SLOVENSKI STANDARD
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Kakovost vode - Navodilo za ocenjevanje biovolumna alg

Water quality - Guidance on the estimation of microalgal biovolume

Wasserbeschaffenheit - Anleitung zur Bestimmung des Phytoplankton-Biovolumens

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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 microalgal biovolume

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

Wasserbeschaffenheit - Anleitung zur Abschätzung des Phytoplankton-Biovolumens

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

CEN-CENELEC Management Centre: Avenue Marnix 17, B-1000 Brussels

Contents

Foreword

This document (prEN 16695:2013) has been prepared by Technical Committee CEN/TC 230 “Water analysis”, the secretariat of which is held by DIN.

This document is currently submitted to the CEN Enquiry.

This document has been prepared under a mandate given to CEN by the European Commission and the European Free Trade Association, and supports essential requirements of the Water Framework Directive (2000/60/EC) (WFD), and the Directive on Environmental Quality Standards (Directive 2008/105/EC).

IMPORTANT — This draft European Standard contains in Annex C a very extensive list of common European phytoplankton taxa down to species level and their proposed geometrical shapes to be used for biovolume estimations. This list will be the central subject to discussion during the further revision of the draft, to decide if this list can be reduced for certain taxa to the genus level. The result will be a much shorter list and a more practicable approach for biovolume estimation.

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. Thus, biomass is much more relevant for ecological studies, classification schemes and ecosystem modelling than abundance, particularly because the energy is available in the form of carbon to other trophic levels of food webs. Therefore, it is important to determine the biomass of phytoplankton taxa. Because it is not possible to directly analyse the carbon content on the taxonomic level in natural phytoplankton samples, the biovolume of the phytoplankton cells is a suitable measure to determine the biomass of an ecosystem according to the taxonomic composition. The biovolume is an accurate basis for assessing hazards from algae and cyanobacteria which contain noxious or toxic metabolites, and is used in combination with cell numbers or chlorophyll-a within WHO guidelines and a number of national regulations for risk assessments. An estimation of the carbon content is possible using conversion factors (see Annex C). Neither particle size analysis using laser analysis, nor flow cytometry, nor Coulter Counters, nor chemical analyses of chlorophyll-a as well as total carbon do allow statements on the taxon level.

The organic substance is not equally distributed within the algal cells. Cells have one or more vacuoles which are filled with liquid. Organic substances are also dissolved in the vacuoles, but by far the largest part is located in the cytoplasm. Especially diatoms have extensive vacuoles which can form up to 90% of the total cell volume in larger species. The cytoplasm is then limited to a narrow region along the cell wall and plasma threads passing through the vacuole. Depending on the aims of the investigation, the determination of biovolume can be carried out by microscopy quantifying either cell numbers or cell volumes or plasma volumes (i.e. cell volume minus vacuole volume). The latter is very difficult to measure with conventional microscopy, so that in the routine monitoring the total cell volume is determined. For the estimation of the carbon biomass, the different sizes of the vacuoles are taken into account by applying different conversion factors and regarding the dependency on the cell size (see Annex C).

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]). Main objective of this document is the standardisation of the procedure for determining the phytoplankton biovolume in order to achieve comparability of data on an international level. For that purpose, the microscopic determination of the biovolume in phytoplankton samples in sedimentation chambers (according to Utermöhl) using an inverted microscope will be described in detail. The use of a standard catalogue containing basic, composed and fractional geometrical shapes will be 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 justifying the expense. But the usage of agreed geometrical shapes and the application of the respective equations will improve the comparability of phytoplankton data and will be an important step forward to the implementation of quality assurance measures in phytoplankton analysis.

1 Scope

This European Standard describes a general procedure for determination or estimation of biovolume of marine and freshwater phytoplankton taxa using inverted microscopy (Utermöhl technique).

The determination of phytoplankton abundance and composition according to EN 15204 is a precondition for the calculation of the biovolume of a phytoplankton sample.

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 14996, *Water quality — Guidance on assuring the quality of biological and ecological assessments in the aquatic environment.*

EN 15204, *Water quality — Guidance standard for the routine analysis of phytoplankton abundance and composition using inverted microscopy (Utermöhl technique).*

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 a single taxon including the cell wall (if existing) exclusive of lorica and/or mucilaginous envelopes and cell surface structures such as spines, bristles and scales

3.3
cell biovolume
total volume of a single cell including the cell wall (if existing) exclusive of lorica and/or mucilaginous envelopes and cell surface structures such as spines, bristles and scales

3.4
plasma biovolume
volume of cytoplasm of a single cell (total cell biovolume minus vacuole volume)

4 Principle

For every phytoplankton taxon, a preferably simple (i.e. with as few dimensions as possible) and best fitting geometrical shape is assigned (for the catalogue of geometrical shapes see Annex A). If it is not possible to describe the actual shape with a simple basic geometrical shape, then composite shapes (e.g. cone with half sphere) or fractional shapes (e.g. half sphere) are used. In most cases, the assignment of a geometrical shape should be based on a single cell, but for some colony-forming species where individual cells are hardly to be distinguished or have very complex contours it can be expedient to assign a geometrical shape based on the shape of the whole colony.

Taxon lists describing the preferred geometrical shapes have been published before (see e.g. [1], [3], [5]), based on specific taxonomical levels or for particular areas. This guidance document will provide harmonised geometrical shapes for microalgae spread in European marine, brackish, and freshwater systems. Annex C contains an alphabetical list for genera, species, subspecies, forms, and varieties with the assigned geometrical shapes.

The dimensions needed for the biovolume determination are analysed in the sedimentation chamber by measuring the cells or counting units of the relevant phytoplankton taxa, using inverted microscopy and an eyepiece micrometre or image analysis software. The results are reported in micrometres (µm). The biovolume per taxon and sample is calculated by multiplying the average cell volume of the taxon by the number of individuals (cells/ml or cells/l) or counting units (e.g. number of 100 µm filament pieces/l).

For taxa with a high variability in cell size (e.g. several diatoms, different stages in life cycle) it is advised to determine reasonable size classes first and then assign the individuals to both the taxon and the size class. The appropriate number of size classes depends on the size variation of each taxon. A mean cell volume for each size class is determined. In routine programmes, standard size classes should be used (e.g. [5]). If regional lists of size classes were available they should be used instead [5].

Different taxa are quite variable in appearance. Thus, few species sometimes can be rather spherical (sphere), in the other cases rather elliptical (prolate spheroid). Moreover, different stages within the life cycle (e.g. cysts) may have various forms. In addition, amoeboid taxa are existing. In the list of Annex C, the assignments of the geometry have been made according to the common vegetative form. If a corresponding taxon in the sample to be analysed shows a different shape deviating from the list, an appropriate geometrical shape will have to be selected according to Annex A.

5 Procedure

5.1 Determination of required dimensions

The required dimensions (e.g. diameter, height, length, width etc.) of the relevant geometrical shape shall be measured for the taxon of interest. At least 20 individuals per taxon should be measured to ensure that the standard error will be generally < 10 %.

Depending on the cell size variation, the number of measured cells/counting units should be increased up to 50 individuals ([3], [7]).

If the variability of a taxon is negligibly small, the number of cells measured may be reduced to 5 to 10 cells. In this case, statistical analysis should verify that the standard error is < 10 %. For that purpose, the standard deviation can be calculated according to Equation (1):

$$s = \sqrt{\frac{\sum (x - \bar{x})^2}{n - 1}} \qquad (1)$$

where

- s is the corrected sample standard deviation;
- x is the sample value;
- $\bar{x}$ is the mean values of all samples;
- n is the number of the respective counted samples.

From the standard deviation s, the standard error of the mean, $\sigma_{\bar{x}}$, can be calculated using Equation (2):

$$\sigma_{\bar{x}} = \frac{s}{\sqrt{n}} \qquad (2)$$

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 Equation (3):

$$\sigma_{rel} = \frac{\sigma_{\bar{x}}}{\bar{x}} \cdot 100 \quad (3)$$

From the results, the 95 % confidence limits can be determined using Equation (4) and (5):

$$\bar{x}_u = \bar{x} + (\sigma_{\bar{x}} \cdot 1{,}96) \quad (4)$$

$$\bar{x}_l = \bar{x} - (\sigma_{\bar{x}} \cdot 1{,}96) \quad (5)$$

where

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

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

Since the required number of individuals to be measured is highly dependent on the variability of the size for a certain taxon, it is recommended to calculate the biovolume after each measurement, in order to allow continuous statistical analysis and check the precision. The confidence limits should be set to 95 % as a measure for precision. By this, only as many cells as necessary for achieving that limit must be counted and thus, the amount of laboratory work will be minimised.

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

The required dimensions for determination of the biovolume shall be measured using an eyepiece micrometre or image analysis software. Prior to measurement, both systems shall be calibrated with a standardised object micrometre for every microscope and all objectives and oculars used.

Measuring a high number of cells for every taxon in every sample is a time consuming procedure. For routine monitoring programmes mean cell volumes, used for a particular project and area and calculated from own measurements, may be used. These mean cell volumes shall be checked regularly by measuring actual cell dimensions (see 6.2) and calculating actual biovolumes (see 6.3). For taxa having a high variability in cell sizes and representing more than 50 % of total biovolume, these checks are compulsory.

If taxa show high variability of sizes within size classes, determine and use mean cell volumes per size class. If the cells are counted directly into size classes, all individuals shall be measured while counted. In this case a separate measurement of dimensions is not necessary.

5.2 Calibration of the eyepiece micrometre

The scale of commercially available standardised object micrometres has a length of 1 mm and is divided into 100 equal parts. The distance between the graduation lines is 10 µm. By putting the scale of the eyepiece micrometre over the scale of the object micrometre, the scale value (S) = calibration factor of the eyepiece micrometre can be determined for each objective magnification as follows:

$$S = \frac{n_{obj} \cdot 10\mu m}{n_{eye}} \quad (6)$$

Where

S is the scale value (calibration 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

The calibration 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 standardised object micrometre for every objective.

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

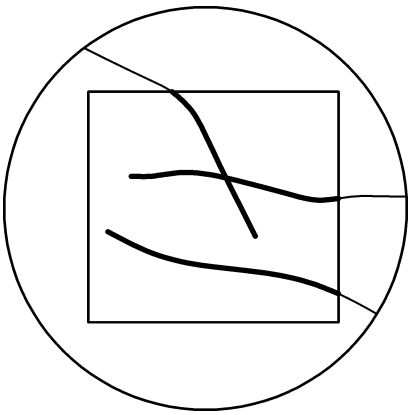
5.3 Measurement

Measurement of the cells/counting units can be carried out parallel to the counting process or in a separate step. 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 a 100 and a 1 000 times, in order to obtain corresponding precision.

By rotating the ocular with the eyepiece micrometre and moving the sedimentation chamber with the microscope stage, the scale of the eyepiece micrometre is put over the required dimension of the cell/counting unit to be measured. The number of covered graduation lines is read and with the help of the calibration factor (see 6.1) the length of the measured dimension is calculated by multiplication. 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 image analysis software is used, the corresponding details of the operating manual shall be followed.

For some particular taxa, it is often not possible to measure all dimensions needed for calculation of the cell volume (“hidden dimensions“, e.g. height of prismatic cells of different shapes or small diameter of elliptical cells). Then it is necessary to estimate the length of missing dimensions as a proportion of the visible dimensions. The size of the relation to be assessed can be obtained from measurements of other samples if the position of the cells allows this. Alternatively, corresponding nominal values shall be taken from the literature.

With some filamentous taxa (e.g. cyanobacteria), it is often difficult to distinguish between individual cells, especially when the cells are directly interconnected without any gaps. In such cases filament pieces of a fixed length, e.g. 100 µm, shall be counted and measured (100 µm length and diameter). An alternative and more precise method 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 finishing the transect. Afterwards, the diameter of at least 20 filaments shall be measured and the median filament diameter is calculated. To obtain the biovolume of the respective taxon, the sum of total filament lengths shall be multiplied by the median of filament diameter.



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

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

There are also colony-forming species existing, in which single cells can be distinguished. However, due to uneven and complex geometries these are often difficult to be classified and measured. In such cases, it may be easier to classify and measure the entire colony with its geometry. In Annex B, this is specified for the respective taxa.

Some taxa show very complex cell outlines requiring a composition of multiple geometrical shapes and thus, the application of complicated equations for the 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 efforts for the determination. However, depending on the required precision, more complex and thus more precise geometric subdivisions shall be applied to those taxa. Another possibility is the resorting to already determined cell volumes from literature (mean standard biovolume), bearing in mind that the size of the cells depends on a number of environmental factors and thus, may vary widely. In any case, everything shall be documented in the protocol.

5.4 Calculation of biovolume

The cell 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.). 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, but the apical ends of the cell are rounded. For such cases, the cell volume shall be multiplied by a correction factor, which is given in Annex C for relevant taxa.

The average cell volumes of the various taxa shall be calculated as the median of all individual cell volumes. The median is used, because it is more robust to extreme values compared to the arithmetic mean. These calculations shall be carried out for every phytoplankton taxon identified.

The biovolume per taxon is calculated by multiplying the number of cells/l (or cells/ml) or counting units (e.g. number of 100 µm filament pieces/l) with the median of the determined taxon-specific cell volumes (µm^3):

$$V_{\text{bio},i} = \frac{n_i \cdot \tilde{V}_i}{10^9}$$

where

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

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

$\tilde{V}_i$ is the median of the cell volumes of taxon i in cubic micrometres (µm^3);

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

The specific cell volumes are expressed in cubic micrometres (µm^3) without decimal places or for picoplankton with two significant places. The biovolume of a single taxon and the complete sample is given in cubic millimetres per litre (mm^3/l) with three significant places.

NOTE 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 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 correction factors has not the highest priority. In other cases where it might be mandatory to determine the exact biomass, correction factors should be derived from comparisons between fixated and living organisms. This has to be noted down in the protocol.

5.5 Biovolume biomass relations

Assuming a density of organisms being equal to water (1,0 g/cm^3, [4]), the biomass (wet weight) may be estimated as follows:

1 mm^3/l = 1 cm^3/m^3 = 1 mg/l;

1 mm^3/m^3 = 10^6 µm^3/l = 1 µg/l.

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

6 Quality Assurance

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

Annex A
(informative)

List of geometrical shapes

Table A.1 — Geometrical shapes (1 of 15)

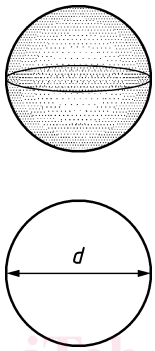
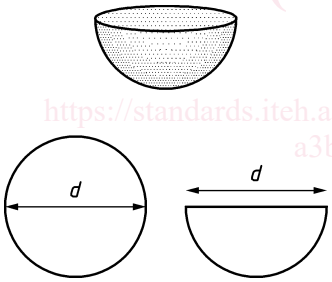
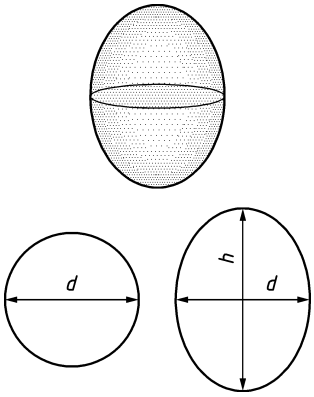
ID	Geometrical shape *(Synonyms)*	Information	Volume equation
1	**Sphere**  	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: diameter (d)*	$V = \frac{1}{6} \cdot \pi \cdot d^3$ For radius $r = \frac{d}{2}$: $V = \frac{4}{3} \cdot \pi \cdot r^3$
2	**Half sphere** *hemisphere*  	*fraction shape* *basic shape: sphere* *Volume calculation precision: exact* **Dimensions:** *A: diameter (d)*	$V = \frac{1}{12} \cdot \pi \cdot d^3$ For radius $r = \frac{d}{2}$: $V = \frac{1}{2} \cdot \frac{4}{3} \cdot \pi \cdot r^3$
3	**Prolate spheroid** *rotational ellipsoid* *ellipsoid of revolution*  	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: diameter (d)* *B: height (h)*	$V = \frac{1}{6} \pi \cdot d^2 \cdot h$ For radius $r = \frac{d}{2}$: $V = \frac{4}{3} \cdot \pi \cdot r^2 \cdot \frac{1}{2} \cdot h$

Table A.1 (2 of 15)

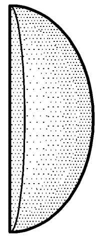
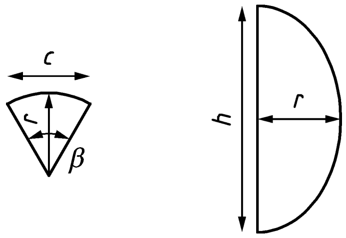
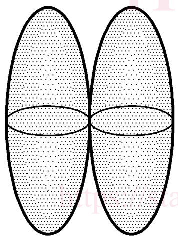
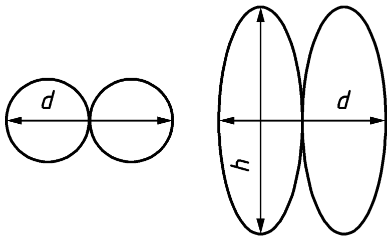
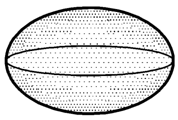
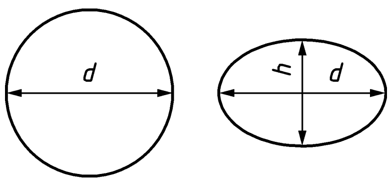
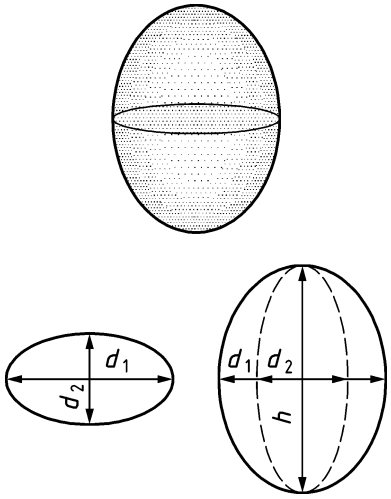
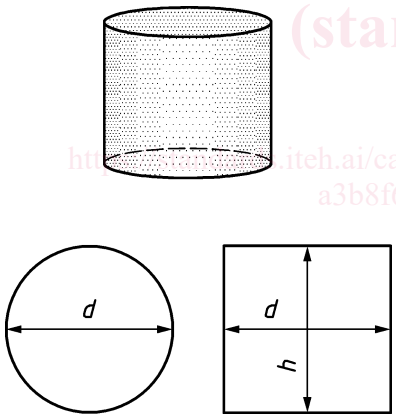
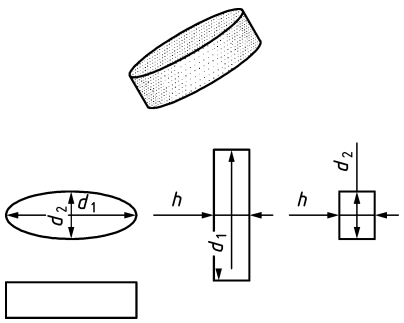
ID	**Geometrical shape** *(Synonyms)*	**Information**	**Volume equation**
4	**Cymbelloid** *Cymbella-shape* *segment of prolate spheroid*  	*fraction shape* *basic shape: prolate spheroid* *Volume calculation precision: exact* **Dimensions:** *A: height (h)* *B: radius (r)* *C: width of "wedge" (c)* *D: angle at "wedge" end (β)*	$V = \frac{2}{3} \cdot \pi \cdot r^2 \cdot h \cdot \frac{\beta}{360}; \sin\frac{\beta}{2} = \frac{c}{2 \cdot r}$ $V = \frac{2}{3} \cdot \pi \cdot r^2 \cdot \frac{1}{180} \cdot \arcsin\left(\frac{c}{2 \cdot r}\right)$
5	**Double prolate spheroid**  	*composed shape* *basic shape: prolate spheroid* *Volume calculation precision: exact* **Dimensions:** *A: total diameter (d)* *B: height (h)*	$V = \frac{1}{12} \cdot \pi \cdot d^2 \cdot h$ For radius $r = \frac{d}{2}$: $V = 2 \cdot \frac{4}{3} \cdot \pi \cdot r^2 \cdot \frac{1}{2} \cdot h$
6	**Oblate spheroid** *rotational ellipsoid* *ellipsoid of revolution*  	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: diameter (d)* *B: height (h)*	$V = \frac{1}{6} \cdot \pi \cdot d^2 \cdot h$ $V = \frac{4}{3} \cdot \pi \cdot r^2 \cdot \frac{1}{2} \cdot h$

Table A.1 (3 of 15)

ID	Geometrical shape *(Synonyms)*	Information	Volume equation
7	**Ellipsoid** *tri-axial ellipsoid,* *flattened ellipsoid* 	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: large diameter (d_1)* *B: small diameter (d_2)* *C: height (h)*	$V = \frac{1}{6} \cdot \pi \cdot d_1 \cdot d_2 \cdot h$ For radius r: $V = \frac{4}{3} \cdot \pi \cdot r_1 \cdot r_2 \cdot r_3$ with $r_1 = \frac{d_1}{2}$; $r_2 = \frac{d_2}{2}$; $r_3 = \frac{h}{2}$
8	**Cylinder** *circle based cylinder* 	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: diameter (d)* *B: height (h)*	$V = \frac{1}{4} \cdot \pi \cdot d^2 \cdot h$ For radius $r = \frac{d}{2}$: $V = \pi \cdot r^2 \cdot h$
9	**Elliptic cylinder** *prism on elliptic base* *oval cylinder* 	*basic shape* *Volume calculation precision: exact* **Dimensions:** *A: large diameter (d_1)* *B: small diameter (d_2)* *C: height of cylinder (h)*	$V = \frac{1}{4} \cdot \pi \cdot d_1 \cdot d_2 \cdot h$ For radius r: $V = \pi \cdot r_1 \cdot r_2 \cdot h$ with $r_1 = \frac{d_1}{2}$; $r_2 = \frac{d_2}{2}$