Biology of Waters and Associated Materials

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Enumeration and Identification of Algae

2024

The Enumeration and Identification of Algae

This booklet contains three methods for the enumeration and identification of algae in surface, ground, and drinking water. It also includes information on the verification of these methods along with a method for estimating phytoplankton biovolume.

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Within the series of SCA publications, there are separate booklets, each dealing with different topics concerning biology methods for drinking water, including:

The Microbiology of Water and Associated Materials

- Practices and Procedures for Laboratories

The Microbiology of Sewage Sludge

- Part 1 – An overview of the treatment and use in agriculture or sewage sludge in relation to its impact on the environment and public health
- Part 2 – Practices and procedures for sampling and sample preparation

Contents

About this series

Introduction

This booklet is part of a series intended to provide authoritative guidance on recommended methods of sampling and analysis for determining the quality of drinking water, ground water, river water and sea water, wastewater, and effluents as well as sewage sludges and biota.

In addition, short reviews of the most important analytical techniques of interest to the water and sewage industries are included.

Performance of methods

Ideally, all methods should be fully evaluated with results from performance tests. These methods should be capable of establishing, within specified or pre-determined and acceptable limits of deviation and detection, whether or not any sample contains concentrations of parameters above those of interest.

For a method to be considered fully evaluated, individual results from at least three laboratories should be reported. The specifications of performance generally relate to maximum tolerable values for total error (random and systematic errors) systematic error (bias) total standard deviation and limit of detection - often, full evaluation is not possible and only limited performance data may be available.

In addition, good laboratory practice and analytical quality control are essential if satisfactory results are to be achieved.

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The preparation of booklets and their continuing revision is the responsibility of the Standing Committee of Analysts (SCA) - Established 1972 by the Department of the Environment.

At present, there are several working groups, each responsible for one section or aspect of water quality analysis:

1. General principles of sampling and accuracy of results
2. Microbiological methods
3. Inorganic and physical methods, metals, and metalloids
4. Organic methods
5. Biological, biodegradability and inhibition methods
6. Radiochemistry methods

The actual methods and reviews are produced by smaller panels of experts in the appropriate field, in co-operation with the working group and main committee. The names of those members principally associated with these methods are listed at the back of this booklet.

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Users should ensure they are aware of the most recent version they seek.

Warning to users

The analytical procedures described in this booklet should only be carried out under the proper supervision of competent, trained analysts in properly equipped laboratories.

All possible safety precautions should be followed, and appropriate regulatory requirements complied with. This should include compliance with the Health and Safety at Work etc. Act 1974 and all regulations made under the Act, and the Control of Substances Hazardous to Health Regulations 2002 (SI 2002/2677). Where particular or exceptional hazards exist in carrying out the procedures described in this booklet, then specific attention is noted.

Numerous publications are available giving practical details on first aid and laboratory safety.

These should be consulted and be readily accessible to all analysts. Amongst such resources are:

HSE: Information about health and safety at work

RSC: Laboratory best practices

The Approved List of Biological Agents. (2023) Advisory Committee on Dangerous Pathogens of the Health and Safety Executive (HSE)

Enumeration and Identification of Algae

1 Introduction

Algae are natural constituents of nearly all surface waters, both flowing and still. As such they play a vital role and may be responsible for a major part of the primary production in an aquatic ecosystem. When nutrients and light are plentiful, blooms may occur which can give rise to water quality and drinking water treatment problems because of colouration of the water, imparted taste, and odour issues, and clogging of treatment filters. Some species produce toxins, which have been associated with fish and other wildlife mortalities e.g. Microcystin. Thus, a knowledge of their abundance, estimated quantity, biomass, and growth potential is of great interest to water quality scientists and engineers.

The purpose of this book is to set down the basic methods for counting and identifying algal cells. The methods are detailed in sections A to C. This book builds on the work completed by previous SCA members in completing and publishing the methods detailed in The Enumeration of Algae, Estimation of Cell Volume, and Use in Bioassays (1990) as part of the Methods for the Examination of Waters and Associated Materials series.

In most field studies of algae there is a requirement to determine cell numbers or biomass. This may be achieved in direct or indirect ways e.g. light microscopy counts, dry weight, organic carbon, cell volume, chlorophyll a content. Among these long-established methods only microscopy allows the user to identify the taxa present. By estimating changes in cell numbers in a succession of samples, understanding is gained of the dynamic aspects of taxa composition in a community. From these numbers it is also possible to estimate by indirect calculations the amount of algal biomass within the sample (Part E).

Novel molecular-based methods are becoming available that can determine algal cell numbers, biomass, or species information. These methods are quick, efficient and do not require the identification expertise of other traditional methods. Molecular-based methods may prove useful for investigative purposes alongside routine algal and water quality analysis once proven.

Sections A to C describe various methods available for estimating algal cell numbers in sufficient detail to allow the user to choose those methods most suited for the populations in their samples. No single method for estimating algal numbers is ideal for all taxa because of the wide range in cell sizes and the great variety of morphology. The chosen method should depend on the purpose and objectives of the algal monitoring, the nature of the samples being analysed and resource available. Each method has an associated error and uncertainty of measurement, so it is important that the user validates each method intended to be used before it is used. Guidance on validation is detailed in Part D.

2 Definitions

Algal object - A unit/cluster of one or more algal or cyanobacteria cells encountered during the phytoplankton analysis that is discrete from other particles in the sample.

Algae free water – Suitably prepared algae free treated water that can be used for reagent preparation and dilutions as part of sample preparation. For example, 0.45 µm membrane filtered deionised water.

Cyanobacteria – Also known as blue/green algae are organisms in the domain bacteria able to carry out oxygenic photosynthesis.

Microscope counting field – Defined area e.g. square in the microscope field of view that is used for enumeration.

Phytoplankton – Community of free-living, suspended, mainly photosynthetic organisms in aquatic systems comprising Cyanobacteriota (Cyanobacteria) and algae.

A Utermöhl Method

A1 Introduction

This method is based on the settling technique as defined by Utermöhl [1], providing an estimation of the abundance and taxonomic composition of phytoplankton by using inverted light microscopy and sedimentation chambers.

Owing to the great variety of size and morphology present within the algae community it is unlikely that one specific concentration method or microscopic magnification is optimal for all taxa, so it is important that the user validates their choice of concentration and enumeration method as detailed in Part D to ensure it is reliable for the material they are attempting to process.

A2 Scope

This method is suitable for all water types from surface water, through all stages of treatment, to the final treated water.

Users intending to employ these methods should verify their performance under their own laboratory conditions over the range of variables encountered in the course of the laboratories routine testing (for example, sample matrices) to identify any adverse impacts on the results achieved. Guidance on appropriate validation of these techniques can be found in Part D.

A3 Principle

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

A4 Limitations

No single method for estimating algal numbers is ideal for all taxa because of the wide range in cell size (<1 to >200 µm) and the great variety of morphology (single cell, colony, filament etc).

Difficulties in settling with certain buoyant taxa such as *Microcystis* or *Anabaena* can be seen when using the Utermöhl method. To overcome this, collapsing of the gas vacuoles can be carried out as detailed in section A8.3.

Algae identification is limited by the skill and knowledge of the analyst performing the method.

A5 Health and Safety

Reagents used in this method are covered by the Control of Substances Hazardous to Health Regulations [2] and appropriate risk assessments should be made before adopting

this method. Standard laboratory microbiology safety procedures should be followed, and guidance is given elsewhere [3] in this series.

When using the reagents required for algae analysis, good chemical handling practice should be adhered to at all times along with using appropriate PPE.

Long periods of microscopic analysis can cause physical fatigue and affect eyesight. Attention should be given to the ergonomics of the microscope and microscopists should take regular breaks, carry out eye exercises and have routine eye checks.

A6 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere [3] in this series. Principally, sedimentation chambers and an inverted microscope with a range of magnifications from 10x - 600x or greater are required.

A6.1 Sedimentation chamber

Sedimentation chambers consist of a vertical column, with a base through which the contents can be observed with an inverted microscope. Sediment chambers are available as a split chamber (unfilled chamber in Figure A1) where the chamber can be removed from the base or a screw in fixed volume (filled chamber in Figure A1) where the chamber is fixed to the base.

Figure A1 – Standard sedimentation chamber.

The thickness of the base plate (a coverslip) should not exceed 0.17 mm as this directly affects image quality. The height of the sedimentation chamber should not be higher than 5 times of the chamber diameter [4].

Sedimentation chambers should be calibrated annually so that the volume of the sub-sample contained can be accurately determined (Appendix 1).

A6.2 Inverted light microscope

For phytoplankton counting, the microscope should be equipped with:

- Binocular, wide-field eyepiece of 10x or 12.5x magnification.
- Range of objective lenses, ideally 10x, 20x, 40x, 60x and 100x, phase and/or DIC objective lenses.
- A mechanical stage.
- One eyepiece should be equipped with an appropriate counting graticule. Typically, a square field or grid e.g. Whipple disc.

NOTE - More information on microscopes is given in Annex A and E of the British Standard BS EN 15204:2006 [4].

A7 Media and reagents

A7.1 Acid Lugol's iodine

To prepare Lugol's iodine solution, all reagents used should be of an appropriate purity and suitable for algal analysis. For best results, preparation of all reagents should be performed according to the manufacturer's instructions.

Commercial formulations of Lugol's iodine are available but may possess minor variations to their formulation. Commercial formulations should be used and stored according to manufacturer's instructions. Variations in the preparation and storage of Lugol's iodine should also be verified.

Acid Lugol's iodine	
Potassium iodine	150 ± 1 g
Iodine	50 ± 1 g
Water (algae free)	1000 ± 20 ml
Glacial acetic acid	20 ± 0.4 ml

Dissolve Potassium iodine and iodine in an appropriate 'algae free' water. Then once dissolved add the glacial acetic acid. The Lugol's iodine should then be store in the dark at room temperature and is stable for six months.

Other preservatives are available and further detailed in British Standard BS EN 15204:2006 [4].

A8 Analytical Procedure

A8.1 Sample Preservation

All samples should be fixed with a suitable preservative. This should be added at the time of sampling or as close to the time of sampling as possible but no later than 24 hr. Longer

times for preservation can be used provided this has been suitably verified under the users laboratory conditions.

It should be noted that certain preservatives can interfere with the identification features of algae, especially the flagella. This is true for the most widely used preservative, Lugol's iodine solution, which otherwise fulfils a multipurpose role:

- Preserving algal features and structures
- Preventing further algae growth
- Killing zooplankton that would consume algae.
- Staining algae and increasing their density which aids sedimentation
- Helps in collapsing gas vacuoles to aid in sedimentation of gas-vacuolate cyanobacteria.

Samples preserved with approximately 1 ml of Lugol's iodine solution per 500 ml of sample should be stored in the dark and refrigerated (5 ± 3 °C), unless the samples are to be analysed within three weeks of being sampled. In this case the samples can be stored in the dark at room temperature.

Samples can be stored refrigerated for up to 12 months when preserved. For long-term storage of preserved samples, it is recommended that they are kept in glass containers. Plastic storage bottles must be checked and topped up with Lugols iodine every 6 to 12 months as most plastics absorb Lugol's iodine.

Figure A2 – Example of samples preserved with Lugol's iodine solution. It is important to ensure quantity of Lugol's iodine added to the sample is correct. Should be straw like in colour (middle sample). Too much iodine (left sample) and this will obscure the feature of the algae that aide in identification. Too little iodine (right sample) and the sample will not be adequately preserved.

A8.2 Acclimatisation

For optimum analysis and ensuring a random distribution of material within the sedimentation chamber, the sample and all equipment should be of a similar temperature. To ensure this a suitable acclimatisation period should be left for the sample and equipment to reach room temperature. This typically takes 18 ± 6 hr but is dependent upon the sample volume and ambient temperature.

A8.3 Collapsing Gas Vacuoles

Gas vacuoles provide buoyancy to certain algae and Cyanobacteria taxa and as such are an impediment to concentration. The sample must be prepared in such a way to collapse the gas vacuoles of any buoyant algae within the sample which can be achieved by creating a sudden change in pressure within the sample. This change in pressure collapses any gas vacuoles in the cells, rendering them amenable to sedimentation.

After ensuring an even distribution of cells by agitation, open the bottle and squeeze to remove as much air as possible from the headspace. Reseal this full-to-brim bottle and drop it from a height of approximately 1 m on to a hard surface up to 3 times. For this pressure shock to be effective the bottle used should comprise a soft walled plastic (e.g. PET1), rather than less flexible high-density polyethylene (HDPE). If samples are taken in a glass bottle, the sample should be transferred to a suitable soft walled plastic bottle to carry out this step.

A8.4 Homogenisation

Resuspension and separation of particles is a key step and can be achieved manually or using an appropriate shaking device.

Shaking devices such as tumbler mixers should be based on a three-dimensional motion to ensure complete mixing.

Manual shaking should be done by mixing the sample using a combination of alternating horizontal rolling and vertical tumbling (turning upside down) of the sample bottle for at least 20 repetitions in a slow manner (over approximately 2 min). These actions should be gentle and not involve any vigorous shaking.

NOTE – Other manual shaking patterns may be suitable, but these must be verified by the user laboratory. The manual shaking method should be described clearly to minimise difference between analysts.

A8.5 Sub-sample Preparation

The exact volume of the sub-sample to be settled depends on the phytoplankton density or Chlorophyl *a* result (if available). For samples where there is a high biomass of phytoplankton a dilution may be necessary. Dilutions can be made by pipetting an appropriate volume into a sedimentation chamber then filling the chamber to the top using suitable algae free water. Caution should be taken to ensure no sample is lost by overfilling the sedimentation chamber.

NOTE – when using pipette tips, it is recommended to cut the orifice to achieve a working diameter of 3 – 4 mm. This ensures large taxa e.g. *Ceratium* spp. are not excluded. However, cutting of the pipette tip impacts the accuracy of the volume pipetted, which should therefore be checked using a suitable balance before carrying out dilution analysis.

Once the sub-sample volume has been chosen, the appropriate sedimentation chamber should be filled either directly from the sample bottle or by pipetting a suitable volume that's then topped up using suitable algae free water.

Place the sedimentation chamber on a horizontal flat surface that is a poor heat conductor such as a thin acrylic plate. Fill the sedimentation chamber in a single attempt ensuring no air spaces at the top of the chamber.

Without trapping air bubbles, close the chamber with a coverslip. If an air bubble is introduced this can be eliminated by topping up with suitable algae free water using a small dropper pipette and then sliding the coverslip back.

The sedimentation should take place at a constant ambient temperature and on a bench that is free of vibrations. Samples should be allowed a settling time of at least 4 hr per cm height [(4)] of the settling chamber (Table A1).

Volume of Chamber (ml)	Height of Chamber (cm)	Settling time (hr)
10	1	4
50	8	32
100	18	72

Table A1 – Examples of settling times for preserved ground, surface, and drinking water samples.

If using a split sediment chamber, after the suitable sedimentation period, discard the unwanted water over a sink by gently sliding the chamber from over the base plate, taking great care to ensure the settled algae are not disturbed. Position a cover slip directly onto the base plate and seal, ideally without introducing air bubbles. If an air bubble is introduced this can be eliminated by topping up with suitable algae free water using a small dropper pipette and then sliding the coverslip back.

Alternatively, sedimentation chambers with a screw in fixed volume tube can be purchased that do not require the removal of unwanted water. Typical volumes are 2.5 ml, 5 ml, 10 ml, and 25 ml. These chambers are filled as above, and a glass cover placed on top in a way as to not create air bubbles. The chambers are then settled according to Table A1 and can be read without the need to remove excess liquid.

The sample can be examined using microscopy as detailed in section A8.6.

NOTE – Sample may be transferred to a counting chamber for enumeration. If this, is the case, section B details the use of Sedgwick Rafter/Haemocytometers.

A8.6 Microscopy

Microscopic quantitative analysis involves recording the taxa observed and the number of algal objects or cells for each taxon, in a known area of the counting chamber. If the area of the counting chamber and the volume settled is known, then the concentration of each individual taxon can be calculated.

Prior to commencing identification and enumeration, the overall distribution pattern should be judged. This can be done on a low magnification using a 4x or 10x objective. A random distribution of particles will display as an irregular pattern with open spaces (Figure A3).

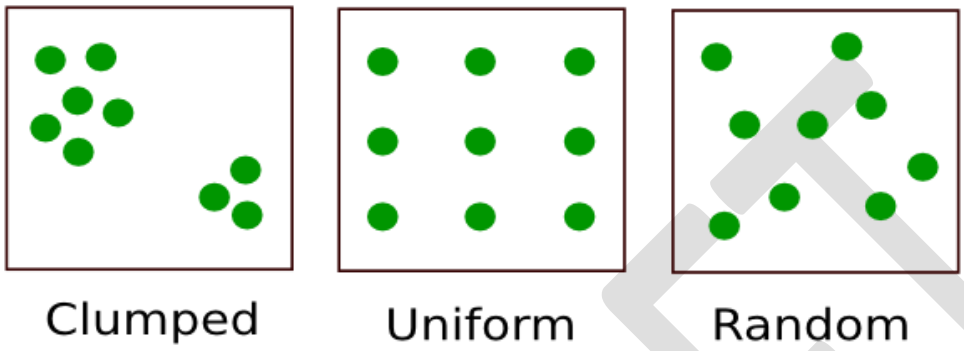

Figure A3 – Type of distribution of particles seen under microscope.

NOTE – A pattern where particles of the same size are near the chamber wall and particles of the same size clumped together, in the middle of the chamber typically indicate a temperature difference between the sedimentation chamber and the sub-sample.

If the counting chamber is considered too overcrowded and difficult to count, the sample should be re-prepared using a diluted sample or a smaller settling volume.

The choice of enumeration methodology depends on the information needed from the analysis and the typical methodologies for enumeration include counting a number of randomly selected fields, counting transects or counting the whole settlement area. Ideally all three methods would be used in combination to ensure effective counting of algal taxa, but this may not always be practical. The chosen enumeration methodology should depend on the purpose and objectives of the algal monitoring, the nature of the samples being analysed and resource available.

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

A8.6.1 Identification

Identification should be carried out to the taxonomic level required for the intended use of the method, typically genus level. It is important to note that it is better to correctly identify algae to a lower taxonomic level than misidentify algae to a higher taxonomic level.

Identification can be somewhat controlled by using reference guides, keys or in-house guides of the typical algae seen in routine samples. See Appendix 2 for further guidance. Photographing or drawing algae may be useful for training purposes or developing in-house training materials.

It is recommended to regularly perform inter and intra-laboratory comparison tests to minimise and highlight identification challenges or differences between analysts.

A8.6.2 Counting the Whole Settlement Area

This count should be carried out at a low magnification, 4x to 10x objective. This counting technique is suited to larger taxa e.g. colonial or filamentous cyanobacteria.

For counting the whole chamber, an eyepiece graticule consisting of two parallel lines (sometimes referred to as an H eyepiece) is used to define the transect. Counting the whole chamber is done by traversing back and forwards across the chamber from top to bottom or vice versa (Figure A4). The analyst should work through the whole chamber systematically, recording the taxa observed and the number of algal objects or cells for each taxon.

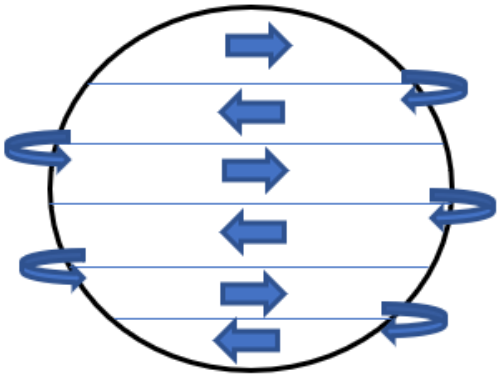

Figure A4. Example counting method for whole chamber.

NOTE – Counting the whole chamber as described above is suitable for larger taxa e.g. *Microcystis* colony and *Ceratium* spp. but is not suitable for counting smaller taxa.

A8.6.3 Counting Transects

This count should be carried out at an intermediate magnification such as 200x. This counting technique is suited to medium taxa e.g. diatoms and greens.

For counting the transects, an eyepiece graticule consisting of two parallel lines is used to define the transect. Counting transects is done by choosing randomly selecting diameter transects (Figure A3) within the counting chamber with the analyst recording the taxa observed and the number of algal objects or cells for each taxon within the transect.

The number of randomly selected transects can be set according to the level of precision or detection needed, A standard accepted number of transects to count is between 3 and 5.

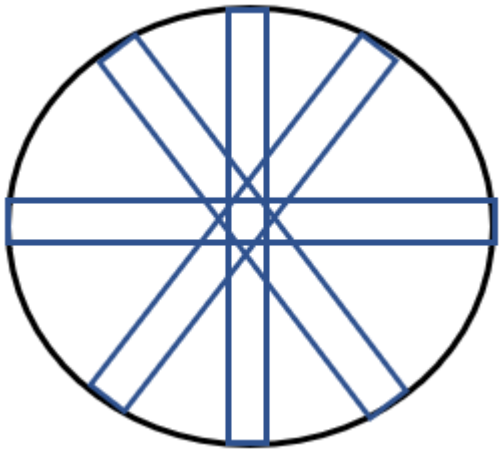

Figure A5. Example of diameter transects that can be counted.

A8.6.4 Randomly Select Fields

This count should be carried out at a high magnification, 400x or greater. This counting technique is suited to small cells e.g. *Rhodomonas* spp.

For randomly selected fields, a square or grid eyepiece graticule is used to define the counting field and a tally is kept of the number of fields counted. Fields should be selected either by the analyst in a pseudo-random fashion or using an electronic stage with built-in random-position controller.

When using a microscope counting field, it is important to ensure a consistent approach when an algal object is crossing the counting field boundaries. A simple rule shall be established by the laboratory to ensure consistent counting results between analysts. For example, algal cells on the south and east that overlap the field of view boundary are included and cells that overlap the south and west boundaries are excluded. This is illustrated in Figure A6.

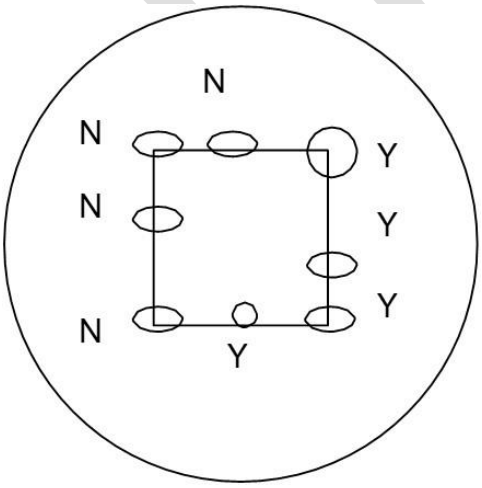

Figure A6. Example of rule to apply for algae crossing the counting field boundaries. Y – counted. N – not counted.

A predefined number of randomly selected fields should be worked through systematically with the analyst recording the taxa observed and the number of algal objects for each taxon, in a known area of the counting chamber.

The number of randomly selected fields to count can be set according to the level of precision or detection needed, as the precision/detection limit is dependent on the number of algal objects/fields. A standard accepted number of fields to count is between 10 and 20 fields of view. This is based on historical water industry laboratory data demonstrating that this provides a representative count of the algae within a typical surface water sample.

NOTE – Counting randomly selected fields is claimed in some scientific studies [4] to provide more accurate data and to be less time consuming.

A9 Calculations

To calculate the algal concentration per ml, first a correction factor should be applied to extrapolate the results to account for the proportion of the counting chamber that is analysed. This correction factor is based on the area screened, the area of the counting chamber and the proportion of the area screened. An example of how this can be calculated is detailed below:

As the area and volume of the whole chamber is known, then the concentration of each individual taxon can be calculated:

$$TC = C \times \left(\left(\frac{A}{G_A} \right) \times n \right)$$

Where:

TC – Total Cells.
C – Cells counted in total fields of view or transects.
A = Area Screened (mm^2) i.e., Area of eyepiece graticule (one field of view).
G_A= Area of Zooplankton chamber (mm^2).
n = number of fields viewed.

Cells per ml is calculated by dividing by the adjusted total cell count (TC) by the volume of sub-sample used:

$$Cells\ per\ ml = \frac{TC}{Subsample\ volume}$$

A10 Expression of results

Counts are expressed in cells per ml.

Algae are typically grouped into Diatoms, Green, Blue/Green (cyanobacteria) and other categories.

A11 Quality assurance

Routine calibration of sedimentation chambers should be carried out so that the volume of the sub-sample can be accurately determined.

Quality control checks for consistency in the analysis method can be done by using duplicate split samples. Split samples typically comprise a sample divided into two sub-samples, each of which is analysed with each batch of routine samples. A duplicate sample could also comprise the same sample enumerated by two different analysts. The duplicate sub-samples can be considered as two halves of a single sample, and the results can be plotted on a chart containing appropriate response limits. More information on duplicate split analysis is given in The Microbiology of Water and Associated Materials (2017) Practices and Procedures for Laboratories.

A blank sample should be carried out routinely to demonstrate effective cleaning of the Utermöhl chambers.

It is recommended to carry out regular inter and intra-laboratory proficiency assessment. This can be done via numerous methods where analysts analyse the same field of views, either using a natural sample or colour photographs. This allows different analysts to carry out standard analysis on the same sample, one after each other and highlights any discrepancies in identification.

A12 References

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6. Deliverable D3.1-4: Guidance document on sampling, analysis and counting standards for phytoplankton in lakes (2011) Ute Mischke, Stephen Thackeray, Michael Dunbar, Claire McDonald, Laurence Carvalho, Caridad de Hoyos, Marko Jarvinen, Christophe Laplace-Treyture, Giuseppe Morabito, Birger Skjelbred, Anne

Lyche Solheim, Bill Brierley and Bernard Dudley.

B Sedgwick Rafter/Haemocytometer method

B1 Introduction

Alternative enumeration methods provide a relatively quick and simple way of enumerating algae if not using a sedimentation chamber with a counting base as detailed in the Utermöhl method.

Algae concentration by centrifugation provides a relatively quick and convenient direct method of preparation for enumeration. The alternative sedimentation method of Utermöhl was developed in response to perceived problems of accuracy resulting from vortex creation in centrifugation tubes, however work following this has shown that such issues can be addressed by careful consideration of centrifugation brake speed, tube shape and pipette flow [(1)]. Further to this, a centrifugation technique can utilise counting chambers thin enough that they do not limit the user to an inverted microscope.

Owing to the great variety of size and morphology present within the algae community it is unlikely that one specific concentration method or microscopic magnification is optimal for all taxa, so it is important that the user validates their choice of concentration and enumeration method as detailed in Part D to ensure it is reliable for the material they are attempting to process.

B2 Scope

This method is suitable for all water types from surface water, through all stages of treatment, to the final treated water.

Users intending to employ these methods should verify their performance under their own laboratory conditions over the range of variables encountered in the course of the laboratories routine testing (for example, sample matrices) to identify any adverse impacts on the results achieved. Guidance on appropriate validation of these techniques can be found in Part D.

B3 Principle

Preserved algae are concentrated and then transferred to a dedicated counting chamber, such as a haemocytometer or Sedgwick-Rafter chamber.

B4 Limitations

Centrifugation rapidly applies a greater force than that used in gravity-based sedimentation, and as such is more likely to damage cells. This has the potential to remove or distort some algae identification features.

Algae identification is limited by the skill and knowledge of the analyst performing the method.

B5 Health and Safety

Reagents used in this method are covered by the Control of Substances Hazardous to Health Regulations and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed, and guidance is given elsewhere in this series.

When using the reagents required for algae analysis, good chemical handling practice should be adhered to at all times along with using appropriate PPE.

Long periods of microscopic analysis can cause physical fatigue and affect eyesight. Attention should be given to the ergonomics of the microscope and microscopists should take regular breaks, carry out eye exercises and have routine eye checks.

B6 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere in this series. Principally, a light microscope and suitable counting chambers are required.

B6.1 Light Microscope

The exact specifications for the light microscope are determined by the prospective taxonomic level the user intends to identify algae to. For most genus level identification, the following specifications are suitable:

10x binocular eyepieces.
10x, 20x, 40x, 60x long working distance objectives.
Capable or either DIC or phase contrast techniques.
A Whipple square grid graticule incorporated into one of the eyepieces.

B6.2 Centrifuge

A centrifuge capable of 2500 rpm (revolutions per minute), with the option to switch off automatic breaking.

B6.3 Measuring Cylinders

Stoppered measuring cylinder at suitable volume for settling samples.

B6.4 Counting chambers

This method details the use of a Sedgewick-Rafter counting chamber (as described in Whipple et al, 1927 [2]), modern versions of which are manufactured with an inbuilt grid pattern to aid enumeration. Each Sedgewick-Rafter counting chamber has a well 50 mm long, 20 mm wide and 1 mm deep, providing an area incorporating 1 ml of liquid, with the squares of the grid thus corresponding to 1 µm of liquid each.

This counting chamber can be substituted for any suitable alternative that enables cells to be counted within a known volume of liquid, such as Fuchs-Rosenthal haemocytometers or Helber chambers.

Counting chamber volumes can be checked/calibrated through weight checks as per guidance Appendix 1.

B7 Media and reagents

Acid Lugol's iodine as detailed in section A8.1

B8 Analytical Procedure

B8.1 Preservation

Refer to section A8.1.

B8.2 Collapsing Gas Vacuoles

Refer to section A8.2.

B8.2 Subsample Preparation

B8.2.1 Centrifugation

Samples volumes of 10-20 ml should be centrifuged for a minimum of 15 minutes utilising at least 1500 rpm [(1)], with larger volumes potentially requiring longer or faster cycles. If the centrifuge has a brake, it should be switched off before starting the process. After centrifugation, the top 90% of liquid should be removed using a gentle siphoning action, which can be achieved from a pipette attached to a vacuum trap, connected to a pump. The remaining concentrate can then be mixed by repeated pipetting motion before transfer to a counting chamber.

Rapid slowing of a centrifuge post spin cycle, and overly fast or unsteady aspiration techniques can both cause errors in this method, so it is essential to avoid the use of quick breaks on centrifuge settings and recommended that precautions such as upturned siphoning tips and steady siphoning flows are utilised to avoid disturbing pelleted cells.

B8.2.2 Measuring Cylinder

The exact volume of the sub-sample to be settled depends on the phytoplankton density or Chlorophyl a result (if available). Once the sub-sample volume has been chosen, the appropriate measuring cylinder chamber should be filled either directly from the well mixed sample or by pipetting a suitable volume into a measuring cylinder and the remaining volume topped up using suitable algae free water.

Place the measuring cylinder on a horizontal flat surface that is a poor heat conductor such as a thin acrylic plate. Allow the sample to settle for a suitable time period (refer to section A8.5).

After suitable settlement time, siphon off the supernatant, taking care not to disturb the concentrate (10 ml if using 100 ml measuring cylinder). Swirl or vortex the concentrate to

randomise the phytoplankton and a suitable volume - typically 1 ml can be used for section B8.3.

B8.3 Counting chamber preparation

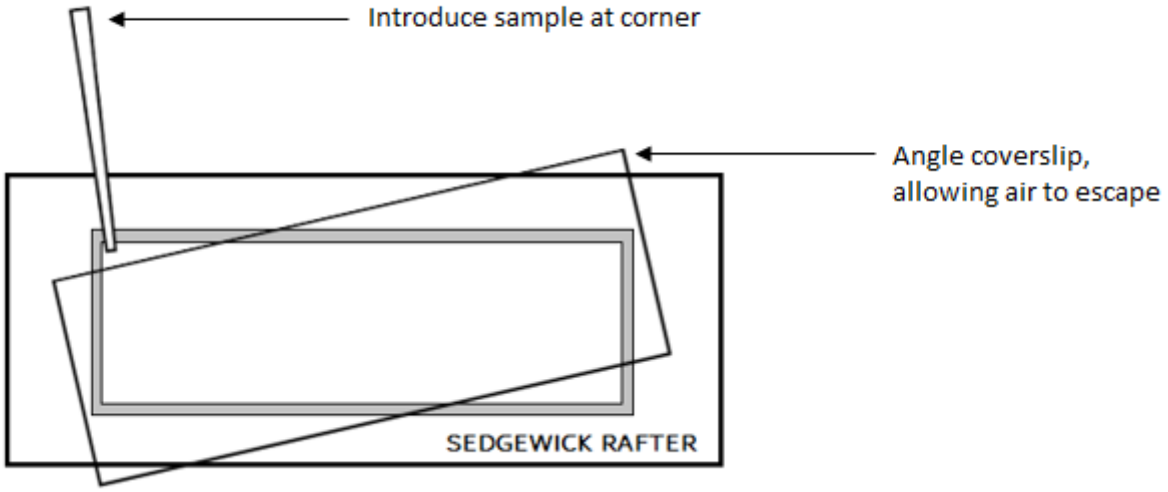

Figure B1. Counting Chamber Preparation

Place a coverslip across the Sedgewick-Rafter chamber at an angle so that two opposing corners of the well are both uncovered (see figure B1). After thoroughly mixing, transfer the concentrated sample into the well through one of the gaps left by the jaunted coverslip. Air should escape from the opposing gap, allowing a slide to be prepared without bubbles. Once the well is full, rotate the coverslip to wholly cover it. A coverslip that floats and moves too freely is an indication that the well has been overfilled; it is important that the coverslip is held onto the cell walls by surface tension as overfilling invalidates attempts at volume-based calculations of cell density.

Leave the counting chamber to stand for at least 15 min once prepared to allow the algae to settle on the slide.

B8.4 Microscopy

The chamber is scanned under a low power objective, aiming to observe a random distribution of algae on the slide before counting. With this satisfied a microscopist can count the algae present within a set number of counting chamber squares, representing a known volume of liquid.

B8.5 Identification

Identification should be carried out to the taxonomic level required for the intended use of the method, typically genus level. It is important to note that it is better to correctly identify algae to a higher taxonomic level than misidentify algae at a lower taxonomic level.

Identification can be somewhat controlled by using reference guides, keys or in-house guides on the typical algae seen in routine samples.

It is recommended to regularly perform inter and intra-laboratory comparison tests to minimise and highlight identification challenges or differences between analysts.

B8.6 Enumeration.

Count the cells of algae belonging to each taxon in a set area of the counting chamber. The size of this area should vary depending on the cell density and counting chamber chosen; for a Sedgwick Rafter chamber twenty of its ruled squares would normally suffice while fewer could be screened for expediency if the cell density ensures over 100 objects are still counted in the reduced area. Avoid utilising the gridded squares at the edges of the well as these are prone to 'edge effects' since they tend to not wash as easily as the main area of the slide. Some members of buoyant taxa like *Woronichia* may not all settle to the bottom of the chamber, but rather float to the base of the coverslip regardless of steps taken in B8.2. Take care to adjust focus while screening to incorporate this area in any count.

If the Sedgwick Rafter chamber is considered too overcrowded and difficult to count, the sample should be re-prepared using a diluted sample.

B9 Calculations

$$T_c = C_r \times \left(\frac{1000}{x}\right) \times \left(\frac{V_1}{V_0}\right)$$

Where:

T_c = Cells per ml
C_r = Cells counted
x = Number of counting chamber squares screened
V_1 = Volume of liquid retained after subsample preparation e.g. centrifugation
V_0 = Volume of liquid prepared e.g. centrifuged or settled.

B10 Expression of results

Counts are expressed in cells per ml.

Algae are typically grouped into Diatoms, Green, Blue/Green (cyanobacteria) and other categories.

B11 Quality assurance

Quality control checks for consistency in the analysis method can be done by using duplicate split samples. Split samples typically comprise a sample divided into two sub-samples, each of which is analysed with each batch of routine samples. A duplicate sample could also comprise the same sample enumerated by two different analysts. The duplicate sub-samples can be considered as two halves of a single sample, and the results can be plotted on a chart containing appropriate response limits. More information on duplicate split analysis is given in The Microbiology of Water and Associated Materials Practices and Procedures for Laboratories [3].

Blank samples should be carried out routinely to demonstrate effective cleaning of the counting chambers and centrifuge tubes.

It is recommended to carry out regular inter and intra-laboratory assessment. This can be done via numerous methods where analysts analyse the same field of views, either using a natural sample or colour photographs. This allows different analysts to carry out standard analysis on the same sample, one after each other and highlights any discrepancies in identification.

B12 References

1. Ballantine, D., 1953. Comparison of different methods of estimating nanoplankton. J. Mar. Biol. Ass. UK., 32, 129—147.

2. Whipple, G C., Fair, M. and Whipple, M C., The microscopy of drinking water. 4th Edn. 1927. Chapman and Hall, London.

3. Standing Committee of Analysts, The Microbiology of Water and Associated Materials (2017). Water Quality and Public Health. Practices and Procedures for Laboratories, in this series, Environment Agency.

C Filtration Method

C1 Introduction

This method is based on the filtration application as previously described by Millipore Filter Corporation [1]. It provides an estimation of abundance and taxonomic composition of phytoplankton by using microscopical examination of a concentrated preparation derived from filtration of a known volume of sample.

Owing to the great variety of size and morphology present within the algae community it is unlikely that one specific concentration method or microscopic magnification is optimal for all taxa, so it is important that the user validates their choice of concentration and enumeration method as detailed in Part D to ensure it is reliable for the material they are attempting to process.

C2 Scope

This method is suitable for all water types from surface water, through all stages of drinking water treatment, to the final treated water.

Users intending to employ these methods should verify their performance under their own laboratory conditions over the range of variables encountered in the course of the laboratories routine testing (for example, sample matrices) to identify any adverse impacts on the results achieved. Guidance on appropriate validation of these techniques can be found in Part D.

C3 Principle

Preserved algae are concentrated in a single plane by filtration onto a membrane. The membrane is then dried and mounted on a slide with a transparency medium. The slide is examined with a light microscope under a range of magnifications. The observed algae are identified and counted so that the number of cells per taxa per millilitre of sample can be calculated.

C4 Limitations

High suspended solids will dictate that only a smaller volume of sample can be filtered. Solids may still obscure algae in the preparation, and the large calculation factors that apply to very small volumes can potentially distort the result.

Preparation to slide can impact on algae morphology. The filtration stage may cause fragile cells to fragment, while the drying stage could lead to cell distortion and shrinkage. For example, colonial forms may disintegrate to unicells, and flagella, spines and plates maybe shed. Any change in the appearance of algae could increase the potential for miss-identification. Where algae cannot be readily identified from the membrane preparation, reference could be made to live, or a gently pre-concentrated representative sub sample. Vigorous shaking of the sample may cause colonial forms to fragment, which could impact on identification and enumeration.

Variation in results is likely to increase at small volumes, lower population densities and with multicellular forms that vary in size and density.

Algae identification is limited by the skill and knowledge of the analyst performing the method.

C5 Health and Safety

Reagents used in this method are covered by the Control of Substances Hazardous to Health Regulations [2] and appropriate risk assessments should be made before adopting this method. Standard laboratory microbiology safety procedures should be followed, and guidance is given elsewhere [3] in this series.

When using the reagents required for algae analysis, good chemical handling practice should be adhered to at all times along with using appropriate PPE.

Long periods of microscopic analysis can cause physical fatigue and affect eyesight. Attention should be given to the ergonomics of the microscope and microscopists should take regular breaks, carry out eye exercises and have routine eye checks.

C6 Apparatus

Standard laboratory equipment should be used which conforms to the performance criteria outlined elsewhere in this series. Principally a compound light microscope and suitable filtration system is required.

C6.1 Compound light microscope

The exact specifications for the light microscope are determined by the prospective taxonomic level the user intends to identify algae to. For most genus level identification, the following specifications are suitable:

10x binocular eyepieces.
10x, 20x/25x, 40x objectives.
Capable of DIC and/or phase contrast techniques.
A Whipple square grid graticule incorporated into one of the eyepieces.

C6.2 Filtration system

A suitable filtration system consisting of a membrane filter holder, with capacity for largest sample volume analysed i.e. 100 ml, and of diameter compatible with membrane type used i.e. 47 mm. Connected to a waste filtrate collection vessel, and source of vacuum. The vacuum source should not exceed 70 kPa. The filtration apparatus should be made of a material easily cleaned between samples such as glass.

C6.3 Filtration membranes e.g. 47 mm diameter, gridded, cellulose membranes, pore size 0.45 μm.

C6.4 Sample measuring devices e.g.µ pipettes or measuring cylinders capable of 0.1 ml, 1 ml, 5 ml, 10 ml, 25 ml, 100 ml, dependent on the range of volumes required for analysis.

C.6.5 Petri dishes or similar for holding membranes during drying. Need to be clean but absolute sterility is not essential.

C6.6 Membrane forceps, and scissors if a membrane section is cut.

C6.7 1 litre bottle with jet nozzle, or similar, for dispensing iodine rinsing water.

C6.8 Glass slides and coverslips of thickness and quality compatible with microscope and membrane, e.g. coverslips 22 x 40 mm and 0.1 - 0.2 mm thick, slides 26 x 76 mm and 1.0 - 1.2 mm thick.

C6.9 Slide mounting and slide storage boxes/trays.

C6.10 Drying incubator with temperature monitoring. Set at a temperature suitable for drying membranes e.g. <40 °C.

C6.11 Counting devices e.g. tally counters.

C7 Media and reagents

C7.1 Acid Lugol's iodine as detailed in section A8.1.

C7.2 Diluted Acid Lugol's iodine wash, prepared from approximately 1ml of C7.1 per 1000 ml of suitable algae free water. Concentration is not critical as used for wetting and rinsing stages. The iodine is added to prevent biofilm contamination of the wash bottle.

C7.3 Transparency medium / immersion oil with refractive index compatible with brand of membrane used. When the oil is applied to a dried membrane it will turn from opaque to transparent, allowing it to be viewed under light microscope. Product suitability should be assessed by the user.

C7.4 Slide sealant e.g. clear nail varnish.

C8 Analytical Procedure

Note: Algae analysis does not require the strict aseptic technique used for most other Microbiology methods. However, care should be taken to ensure algal are not carried over from one sample to another resulting in cross contamination.

C8.1 Preservation

As detailed in section A8.1.

C8.2 Concentration by Filtration

The sub sample should be taken and filtered aiming to achieve a random distribution of algae on the membrane surface.

The exact volume of the sub-sample to be filtered depends on the phytoplankton density. Suitable volume can be judged from sample turbidity and experience of the algal population for that sampling point. Volumes may require further reduction for samples with high suspended solids. Typical volumes include:

Volume (ml)	Dispensed by	Water sample type
100	Measuring cylinder	Treated waters with very low algae populations
25	Measuring cylinder	Low algae populations
10 or 5	Pipette	Medium algae populations
1	Pipette	High algae populations
0.1	Pipette	Extreme algae populations / blooms

Table C1 – Examples of volumes to be analysed using filtration method.

Place a new membrane into the clean filtration apparatus, check the membrane is lying flat on the support. Check the membrane is filtering evenly by adding enough diluted Acid Lugol's iodine wash to cover the surface, apply the vacuum briefly to pull the wash through. Areas of the membrane which have permitted flow will be stained brown by iodine, whereas areas that remain white have not and the membrane should be discarded.

Gently but thoroughly mix the sample bottle by inversion, immediately collect the required volume for filtration by pipette or measuring cylinder. Add this sub sample to the filtration apparatus with the membrane. The sub sample should be mixed within the filtration apparatus to ensure a random distribution of algae on the membrane. For example, this can be done using a jet of iodine wash to mix the sample.

Allow the sample to stand in the filtration apparatus for a minimum of 15 seconds while the algae start to settle at random on the membrane. Apply the vacuum for just long enough to pull the sample through.

Transfer the membrane to half a labelled Petri dish, taking care not to disturb the algae on the surface. Immediately place in the drying incubator to limit the risk of airborne contaminants e.g. dust falling on the preparation.

Dry the membranes at <40 °C, the time required will be dependent on number and size of the membranes, 1 hr is typically adequate.

C8.3 Slide Preparation

During the slide mounting procedure care is required not to disturb the algae on the surface of the membrane and to maintain cleanliness to produce a good quality slide.

Transfer the dry membrane or a suitable portion e.g. 8 x 4 grid square portion of the membrane to a microscopy slide. Apply a few drops of the transparency medium to the surface of the membrane, enough to clear the membrane without leaving excess that will

leak from the slide mount. If the membrane does not clear it is likely it was not dry enough, and the sample should be re-filtered.

Lower a cover slip over the membrane and allow the transparency medium to spread for a few minutes.

Seal around the edges of the coverslip with clear nail varnish. Label the slide with sample details, it is essential to know the sample volume filtered.

The slides may be examined immediately or if they are to be retained and read at a later date, they should be stored at room temperature and protected from dust.

C8.4 Microscopy

C8.4.1 Enumeration

Microscopic quantitative analysis involves recording the taxa observed and the number of algal objects for each taxon, in a known area of the slide preparation. If the area examined and volume filtered is known, then the concentration of each individual taxon can be calculated.

The choice of enumeration methodology depends on the information needed from the analysis and may include counting a number of randomly selected whole membrane grid squares, counting transects of membrane grid squares or counting whole fields of view.

The slides are examined using a compound light microscope set for phase contrast, using a range of magnification objectives. A predefined number of randomly selected areas should be examined systematically with the analyst recording the taxa observed and the number of algal objects for each taxon.

For example:

Using 10x objective, scan 10 whole membrane grid squares, recording the larger taxa e.g. colonial or filamentous cyanobacteria.

Using 20x objective, scan 10 transects across a grid square, recording the medium size taxa e.g. diatoms and greens

Using 40x objective, observe 20 fields of view, recording the smallest taxa e.g. *Rhodomonas.*

The type of algal objects counted are dependent on the form and abundance of the taxon. Algal objects may be counted directly as individual cells or to make the task easier for the analyst in terms of whole colonies, whole filaments, Whipple grid squares or lengths, all of which can be multiplied up to cells using an estimation of average cells per algal object.

If the slide is considered too overcrowded and difficult to count, it should be re filtered using a smaller volume.

C8.4.2 Identification

A gently pre-concentrated representative sub sample can be used to aid identification when algae morphology has been changed by the filtration and drying process. To make, filter some sample to concentrate the algae on a membrane. Transfer the membrane to a support (upside down Petri dish), and with a Pasteur pipette place a drop of sample onto the membrane. Using the flat edge of forceps, gently scrape the sample drop over the surface of the membrane to re suspend the concentrated algae. Pour off onto a slide and add cover slip. If the slide is required for a longer timeframe seal with nail varnish to prevent desiccation.

Identification should be carried out to the taxonomic level required for the intended use of the method, typically genus level. It is important to note that it is better to correctly identify algae to a lower taxonomic level than misidentify algae to a higher taxonomic level.

Identification can be somewhat controlled by using reference guides, keys or in-house developed guides on the typical algae seen in routine samples. See Appendix 2 for further guidance.

It is recommended to regularly perform inter and intra-laboratory comparison tests to minimise and highlight identification challenges or differences between analysts.

C9 Calculations

$$T_c = C_r \times (AF/AE) \div V$$

Where:
T_c = Cells per ml
C_r = Cells counted
AF = Total effective filtration area of the membrane (mm^2)
AE = Total area of the membrane examined (mm^2)
V = Volume filtered (ml)

Worked examples are shown in Appendix 3.

When a pre-defined set of sample volume, area of membrane examined, and objectives are used for a specific microscope and membrane type, during analysis, it may be useful to calculate constants which can simply be used to multiply up the cells counted to cells per ml.

C10 Expression of results

Counts are expressed in cells per ml.

Algae are typically grouped into Diatoms, Green, Blue/Green (cyanobacteria) and other categories.

C11 Quality assurance

Quality control checks for consistency in the analysis method can be done by using duplicate split samples. Split samples typically comprise a sample divided into two sub-samples, each of which is analysed with each batch of routine samples. A duplicate sample could also comprise the same sample enumerated by two different analysts. The duplicate sub-samples can be considered as two halves of a single sample, and the results can be plotted on a chart containing appropriate response limits. More information on duplicate split analysis is given in The Microbiology of Water and Associated Materials (2017) Practices and Procedures for Laboratories.

Blank samples should be carried out routinely to demonstrate effective cleaning of the equipment used in the filtration process.

It is recommended to carry out regular inter and intra-laboratory assessment. This can be done via numerous methods where analysts analyse the same field of views, either using a natural sample or colour photographs. This allows different analysts to carry out standard analysis on the same sample, one after each other and highlights any discrepancies in identification.

C12 References

1. Anon., 1971. Analysis of algae, Millipore Corporation Applications Procedure 305. Millipore Corporation, Cat. No. LAP305.

2. The Control of Substances Hazardous to Health Regulations (2002). Statutory Instrument 2002 No. 2677.

3. Standing Committee of Analysts, The Microbiology of Water and Associated Materials (2017). Water Quality and Public Health. Practices and Procedures for Laboratories, in this series, Environment Agency.

D Technical protocol for the characterization and verification of Algae enumeration and identification methods

Technical criteria and requirements described in this part of the booklet may be used for the characterisation and validation/verification of methods used for the identification and enumeration of algae. Each water type intended to be analysed by the laboratory should be used at each verification step described below.

For verification, all requirements stated in this part of the booklet are applicable. For method comparison studies, simplified requirements can be used for verification.
For carrying out statistical analysis it is advised to use the algal object count rather than the algal cell count. This reduces the skewing of the data from large filamentous algae e.g. *Aphanizomenon* spp. A definition of algal objects is provider earlier in this book within the definitions section.

D1 Sample Preparation

D1.1 Random Distribution

The preparation of the sub-sample for analysis is crucial, as it affects the final distribution of settled particles which impacts the enumeration of the sample. The objective of this validation step is to assess if algal objects, as a whole can be considered to be randomly distributed across the counting area following sample preparation.

A minimum of 11 samples should be prepared as per the chosen standard method detailed in Parts A, B or C. Each sample should then be analysed by a single analyst using the standard algal microscopy method, identifying, and enumerating algal objects across the entire counting area. This should be done to ensure the chosen fields of view are spread out across the counting area. To aid this, two perpendicular transects or diagonals across the slide/chamber/filter can be used as show in Figure D1.

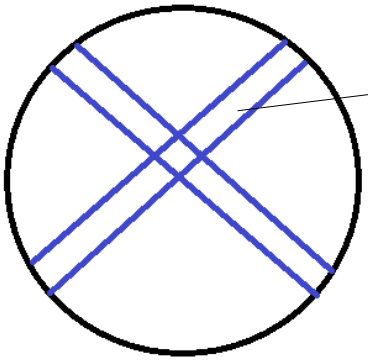

Figure D1 – An example of two diagonals of a counting chamber.
The variance to mean ratio (VMR) should be calculated for each sample to determine if the total algal objects, can be considered randomly distributed across the area of the chamber counted. This can be calculated by:

$$VMR = \frac{S^2(n-1)}{\bar{x}}$$

n is the number of fields. n – 1 is also known as degrees of freedom (DoF).
$\bar{x}$ is the mean number of algal objects.
s^2 is the variance of the number of algal objects.

The critical values of VMR can be found in standard chi-squared tables. Agreement with a Poisson series is accepted at the 95% probability (P > 0.05) level if the VMR lies between the appropriate 5% significance levels (Q = 0.975 and Q = 0.025) for n - 1 degrees of freedom (available in standard statistical textbooks).

An example is detailed below:

Raw water sample prepared as per method A and 20 fields of view enumerated and algal objects identified across diagonal transects as detailed in Figure D1.

Field of View	Algal Objects
1	63
2	77
3	56
4	60
5	55
6	50
7	60
8	40
9	55
10	69
11	53
12	48
13	55
14	56
15	49
16	67
17	55
18	59
19	66
20	78

Calculations	
Number of Fields (n)	20
Mean ($\bar{x}$)	59
Variance (s^2)	89.10
Variance to Mean Ration (VMR)	28.91
Upper limit for 19 DoF (Q = 0.025)	32.85
Lower limit for 19 DoF (Q = 0.975)	8.91
VMR within limits	Yes

As the VMR is within the 5% significance levels it can be deemed that algal objects are randomly distributed across the area of the chamber.

Table D1 – Example of variance to mean ratio calculation for one sample.

Determine the variance to mean ratio for each sample analysed (minimum of 11). The stage is deemed acceptable if 90% of samples analysed pass the variance to mean assessment.

D1.2 Random Order of Occurrence

Where more than one taxon is being counted, a second step in the sample preparation verification stage is to test whether the probability of counting a specific taxon is independent of the preceding counted taxon (serial randomness). The method from D1.1 can be used to generate data for this analysis. The enumeration and identification method from D1.1 should be modified to ensure consistency in starting from the same position within the field of view and recording the identified algae in the sequence they were identified.

The data generated for the two most abundant algal objects can then be analysed using a run test [1] (a nonparametric test that can be used to determine whether a sequence of sample data is random). An example is detailed below:

A raw water sample prepared as per method A and 20 fields of view enumerated and algal genera identified. The top two abundant algae identified in the transect counted as part of the random distribution assessment (D1.1) are used for the assessment.

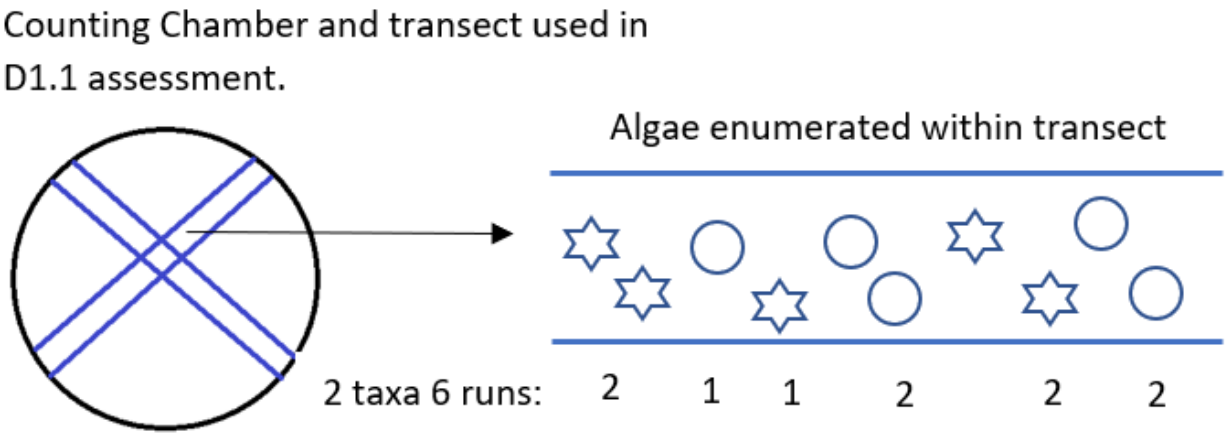

Figure D2 – An example of collecting algal data for a Run-test.

Two most abundant algae:

Monoraphidium

Scenedesmus

Order of two most abundant taxa counted in a sample as illustrated in Figure D2:

Algal Objects
Monoraphidium
Monoraphidium
Scenedesmus
Monoraphidium
Scenedesmus
Scenedesmus
Monoraphidium
Monoraphidium
Scenedesmus
Scenedesmus

Run Test		
R=	6	Runs*
n0=	5	5 *Monoraphidium*
n1=	5	5 *Scenedesmus*
n=	10	10 total algal objects
Expected (R)=	6.00	Calculated
Variance (R)=	2.22	Calculated
StDev (R)=	1.49	Calculated
Z=	0.00	Calculated
Z crit =	1.96	Critical value of the test [1]
Acceptable	Yes	Z<Z crit = acceptable

*A run is a sequence of data having the same characteristic i.e. algae identified. In this example there are 6 runs as there has been 6 changes in the algae identified as illustrated in Figure D2.

Table D2 - Example of random order of occurrence calculation for one sample. Further details on this analysis are in BS EN 15204 [1].

Calculations in Table D2:

Expected (R) = 1+2*n0*n1/n
Variance (R) = 2*n0*n1*(2*n0*n1-n)/n^2/(n-1)
StDev (R) = $\sqrt{Variance}\ (R)$
Z = (R – Expected (R))/StDev (R)

This stage is deemed acceptable if 90% of samples analysed pass the random order of occurrence assessment.

NOTE – There may be certain algae e.g. filamentous algae that exhibit a non-random order of occurrence and this and this should be considered when assessing the validation outcome of this work.

D2 Repeatability and Reproducibility

For the assessment of repeatability and reproducibility all typical water types (matrixes) intended to be analysed by the laboratory should be assessed.

D2.1 Repeatability

A minimum of 11 samples per water type should be prepared in duplicate as per the chosen standard method as detailed in Parts A, B or C. Each pair of the duplicate samples should be analysed by a single analyst. Repeatability for total algal objects is assessed using the standard Chi^2 test as detailed in Microbiology of Drinking Water [(2)].

D2.2 Reproducibility

A minimum of 11 samples per water type should be prepared in duplicate as per the chosen standard method as detailed in Parts A, B or C. For each duplicate sample, the paired samples should be analysed by different analysts under different conditions i.e., time. Repeatability for total algal objects is assessed using the standard Chi^2 test as detailed in Microbiology of Drinking Water [(2)].

D2.3 Phytoplankton Composition

The repeatability and reproducibility assessment should also evaluate whether different counts result in the same proportion of taxa (phytoplankton composition). To test whether different sub-samples are homogeneous with respect to the proportion of the taxa. A multinomial homogeneity test can be used to assess this [(1 and 3)], using the data generated in D2.1 and D2.2. Ideally, a multinomial homogeneity test should be carried out on samples where the algal objects is ≥200. The analysis can be carried out on samples with less algal objects, but results should be interpreted with caution.

A multinomial homogeneity test compares observed data (data from D2.1 and D2.2) to calculated expected values based on statistically expected variation. An example is detailed below:

Observed Data (Analysed sample from D2.1)

Sample Description	Number of Algal Objects				
	Diatoms	Cyanobacteria	Green	Other	Total
Raw Water Reservoir – Duplicate 1	6	51	53	42	152
Raw Water Reservoir – Duplicate 2	8	97	131	31	267
Total per classification	14	148	184	73	419

Expected (Calculated)

Sample Description	Number of Algal Objects				
	Diatoms	Cyanobacteria	Green	Other	Total
Raw Water Reservoir – Duplicate 1	5	54	67	26	152
Raw Water Reservoir – Duplicate 2	9	94	117	47	267

Table D3 - Example of multinomial homogeneity calculation for one sample. Further details on this analysis are in BS EN 15204 (1).

Expected value = (a*b)/c
Where:
a – relevant total per classification.
b – total of relevant sample.
c – total of total per classification.

For example, expected Diatom count for duplicate 1 would be calculated as:

(14*152)/419 = 5.

A Chi^2 assessment can then be carried out for the dataset using the following formula:

$$x^2 = \sum_{i=1}^{k} \frac{(o_i - e_i)^2}{e_i}$$

Where:
o_i – observed count for category e.g. Diatoms.
e_i – calculated expected count for category e.g. Diatoms.
k = number of categories e.g. Diatoms, Green, Cyanobacteria, Others.
An example is detailed below calculating the Chi^2 as per the formula above for each category and sample.

Sample Description	Number of Algal Objects			
	Diatoms	Cyanobacteria	Green	Other
Raw Water Reservoir – Duplicate 1	0.17	0.13	2.83	9.09
Raw Water Reservoir – Duplicate 2	0.10	0.08	1.61	5.18

Table D4 - Example of multinomial homogeneity calculation for one sample. Further details on this analysis are in BS EN 15204 (1).

Chi^2 test statistic (x^2) is then the sum of the calculation for each category and sample = 19.19.

The Chi^2 test statistic can then be compared to Chi^2 critical value at 0.05 probability for the degrees of freedom (k-1) which is available in most statistical textbooks.

In the worked example the Chi^2 critical value is 7.81 (p =0.05 and DoF (k-1) = 3). As the Chi^2 test statistic is greater than the Chi^2 critical value, the samples are not homogeneous with respect to the proportion of the taxa identified.

This stage is deemed acceptable if 90% of samples analysed pass the multinomial homogeneity test assessment.

D3 Measurement Uncertainty

Uncertainty associated with the result of algal analysis encompasses the uncertainties of the whole measurement process such as storage, sub-sampling, homogeneity, identification, and quantification. As part of the verification exercise a qualitative and an estimation of uncertainty should be calculated.

D3.1 Qualitative Uncertainty

Qualitative uncertainty refers to mis- and non-identification of taxa. This can be determined using inter- and intra-laboratory comparisons where the taxa in the sample is known. Misidentified taxa are considered algal taxa identified by a minority of participants or when an algal object is identified by the majority of participants and not by a minority in inter- and intra-laboratory comparisons.

This qualitative uncertainty can then be determined as a maximum percentage of misidentifications. Typical misidentification percentages are <5%.

D3.2 Estimation of Uncertainty.

Calculating a quantitative uncertainty is challenging as it is dependent on the abundance of specific taxa within the sample and cannot be expressed as a single value for algal analysis. As such, an estimation of the impact of uncertainty can be made using the index of dispersion method as used in microbiological analysis detailed in the Microbiology of Drinking Water series. This estimation provides information to understand if the stages of the chosen algal enumeration and identification method are in a state of statistical control. Repeatability and reproducibility total algal object data generated in section D2 can be used to carry out the index of dispersion analysis as detailed in BS 8496 [(4)].

If the index of dispersion indicates that uncertainty is having an impact on the method, then this should be investigated further. As part of the investigation, it may be of benefit to review the individual stages of the method to ensure they are controlled appropriately.

D4 Limit of Detection

The limit detection is theoretical and is determined by Poisson statistics. The limit of detection is defined as the minimum concentration of a specific taxon or group at which it will be detected with a 90% probability. The detection limit can be calculated using the below calculation:

$$n_{det} = -\ln(\alpha) \times \left(\frac{f_{total}}{V \times f_{counted}}\right)$$

Where:
n_{det} is the detection limit.
-ln is the negative natural logarithm.
α is the level of significance (0.01).
f_{total} is the total number of microscope fields in the counting chamber.
$f_{counted}$ is the number of microscope fields typically analysed.
V is the volume of the sub-sample analysed.

The limit of detection is theoretical and is calculated for awareness and to provide an understanding of the potential limitations of the chosen method. This method of determining the limit of detection does not take account of the algal identification skills of the analyst.

D5 Proficiency Testing

As part of the verification a proficiency testing sample should be carried out to assess the method performance. This can be achieved through an inter- or intra-laboratory scheme.

D6 References

1. BS EN 15204:2006. Water Quality. Guidance standard on the enumeration of phytoplankton using inverted microscopy (Utermöhl technique).

2. Standing Committee of Analysts, The Microbiology of Water and Associated Materials (2017). Water Quality and Public Health. Practices and Procedures for Laboratories, in this series, Environment Agency.

3. Mosimann, J.E. (1965). Statistical methods for the pollen analyst: multinomial and negative multinomial techniques. In: Handbook of paleontological techniques. W.H. Freeman and company, pp.636-673.

4. BS EN 8496:2017. Water quality, Enumeration of micro-organisms in water samples. Guidance on the estimation of variation of results with particular reference to the contribution of uncertainty of measurement.

E Estimation of phytoplankton biovolumes

E1 Introduction

The method of calculating of algal biovolumes is based on cell measurements. The cell dimensions are used to calculate biovolumes using geometric formula.

Calculating biovolumes, as well as cell densities, for different taxa and whole samples has several different uses and benefits. For example, biovolume information can be used for estimating growth cycles and the periodicity of algal populations. Biovolume results can also provide useful information in determining the efficiency of water treatment through the different stages from surface water abstraction (rivers or reservoirs) through to the final treated drinking water. Cell densities (as cells/ml) are traditionally used when reporting on algal issues in the UK whereas biovolumes can provide more useful information on potential treatment challenges for water quality, engineering, and production teams.

Algal biovolumes also allow the standardisation in reporting and interpreting results and the potential impact of algae in surface waters. Algal concentrations may not always provide clear information on the challenge algae may pose to water treatment processes. For example, *Aphanizomenon* cells are very small (80µm^3) whereas *Ceratium* spp. is a very large cell (44,000 µm^3) and understanding this may be useful for water quality teams in understanding potential challenging to water treatment works.

The method is based that described by Brierley *et.al*. [(1)] and in the CEN standard [(2)] for estimation of biovolumes.

E2 Scope

This method is suitable for all water types from surface water, through all stages of treatment, to the final treated water.

E3 Principle

Biovolumes for algal taxa are calculated by assigning simple geometric shapes to each cell, filament, or colony.

Due to the wide variety of sizes and morphologies of algal forms, from variations in single cells, filament, and colonial taxa, it is difficult to define fixed biovolumes for each taxa. A range of methods is required to estimate biovolumes for algal taxa and total biovolumes in water samples. The method involves measuring appropriate dimensions and using these to calculate the biovolume.

The use of fixed biovolumes for taxa that do not exhibit a great variety in biovolume is currently being investigated for the most common phytoplankton taxa in the UK.

For those taxa with variable dimensions or for colonies and filaments it is necessary to undertake measurements as part of the counting procedure. Measurements of the required cell dimensions (length, width, diameter) are made at an appropriate magnification using a calibrated ocular eyepiece, e.g. a Whipple Graticule.

E4 Limitations

Measurement of algae dimensions is limited by the skill and knowledge of the analyst performing the measurements.

E5 Apparatus

Standard laboratory equipment required for algal method of analysis (Parts A-C) should be used. Principally a microscope with a calibrated measuring graticule inserted into one of the eyepieces. A Whipple graticule is ideal.

A calibration look-up chart for the graticule being used should be calculated for each magnification being used. An example is illustrated in Figure E1 below:

Magnification	40x	64x	200x	320x	400x	640x
Whole graticule	1700 µm	1060 µm	340 µm	212.5 µm	170 µm	106.25 µm
1 large unit	170 µm	106 µm	34 µm	21.25 µm	17 µm	10.625 µm
1 small unit	34 µm	21.2 µm	6.8 µm	4.25 µm	3.4 µm	2.125 µm

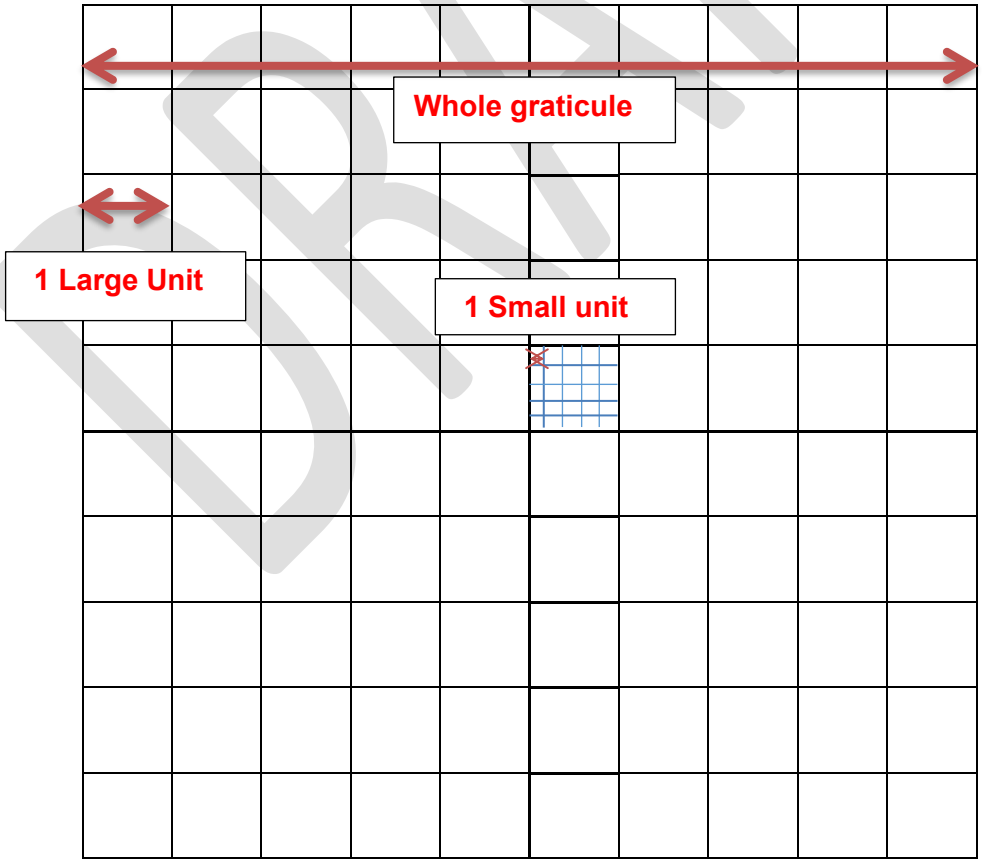

Figure E1 - A calibration look-up chart for eyepiece measuring graticule and a Whipple graticule grid with examples of measurements.

E6 Calculation Procedure

As part of the During the enumeration process, it is necessary to measure the dimensions of algal units (cells, colonies, or filaments) of all or taxa of importance/interest all taxa present in the sample should be measured.

Measuring the algal units is done at the relevant magnification for the different taxa.
It is recommended that the eyepiece with the graticule is rotated so that the scale is put over the required cell dimension and the measurement made by taking the ocular measurement and multiplying by the calibration factor for that magnification and eyepiece combination. Required cell dimensions (length, width, diameter) are measured. The cell dimensions that are needed is dependent on the geometric formula used for different taxa.

The linear dimensions of at least 10 individual units (cells, colonies, or filaments) of all taxa observed when enumerating the sample should be measured. For taxa of more variable size, ideally at least 30 individual units should be measured and for taxa that are very variable (e.g. *Microcystis* colonies) then up to 50 cells/units should be measured.

To determine the standard biovolume for each taxa, the mean cell dimensions for each taxa can be calculated and biovolume calculated using the taxa geometric formula (Table E1).

Biovolume shape	**Formula**	**Taxon examples**
CIRCLE BASED CYLINDER -	π*L*D*D/4	*Aphanizomenon*
CIRCLE BASED CYLINDER - SHORT	π*H*D*D/4	Centric diatoms
CIRCLE BASED ELLIPSE	π*L*D*D/6	
OVAL BASED CYLINDER	π*L*D*H/4	
OVAL BASED ELLIPSE	π*L*D*H/6	
CONE	π*L*D*D/12	*Mallamonas akrokomos*, horn of
CONE + HEMISPHERE	(π*D*D)12*(D/2+ L)	*Rhodomonas, Mallamonas caudata*
DOUBLE CONE	π*L*D*D/12	*Ankistrodesmus, Closterium*
CUBOID/RECTANGLE	L*D*H	*Tabellaria*, pennate diatoms,
CUBOID/RECTANGLE * 0.5	0.5*L*D*H	*Nitzschia acicularis*
SPHERE	π*D*D*D/6	*Microcystis, Sphaerocytis*, picoplankton
0.2 SPHERE	0.2*π*D*D*D/6	*Woronichinia*
0.25 SPHERE	0.25*π*D*D*D/6	*Eudorina*
0.5 SPHERE	0.5*π*D*D*D/6	*Aphanothece, Aphanocapsa*
0.75 * SPHERE	0.75*3π*D*D*D/6	*Snowella, Gomphosphaeria*

Table E1 - Algal Biovolume formula. L = length (µm), D = Diameter or width (µm) H = Depth or height (µm) and P = Numbers of arms/branches in Staurastrum half cell

Appendix 4 provides a list of common taxa within the UK and includes Whitton Codes, accepted names, agreed biovolume shape, biovolume formulae and biovolume ranges where available and taken from the published literature. This appendix should be used as a guide for carrying out the correct biovolume calculation.

To calculate biovolumes of taxa that are variable in size, whether they are cells (e.g. *Pediastrum* cells), filaments (e.g. *Aphanizomenon)* or colonies (e.g. *Asterionella,*

Microcystis, Woronichiania) it is necessary to develop agreed general rules. Below are some examples of these types of rules:

- For the simpler colonies- e.g. *Scenedesmus,* count the number of cells in 20-30 colonies and measure the diameter and length of minimum of 30 cells. Calculate the average cell dimensions per cell, average number of cells per colony and the average biovolume per cell. Multiply the average biovolume per cell by the cell count to calculate total biovolume.

- For filamentous forms, e.g. *Aphanizomenon,* measure filament length and filament/cell diameter and then calculate filament biovolume and determine the average biovolume per filament for approximately 30 filaments chosen at random.

- Some taxa are more difficult, e.g. spiral filaments such as *Anabaena*. For these, the average number of cells per gyre is counted and then the number of gyres per filament is estimated. The two numbers are multiplied together to give the estimated number of cells per filament. The cell volume is calculated by measuring the diameter of each cell and using formula for a sphere (πr^2).

If the colony is very large and/or cells are very small (e.g. *Microcystis, Woronichinia*) cell numbers may have to be estimated. This is best done by estimating cell numbers in a more restricted area of the colony and estimating how many similar areas are contained within the counting field.

It is recommended that the process of calculating cell and total biovolumes is automated and included with the input of cell count information from typical enumeration method. An automated process can then calculate cells/ml and biovolume/ml (μm^3/ml) for each taxa and for total biovolume in the sample.

An example of this type of system was developed by the UK Regulatory Agencies and UKCEH and a screen shot in shown in Figure E2:

Phytoplankton Counter Spreadsheet CEHv2.00

Developed by Laurence Carvalho[1], Bill Brierley[2] Bernard Dudley[1] and Deena Mobbs[1]

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[2]Environment Agency, Phoenix House, Global Avenue, Leeds LS11 8PG, UK
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Project funders/partners: Centre for Ecology & Hydrology, SNIFFER (WFD80) & Environment Agency
WFD80: Phytoplankton Classification Tool (Phase 2) (June, 2007)

This spreadsheet is the counter form to be used for recording phytoplankton data from counts from UK and Irish lakes. It allows the recording of more than 230 commonly recorded taxa in the UK, Ireland and Central Europe. A macro is also embedded to allow easy transfer of data to the UK and Ireland Phytoplankton database.

For further information, see Carvalho et al. (2007) Phytoplankton Classification Tool (Phase 2). Final Report to SNIFFER (Project WFD80), Edinburgh, June 2007, 98 pp

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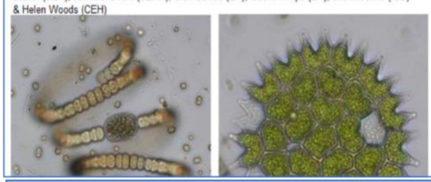
The following people have provided additional inputs to the design or content of the sheet: David Leaver (CEH), Jan Krokowski (SEPA), Sian Davies (EA), Geoff Phillips (EA), Ute Mischke (IGB) & Helen Woods (CEH)



A	B	F	G	H	I	J	K	L	M	N	O	P	Q	R	S	T	U	V	W	X
Lake Name:	<EnterLakeName>	x40	Sample Point Cd	XXXXXX																
UK Lake Cod	XXXXX		Sample ID:	XXXXXX																
Location:			Sample Quality:	100	Good quality - no obvious benthic contamination															
Location Cod	02		Preservative:	Lugols																
Sampling Dat	XX/XX/2013		Sample Comments:																	
Analysis Date	XX/XX/2013					Colony/Filament Biovolume							Cell biovolume							
Code	Taxon	No's per ml	Biovolume Type Code (CE/CO/FI)	Biovolume Units Count	Biovolume (μm^3) per ml	Biovolume formula (μm^3)	Length (L)	Diameter / Width (D)	Depth (H)	Minimum Biovolume	Typical Biovolume	Maximum Biovolume	Biovolume (formula) um^3	Length (L)	Diameter / Width (D)	Depth (H)	Mean Staurastrum or ceratium cell volume	Minimum Biovolume	Typical Biovolume	Maximum Biovolume
01430050	Lyngbya contorta					0.00							0.0							
01460000	Merismopedia					0.00							0.0							
01460050	Merismopedia warmingiana					N/A							0.0							
01490000	Microcystis					0.00							0.0							
01490010	Microcystis aeruginosa					0.00							0.0					30		280
01490020	Microcystis flos-aquae					0.00							0.0					20		70
01490030	Microcystis wesenbergii					0.00							0.0					30		180
01530000	Oscillatoria					0.00							0.0							
01530010	Oscillatoria agardhii					0.00							0.0					30		120
01530012	Oscillatoria agardhii var. isothrix					0.00							0.0					100		430
01530160	Oscillatoria limnetica (Pseudanabaena limnetica)					0.00							0.0					10	34	70
01530170	Oscillatoria limosa					0.00							0.0					236		860
01530230	Oscillatoria redekei					0.00							0.0					10		70
01580000	Pseudanabaena					0.00							0.0							
01750000	Snowella					0.00							0.0							
01750010	Snowella lacustris					0.00							0.0					3		30
01750020	Snowella septentrionalis					N/A							0.0							
01750030	Snowella atomus					N/A							0.0							
01780000	Woronichinia					0.00							0.0							
01780010	Woronichinia naegeliana					0.00							0.0					32		91
04020000	Euglena					N/A							0.0							
04070000	Phacus					N/A							0.0							
04090000	Strombomonas					N/A							0.0							
04100000	Trachelomonas					N/A							0.0							
05020000	Chroomonas					N/A							0.0							
05020010	Chroomonas acuta					N/A							0.0						301	
05040000	Cryptomonas					N/A							0.0							
05040001	Cryptomonas (small) Length <20 µm					N/A							0.0							
05040002	Cryptomonas (medium) Length 20-30 µm					N/A							0.0							
05040003	Cryptomonas (large) Length >30 µm					N/A							0.0							
05040030	Cryptomonas erosa					N/A							0.0							
05040040	Cryptomonas marssonii					N/A							0.0							

Information | Instructions | Raw data | Database entry | DatabaseSample | Quality codes

Figure E2 – Example of automated biovolume calculation spreadsheet.

E7 References

1. B. Brierley, L. Carvalho, S. Davies, J. Krokowski (2014). Guidance on the quantitative analysis of phytoplankton in freshwater samples. Report to SNIFFER (Project WFD80).

2. BS EN 16695:2015. Water quality. Guidance on the estimation of phytoplankton biovolume (BSI). 2015.BS EN 16695.

Appendix 1 Calibration of Sedimentation Chambers (gross error check)

If the chambers can be uniquely identified, it is recommended to calibrate a minimum of 10% annually. If they cannot be uniquely identified, then calibration should occur on all chambers available annually.

1.1 Apparatus required

Calibrated balance capable of reading mass of interest. It is suggested this is capable of a minimum of 2 decimal places.

Thermometer
DI water
Suitable sedimentation chambers e.g. Utermöhl chambers
Coverslips both top and bottom

1.2 Procedure

Insert a base coverslip into the chamber and select a top coverslip. Weigh the chamber and top coverslip together and record weight (A).

Fill chamber with DI water and place top coverslip on as per use for analysis. Reweigh filled chamber and record weight (B).

Calculate B-A which will give volume of water within chamber. This value is then corrected for the mass of distilled water for the recorded temperature, resulting in a corrected volume value (C).

Calculate C as a percentage of the nominal volume, e.g. 10 ml. Rejection or acceptance is based upon volume being within 10% of the nominal volume.

A worked example is detailed below:

Utermohl chamber calibration raw data

									Balance	00011	Distilled water mass	0.99777
Date	Analyst	Nominal volume	Dry weight (g)	Wet Weight (g)	Difference (g)	Corrected value	Variance from nominal	% variance				
21/12/2020	CRS	10	61.55	71.98	10.43	10.41	0.41	4.07				
21/12/2020	CRS	10	59.81	69.70	9.89	9.87	-0.13	-1.32				
21/12/2020	CRS	10	58.53	69.47	10.94	10.92	0.92	9.16				
21/12/2020	CRS	10	59.83	69.85	10.02	10.00	0.00	-0.02				
21/12/2020	CRS	10	59.71	69.56	9.85	9.83	-0.17	-1.72				
21/12/2020	CRS	10	63.02	73.05	10.03	10.01	0.01	0.08				
21/12/2020	CRS	10	59.60	69.54	9.94	9.92	-0.08	-0.82				
21/12/2020	CRS	10	64.71	74.62	9.91	9.89	-0.11	-1.12				
21/12/2020	CRS	10	59.39	69.55	10.16	10.14	0.14	1.37				
21/12/2020	CRS	10	59.93	70.05	10.12	10.10	0.10	0.97				
21/12/2020	CRS	10	60.26	70.36	10.10	10.08	0.08	0.77				
21/12/2020	CRS	10	59.52	69.48	9.96	9.94	-0.06	-0.62				
21/12/2020	CRS	10	61.34	72.12	10.78	10.76	0.76	7.56				
21/12/2020	CRS	10	60.57	70.38	9.81	9.79	-0.21	-2.12				
21/12/2020	CRS	10	58.95	69.16	10.21	10.19	0.19	1.87				
21/12/2020	CRS	10	59.63	70.12	10.49	10.47	0.47	4.67				
21/12/2020	CRS	10	58.26	68.08	9.82	9.80	-0.20	-2.02				
21/12/2020	CRS	10	59.67	69.76	10.09	10.07	0.07	0.67				
21/12/2020	CRS	10	60.86	71.24	10.38	10.36	0.36	3.57				
21/12/2020	CRS	10	61.24	72.17	10.93	10.91	0.91	9.06				

Appendix 2 Algal Reference Guides and Keys

Book or Website	ISBN	Comment
John et al. The Freshwater Algal Flora of the British Isles, An identification Guide to Freshwater and Terrestrial Algae.	ISBN 0 521 77051 3	Core text for UK
https://www.algaebase.org/	N/A	Core website
https://www.nhm.ac.uk/our-science/data/algaevision.html	N/A	Core website
https://diatoms.org/	N/A	Diatom specific
Belcher and Swale. An illustrated guide to river phytoplankton. Culture Centre of Algae and Protozoa.	ISBN 0 11 886602 8	Beginners guide
Barber and Hayworth. A guide to the morphology of the Diatom Frustule, FBA scientific publication No. 44.	ISBN 0 900386 42 8	Diatom Specific
Kelly. Identification of common benthic diatoms in rivers, Field Studies Council AIDGAP guides, Volume 260.	ISBN 9781851532087	Diatom Specific
Prescott. Algae of the Western Great Lakes Area	ISBN 0 697 04552 8	
West and Fritsch. A Treatise on The British Freshwater Algae, Cambridge University press.	ISBN 1372758798	Good illustrations and simple line drawings
Belcher and Swale. A beginners guide to Freshwater Algae, Institute of terrestrial Ecology.	ISBN 0 11 881393 5	
Bellinger. A key to the identification of the more common Algae found in British Freshwaters, The society for water treatment and examination.	ISBN 0521770513	Beginners guide
Pentecost. Introduction to Freshwater Algae, Richmond publishing.	ISBN 0 85546144 6	
Lind and Brook. Desmids of the English Lake District, FBA scientific publication No. 42.	ISBN 0 900386 40 1	
Lund and Lund. Freshwater Algae Their microscopic world explored, Biopress Ltd.	ISBN 0 948737 25 5	
Prescott. The Freshwater Algae.	ISBN 0 697 04859 6	

This does not constitute an endorsement of these guides and books but serves only as an illustrative example of the type of guides available. Equivalent guides and books may be available.

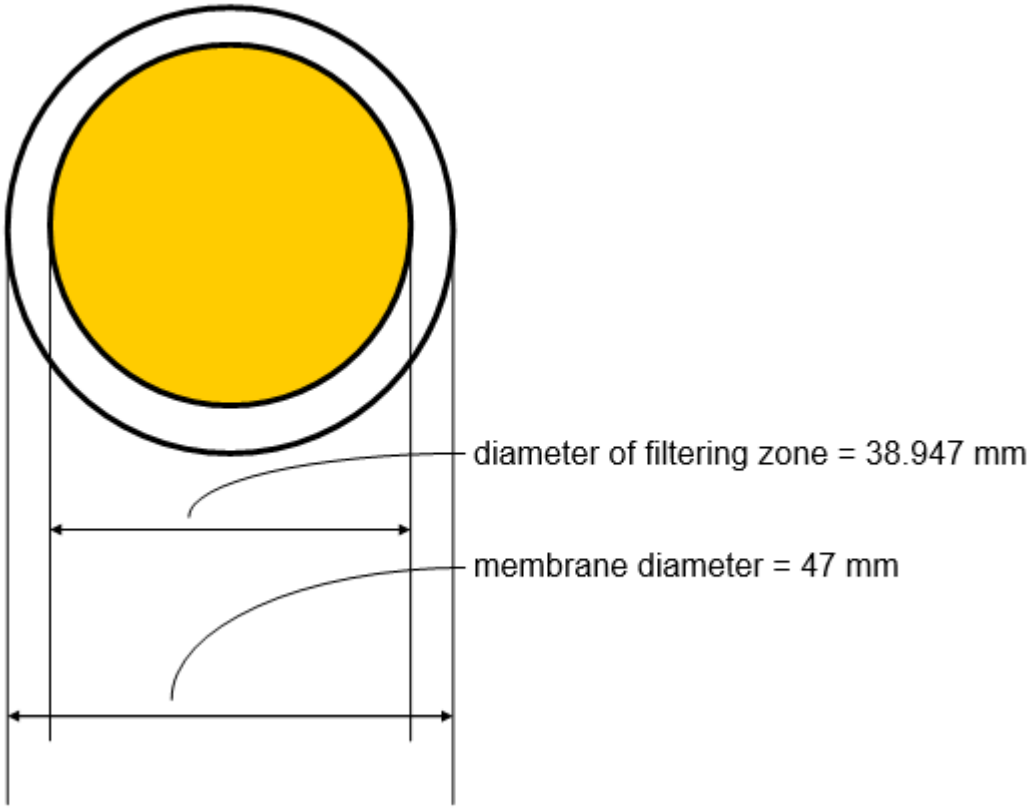
Appendix 3 Filtration Method Calculation Examples

Calculation of Total effective filtration area of the membrane (mm^2)

Filter approximately 25 ml of a preserved sample containing abundant algae. Dry the membrane but do not mount to slide. Measure the diameter of the effective filtration area in mm, the section of the membrane-stained brown with iodine. Measurements could be made while viewing the membrane under a stereo microscope.
The Total effective filtration area of the membrane is calculated from

$$\pi \times \left(\frac{\text{diameter}}{2} \right)^2$$

For example:



Total effective filtration area of the membrane = 1191.5 mm^2
Consideration should be given to membrane brands where algae do not settle on the printed grid lines and if deductions of the grid line area is therefore required to calculate the effective membrane area.

Worked Example 1:

25ml of a raw water was filtered by the described method. The 10x objective was used and 144 *Aphanizomenon flos-aquae* filaments in ten randomly selected squares were counted. The *A. flos-aquae* filaments were all approximately of the same length and the number of cells per average filament was estimated at a higher magnification to be 30.

Volume filtered = 25 ml
Whole membrane grid squares counted = 10
Average width of membrane grid square = 2.999 mm
Total algal objects (filaments) counted = 144
Average cells per algal object (filament) = 30
Total effective filtration area of the membrane = 1191.5 mm^2
From this data the following can be calculated:
Cells counted = 144 x 30 = 4320
Total area of the membrane examined = 2.999 x 2.999 x 10 = 89.94 mm^2
Cell per ml =4320 x (1191.5 ÷ 89.94) ÷ 25 = 2289 A. *flos-aquae* cells per ml

Worked example 2:

10 ml of a raw water was filtered by the standard method. Scans were made across ten randomly selected grid squares using the x25 objective. 87 individual cells of the flagellate *Trachelomonas* were identified and counted.

Volume filtered = 10 ml
Membrane grid squares transect scanned = 10
Average width of membrane grid square = 2.999 mm
Diameter of field of view with x25 objective = 0.48 mm
Total algal objects (cells) counted = 87
Average cells per algal object (cells) = 1
Total effective filtration area of the membrane = 1191.5 mm^2
From this data the following can be calculated:
Cells counted = 87 x 1 = 87
Total area of the membrane examined = 2.999 x 0.48 x 10 = 14.40 mm^2
Cell per ml =87 x (1191.5 ÷ 14.40) ÷ 10 = 720 Trachelomonas sp cells per ml

Worked Example 3:

5 ml of a raw water was filtered by the standard method. *Crucigenia* colonies were identified and counted in 20 randomly selected fields of view using 40x objective. The *Crucigenia* averaged 4 cells per colony.
Volume filtered = 5 ml
Number of fields of view counted = 20
Diameter of field of view with x40 objective = 0.22 mm
Total algal objects (colonies) counted = 164
Average cells per algal object (colony) = 4
Total effective filtration area of the membrane = 1191.5 mm^2
From this data the following can be calculated:
Cells counted = 164 x 4 = 656

Area of field of view at x40 calculated from

$$\pi \times \left(\frac{\text{diameter}}{2} \right)^2$$

= 3.142 x (0.22 ÷ 2)^2 = 0.038 mm^2
Total area of the membrane examined = 0.038 x 20 = 0.76 mm^2
Cell per ml =656 x (1191.5 ÷ 0.76) ÷ 20 = 51423 *Crucigenia* cells per ml.

Appendix 4 Biovolume Calculation Guidance

Whitton Code	Accepted name	Colony biovolume	Cell biovolume formula	Minimum Biovolume	Typical Biovolume	Maximum Biovolume
12010010	*Acanthoceras zachariasii*		Sphere			
17020010	*Actinastrum hantzschii*		Cone			
1020040	*Anabaena catenula*	Circle based ellipse	Circle based ellipse			
1020042	*Anabaena catenula var. solitaria*	Circle based ellipse	Sphere			
1020050	*Anabaena circinalis*	Circle based ellipse	Sphere			
1020090	*Anabaena flos-aquae*	Circle based ellipse	Circle based ellipse			
1020000	*Anabaena sp.*	Circle based ellipse	Circle based ellipse			
1020140	*Anabaena spiroides*	Circle based ellipse	Sphere			
1020190	*Anabaena viguieri*	Circle based ellipse	Sphere			
17050030	*Ankistrodesmus falcatus*		Cone			
17050050	*Ankistrodesmus fusiformis*		Cone			
17050000	*Ankistrodesmus sp.*		Cone			
17060020	*Ankyra judayi*		Cone	234	1021	1299
1040020	*Aphanizomenon flos-aquae*	Circle based ellipse	Circle based cylinder - long			
1040040	*Aphanizomenon issatschenkoi*	Circle based ellipse	Circle based cylinder - long		309	
1040000	*Aphanizomenon sp.*	Circle based ellipse	Circle based cylinder - long			
1050020	*Aphanocapsa delicatissima*	0.5 sphere	Sphere			
1050030	*Aphanocapsa elachista*	Sphere	Sphere			
1050000	*Aphanocapsa sp.*	0.5 sphere	Sphere			
1060020	*Aphanothece clathrata*	0.5 sphere	Circle based ellipse		105	
1060000	*Aphanothece sp.*	0.5 sphere	Circle based ellipse			
13080010	*Asterionella formosa*		Cuboid/rectangle		270	1400
12030060	*Aulacoseira granulata*	Circle based cylinder - long	Circle based cylinder - long	46		260

12030062	*Aulacoseira granulata v. angustissima*	Circle based cylinder - long	Circle based cylinder - long			
12030070	*Aulacoseira islandica*	Circle based cylinder - long	Circle based cylinder - long			
12030080	*Aulacoseira italica*	Circle based cylinder - long	Circle based cylinder - long			
12030084	*Aulacoseira italica v. tenuissima*	Circle based cylinder - long	Circle based cylinder - long	80		400
12030000	*Aulacoseira sp.*	Circle based cylinder - long	Circle based cylinder - long	20		180
9030010	*Bitrichia chodatii*		Circle based ellipse			
9030020	*Bitrichia longispina*		Circle based ellipse			
9030000	*Bitrichia sp.*		Circle based ellipse		2.15	
17080010	*Botryococcus braunii*	Circle based ellipse	Circle based ellipse			
17080000	*Botryococcus sp.*	Circle based ellipse	Circle based ellipse	0.4		3.3
16060000	*Carteria sp.*		Circle based ellipse			
6020020	*Ceratium cornutum*			31		504
6020030	*Ceratium furcoides*				30	
6020040	*Ceratium hirundinella*					
16180000	*Chlamydomonas sp.*		Circle based ellipse	11	14.3	17
9050030	*Chromulina nebulosa*		Circle based ellipse			
9050000	*Chromulina sp.*		Circle based ellipse			
1130020	*Chroococcus dispersus*		Sphere			
1130060	*Chroococcus minutus*		Sphere			
1130000	*Chroococcus sp.*		Sphere	30		280
5020010	*Chroomonas acuta*		Oval based ellipse	20		70
5020000	*Chroomonas sp.*		Oval based ellipse	30		180
8010010	*Chrysochromulina parva*		Oval based ellipse			
9130000	*Chrysococcus sp.*		Sphere	30		120
9150000	*Chrysolykos sp.*		Circle based ellipse	100		430

9170000	*Chrysopyxis sp.*		Circle based ellipse	10	34	70
17170010	*Closteriopsis acicularis*		Cone	236		860
17170020	*Closteriopsis longissima*		Cone	10		70
17170000	*Closteriopsis sp.*		Cone			
27040030	*Closterium aciculare*		Cone	3		30
27040040	*Closterium acutum*		Cone			
27040044	*Closterium acutum v. variabile*		Cone	32		91
27040340	*Closterium kuetzingii*		Cone			
27040500	*Closterium parvulum*		Cone			
27040000	*Closterium sp.*		Cone			
17200010	*Coelastrum astroideum*	Sphere	Circle based ellipse			
17200020	*Coelastrum microporum*	Sphere	Sphere		301	
17200000	*Coelastrum sp.*	Sphere	Sphere			
17200070	*Coelastrum sphaericum*	Sphere	Sphere			
1150010	*Coelosphaerium kuetzingianum*	0.2 sphere	Sphere			
1150000	*Coelosphaerium sp.*	0.2 sphere	Sphere			
17210010	*Coenochloris fottii*		Circle based ellipse			
17230020	*Coenocystis planktonica*		Circle based ellipse			
27050000	*Cosmarium sp*		Oval based ellipse			
17250000	*Crucigenia sp.*		Oval based ellipse			
17250030	*Crucigenia tetrapedia*		Cuboid/rectangle	84	128	150
5040030	*Cryptomonas erosa*		Oval based ellipse	35	105	183
5040040	*Cryptomonas marssonii*		Oval based ellipse		25560	
5040050	*Cryptomonas ovata*		Oval based ellipse		18600	
5040000	*Cryptomonas sp.*		Oval based ellipse		44000	70000
5040003	*Cryptomonas sp. (large) Length >30µm*		Oval based ellipse			
5040002	*Cryptomonas sp. (medium) Length 20-30 µm*		Oval based ellipse			
5040001	*Cryptomonas sp. (small) L<20µm*					

			Oval based ellipse	9905		25000
12070000	*Cyclotella sp.*		Circle based cylinder - short			
13260042	*Diatoma elongatum*		Cuboid/rectangle	21120	57000	99700
13260000	*Diatoma sp.*		Cuboid/rectangle	41000	64500	103000
13260040	*Diatoma tenuis*		Cuboid/rectangle		18560	
17330040	*Dictyosphaerium pulchellum*	0.25 sphere	Sphere	40	75	125
17340000	*Didymocystis sp*		Circle based ellipse			
17350020	*Didymogenes palatina*		Circle based ellipse	467	887	970
9230030	*Dinbryon crenulatum*		Circle based ellipse	99	112	158
9230010	*Dinobryon bavaricum*		Circle based ellipse			
9230050	*Dinobryon divergens*		Circle based ellipse		183	
9230070	*Dinobryon sertularia*		Circle based ellipse			
9230080	*Dinobryon sociale*		Circle based ellipse			
9230000	*Dinobryon sp.*		Circle based ellipse			
9230090	*Dinobryon suecicum*		Circle based ellipse			
25010010	*Elakatothrix gelatinosa*		Cone + hemisphere			
9250000	*Epipyxsis sp.*		Circle based ellipse			
27110000	*Euastrum sp.*		Oval based ellipse			
16260010	*Eudorina elegans*	0.25 sphere	Sphere			
4020000	*Euglena sp.*		Oval based ellipse			
13370030	*Fragilaria capucina*		Cuboid/rectangle			
13370040	*Fragilaria crotonensis*		Cuboid/rectangle			
13370000	*Fragilaria sp.*		Cuboid/rectangle			
6050000	*Glenodinium sp*		Oval based ellipse			
17420000	*Gloeocystis sp.*		Sphere	22		1000
17430020	*Golenkinia radiata*		Sphere	1000		20000
17430000	*Golenkinia sp.*		Sphere			
17440020	*Golenkiniopsis longispina*		Sphere			
1320010	*Gomphosphaeria aponina*	0.75 * sphere	Circle based ellipse			

1320000	*Gomphosphaeria sp.*	0.75 * sphere	Circle based ellipse			
27130000	*Gonatozygon sp.*		Circle based cylinder - long			
7010010	*Gonyostomum semen*		Cone + hemisphere			
6070110	*Gymnodinium helveticum*		Oval based ellipse			
6070000	*Gymnodinium sp.*		Oval based ellipse	329	580	? 2200
9290000	*Kephyrion sp.*		Circle based ellipse			
25030010	*Koliella longiseta*		Cone	424	597	3816
25030000	*Koliella sp.*		Cone		377	575
17540040	*Lagerheimia genevensis*		Circle based cylinder - long	254	487	? 3185
17540000	*Lagerheimia sp.*		Circle based cylinder - long	35	540	5828
12000003	*Large centric diatom (>20 µm diam.)*		Circle based cylinder - short	377		615
13000003	*Large pennate diatom >20 µm*		Cuboid/rectangle			
9310030	*Mallomonas akrokomos*		Cone + hemisphere			
9310080	*Mallomonas caudata*		Cone + hemisphere	1501	4671	14223
9310000	*Mallomonas sp.*		Circle based ellipse			
12000002	*Medium centric diatom 10-20µm*		Circle based cylinder - short			
13000002	*Medium pennate diatom 10-20 µm*		Cuboid/rectangle	141		1884
12110000	*Melosira sp.*	Circle based cylinder - long	Circle based cylinder - long	1207		3418
12110080	*Melosira varians*	Circle based cylinder - long	Circle based cylinder - long	114	480	983
1460000	*Merismopedia sp.*	Cuboid/rectangle	Circle based ellipse			
17570010	*Micractinium pusillum*		Sphere	60	204	2993
17570000	*Micractinium sp*		Sphere	320	550	1482
1490010	*Microcystis aeruginosa*		Sphere			

1490020	*Microcystis flos-aquae*		Sphere	81	388	1011
1490000	*Microcystis sp.*		Sphere	169	640	2228
1490030	*Microcystis wesenbergii*		Sphere			
17580010	*Monoraphidium arcuatum*		Cone			
17580020	*Monoraphidium contortum*		Cone	37	200	912
17580030	*Monoraphidium convolutum*		Cone			
17580040	*Monoraphidium griffithii*		Cone	544	880	2700
17580050	*Monoraphidium irregulare*		Cone		158	
17580070	*Monoraphidium komarkovae*		Cone	920	1600	9800
17580080	*Monoraphidium minutum*		Cone			
17580110	*Monoraphidium pusillum*		Cone	828		2185
17580000	*Monoraphidium sp.*		Cone	440	1185	7349
17580120	*Monoraphidium tortile*		Cone		2402	
90000003	*Nanoplankton - unidentified single cells, 2–20 µm diam.*		Sphere			
13520000	*Navicula sp.*		Cuboid/rectangle			
13540020	*Nitzschia acicularis*		Cuboid/rectangle * 0.5			
13540000	*Nitzschia sp.*		Cuboid/rectangle			
9350000	*Ochromonas sp.*		Circle based ellipse	49		2078
17640130	*Oocystis borgei*		Circle based ellipse		509	
17640050	*Oocystis lacustris*		Circle based ellipse	31		205
17640000	*Oocystis sp.*		Circle based ellipse			
1530010	*Oscillatoria agardhii*	Circle based cylinder - long	Circle based cylinder - long	17		181
1530012	*Oscillatoria agardhii var. isothrix*	Circle based cylinder - long	Circle based cylinder - long	16		132
1530160	*Oscillatoria limnetica*	Circle based cylinder - long	Circle based cylinder - long	3		71
1530170	*Oscillatoria limosa*	Circle based cylinder - long	Circle based cylinder - long			

1530230	*Oscillatoria redekei*	Circle based cylinder - long	Circle based cylinder - long		52	
1530000	*Oscillatoria sp.*	Circle based cylinder - long	Circle based cylinder - long			
16470010	*Pandorina morum*	Sphere	Sphere			
16470000	*Pandorina sp.*	Sphere	Sphere			
17680020	*Pediastrum biradiatum*	Circle based cylinder - short				
17680030	*Pediastrum boryanum*	Circle based cylinder - short		31	258	716
17680050	*Pediastrum duplex*	Circle based cylinder - short		19		293
17680080	*Pediastrum simplex*	Circle based cylinder - short		58	95	130
17680000	*Pediastrum sp.*	Circle based cylinder - short				
17680090	*Pediastrum tetras*	Circle based cylinder - short		4000		32226
9360000	*Pedinella sp.*		Oval based ellipse			
6110050	*Peridinium cinctum*		Oval based ellipse	15		189
6110000	*Peridinium sp.*		Oval based ellipse	15		167
6110100	*Peridinium willei*		Oval based ellipse			
4070000	*Phacus sp.*		Oval based ellipse	19	21	24
90000000	*Picoplankton - unidentified single cells <2 µm diam.*		Sphere			
17690010	*Planktosphaeria gelatinosa*		Sphere			
9430000	*Pseudokephyrion sp.*		Circle based ellipse	448		1732
9550000	*Pseudopedinella sp.*		Sphere		523	
17780000	*Quadrigula sp.*		Cone			

5100010	*Rhodomonas lacustris*		Cone + hemisphere	12	35	144
5100012	*Rhodomonas lacustris var nannoplanctica*		Cone + hemisphere			
5100020	*Rhodomonas lens*		Cone + hemisphere	31		485
5100000	*Rhodomonas sp*		Cone + hemisphere			
17810030	*Scenedesmus acuminatus*		Circle based ellipse	26		121
17810080	*Scenedesmus armatus*		Circle based ellipse	4		68
7810160	*Scenedesmus communis*		Circle based ellipse		353	
17810220	*Scenedesmus falcatus*		Circle based ellipse	44		107
17810340	*Scenedesmus opoliensis*		Circle based ellipse	31		421
17810000	*Scenedesmus sp.*		Circle based ellipse	44		283
17830030	*Schroederia setigera*		Cone	32		103
17830000	*Schroederia sp.*		Cone		124	
12000004	*Very small centric diatom (<5 µm diam.)*		Circle based cylinder - short	3		102
12000001	*Small centric diatom (5-<10 µm*		Circle based cylinder - short			
13000001	*Small pennate diatom <10 µm*		Cuboid/rectangle	73	110	411
1750010	*Snowella lacustris*	0.75 * sphere	Circle based ellipse		1415	
1750000	*Snowella sp.*	Sphere	Sphere			
17910020	*Sphaerocystis schroeteri*		Sphere			
17910000	*Sphaerocystis sp.*		Sphere			
9450000	*Spinifertomonas sp.*		Sphere			
27360040	*Spondylosium planum*		Oval based ellipse			
27380330	*Staurastrum cingulum*			187	643	8181
27380840	*Staurastrum longipes*					
27380860	*Staurastrum lunatum*					
27380000	*Staurastrum ophiura*			11		394
27381120	*Staurastrum planctonicum*			19		289
27370000	*Staurastrum sp.*					
27381460	*Staurastrum tetracerum*			28		430

27390190	*Staurodesmus incus*					
27390000	*Staurodesmus sp.*			36	147	793
12180000	*Stephanodiscus sp.*		Circle based cylinder - short			
9480000	*Stichoglea sp.*		Circle based ellipse		69	
13810010	*Synedra acus*		Cuboid/rectangle			
13810120	*Synedra nana*		Cuboid/rectangle	139		905
13810000	*Synedra sp.*		Cuboid/rectangle	30		387
13810180	*Synedra ulna*		Cuboid/rectangle			
9530000	*Synura sp.*		Circle based ellipse	9		113
13820010	*Tabellaria fenestrata*		Cuboid/rectangle	29		157
13820020	*Tabellaria flocculosa*		Cuboid/rectangle			
13820022	*Tabellaria flocculosa var. asterionelloides*		Cuboid/rectangle	41	218	247
13820000	*Tabellaria sp.*		Cuboid/rectangle			
17960010	*Tetraedron caudatum*		Cuboid/rectangle	11	45	130
17960030	*Tetraedron minimum*		Cuboid/rectangle		570	916
17960000	*Tetraedron sp.*		Cuboid/rectangle		377	
17970000	*Tetrastrum sp.*		Cone + hemisphere	21436		95529
17970050	*Tetrastrum staurogeniaeforme*		Cone + hemisphere	8150		33809
17970060	*Tetrastrum triangulare*		Cone + hemisphere			
4100000	*Trachelomonas sp.*		Circle based ellipse			
18010010	*Treubaria setigera*		Circle based cylinder - short			
90000004	*Unidentified cells >20 µm diam.*		Sphere	129	154	262
17000001	*Unidentified colonial greens.*		Sphere		22763	
1000000	*Unidentified cyanophytes - colonial algae <2 µm diameter.*		Sphere	1916		15215
90000005	*Unidentified flagellates 2 – 20*					

	µm diam.		Sphere		1767	
17000000	*Unidentified small green round cells (sgrt)*		Sphere		7503	
9540000	*Uroglena sp.*		Circle based ellipse		3031	
12200000	*Urosolenia*		Cone		1608	
12200010	*Urosolenia eriensis*		Cone			
12200020	*Urosolenia longiseta*		Cone		48444	
16770010	*Volvox aureus*		Circle based ellipse			
16770010	*Volvox sp.*		Circle based ellipse			
1780010	*Woronichinia naegeliana*	0.2 sphere	Circle based ellipse			
1780000	*Woronichinia sp.*	0.2 sphere	Circle based ellipse			
27430020	*Xanthidium antilopaeum*		Oval based ellipse	163	323	696
27430000	*Xanthidium sp.*		Oval based ellipse			

Members assisting with these methods.

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Correspondence

However well procedures may be tested, there is always the possibility of discovering hitherto unknown problems. Analysts with such information are requested to contact the Secretary of the Standing Committee of Analysts:

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Amendment History

Enumeration and Identification of Algae is a new book. Therefore, there isn't an amendment history in this occasion.

standing
committee of
analysts