers many examples (e.g. [11] and [30]). In a few cases (such as the Cyanobacteria family Rivulariaceae), the morphology is strongly influenced by the environment and an understanding of this can lead to more precise interpretations of data;

— the way in which a species name is used can vary between Floras and can, over time, deviate from that originally intended and different Floras can use the same binomial (the name of a species that consists of a generic name and a specific epithet) to imply different ‘concepts’ of the species. For this reason it is important that the names of any Floras applied, along with the authorities for any species, are recorded;

— occasionally, individuals are found which do not correspond to descriptions in the principal Floras. In this case, the analyst should make a personal decision based on a wider range of Floras as well as publications in scientific journals. Actual specimens and/or drawings and photographs should be sent to expert phycologists for confirmation and the sample records should record both the identity of the organism along with the authority for this decision. If no decision can be made, then the organism should be referred to in the species list at the lowest taxonomic level to which it can be reliably identified along with a number, corresponding to the serial number of the photograph or the drawing in the personal collection.

Annex E
(informative)

Use of conventional compound microscopes

E.1 Introduction

The Utermöhl technique, using an inverted microscope as described in this guidance standard, is the recommended method for analysing phytoplankton abundance and composition. However, where an inverted microscope is not available, phytoplankton may be enumerated using a conventional (upright/non-inverted) compound microscope with one of several alternative counting chambers, e.g. Sedgwick-Rafter, Lund and Palmer-Maloney chambers. Much of this guidance standard is applicable to these methods, the exception being the descriptions of the chambers themselves (see 5.2) and the preparation of sub-samples and filling of the chambers (see 6.5) described in this annex. The distribution of algal objects for most algal taxa within these chambers is random and, therefore, a number of random fields can be counted in order to obtain a quantitative estimate. The distribution of algal objects within the chambers should be checked using the procedures outlined in Annex F.

E.2 Counting chambers

E.2.1 Sedgwick-Rafter chamber

This comprises a glass/plastic microscope slide on top of which is a rectangular raised rim, 50 mm × 20 mm × 1 mm (e.g. [7], [13] and [34],). When covered by a rectangular cover glass, the chamber created is 1 mm deep, encloses an area of 1 000 mm^2 and holds a nominal volume of 1 ml. The base of the chamber may be ruled into 1 000 units of 1 mm^2, although for phytoplankton enumeration, a calibrated counting-graticule is required to delimit counting fields. The chamber is best suited for counting large algae at low concentrations. As high magnifications (e.g. using a × 40 objective lens) are difficult to achieve due to the thickness of the chamber, accurate identification of organisms smaller than 10 µm to 15 µm is difficult or impossible. The depth of the chamber can be reduced, if necessary, by grounding down the rim of the chamber and then determining the new volume as the weight of water required to fill it completely.

E.2.2 Lund chamber

This is a simple counting chamber that can be made in the laboratory, if not commercially available [13], [16]. It comprises a glass microscope slide to which a strip of glass or brass (approximately 55 mm long, 3 mm wide and 0,5 mm deep) is cemented along each of the longer sides. A standard 50 mm x 22 mm glass cover slip is then placed across these supporting strips to create a chamber with open ends (of variable volume, but in the region of 0,5 ml to 0,6 ml). The cover slip is not fixed permanently and can be removed for cleaning. As microscope objectives up to × 32 or × 40 (with a long working distance) can be used, identification over a range of phytoplankton sizes is possible, although difficulties may be experienced in achieving a random distribution if the sample contains large algae (e.g. large dinoflagellates, large colonial or filamentous species).

E.2.3 Palmer-Maloney chamber

The Palmer-Maloney chamber [22] comprises a glass microscope slide onto which is sealed a shallow, disc-shaped chamber (diameter 17,9 mm, depth 0,4 mm) with two narrow (2 mm wide) channels/slots through the wall of the chamber opposite each other. When full, the chamber holds a volume of 0,1 ml. Objectives up to × 40, with a long working distance, can be used, making it useful for counting nanoplankton. For routine counts of large phytoplankton present at low concentration, however, a chamber holding a larger volume of sample (e.g. Sedgwick-Rafter chamber) may be more appropriate.

E.2.4 Modified sedimentation chambers

Special sedimentation chambers with a removable sedimentation column and an extremely shallow base tray may be used with a conventional microscope. An example of such a modified chamber, which can be constructed in the laboratory, is described by Hamilton *et al.* (2001) [8]. The Hamilton chamber consists of a 1 mm thick glass base (either rectangular, 75 mm × 51 mm, or circular, 70 mm) to which a piece of styrene cut to the same size as the base plate (approximately 130 µm thick, or 260 µm if large colonial or mat-like algae are present), is adhered. A 26 mm circular section is cut from the centre of the styrene before it is attached to the base plate, creating a shallow chamber, approximately 130 µm (or 260 µm) deep and 26 mm in diameter. The sub-sample is prepared and the chamber filled in the same way as a conventional sedimentation chamber, the column being removed and replaced with a rectangular cover slip once the algae have settled. If the cover slip is sealed with a suitable sealant (e.g. corn syrup or petroleum jelly), the sample can be kept intact for weeks without evaporation. The chamber can be used with objectives up to × 40 magnification and even works well with a × 50 oil immersion objective, so it is suitable for enumerating and identifying nanoplankton.

As the operation of such modified sedimentation chambers is in agreement with the guidance in the normative part of this European Standard, the following sections refer only to Sedgwick-Rafter, Lund and Palmer-Maloney chambers.

E.3 Calibration

Algal enumeration is based on counting the number of algal objects in a known number of randomly-selected fields of known size, within a chamber of known area and volume (except for populations of large algae at low concentration, when it may be more appropriate to count the entire chamber).

Guidance on calibration of microscope counting fields is given in 5.3.

For calibration of chambers, the volume can be determined by weighing the (dry) slide and cover slip (in g), then filling the chamber with deionised (or similar) water and weighing it again: the difference between the weights is equal to the volume of the chamber in millilitres, assuming 1 g of deionised water weighs 1 ml. The chamber can then be re-filled a further 4 times and a mean volume determined. Such calibration is essential for use of the Lund chamber, where the volume varies depending on the precise construction of each chamber, but can also be used to confirm the volume of Sedgwick-Rafter (or Palmer-Maloney) chambers, even when these have a nominally standard volume. The area of the chamber is determined (or confirmed) by direct measurement: for the Lund chamber, the chamber area is defined as the mean width between the glass strips (in mm) multiplied by the length of the cover slip. When using more than one chamber of the same type within a laboratory, each chamber should be calibrated and marked to distinguish it (e.g. A, B, C), and a record kept of the areas and volumes of each chamber.

E.4 Sub-sample preparation

The chamber is filled by placing the cover slip/glass over the rim of the chamber, using a Pasteur/large-bore pipette (without pipette bulb) to introduce an aliquot of sample into the chamber. This should be done gently and continuously by capillarity (not “forced” in by using a pipette bulb), to avoid the occurrence of air bubbles. For Sedgwick-Rafter and Lund chambers, the cover slip/glass is initially placed diagonally across the rim, so that there is a slight opening at opposite ends: as the aliquot is introduced via one of these ends, air is forced out of the other end, further reducing the risk of air bubbles forming. The cover slip/glass is then moved carefully into position and filling completed. For Palmer-Maloney chambers, the aliquot is delivered via one of the two channels in the rim, these performing the same function. If air bubbles occur, the chamber should be re-filled. When filling is compete, the cover slip/glass should fit tightly.

NOTE 1 Use of a calibrated pipette to deliver a known volume of sample to chambers such as the Sedgwick-Rafter chamber can avoid variations in chamber volume due to variations in filling technique (although see 6.5 on use of pipettes).

Once the chamber has been filled, it should be set aside to allow the algae to settle on the bottom of the chamber. The settling time depends on the depth of the chamber, the taxa present and the preservative used and should be determined at the validation stage when a new procedure is being established (as a guide, 7 min to 15 min is usually sufficient for the Sedgwick-Rafter chamber whereas 2 min to 5 min should be sufficient for the Lund chamber).

NOTE 2 For any partially or fully open counting chamber (the Lund chamber has open ends), evaporation during the settling time can be prevented by placing the counting chamber into a Petri dish or other closed container with a damp piece of tissue to maintain a saturated atmosphere (or placing it onto a raised support within the Petri dish and adding a few drops of water to the bottom of the dish).

Once the algae are settled on the bottom of the chamber, it should be examined under low power to ensure that the distribution of algae is obviously non-random. If the distribution appears to be satisfactory, counting can begin.

If the algal concentration is too low for effective counting, a sample can be pre-concentrated by sedimentation (see B.3 for other methods). An aliquot of the sample is poured into a graduated glass measuring cylinder, allowed to stand to allow the algae to settle (at least 4 h per cm, see 6.5 for further guidance), and then the supernatant is carefully siphoned off to leave a known volume of concentrated sample. This can then be counted.

E.5 Counting procedure

The general guidance provided in Clause 7 applies.

Efficiency of counting is increased if more than one chamber is used simultaneously, so that one chamber can be counted whilst another (or others) are settling.

Accuracy of Sedgwick-Rafter chambers (at least) can be increased by counting a small number of fields in several chambers, rather than a large number of fields in one chamber. The optimum number of fields per chamber and the optimum number of chambers (taking into account the efficiency of the method in terms of precision and time taken) can be derived from the time taken for preparing and cleaning the slide, the time taken to count one field, the between-chamber variance and the within-chamber variance [36].

Evaporation from the open ends of the Lund chamber during counting may distort the distribution of algae within the chamber. If this happens, the concentration of the sample may need to be adjusted and the chamber re-filled, to allow faster counting, or more than one chamber counted with only a small number of fields counted in each chamber.

Annex F
(informative)

Statistical procedure

F.1 General

This annex provides some basic statistical tests which can be used to validate analytical protocols and to assess measurement uncertainty. It should be noted that the tests described in F.2, F.3 and F.4, though simple and practical to use, have a relatively low power and therefore the uncertainty associated with microscopic counting level could be underestimated. Nevertheless, the tests are effective to bring analyses under a state of statistical control and to provide an estimate of measurement uncertainty.

When dealing with colonies, the procedure set out below refers to the number of colonies and not to the number of cells. When analysing the mean number of cells per colony with unknown distribution pattern, the central limit theorem should be applied. The number of cells in at least 30 replicate colonies should then be counted in order to estimate the mean.

F.2 Variance to mean ratio

The variance to mean ratio (I), which gives a good approximation of χ^2 for n-1 degrees of freedom (ν) is calculated according to:

$$I = \chi^2 = \frac{s^2(n-1)}{\bar{x}} \qquad \text{(F.1)}$$

where

n is the number of fields;

$\bar{x}$ is the mean number of objects;

s^2 is the variance of the number of objects.

For $\nu < 30$ the critical values of χ^2 can be found in standard chi-squared tables included in most statistical textbooks. Agreement with a Poisson series is accepted at the 95 % probability (P > 0,05) level if the χ^2 value lies between the appropriate 5 % significance levels (Q = 0,975 and Q = 0,025) for n-1 degrees of freedom.

For $\nu \geq 30$ agreement with Poisson is accepted at the 95 % probability level when:

$$|d| = \sqrt{2\chi^2} - \sqrt{2\nu - 1} < 1{,}96 \quad (P > 0{,}05) \qquad \text{(F.2)}$$

If the sample is large enough, the result should be checked with a goodness-of-fit test using χ^2 when n/k is large (k is number of categories and n is number of objects) or the G statistic when n/k is small (see statistical textbooks).

When dealing with slightly contagious distributions ($\sigma^2 > \mu$) a Poisson approximation is still applicable when μ is small (< 5) and $1/k$ (index of dispersion: $k = \frac{\bar{x}^2}{s^2 - \bar{x}}$) is fairly small (< 0,2). The maximum allowable variances are given in Table F.1.

Table F.1 — Maximum allowable variance for Poisson approximation (μ = mean, σ^2 = variance)

μ	0,5	1	2	3	4	5
σ^2	0,55	1,20	2,80	4,80	7,20	10,00

F.3 Serial randomness

A Run test can be used to test whether the separate taxa (species) are randomly distributed in relation to each other. When taxa are randomly distributed, the probability of counting a specific taxon is independent of the preceding counted taxon. This so-called serial randomness can be tested with a Run-test. In this case a run is a sequence of identical algae observed in a small diagonal band bound on either side by another algae or no algae (Figure F.1).

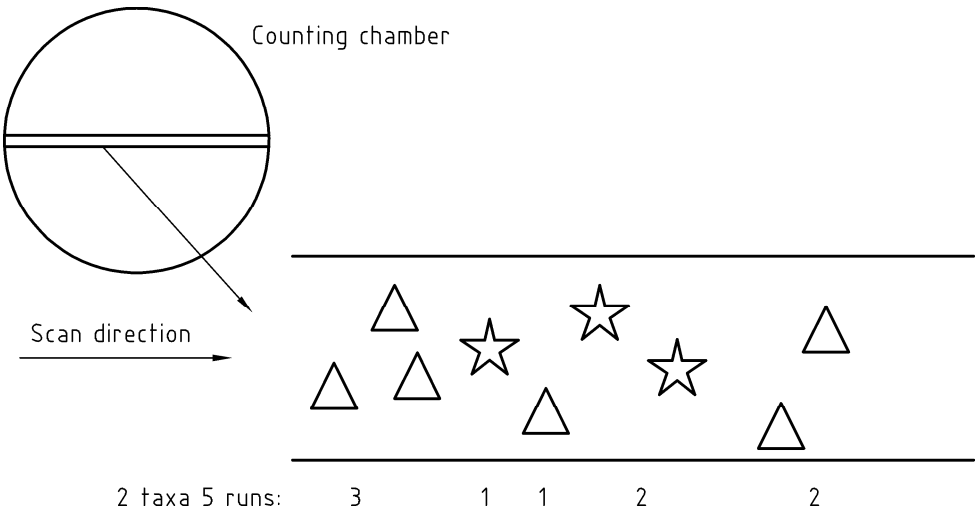


Figure F.1 — Illustration of collecting algal data for a Run-test

The Run test can be applied to two or more taxa. When one taxon has an abundance of at least 30 objects in the scan, the expected mean amount of runs is calculated according to:

$$\mu_u = \frac{N(N+1) - \sum n_i^2}{N} \qquad \text{(F.3)}$$

where

N is the total number of objects;

n_i is the number of objects of taxon i.

The mean μ_u has a standard deviation of

$$\sigma_u = \sqrt{\frac{\sum n_i^2 \left[\sum n_i^2 + N(N+1)\right] - 2N\sum n_i^3 - N^3}{N^2\ (n-1)}} \qquad \text{(F.4)}$$

The statistic

$$Z_c = \frac{|u - \mu_u| - 0{,}5}{\sigma_u} \qquad \text{(F.5)}$$

in which u is the observed number of runs, may be considered as a normal deviate with $Z_{\alpha(2)}$ as the critical values (Z_c) of the test ($Z_{0,05(2)} = t_{0,05(2),\,\nu} = 1{,}96$).

F.4Multinomial homogeneity test

A multinomial test can best be performed by counting approximately 200 algae in each sample. To test whether N samples are homogeneous with respect to the proportion of the taxa, χ^2 should be calculated according to the method of Mosimann [18] by:

Table F.2 — Multinomial homogeneity test

Sample No	Number of objects x_1	Number of objects x_2	Number of objects	Number of objects x_i	Total objects
Sample 1	x_{11}	x_{21}	…	x_{i1}	n_1
Sample 2	x_{22}	x_{22}	…	x_{i2}	n_2
Sample ...	…	…	…	…	…
Sample N	x_{1N}	x_{2N}	…	x_{iN}	n_N
Total	$x_{1'}$	$x_{2'}$	…	$x_{i'}$	n'

$$\begin{aligned}\chi^2 = {} & \frac{(x_{11} - n_1\hat{p}_1)^2}{n_1\hat{p}_1} + \frac{(x_{21} - n_1\hat{p}_2)^2}{n_1\hat{p}_2} + \ldots\ldots + \frac{(x_{k1} - n_1\hat{p}_k)^2}{n_1\hat{p}_k} \\ & + \frac{(x_{12} - n_2\hat{p}_1)^2}{n_2\hat{p}_1} + \frac{(x_{22} - n_2\hat{p}_2)^2}{n_2\hat{p}_2} + \ldots\ldots + \frac{(x_{k2} - n_2\hat{p}_k)^2}{n_2\hat{p}_k} \\ & + \frac{(x_{1N} - n_N\hat{p}_1)^2}{n_N\hat{p}_1} + \frac{(x_{2N} - n_N\hat{p}_2)^2}{n_N\hat{p}_2} + \ldots\ldots + \frac{(x_{kN} - n_N\hat{p}_k)^2}{n_N\hat{p}_{k1}}\end{aligned} \qquad \text{(F.6)}$$

where

$\hat{p}_i = x_i' / n'$ and (df = (N - 1)(k - 1))

$$\sum x_1^2 / n_1 = \left(x_{11}^2 / n_1\right) + \left(x_{12}^2 / n_2\right) + \ldots\ldots + \left(x_{1N}^2 / n_N\right) \qquad \text{(F.7)}$$

The critical value of χ^2 can be found in most statistical textbooks. 'Rare' taxa included in the counts should be grouped according to the rule that a minimum count of 1 is allowable if less than 20 % show an abundance of < 5. In the table above, a 'sample' might be a replicate sub-sample, or a replicate counting of the same sub-sample (counting chamber). However, this test can also be used to compare different samples from the same water body, in order to validate the sampling method (not covered in this European Standard).

NOTE The above equation has the advantage that the contribution of each term (taxon or group) to the χ^2 value can be judged. Each term actually represents the relative deviation from the 'true' proportion for that particular taxon or group.

If only one proportion is of importance, a normal approximation might be used to test differences among proportions [37]. In this test χ^2 is calculated as:

$$\chi^2 = \sum_{i=1}^{k} \frac{(x_i - n_i \bar{p})^2}{n_i \bar{p}\bar{q}} \qquad \text{(F.8)}$$

where

$\bar{p}$ is the overall mean proportion of taxon or group i, $\bar{q}$ = 1- $\bar{p}$ and χ^2 has k-1 degrees of freedom.

F.5Central limit theorem

F.5.1 General

As a general rule, if random samples of size n are drawn from a non-normal population, the distribution of the sample means will tend towards normal as n increases in size. This is the central limit theorem and it applies to a Poisson series when $n\bar{x}$ > 30, where n is the number of fields and $\bar{x}$ is the mean number of a taxon or group per field.

F.5.2 Confidence limits

$n\bar{x}$ > 30 the 95 % confidence limits for the estimate of the mean are calculated according to:

$$\bar{x} \pm t_{0,05(2),\nu} \sqrt{\frac{\bar{x}}{n}} \qquad \text{(F.9)}$$

where

n is number of fields counted per chamber;

$\bar{x}$ is the mean number of a particular alga or group per field;

ν is n-1.

Those taxa which do not fulfil the constraint of $n\bar{x}$ > 30 can be added together as a group, for which $n\bar{x}$ > 30. This allows the measurement uncertainty for this group to be computed.

The confidence limits represent only the uncertainty in the estimation of the mean for a particular sub-sample. The reproducibility error should be computed by counting different chambers etc. and then calculating the overall variance. Then the confidence limits can be calculated according to:

$$\bar{x} \pm t_{0,05(2),\nu} \sqrt{\frac{s_p^2}{n+m}} \qquad \text{(F.10)}$$

where

ν is (n-1)+(m-1);

n is the number of fields counted per chamber;

m is the number of chambers;

s_p^2 is the overall variance which is the sum of $\bar{x}$ and the reproducibility variance s_R^2.

The variance s_p^2 should encompass, if relevant, different analysts, days, chambers etc.

F.6 Confidence limits for Poisson counts

The best approach to calculate confidence limits for the total count irrespective of the number of fields or objects counted is:

Lower 1-α confidence limit:

$$L_1 = \frac{\chi^2_{(1-\alpha/2),\nu}}{2} \qquad \text{(F.11)}$$

where

ν is $2x$;

x is the number of objects counted.

Upper 1-α confidence limit:

$$L_2 = \frac{\chi^2_{(\alpha/2),\nu}}{2} \qquad \text{(F.12)}$$

where

ν is $2(x+1)$.

This approach for calculating the confidence limits for the Poisson parameter results in an asymmetrical confidence interval.

When a significant part of a chamber has been screened (for instance for large diatoms or desmids) the Poisson series is still applicable. It is recommended to calculate the confidence limits according to the method above for calculating confidence limits for the Poisson parameter.

Once confidence limits for a count have been calculated, they can be expressed in the same units as the count itself (e.g. cells per ml) by multiplying by the ratio between the total area of the chamber and the area counted, and then dividing by the sub-sample volume in the chamber.

F.7 Confidence limits for proportions

If data are binomial by nature and n is fairly large, approximate 95 % confidence intervals for the proportion of a particular taxon or group can be computed according to [18] by:

$$p_{L/U} = \frac{\hat{p} + [3{,}84/(2n)] \pm (1{,}96)\sqrt{[\hat{p}(1-\hat{p})/n] + [3{,}84/(4n^2)]}}{1 + (3{,}84/n)} \qquad \text{(F.13)}$$

where

$\hat{p}$ is the counted proportion of a category (taxon);

n is the total number of observations.

In general, when there are more than two categories, confidence limits should be estimated simultaneously using the multinomial distribution. However, for the purpose of estimating measurement uncertainty the binomial approximation is preferable as it is easier to calculate. In principle, each category by itself has a binomial distribution since the remaining categories can always be considered as a single category.

F.8 Calculating measurement uncertainty

Irrespective of the central limit theorem (F.5), confidence limits for a single count can best be calculated directly (F.6). Confidence limits of proportions can be estimated by a normal approximation (F.7). These confidence limits should be combined with a reproducibility estimate by standard propagation rules. However, a constant multiplication factor based on reproducibility experiments can be simple and effective. Any indication of a systematic deviation from some true value as might be obtained by inter-laboratory studies can be reported separately.

A variety of suitable approaches to calculate the measurement uncertainty exist, which are not part of this guidance. Especially the EURACHEM/CITAC-Guide is useful in cases where sufficient previous data are not available, and therefore the mathematical analytical approach according to GUM with all different steps should be used. For more detailed instructions see ISO/TS 21478 and special technical literature, i.e. NORDTEST Technical Report 537.

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