SLOVENSKI STANDARD
SIST EN 16695:2015

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Kakovost vode - Navodilo za ocenjevanje biovolumna mikroalg

Water quality - Guidance on the estimation of microalgal biovolume

Wasserbeschaffenheit - Anleitung zur Abschätzung des Phytoplankton-Biovolumens

Qualité de l'eau - Lignes directrices pour l'estimation du biovolume des microalgues

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13.060.70	Preiskava bioloških lastnosti vode	Examination of biological properties of water

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Water quality - Guidance on the estimation of phytoplankton biovolume

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European foreword

This document (EN 16695:2015) 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of an identical text or by endorsement, at the latest by March 2016 and conflicting national standards shall be withdrawn at the latest by March 2016.

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Introduction

The abundance or number of counting units of individual phytoplankton taxa does not necessarily reflect the real ratio of single taxa to the complete biomass of a phytoplankton community. Few big cells/counting units can contribute far more biomass to the system than many small ones. Hence, abundance data alone is often not an ideal measurement of population size. Biomass estimations give very important information for ecological studies, classification schemes and ecosystem modelling. Therefore, it is necessary to determine the biomass of phytoplankton taxa, particularly because phytoplankton delivers energy in the form of carbon, to other trophic levels of food webs. It is not possible to directly analyse the carbon content on the taxonomic level in natural phytoplankton samples, therefore the biovolume of the phytoplankton taxa is a suitable measure to determine the biomass of an ecosystem according to the taxonomic composition. Neither particle size analysis using laser analysis, nor flow cytometry, nor Coulter Counters, nor chemical analyses of chlorophyll-a concentration as well as total carbon allow statements on the taxon level. An estimation of the carbon content is possible using conversion factors (see Annex C).

Further, the biovolume is a quantitative basis for assessing hazards from those algae and cyanobacteria, which (can) contain noxious or toxic metabolites, and is used in combination with cell numbers or chlorophyll-a concentration within WHO guidelines and national regulations for risk assessments.

Up to now, various guidelines for estimating the biovolume of microalgae have been used in different national and international monitoring programs (e.g. [1], [2], [3], [4]). The main objective of this document is the standardization of the procedure for determining the phytoplankton biovolume in order to achieve comparability of data. For this reason, the estimation of the biovolume in phytoplankton samples in sedimentation chambers (according to Utermöhl) using an inverted microscope will be described in detail.

This European Standard is also applicable for image analysis of pictures derived from microscope and flow cytometry cameras. The use of a standard catalogue containing basic and some composed geometrical shapes is recommended. Of course, such a standard list will not reflect the variety of all naturally existing shapes and will not match the exact biovolume values of each taxon. It will always be a compromise between accuracy and efficiency. However, the usage of agreed geometrical shapes and the application of the relevant formulae will improve the comparability of phytoplankton data and will be an important step forward for the implementation of quality assurance measures in phytoplankton analysis.

1 Scope

This European Standard specifies a procedure for the estimation of biovolume of marine and freshwater phytoplankton taxa using inverted microscopy (Utermöhl technique according to EN 15204), in consideration of some heterotrophic protists (< 100 µm) that are not considered in routine zooplankton analysis and benthic microalgae, which can be found in pelagic water samples.

This European Standard describes the necessary methods for measuring cell dimensions and for the calculation of cell or counting unit volumes to estimate the biovolume in phytoplankton samples. This shall be done using harmonized assignments of geometrical shapes to avoid errors.

2 Normative references

The following documents, in whole or in part, are normatively referenced in this document and are indispensable for its application. For dated references, only the edition cited applies. For undated references, the latest edition of the referenced document (including any amendments) applies.

EN 15204, *Water quality - Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique)*

EN 15972, *Water quality - Guidance on quantitative and qualitative investigations of marine phytoplankton*

EN 16698, *Water quality - Guidance on quantitative and qualitative sampling of phytoplankton from inland waters*

3 Terms and definitions

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

3.1
biomass
total mass of living organic matter within a system or taxon

3.2
biovolume
total volume of (living) organisms within a system or taxon

Note 1 to entry: The biovolume is usually expressed in cubic millimetres per litre (mm^3/l).

3.3
cell volume
counting unit volume
total volume of a single cell or one counting unit

Note 1 to entry: The cell volume or counting unit volume includes the cell wall (if existing) but excludes lorica and/or mucilaginous envelopes and cell surface structures such as spines, bristles and scales.

Note 2 to entry: The cell volume or counting unit volume is usually expressed in cubic micrometres ($µm^3$).

4 Principle

Generally, the estimation of the total or taxon specific biovolume in phytoplankton samples of natural communities or cultures is based on measurements of a representative number of individuals. By

multiplying the average or median cell or counting unit volume with the abundance, the total biovolume of each taxon in the sample is determined.

Three approaches are feasible:

1) **Estimation by representative measurement:** A representative number of individuals (in most cases single cells) or counting units of all recorded or dominating taxa is measured in each sample or a specified number of samples within a comparable series. These data are used to calculate the average or median cell or counting unit volume of each taxon using the applied geometrical formulae.

2) **Estimation using size classes based on representative measurements:** For taxa with a high variability in cell size (e.g. several diatoms, different stages in life cycle) reasonable size classes can be determined first, and then the individuals are assigned to both the relevant taxon and size class. Basis for the definition of the size classes are measurements in the same manner as described in (1).

3) **Estimation using standard volumes based on representative measurements:** A reasonable general standard cell or counting unit volume is defined for each taxon once. These standard values are determined by representative measurements and calculated by the formula of the assigned geometrical shapes as described in (1).

A geometrical shape shall be assigned to each taxon in all approaches to calculate the cell or counting unit volume. Thus, to harmonize these approaches the geometrical shapes are pre-assigned to all taxa (see Annex D). These shapes have been chosen to reflect the corresponding taxa shapes as accurately as possible, and to allow effective taxa measurement with little effort (i.e. with as few dimensions as possible; usually only two are necessary). Seventeen different geometrical shapes are utilized (for the catalogue of geometrical shapes see Annex A). If it is impossible to describe the actual shape of a taxon with a simple basic geometrical shape, composite shapes (e.g. cone with half sphere) are used. If the actual geometry of taxa does not fit exactly to the assigned shape, a "geometry correction factor" is used for the final cell or counting unit volume calculation.

Taxon lists describing the preferred geometrical shapes have been published before (see e.g. [1], [3], [4]), based on specific taxonomical levels or for particular areas. This guidance document provides harmonized geometrical shapes for phytoplankton organisms spread across European marine, brackish, and freshwater systems. Annex D contains an alphabetical list of genera with the assigned geometrical shapes. If there are divergent forms on species, subspecies, form, or variety level within a genus they are listed as well.

5 Equipment and preservatives

The following equipment is required for biovolume analysis of phytoplankton samples.

5.1 Inverted microscope equipped with a condenser featuring a numeric aperture (NA) of at least 0,5 and plan objectives with a NA of 0,9 or more allowing for total magnification between 63× and 400× at a minimum. The microscope should have binocular, bright field (additional phase contrast is useful), 10× or 12,5× eyepieces.

Though inverted microscopy is the recommended method for analysing of phytoplankton, conventional (non-inverted) compound light microscopes may also be used for measuring phytoplankton under some conditions.

5.2 Calibrated object micrometre.

5.3 Eyepiece (ocular) micrometre.

5.4 Counting-graticule.

5.5 Sedimentation chambers according to EN 15204.

5.6 Image analysis software, if available.

5.7 Sampling bottles according to EN 15204.

5.8 Preservatives, acidic Lugol's iodine and/or alkaline Lugol's iodine according to EN 15204.

6 Procedure

6.1 Sampling and sample preparation

The sampling and determination of phytoplankton abundance and composition is a precondition for the calculation of the biovolume of a phytoplankton sample. Sampling shall be carried out according to EN 16698 for freshwater samples and EN 15972 for marine samples. For counting and species determination, see EN 15204.

The dimensions needed for the biovolume estimation of the relevant phytoplankton taxa are analysed in sedimentation chambers, which are prepared in the same manner as for counting and species determination (see EN 15204), using an inverted microscope and an eyepiece micrometre or image analysis software.

For specific scientific purposes, measurements can be carried out also with a conventional (non-inverted) compound light microscope.

6.2 Calibration of the eyepiece micrometre, counting-graticule and image analysis software

The required dimensions for estimation of the cell or counting unit volume shall be measured using an eyepiece (ocular) micrometre or an image analysis software. For the application of size classes a calibrated counting-graticule can also be used.

Prior to measurement, all systems shall be calibrated with a calibrated object micrometre for every microscope and all objectives and eyepieces used.

The scale of commercially available calibrated object micrometres has a length of 1 mm (or 2 mm) where the millimetre is divided into 100 equal parts. The distance between the graduation lines is 10 µm. By aligning the scale of the eyepiece micrometre with the scale of the object micrometre or the grid boxes of the counting-graticule, the scale value (S) or conversion factor of the eyepiece micrometre can be determined for each magnification as follows:

$$S = \frac{n_{\text{obj}} \times 10}{n_{\text{eye}}} \tag{1}$$

where

S is the scale value (conversion factor) for the eyepiece micrometre in micrometres (µm);

n_{obj} is the number of graduation lines of the object micrometre;

n_{eye} is the number of graduation lines of the eyepiece micrometre or the number of grid boxes of the counting-graticule.

The conversion factor should be specified with up to two decimal places. The intervals between the graduation lines of the scale shall be separately determined with the calibrated object micrometre for every objective used.

If image analysis software is used, this equipment shall be calibrated with the calibrated object micrometre separately for every level of magnification, following the instructions in the operating manual of the software.

6.3 Statistical requirements for determination

The required dimensions of the relevant geometrical shape shall be measured for each taxon of interest. At least 20 individuals per taxon should be measured to ensure that the standard error of cell or counting unit volume will be generally < 10 %.

For taxa, which are very variable in size, the number of measured cells/counting units should be increased until the standard error is < 10 %, to a maximum of 50 individuals ([3], [5]).

Where the size variability of a taxon is small, the number of measured cells may be minimized to only 5 to 10 individuals. In all cases, it is advisable to check that the standard error of cell or counting unit volume is low. The standard deviation and standard error can be calculated according to Formulae (2) and (3) as follows:

$$s = \sqrt{\frac{\sum_{i=1}^{n}(x_i - \bar{x})^2}{n-1}} \tag{2}$$

where

s is the taxon volume standard deviation;

x_i is the volume of a single cell/counting unit of the taxon;

$\bar{x}$ is the mean volume of all measurements of the taxon;

n is the number of the respective measured cells of the taxon.

From the standard deviation s, the standard error of the mean $\sigma_{\bar{x}}$ can be calculated using Formula (3):

$$\sigma_{\bar{x}} = \frac{s}{\sqrt{n}} \tag{3}$$

To obtain the percentage range σ_{rel} of the standard error, divide the standard error of the mean $\sigma_{\bar{x}}$ by the mean value $\bar{x}$ of the samples using Formula (4):

$$\sigma_{rel} = \frac{\sigma_{\bar{x}}}{\bar{x}} \times 100 \tag{4}$$

From the results, the 95 % confidence limits can be determined using Formulae (5) and (6):

$$\bar{x}_u = \bar{x} + (\sigma_{\bar{x}} \times 1{,}96) \tag{5}$$

$$\bar{x}_l = \bar{x} - (\sigma_{\bar{x}} \times 1{,}96) \tag{6}$$

where

$\overline{x}_u$ is the upper 95 % confidence limit;

$\overline{x}_l$ is the lower 95 % confidence limit.

Since the required number of individuals to be measured for any taxon is dependent upon the size variability of that taxon, it is helpful to calculate the cell or counting unit volume after each measurement. This will allow continuous statistical analysis and check of precision, thus minimizing the number of measurements required. Ideally, the confidence limits should be set at 95 % as a measure for precision. By ensuring that only as many cells as necessary for achieving this limit will be counted, the amount of laboratory work will be minimized.

If not enough cells/counting units of a taxon are present in the sample in order to achieve the minimum statistical requirements described, all of the (few) cells of this taxon shall be measured or a mean standard biovolume may be used (see below).

Measuring a high number of cells for every taxon in every sample is a time consuming procedure. For routine monitoring programmes mean cell or counting unit volumes calculated from own measurements, for a particular project and area, may be used. These mean volumes shall be checked regularly by measuring actual cell dimensions (see 6.4) and calculating actual cell and counting unit volume (see 6.5). For taxa with a high variability in cell size and representing more than 50 % of the total biovolume, these checks are strongly advised.

6.4 Measurement

6.4.1 General

Measurement of the cells/counting units can be carried out in a separate step or parallel to the counting process. Depending on the cell size of the taxa, the determination of the required dimensions (e.g. diameter, height, length, width, etc.) should be carried out at magnifications between 63× and 1 000×, in order to obtain corresponding precision.

The so-called empty magnification, which does not reveal any new detail, should be avoided. Therefore, the total magnification of the microscope should preferably be higher than 500× but smaller than 1 000× of the NA of the used objectives to work in the area of useful magnification.

It is important that the cells to be measured are chosen randomly to avoid a discrimination of special size classes. This can be achieved by selecting the cells from randomly distributed visual boxes all over the sedimentation chamber.

If single cells can clearly be distinguished in chain-building or filamentous species or other colony forming taxa, only one cell per chain, filament or colony should be measured. Filamentous algae often form lumps and are distributed very unevenly in the chamber. Also in these cases, it shall be ensured that cells from different lumps are measured.

By rotating the eyepiece micrometre and moving the sedimentation chamber with the microscope stage, the scale of the eyepiece micrometre is placed over the required dimension of the cell/counting unit to be measured. The number of covered graduation lines is read, and with the application of the conversion factor (see 6.2), the length of the dimension is calculated by multiplication.

The measurement results should be reported in micrometres, with up to two decimal places. If the end of the cell dimension to be measured is between two graduation lines of the ocular scale, the share is to be estimated to a maximum of one decimal place.

When using the size classes approach based on representative measurements, the calibrated counting-graticule may also be used for the assignment of the individuals to the corresponding size classes.

When image analysis software is used, the corresponding details of the operating manual shall be followed.

6.4.2 Using size classes based on representative measurements

The appropriate number of size classes depends on the size variation of each taxon. The size classes, for example, can be derived from cluster analyses applied to a representative dataset of measured cells of the taxon (see 6.4.1).

For each size class the average cell or counting unit volume is calculated using the formula of the taxon specific geometrical shape with mean dimension lengths. By multiplication with the respective abundance and addition of all size classes of a taxon, the total taxon specific biovolume in the sample is determined. In routine monitoring programmes, standard size classes should be used (e.g. [4]). If regional lists of size classes are available, they should be used instead [4]. It is recommended to create a new size class, when the biovolume of individuals significantly exceeds the biovolume of existing size classes.

6.4.3 How to deal with hidden dimensions

For some particular taxa, it is often not possible to measure all dimensions needed to calculate the cell volume ("hidden dimensions", e.g. height of prismatic cells of different shapes or small diameter of elliptical cells) during routine analysis. Then it is necessary to estimate the length of missing dimensions as a proportion of one of the visible dimensions using a calculated species-specific factor. The visible to hidden size relationship can be obtained from measurements of other samples from the same sample series or from the same area if the position of the cells allows this. Measurements should be used preferentially over estimates. The taxon list in Annex D gives suggestions for the dimension relations for most of the taxa, which usually have a "hidden dimension". For those taxa, where no suggestion for the "hidden dimension" is given, corresponding nominal values shall be taken from the literature if available. If the "hidden dimension" shall be determined exactly for a special problem, the cells can be turned around in paraffin oil. With special microscopes, it is also possible to measure the distance between focus of the upper and lower end of the "hidden dimension".

6.4.4 Measurement of filamentous taxa

With some filamentous taxa (e.g. cyanobacteria), it is often difficult to distinguish individual cells within a filament, especially when the cells are directly connected without any gaps. In such cases, filament pieces of a fixed length, e.g. 100 µm or 10 µm, can be counted and measured (100 µm or 10 µm length and diameter), and multiplied by the total enumeration of this counting unit in the sample.

Alternatively, mean dimensions of filaments can be measured to calculate the volume of one filament, a value that is then multiplied by the number of filaments in the sample.

A third method, which is more precise for filamentous forms, particularly those, which have no distinct boundaries between cells, is as follows: Instead of counting individual filaments, the total length of the fraction of each filament that is within the boundaries of a counting grid shall be measured, ignoring the fraction outside of the grid boundaries (see Figure 1). The sum of the total length of all fractions of filaments within the grid shall be calculated after counting of the transect is completed. Afterwards, the diameter of at least 20 filaments shall be measured and the mean filament diameter is calculated. To obtain the biovolume of the respective taxon, the sum of total filament lengths (h) shall be multiplied with the square of the median filament diameter and the factor of $\pi/4$, because the filament is a cylinder with the volume:

$$V = \frac{1}{4} \times \pi \times d^2 \times h \qquad (7)$$

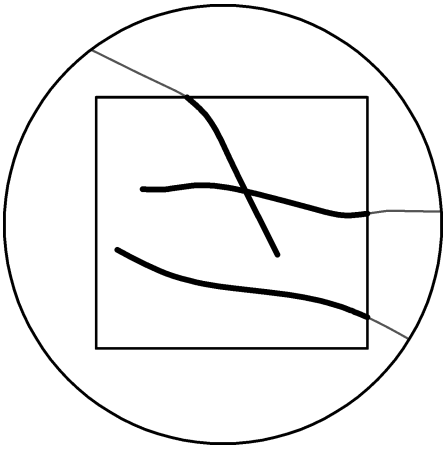

Figure 1 — Determining biovolume for filaments without distinct cell boundaries using a counting grid

NOTE The lengths of all fractions within the counting grid (black rectangle) are measured, excluding fractions outside of the counting grid boundaries [6].

6.4.5 Measurement and counting of colony- and coenobium-forming taxa

In most cases, the assignment of a geometrical shape should be based on the shape of an individual cell. For some colony- and coenobium-forming species where individual cells are difficult to distinguish or have very complex contours, it can be expedient to assign a geometrical shape based on the shape of the whole colony or coenobium or to use ultrasonic treated samples (where the association of cells have become disintegrated into individual cells) for counting and measuring the cells.

For example in some species, small individual cells are aggregated into compact spatial colonies. The individual cells are usually indistinguishable, and the number may be very difficult to assess in these colonies or coenobia. In such cases, the estimation of the volume can be based on the geometry of the entire colony or coenobium, in particular during the analysis of routine monitoring samples. For some taxa an additional geometrical shape correction factor shall be taken into account for the volume calculation. The same applies for colony- or coenobium-forming taxa with very complex contours of individual cells. Table 1 lists examples. In Annex D, it is specified for the respective taxa if the entire colony or coenobium with its geometry should be measured.

Table 1 — Geometrical shapes and correction factors for some colony- and coenobium-forming taxa.

Taxon name	Geometry based on colony	Geometrical shape correction factor	Hidden dimension factor
Woronichinia	sphere	0,2	-
Coelosphaerium	sphere	0,2	-
Snowella	sphere	0,75	-
Botryococcus	spheroid	-	-
Eudorina	sphere	0,25	-
Pandorina	sphere	-	-
Coelastrum	sphere	-	-
Pediastrum	cylinder	species dependend	height of colony = height of single cell (factor see Annex D)
Crucigenia	cuboid	species dependend	third edge length (height) = 0,5×second edge length (width)

A more precise method to achieve the biovolume of such colonial forms, especially the cyanobacterial genera *Microcystis, Aphanothece, Aphanocapsa*, and the *Dolichospermum* species forming "ball of yarn" colonies, is to measure cell dimensions and the greatest axial linear dimension of the colony in the sedimented sample, and then separate the colonies by ultrasonic treatment, and count the individual cells in the treated sample [7].

6.4.6 Measurement of complex geometrical shapes

Some taxa show very complex cell outlines requiring a composition of multiple geometrical shapes and thus, the application of complicated formulae for biovolume calculations. In order to ease that work, some simplified combined forms have been assigned to these taxa that can be easily measured and will require only minimum effort for the estimation. However, depending on the required precision, more complex and thus more precise geometrical subdivisions shall be applied to those taxa. Another possibility is to resort to pre-determined cell volumes from literature (mean standard cell or counting unit volume), bearing in mind that the size of the cells depends on a number of environmental factors and thus, may vary widely. On the other hand, cell volume estimation for some species is easier on half-cell basis (for example in some desmid genera like *Cosmarium* and *Staurastrum*).

In any case, everything shall be documented in the protocol. As an example, Annex E shows how to measure the needed dimensions for four taxa from the conducted interlaboratory comparison as well as for two additional taxa, which are not easy to measure.

6.5 Calculation of biovolume

The cell or counting unit volume is calculated on the basis of the taxon-specific geometrical shape and the linear dimensions determined for the individual cell or counting unit (e.g. diameter, height, length, width, etc.; see Annex A). For some taxa, the associated geometrical form will not fit exactly to the actual cell shape, for example, if a cuboid is assigned to a pennate diatom with rounded apical cell ends. For such cases, the cell volume shall be multiplied by a correction factor, which is given in Annex D for relevant taxa.

The average cell or counting unit volumes of the various taxa shall be generally calculated as the median of all individual cell or counting unit volumes.

NOTE If the individual volumes per taxon are normally distributed (according to Chi-squared test or Kolmogorov-Smirnov test), the arithmetic mean can be used for the calculation of the average volume instead of the median. For answering specific questions in ecology, it can be necessary to use also the arithmetic mean for non-normally distributed values.

These calculations shall be carried out for every phytoplankton taxon recorded in all or selected samples using the "representative measurement" approach (see Clause 4) or basically once for characteristic samples using the "standard factor" approach (see Clause 4). If applying the "size class" approach (see Clause 4 and 6.4.2) the average cell or counting unit volume for each size class is calculated using the mean of the upper and lower dimensions of size class borders.

The biovolume per taxon in a sample is calculated by multiplying the number of cells/l (or cells/ml) or counting units (e.g. number of 100 µm filament pieces per liter) with the median (or mean) of the determined taxon-specific cell or counting unit volumes (µm³) as measured by one of the three methods listed above:

$$V_{\mathrm{bio},i} = \frac{n_i \times \tilde{V}_i}{10^9} \tag{8}$$

where

$V_{\mathrm{bio},i}$ is the biovolume of taxon or size class i in cubic millimetres per litre (mm^3/l);

n_i is the number of cells (or number of counting units) of taxon or size class i per litre (l^{-1});

$\tilde{V}_i$ is the median (or mean) of the cell or counting unit volumes of taxon or size class *i* in cubic micrometres (µm³).

The total biovolume to be determined for each sample results from the sum of the biovolume determined for each phytoplankton taxon or size class.

Statistical performance data from the conducted European wide interlaboratory comparison for validation are listed in Annex B.

6.6 Biovolume biomass relations

In phytoplankton ecology, the biomass is usually expressed as chlorophyll-a concentration (µg/l), biovolume (mm³/l) or carbon content (µg/l). Assuming the density of organisms being equal to the density of water (1,0 g/cm³, [8]), the biomass as wet weight may be estimated as follows:

1 mm³/l (biovolume) = 1 cm³/m³ (biovolume) = 1 mg/l (wet weight);

1 mm³/m³ (biovolume) = 10^6 µm³/l (biovolume) = 1 µg/l (wet weight).

NOTE For the estimation of the carbon content, see Annex C.

6.7 Reporting

The specific cell or counting unit volumes are expressed in cubic micrometres (µm³) without decimal places or for picoplankton with two significant places (e.g. 4,2 µm³). The biovolume of a single taxon and the complete sample is given in cubic millimetres per litre (mm³/l or mm³/ml) with three significant digits or places (e.g. 12,5 mm³/l, 3,75 mm³/l, 0,138 mm³/l, 0,004 mm³/l).

As an example, the taxon specific part of a sample report can contain information as shown in Table 2.

Table 2 — Example report for taxon specific sample part.

Taxon name	Abundance n/l	Geometry	Average cell volume µm³	Total biovolume mm³/l	Carbon content (according to ...) µg/l
Thalassiosira nordenskioeldii	12 109	cylinder	9 817	0,119	6,53
Odontella aurita	265 725	elliptic cylinder	14 091	3,74	188,5
....					
Sum of sample 5	**765 180**			**4,43**	**231,2**

Some taxa (e.g. flagellates without solid cell wall) are inclined to shrink during the fixation process. Shrinkage depends on many influencing factors (preservative, life stage, physiological status, species, etc., even diatoms can shrink). Often it is difficult to find good correction factors for this process in the literature. As a consequence, applying a general correction factor to preserved material can lead either to overestimation or to underestimation of the biovolume. For monitoring activities with the objective of detecting trends, the exact compensation by preservation correction factors is not of the highest priority. In other cases where it might be mandatory to determine the exact biomass, correction factors for influence of fixation should be derived from comparisons between fixated and living organisms. This shall be noted in the protocol.

7 Quality Assurance

The quality assurance associated with this European Standard should be in accordance with EN 14996.