Appendix 4

PRESERVATIVES AND METHODS FOR ALGAL CELL ENUMERATION

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1 INTRODUCTION

There are multiple ways to preserve phytoplankton samples and determine the algal species composition and abundance. This Appendix provides details on some of the most common methods. Additional relevant publications are included in Section 5, References. One of the most useful is Karlson et al. (2010), which can be downloaded at http://hab.ioc-unesco.org/index.php?option=com_oe&task=viewDocumentRecord&docID=5440.

2 MISCELLANEOUS SUPPLIES, RECIPES, AND PROTOCOLS

2.1 Preservatives

Lugol's solution has proved to be a suitable preservative for many phytoplankton samples. The solution preserves the shape of the algal cells; for flagellated species, the flagella remain attached to the cells and the iodine staining of the algal cells gives a good contrast between the organisms and the background when using phase-contrast microscopy. For seawater samples, neutral or alkaline Lugol's solution is most appropriate because calcareous flagellates (coccolithophorids) can comprise a considerable proportion of the algal biomass. If acidic Lugol's solution is used, a parallel sample fixed in alkaline Lugol's or with hexamine buffered formalin should be taken because the acid dissolves the calcareous casings (coccoliths) which are the basis for species determination of coccolithophorids. Diatoms, which have silicate cell walls, preserve well in acidic Lugols. In samples fixed with alkaline Lugol's solution, fungal infections may occur, and for long-term storage, such samples should be post-fixed by adding a few drops of concentrated formalin solution. If the samples are to be stained with Calcofluor to enhance the pattern of cellulose plates on

armored dinoflagellates, it is an advantage to use neutral or basic Lugol's solution or a buffered formaldehyde solution, because acid prevents staining of the cellulose.

If cells are rugged and do not distort, a 2-5% formaldehyde solution is generally used, working from a stock formalin sample (37% formaldehyde) that is often acidified with acetic acid. Recipes for these fixatives follow:

Acidic Lugol's solution: Potassium iodide (KI) 100 g, Distilled water 1000 mL, Iodine (I_2) 50 g, Acetic acid glacial (100 % CH3COOH) 100 g, Neutral Lugol's solution, Potassium iodide (KI) 100 g, Distilled water 1000 mL, Iodine (I_2) 50 g.

Alkaline (basic) Lugol's solution: Potassium iodide (KI) 100 g, Distilled water 1000 mL, Iodine (I_2) 50 g, Sodium acetate (CH_3COONa) 100 g.

Neutral, weakly basic formalin solution: Analytical quality formalin is diluted to 20 % (1 part concentrated solution to 1 part distilled water). 100 g hexamethylenetetramine (hexamine) $C_6H_{12}N_4$ is added to 1 L of the 20 % formaldehyde solution.

Acidic formalin: Equal amounts of analytical quality formalin (formaldehyde 37 %) and concentrated acetic acid are mixed.

2.1.1 Advantages of Lugol's solution

Lugol's enters the cell more quickly than formaldehyde, leaving shock-sensitive organisms better preserved in the sample. The specific weight of organisms is increased by iodine, resulting in faster settling times. Detection of phytoplankton cells is made easier by the enhanced contrast between organisms and the surrounding fluid. The iodine stains starch, aiding recognition of those groups of algae (e.g. Chlorophyta) which use this as a storage compound.

2.1.2 Advantages of formaldehyde

Formaldehyde is a good fixing and preserving agent for cells that have a relatively rigid cell wall, as the cell wall structures and other characteristics such as eye spots remain visible. When stored properly in appropriate bottles, samples will stay in good condition for many years without attention. Autofluorescence of chlorophyll-*a,* though decaying, remains intact for at least several days if the samples are stored in constant dark.

2.2 Sample storage

Preserved phytoplankton samples should be stored in the dark at room temperature to prevent photo-oxidation. The maximum storage time for iodine (Lugols)-fixed samples that have been stored in a dark and cool place (1 to 5 °C) is 12 months. During this period, the samples should be checked every second month and topped up with Lugol's solution if necessary (the sample should be golden brown in color, like tea). Reference material should be collected and stored in suitable separate sample bottles. For all phytoplankton samples to be stored for more than 3 to 4 months, dark glass bottles are the preferred container.

2.3 Settling chambers

If an inverted microscope is to be used (which is highly recommended), consider purchasing the Hydrobios Combined Plate Chamber (Utermöhl-Chamber) set or the equivalent, with one cylinder each of 10, 50, and 100 mLs (Figure 1). Three cover plates are also needed. There

are two versions of the chambers. The sliding version is desirable since the main part of the chamber is removed during analysis. This makes it possible to have better illumination and to work at higher magnifications. The non-sliding version can be rotated, which is useful when analyzing several transects (diameters).

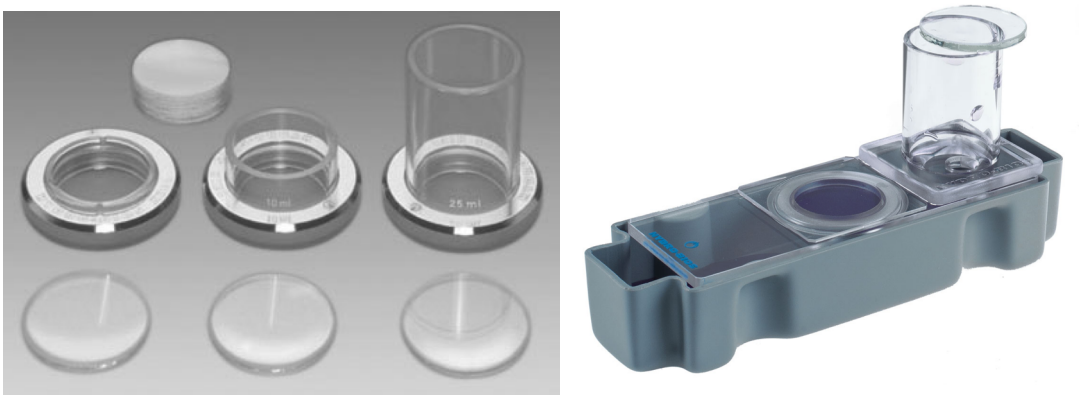


Figure 1. Settling chambers for the Utermöhl technique. Left: chambers and chimneys for different volumes. Right: a stand for the settling chambers that will collect the water sample when the chimney is moved sideways, away from the observation chamber. Square cover slip for the observation chamber is at left. Photos: Hydrobios. http://www.hydrobios.de/product/combined-plate-chamber/

2.4 Inverted microscope equipped with phase contrast and fluorescence

Inverted microscopes are very useful for phytoplankton counting, since samples can be settled and counted directly in the settling chamber. If an inverted microscope is not available, then it is also possible to use a standard microscope, but with a Sedgewick Rafter slide (1 mL capacity; Figure 2) for counting. In this instance, the water sample needs to be settled in centrifuge tubes or graduated cylinders, the overlying water removed using aspiration, and then a subsample taken from the residue (after mixing) for counting, taking careful consideration of the initial and final volumes. More details on filling the Sedgewick Rafter slide are given below. It is useful to have a microscope fitted with a digital camera to take pictures. If the camera can take pictures under fluorescence illumination, this is preferred.

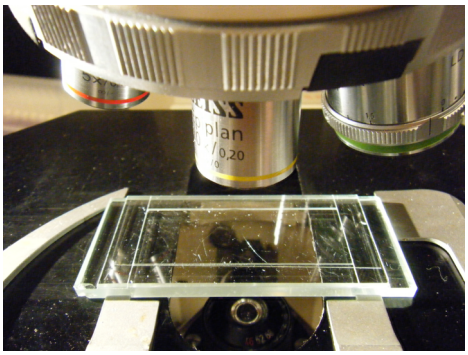


Figure 2. Sedgewick Rafter counting slide. Photo: D. Kulis.

2.5 Software that aids counting and data processing

There are free (e.g. Plankton Toolbox; Karlson et. al. 2015) and commercial software that can aid in the process of counting phytoplankton samples. The Plankton Toolbox provides a counting module that may be used on a computer placed next to the microscope during analysis of samples. The software facilitates the counting of many species concurrently, and also helps in calculations of the cell volumes of the organisms. Cell volumes are used to calculate the biomass of the plankton.

Figure 3. Single-position counter.

2.6 Manual cell counter

Manual counters are very useful, but surprisingly expensive. A multi position counter is the most useful – one on which the worker

can keep count of 5 or more species at the same time, but smaller, single counters are also useful (Figure 3).

2.7 Qualification requirements for staff

Technical staff should have competence in identification of marine phytoplankton, preferably through formal training courses in marine botany (algal flora) at the university level or through practical experience in phytoplankton identification. Short courses are offered, such as those given by the IOC HAB Programme:

http://hab.ioc-unesco.org/index.php?option=com_content&view=article&id=32&Itemid=0

A database of pictures of the species present in the samples is useful and can clarify doubts in identification for analysts with less experience. A limited HAB species image database with some species description details is found in Appendix 1.

2.8 Assistance with species identification

The best advice to plant operators seeking to mitigate the effects of a specific algal bloom is to collect samples and identify the causative organism, hopefully to the species level, but at least to genus. With some training and modest microscope facilities, this can be done on site (Chapter 3). There are also outside experts and services that will do this type of work on demand, but this will cost time. The Intergovernmental Oceanographic Commission (IOC) Science and Communication Centre on Harmful Algae, University of Copenhagen, Denmark http://hab.ioc-unesco.org/ can offer assistance in identification of microalgae. Small samples with a limited number of species will be examined free of charge while analysis of a larger number of samples may incur a charge. There are also several other institutes, universities and companies that have the capability to analyze phytoplankton samples.

3 ENUMERATION OF PHYTOPLANKTON USING INVERTED MICROSCOPY (THE UTERMÖHL TECHNIQUE)

The most common method for counting phytoplankton uses an inverted microscope (i.e., one in which the sample sits above the microscope optics) and the Utermöhl method for settling and concentrating the sample. The following sections describe this procedure in detail. For facilities that only have a standard microscope, another cell counting method using the Sedgewick Rafter slide is described in section 4 below.

3.1 Equipment and reagents

Sample containers must be clean, not permeable, watertight, wide mouth, and typically are 100-250 mL in volume. Sample containers must not be completely filled, as some air at 20% of the total capacity will facilitate the homogenization of the sample before settling.

Settling chambers consist of a cylinder and a thin base (no more than 0.17 mm) allowing observation from below using the inverted microscope. The chambers must be cleaned between samples using a thin paintbrush, detergent and distilled water. The settling chambers must be calibrated.

The inverted microscope should be equipped with phase contrast and/or Nomarski (Differential Interference Contrast = DIC), and if possible a digital camera. The oculars must have a micrometer and a reticule. The microscope must be calibrated for each objective. Fluorescence is a useful capability on the microscope.

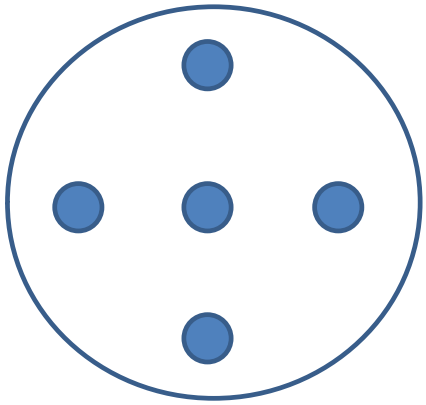
3.2 Procedure

1. For monitoring of phytoplankton it is important to analyze the samples rapidly, so it is better to maintain the samples close to room temperature during transport by keeping the

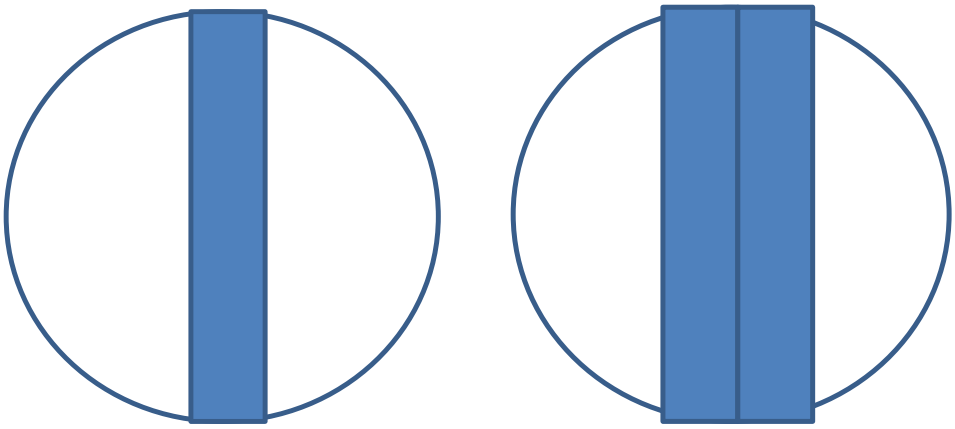
samples inside a cooler with cooling blocks. On arrival to the laboratory, the samples should be placed on the bench. A portion of the sample is saved for live examination and another portion preserved (if this was not done in the field) and either stored or settled. To demonstrate that the temperature in the room and the temperature in the sample are close, a thermometer for the air temperature in the laboratory and other thermometer to measure temperature in the water sample are used. In a notebook, records are kept of the code of the sample, the complete reference of the sample, the date and hour of settling, the number and volume of the settling chamber, temperature in the laboratory and in the sample, and the analyst who did the settling.

2. During sample storage, suspended particles settle out and (small) algae become indiscernible by incorporation in detritus aggregates or by adhesion to other large algal cells. Resuspension and separation of particles before settling them in the Utermöhl chamber can be achieved by shaking the sample as gently as possible. The method used for manual shaking should be described clearly in order to minimize differences between operators. A combination of alternating horizontally rolling and vertical turning upside down of the sample bottle for a specific number of times provides better mixing than straightforward shaking. The homogenization of the samples must be gentle, combining circular horizontal movements with vertical movements.
3. One subsample is maintained alive for *in vivo* examination and another is preserved in Lugols. Samples must be preserved as soon as possible after collection. Live samples must be observed within 36 h. Preserved samples must be stored in the dark; room temperature is appropriate if the analysis is done within 3 weeks. Otherwise, refrigerate at 4^{o}C. Lugol's- fixed samples can be stored for 1 year in the dark and at temperatures between 1-5ºC; for longer periods of storage it is necessary to add formaldehyde.
4. The volume of the settling chamber must be adapted to the type of waters to be analyzed: 100 mL for oligotrophic waters, 50 mL for mesotrophic, 5-25 mL for eutrophic. For nanoplankton and picoplankton the settling chambers of 100 mL are not adequate and require preconcentration. The settling chambers must be placed on a horizontal surface, all the chamber must be filled with the sample without air spaces, and the chamber must be closed using a glass cover. The bench must be free of vibrations. The recommended settling time depends on the volume (height) of the chamber, 48 h for 100 mL, 24 h for 50 mL, 12 h for 25 mL, 8 h for 10 mL, 3 h for 2 mL. After settling, the cylinder is displaced and a glass cover is placed on top of the chamber.
5. Preserved samples must be acclimated to the ambient temperature, homogenized and transferred directly from the container to the settling chamber. Temperature differences between sedimentation chamber and medium may produce convection currents that have differential effects on the settling of phytoplankton species, depending on their physical properties. Furthermore, bubbles may develop in relatively cold samples as the solubility of gases declines with the gradual rise of the temperature of the sample. When the temperature of the chamber is higher than that of the sub-sample, the larger and heavier particles tend to concentrate towards the chamber wall and the smallest particles more towards the center of the chamber. If the subsample has a higher temperature, the smallest particles collect near the chamber wall.
6. There are three counting strategies; the strategy to choose depends on the abundance and size of each species.

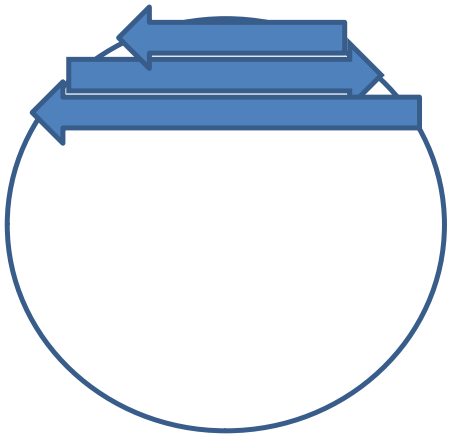
Quantification by fields: 5 fields randomly distributed in the chamber; more fields can be counted to increase the total number of cells counted.



Quantification using transects, 1 or 2 across the middle of the chamber one on the side of the other.



Quantification using the full chamber: counting all cells present in the total volume settled.



For the 3 strategies, it is necessary to define a normal procedure for the quantification of cells at the boundary of the field; for example, do not include cells lying across the top line and the left line but include the cells crossing the lower and the right lines of the reticule.

As a general rule, if a precision of 5% in the estimation of the mean number of cells per sample is required, then 400 objects should be counted. It makes no difference whether 10 fields are counted with 40 objects per field or 80 fields with just 5 objects. It is, however, sometimes difficult to count 400 cells, so in practice, the number of cells counted should be at least 50-100 cells in total for each dominant species. The area to be counted should be adjusted to reach this number of cells. For some species this will not be possible because they are in low density. In that case, the full chamber should be examined.

3.3 Calculations

The following equation related the number of cells per unt volume to the area examined:

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

N is the number of cells per unit volume (e.g., cells/mL)
X is the average number of cells per field, transect or full chamber
A is the area of the chamber
v is the volume of the chamber. If the result is in mL then we use mL, if the result is in L we will use L
a is the area of the total field counted
d is the dilution factor (when the sample is diluted)

Example of calculations for the case of counting 10 cells in a full chamber (area 529.13 mm^2) after settling 50 mL.

$$N = 10 * \frac{529.13 * 1}{529.13 * 50\ mL} = 0.2\ cells/mL = 200\ cells/L$$

4 PHYTOPLANKTON COUNTING USING A SEDGEWICK RAFTER SLIDE

For laboratories that do not have an inverted microscope, an alternative to the Utermohl's technique is to use a Sedgewick Rafter slide (SRS) and a regular compound microscope (see sction 2.4). The Sedgewick Rafter slide holds exactly one mL. The thickness of the slide generally restricts magnification to 200X or lower.

4.1 Procedure

The SRS without grid with cover glasses is available from Wildlife Supply Company, 95 Botsford Place, Buffalo NY 14216 (www.wildco.com). Two styles are available – one without, and one with grids. The latter is useful if only a portion of the slide is to be counted.

1. The preserved sample can be counted directly if the cell concentrations are high enough, or the sample can be concentrated by settling a known volume in a graduated cylinder for several days, with the settled material careful removed from the bottom with a pipette, and its volume measured.
2. Swirl the sample manually or with a mechanical agitator or vortexer to randomize the organisms.

3. Withdraw a 1.0 mL sample and dispense it into the SRS. This is best accomplished by rotating the cover slip so that it sits diagonally across the SRS such that small openings are in each diagonal corner. The sample is pipetted into one open corner, and air exits from the other opening. When the slide is full, straighten the cover slip so there are no more openings. Placement of the cover glass limits the volume to 1 mL.
4. The gridded SRS has 20 rows, 50 columns, and 1000 squares. Using a microscope magnification that allows the cells of interest to be easily identified, move the microscope stage laterally, counting cells within a gridded row. Repeat with a return transect across the slide, either directly below the first transect, or randomly – the latter if only a portion of the slide is to be scanned. Have a consistent policy for cells that fall on a line. For example, do not include cells lying across the top line, but do count those lying across the bottom line. Try to count 100 - 400 algal cells, and calculate cells/mL from that. For example, if you counted 150 cells in 8 rows, you have 150 * 20/8 = 375 cells/mL. This should be multiplied by the concentration factor determined from the settling step in # 1 above, if that was done.
5. Note that if you have a non-gridded SRS, you can still count less than the entire slide. Just move the microscope stage horizontally, counting all cells that are visible in the field of view, and then when the edge of the chamber is reached, slide the field of view vertically to a new, random position, and then scan horizontally to the opposite edge. Repeat with another random scan. Keep track of the number of horizontal scans until you have counted 100 – 400 cells of interest. You can then count how many horizontal scans there are along the vertical edge of the SRS, and use the same approach described above for the gridded SRS to calculate a cell concentration.

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