4

Estimating cell numbers

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4.1 Introduction

Quantification of microalgae is one of the basic routines in HAB monitoring programmes or research projects. Qualitative investigations will reveal which species to take into account, but when assessing risks precise knowledge on the abundance of the harmful species is essential. In general, high concentrations of harmful algae indicate a high risk of harmful effects, whereas the same species may appear in low concentrations with no harmful effects. The harmful concentration of a HAB organism is species-specific. Some algae are harmful at low concentrations, such as species from the genus *Alexandrium,* which may cause accumulation of PSP toxins in shellfish at concentrations of a few hundred cells per litre, whereas other species such as *Karenia* (*Gymnodinium) mikimotoi* can cause fish kills at millions of cells per litre (Zingone and Enevoldsen, 2000). This chapter discusses several microscope-based methods for quantification of microalgae at the species/genus level. Prior to that we present guidelines to sampling and preservation of samples. Furthermore we suggest a simple appropriate statistical routine to calculate the precision of counts as well as how to estimate microalgal biomass using geometric formulae and appropriate conversion factors. A culturing approach (serial dilution culture method) for the estimation of selected HAB species is also presented. If algal data are to be available for analysis, or to be compared with events elsewhere or in the past, it is important to have easy access to validated data. We discuss the use of databases and spreadsheets for data storage and handling as well as quality assurance measures to be taken.

4.2 Planktonic microalgae

4.2.1 Sampling

Qualitative, concentrated samples are best collected by vertically towing a plankton net (mesh size 20 µm) to cover the depth range of interest. The plankton net should be drawn, several times, to the surface of the water, until the water in the sample collector becomes coloured by the algae. Quantitative samples (water/bottle samples) can be collected using a water bottle (e.g. Niskin, Nansen) at different depths to cover the depth range of interest. Depth intervals between sampling should be 2–5 m, dependent on local conditions. The samples from the different depths can be pooled and counted as one sample representative of the whole water column. An alternative to sampling with water bottles is the use of a hose for sampling the whole water column as described by Lindahl (1986), for example.

4.3 Fixation

Immediately after collection the samples must be preserved for later analysis in the laboratory. Microalgal samples should be preserved using either neutral or acidic Lugol's solution (Table 4.1), which produces good preparations for light microscopy and is of low toxicity to humans. If the brownish coloration of the algae, caused by Lugol's imposes a problem in taxonomic investigations, the coloration can be removed by oxidizing the Lugol's using a few drops of a solution of sodium thiosulfate per ml (3 g $Na_2S_2O_3$ for 100 ml of water) of sample (Pomeroy, 1984). Fixation of samples using Lugol's increases the settling velocity of microalgae compared with samples fixed in formaldehyde (Table 4.2).

Table 4.1 Recipes for Lugol's solution (acidic, alkaline or neutral)

Acidic	**Alkaline**	**Neutral**
20 g potassium iodide	20 g potassium iodide	20 g potassium iodide
10 g I_2	10 g I_2	10 g I_2
200 ml distilled water	200 ml distilled water	200 ml distilled water
20 g acetic acid	50 g sodium acetate	

Note: Add approximately 0.5–1.0 ml Lugol's/100 ml sample – the fixed sample must be brownish (the colour of brandy).

Table 4.2 Recipes for formaldehyde fixatives

Neutralized formaldehyde[a]	**Formaldehyde (non-neutralized)**[b]
500 ml 40% formaldehyde	100 ml 40% formaldehyde
500 ml distilled water	700 ml filtered seawater
100 g hexamethylenetetramine	

Note: pH 7.3 to 7.9
Filter after one week

Sources:
a. Edler (1979).
b. Andersen and Sørensen (1986).

Neutralized formaldehyde should be used with care in a fume hood, because of its toxicity (potential carcinogen), and its potential to develop allergic reactions in humans. Edler (1979) recommends the use of a 20% formaldehyde working solution and a final concentration of 5% or more. Andersen and Sørensen (1986) used a working solution of 5% and a final concentration of 1.5% (Table 4.2). Use of fresh formaldehyde that is less than six months old is recommended, especially if non-neutralized because this fixative becomes acidic over time.

4.4 Storage/maintenance of fixed samples

Keep the fixed samples in glass bottles in the dark to prevent the Lugol's from being degraded by oxygen and light. Check fixation each month and add new Lugol's to the samples if they turn 'clear' (non-brownish). Properly fixed samples can last for years.

4.5 Handling of samples

4.5.1 Concentration/dilution of samples

Prior to quantitative analysis it may be useful to either concentrate or dilute the samples to obtain concentrations that can be handled using available techniques. Samples can be concentrated by a factor of 10 to 100 by settling of the cells in a measuring cylinder. Alternatively, cells can be concentrated by gentle filtration using, for example, a plankton net (mesh size 10 μm or 20 μm) or a GFC filter with a vacuum pump. Furthermore, microalgae can also be concentrated using the gentle 'inverted filtration method' described in Sieburth (1979). Note that cells can be lost during the concentration procedure. In most cases it is preferable that algal samples are fixed before concentration. If the microalgae are too concentrated the samples can be diluted with filtered seawater.

4.5.2 Setting up samples for quantification

Samples must be adapted to room temperature to reduce the risk of air-bubble formation in counting cells/chambers. To make sure that the algae are evenly distributed in the sample, turn the bottle upside down some 100 times before subsamples are extracted from the bottle. In the case of counting colonial species such as the cyanobacterium *Microcystis*, the colonies can be fragmented into single cells using sonication for 15–60 s at 20–40 kHz before counting (Cronberg, 1980).

4.6 Counting techniques

Prior to quantitative analysis of harmful algae, qualitative analysis of concentrated plankton is a must to establish which species could be misinterpreted during quantification. The magnification used when counting the different species must be adapted to the size of the species of interest (Table 4.3). Normal light microscopy is useful in general. Phase-contrast light microscopy may be useful in the case of species with delicate structures such as spines, scales, flagella and lorica.

Table 4.3 Recommended microscope working magnifications for counting different-size classes of microalgae

Class	Magnification
0.2–2.0 μm (picoplankton)	1 000×
2.0–20.0 μm (nanoplankton)	100–400×
>20.0 μm (microplankton)	100×

Source: HELCOM (2000).

4.6.1 Choice of method

Microalgae can be quantified by a range of different methods based on compound microscopy, inverted microscopy or epifluorescence microscopy (Table 4.4).

TABLE 4.4 Summary of methods for quantification of harmful microalgae

Methods for quantification of microalgae	Volume (ml)	'Sensitivity' (cells l^{-1})	Preparation time
Compound microscopy			
Drops on slide	0.02	50 000–100 000	1 min
Sedgewick-Rafter cell (counting cell)	1	1 000	15 min
Palmer–Maloney cell (counting cell)	0.1	10 000	15 min
Inverted microscopy			
Utermöhl (sedimentation chamber)	2–50	20–500	2–24 hours
Epifluorescence microscopy			
Counting on filters (fluorochrome: Calco Fluor, DAPI, Acridine Orange, etc.)	1–100	10–1 000	15 min

Source: Andersen (1995).

4.6.2 Compound microscopy

A simple method of quickly estimating the concentration of microalgae is to use the 'drop on slide' method. One drop of seawater is approximately 20 µl or 0.02 ml. The drop is placed on a slide, mounted with a cover slip and counted at appropriate magnification to observe the species of interest. If you find one cell in the drop this is equal to approximately 50 cells ml^{-1} = 50,000 cells l^{-1}, see Table 4.4. This method is only useful to screen for algae in high concentrations.

With concentrations of harmful algae $>10^4$ cells l^{-1} counting using a compound microscope and a counting cell is simple and fast (Table 4.4). If, on the other hand, the cell concentration is $<10^2–10^4$ cells l^{-1}, the cells must be concentrated before counting. This is a time-consuming procedure. In this case counting using either inverted microscopy or epifluorescence microscopy is preferable (see below).

Counting cells are available in several different volumes (Fig. 4.1). A well-known type is the Sedgewick-Rafter cell, with a volume of 1 ml. The bottom plate of the Sedgewick-Rafter cell is divided into 1,000 squares, each representing 1/1,000 of the volume of the cell. Other types of counting cell hold different volumes (Table 4.4). You can make your own counting cells using a slide mounted with a 'frame' on top. The exact volume of the home-made counting cell may be estimated as the difference in weight between the empty counting cell (including the cover slip) and the filled counting cell (including the cover slip) carefully dried on the outside of the chamber using a tissue to remove excess water.

Using a counting cell you can choose either to count the microalgae in the entire bottom of the chamber or only a fraction of the entire bottom. Knowing the fraction of the entire bottom counted and the number of microalgae encountered, the total number of microalgae in the counting cell can be calculated. Remember to incorporate the dilution/concentration factor of the sample when calculating the concentration of the microalgae (cells l^{-1}).

4.6.3 Inverted microscopy

Quantification of harmful algae using inverted microscopy and sedimentation chambers according to Utermöhl (1958) is useful for counting algae in rather low

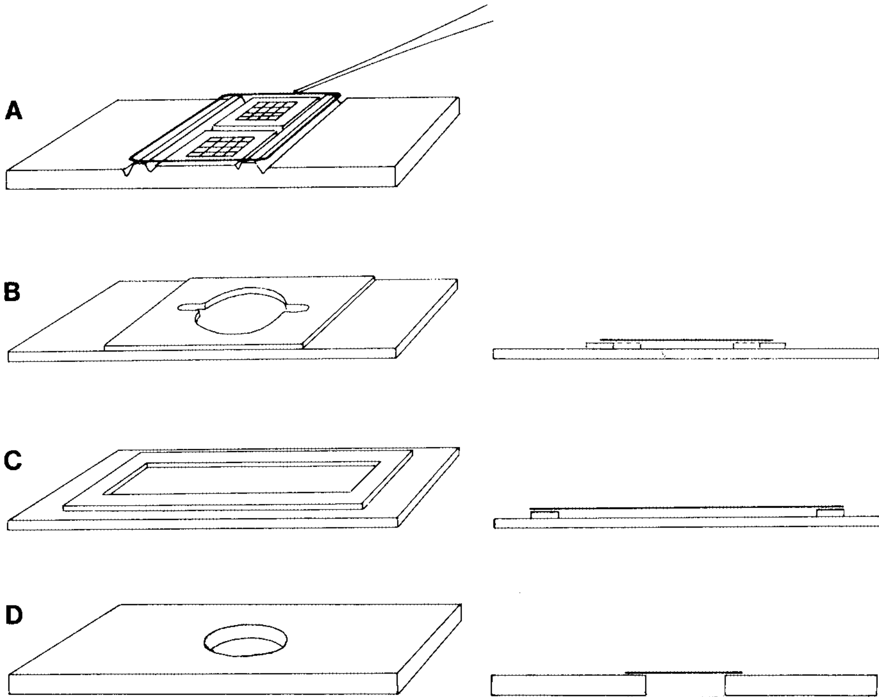

Figure 4.1
Counting cells.
Source: Throndsen (1995).

concentrations (<10^2–10^4 cells l^{-1}) (Sournia, 1978). If concentrations are higher the samples can be diluted using filtered seawater before counting. Equipped with suitable slide holders, an inverted microscope is also excellent for qualitative examination of normal slide preparations or quantitative analysis using counting cells. Sedimentation chambers are available from different companies in a range of volumes from 2.5–50 ml. In order to keep the sedimentation chambers tight, the bottom of the 50 ml settling cylinder may be greased with a thin layer of Vaseline before it is mounted on the plate chamber. Likewise, the top of the settling cylinder may be greased to keep the cover slip in place and to keep the settling cylinder properly sealed and airtight. During filling and sedimentation, the sedimentation chambers should be placed on a horizontal surface to promote a random distribution of the microalgae settling out. Furthermore, it is very important that the supporting surface is vibration-free, as vibration can cause cells to accumulate in ridges (HELCOM, 2000).

Settling of cells in the sedimentation chamber lasts from a few hours to several days depending on the sample volume (the height of the chamber), the fixative used and the linear dimension of cells. In general, small cells have much longer sedimentation times than large cells. As a rule large cells (L >10 µm) must be allowed to settle for at least 12 hours before counting, while smaller cells must be allowed to settle for approximately 24 hours before counting (Table 4.5). Sedimented samples not

counted within a week should be discarded. Sedimentation chambers of 100 ml should be used with caution as convection currents have been reported to interfere with sedimentation of the microalgae in chambers taller than five times their diameter (Nauwerck, 1963; Hasle, 1978).

TABLE 4.5 Minimum sedimentation times for Lugol's fixed samples

Volume of sedimentation chamber (ml)	Approx. height of sedimentation chamber (cm)	Sedimentation time (h)	
		Lugol's (acidic)	Formaldehyde (neutral)
2	1	3	12
10	2	8	24
50	10	24	48

Source: adapted from Edler (1979).

The bottom of the sedimentation chamber is scanned at different magnifications and a preliminary species list is produced. If the distribution of microalgae on the bottom of the chamber is visually non-random, one-sided or in ridges due to factors such as vibration, the sedimentation chamber should be discarded. According to the size of the different species as well as the abundance of the species, a strategy for counting each of the species of interest is set up, including

- choice of magnification;
- the total bottom of the chamber or subsampling units (half the bottom, diagonal counting, counting grids, etc.) (Fig. 4.2);
- approximate number of subsamplings.

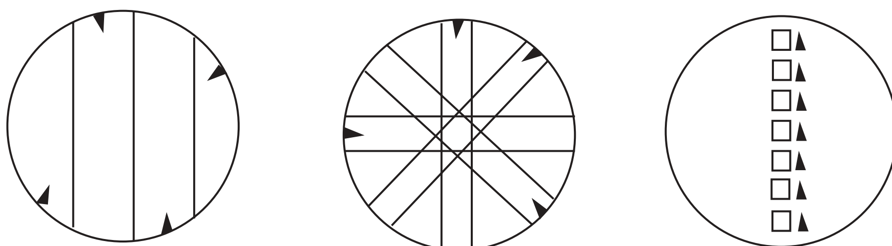

Figure 4.2
Counting strategies using sedimentation chambers. A, counting the entire bottom; B, counting diagonals; C, counting grids (the grid is mounted in one of the eyepieces).
Source: adapted from HELCOM (2000).

The goal of the counting strategy is to obtain a count of 50 to 100 cells of species of interest to assure a proper estimate of the concentration of each. Counting statistics are discussed below. If possible, the individual number of cells in colonial species should be counted. In the case of filamentous species without clearly differentiated cells, such as the genus *Nodularia* or *Trichodesmium*, the number of filaments is counted. In many cases the cells will not be randomly distributed on the bottom of the sedimentation chamber. The larger species often tend to accumulate at the edge of the chamber and the smaller species in the central part (Olrik *et al.*,

1998). Regardless of the counting strategy, it is important to compensate for non-random distribution either by counting diagonals, grids along a diagonal, or the entire bottom of the sedimentation chamber. When counting cells in a diagonal or within a grid you have to make a rule as to which cells are inside/outside the counting area. A simple rule could be that all cells inside or touching the left side of the diagonal are counted while cells outside or touching the right side of the diagonal are omitted from the count.

To calculate the concentration (cells l^{-1}) of the different species in the counting chamber you need to know

V = volume of chamber (ml);

B_a = area of bottom of chamber (mm^2);

B_c = area of part of bottom counted (mm^2);

N = number of cells scored for species of interest;

conversion factor (CF) = B_a/B_c.

The concentration C of species (cells ml^{-1}) is then

$C = N \times (B_a/B_c)/V$.

It is not possible to obtain general conversion factors to be used for all combinations of microscopes, counting magnifications and counting/sedimentation chambers when calculating concentrations from counts such as those done by diagonal counting. This is because the conversion factors are dependent on the magnification used as well as the type of sedimentation chamber. The conversion factors are to be calculated for each microscope and each subsampling area for each magnification, as well as for each type of sedimentation chamber (Table 4.6). Note that the dimensions of commercial chambers vary. It may be necessary to label and calibrate each chamber separately.

4.6.4 Quantitative epifluorescence microscopy

The basic principles of quantitative epifluorescence microscopy are concentration and staining of cells on membrane filters (Fig. 4.3), followed by quantification of cells using an epifluorescence microscope (Fig. 4.4). The method is useful for counting algae in low concentrations ($<10^2$–10^4 cells l^{-1}). If concentrations are high the samples can be diluted using filtered seawater. For thecate dinoflagellates the fluorochrome Calco Fluor White MR2, a specific stain for cellulose, is excellent (Lawrence and Triemer, 1985; Andersen and Kristensen, 1995). For quantification of harmful algae in general, other stains such as Acridine Orange (Andersen and Sørensen, 1986) or DAPI (Porter and Feig, 1980) can be very useful. A great advantage of this methodology is that large volumes (50–100 ml) can be prepared for quantification in a few minutes, and that specific stains, such as Calco Fluor White, allow for counting thecate dinoflagellates in low concentrations in situations where the overall cell concentration and/or the concentration of detritus is very high, because only the thecate dinoflagellates will 'light up' in the preparation.

A normal compound microscope can be transformed into an epifluorescence microscope if it is equipped with a halogen lamp/mercury burner and suitable filter sets for the stains used, see below.

Follow the procedure described in Sections 4.5 and 4.6. For quantitative epifluorescence microscopy the sample does not need to be adapted to room temperature because the formation of air bubbles is not a problem when preparations are made on filters.

The sample is prepared following this procedure:

1. Measure out the volume of sample to be counted using a measuring cylinder. Stains such as Acridine Orange or DAPI can be added directly to the sample at this point. If you use Calco Fluor White for staining thecate dinoflagellates, the stain must be added later, see steps 4 and 5.
2. Add the sample to the filtration unit (pore size of filter e.g. 5 µm). You can use other pore sizes depending on which species are to be concentrated and quantified.

TABLE 4.6 Example of calibration table used for calculating concentrations of microalgae by inverted microscopy and Utermöhl method (area of sedimentation chamber 531 mm^2)

	swi (small window)	**wi (window)**	**dwi (diagonal window)**
	L (mm) Area (mm^2)	*L* (mm) Area (mm^2)	Area (mm^2)
4×	0.202 0 0.040 8	2.02 4.08	31.52
10×	0.081 0 0.006 6	0.81 0.66	12.64
20×	0.040 0 0.001 6	0.40 0.16	6.2
32×	0.020 0 0.000 4	0.20 0.04	3.2
63×	0.008 0 0.000 06	0.08 0.006	1.25
	CF: swi	CF: wi	CF: dwi
4×	13 014	130	16.9
10×	80 455	805	42.0
20×	331 875	3 319	85.7
32×	1 327 500	13 275	166.0
63×	8 850 000	88 500	425.0

Calculation of concentrations using the conversion table:
cells/ml = (cells subsampling unit^{-1} × CF)/volume of chamber.
Calculating concentrations:

Example 1:
Volume of chamber = 100 ml
Counts (entire bottom of chamber) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50/100) = 0.5 cells ml^{-1} = 500 cells l^{-1}

Example 2:
Volume of chamber = 100 ml
Counts (dwi 10×) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50 × 42.0)/100 = 21 cells ml^{-1} = 21,000 cells l^{-1}

3. Turn on the vacuum pump (maximum pressure 200 mm Hg).
4. If you use the stain Calco Fluor White, turn off the vacuum pump when there is approximately 1 ml left in the filtration chimney.
5. Add three to five drops of Calco Fluor working solution (concentration 2 mg l^{-1}).
6. Turn on the vacuum pump again and filter until the filter goes dry.
7. Take off the filter and gently dry the back on a tissue to eliminate surplus water.
8. Mount the filter on a drop of paraffin oil on a slide, add another drop of paraffin oil on top of the filter and put on the cover slip (24 × 24 mm).
9. Observe your preparation using an epifluorescence microscope with an appropriate filter setting for the fluorochrome in use.

4.6.5 Quantitative analysis of samples preserved in acidic Lugol's, formaldehyde or glutaraldehyde using the fluorochrome Calco Fluor

1. Measure out the volume of sample to work on in a measuring cylinder.
2. Adjust the pH of the sample to pH 7 using NaOH before you make your preparation or alternatively adjust the pH when you have completed your filtration procedure and have 1 ml left in the filtration chimney.
3. Add the sample to the filtration unit (pore size of filter e.g. 5 μm).

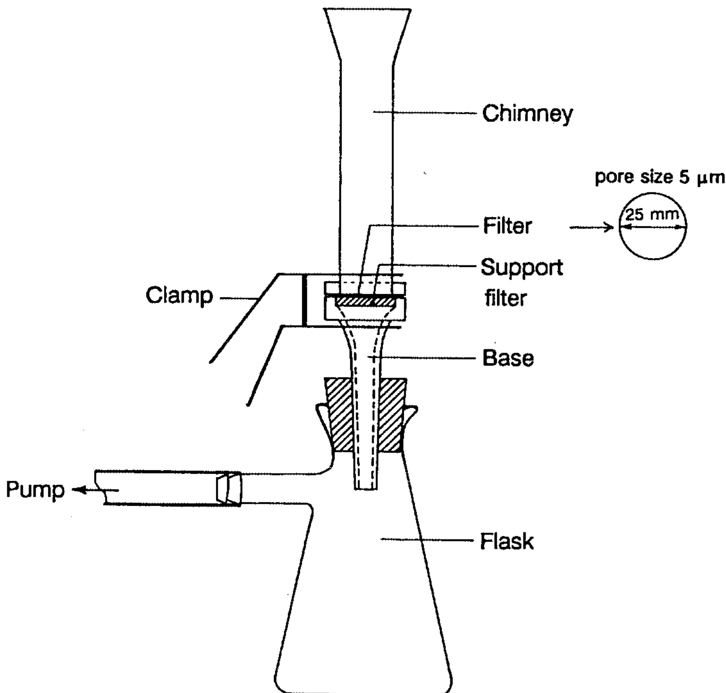

Figure 4.3
Filtration equipment used to concentrate microalgae on polycarbonate membrane filters for quantification using quantitative epifluorescence microscopy.

4. Turn on the vacuum pump (maximum pressure 200 mm Hg).
5. Turn off the vacuum pump when there is approximately 1 ml left in the filtration chimney (adjust pH if necessary – see step 2.).
6. Add three to four drops of Calco Fluor working solution (concentration 2 mg l^{-1}).
7. Turn on the vacuum pump again and filter until the filter goes dry.
8. Take off the filter and gently dry the back on a tissue to eliminate surplus water.
9. Mount the filter on a drop of paraffin oil on a slide, add another drop of paraffin oil on top of the filter and put on the cover slip (24 × 24 mm).
10. Observe your preparation using an epifluorescence microscope.

Note that Calco Fluor will only work at neutral pH (7), otherwise the Calco Fluor will precipitate – and you will see nothing but precipitated Calco Fluor!

The most frequent problems encountered when an epifluorescence method will not work are: (a) the filter set on the epifluorescence microscope does not work with the stain; (b) when using Calco Fluor, the pH of the sample to be analysed is not 7, or the working solution of Calco Fluor is too old.

Here are a couple of scenarios that you may encounter.

Problem # 1: You have made your preparation as described but see no fluorescence. What to do:

(a) Check that your mercury burner is working, if not, change the burner. If it still does not work contact a technician from the company that delivered the microscope.

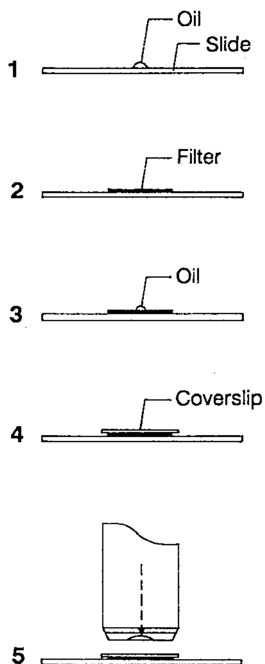

Figure 4.4
Mounting the polycarbonate filter on a slide for observation using quantitative epifluorescence microscopy.

(b) If the burner is working, make sure that you have the correct filter set and that the light from the mercury burner is not blocked and can be seen on the preparation. If the filter set is correct, but no light comes out of the objective, make sure that the light path is not blocked by other filters, etc.

Problem # 2: You have made a Calco Fluor preparation as described and see fluorescence all over the preparation and find it hard to localize the organisms. What to do:

(a) Check that the sample has a pH of 7. If the pH is not 7, adjust the pH as described and try again. If the pH is 7 then the Calco Fluor working solution might be too old. Make a new working solution and try again.

Counting procedure: start observing your preparation at a low magnification, for example using the 10× objective, as it is much easier to find some organisms to observe at low magnification! When you find some, make sure that the stain is working – that is, when using Calco Fluor White, the thecate dinoflagellates must light up blue on a dark background. If you use Acridine Orange the cells will light up orange, yellow and/or green on a dark background. If you use the stain DAPI the cells will light up blue on a dark background. When you have localized the organisms that you want to work on, switch to a higher magnification and adjust the intensity of the fluorescence using the different filters and screens mounted on the microscope.

The calculation is carried out basically as described for the Utermöhl method in Section 4.6.3. To calculate the concentration (cells l^{-1}) of the different species in the filtered sample you need to know:

V = volume of sample concentrated on filter (ml);
B_a = area of filter (mm^2);
B_c = area of part of filter counted (mm^2);
N = number of cells scored for species of interest;
conversion factor (CF) = B_a/B_c.
The concentration C of species (cells ml^{-1}) is then
$C = N \times (B_a/B_c)/V$.

As with the Utermöhl method, it is not possible to obtain general conversion factors to be used for all combinations of microscopes, counting magnifications and filters/filtering units to be used when calculating concentrations from counts such as those done by window counting. This is because the conversion factors are dependent on the magnification used when counting as well as the type of filtering unit. The conversion factors are to be calculated for each microscope and each subsampling area for each magnification, as well as for each type of filtering unit (Table 4.7).

4.7 Epiphytic/benthic microalgae

4.7.1 Epiphytic microalgae

Several methods have been used to sample and quantify epiphytic microalgae which cause ciguatera fish poisoning (Bagnis *et al.*, 1980; Quod *et al.*, 1995; McCafferey *et al.*, 1992). According to Quod *et al.*, epiphytic microalgae, including the

dinoflagellates responsible for ciguatera, can be sampled from macroalgae/sea grasses for quantitative analysis by the following procedure:
1. macroalgae are collected (20 g);
2. macroalgae are vigorously shaken in seawater (say for 30 s);
3. seawater is sieved (mesh size 150 µm);
4. dinoflagellates are investigated/quantified in the fraction <150 µm.

TABLE 4.7 Example of calibration table used for calculating concentrations of microalgae by epifluorescence microscopy (filter area 189 mm²)

	swi (small window)	**wi (window)**	**dwi (diagonal window)**
	L (mm) Area (mm²)	*L* (mm) Area (mm²)	Area (mm²)
4×	0.202 0 0.040 8	2.02 4.08	31.52
10×	0.081 0 0.006 6	0.81 0.66	12.64
20×	0.040 0 0.001 6	0.40 0.16	6.2
32×	0.020 0 0.000 4	0.20 0.04	3.2
63×	0.008 0 0.000 06	0.08 0.006	1.25
	CF: swi	CF: wi	CF: dwi
4×	4 632	46.3	6.04
10×	28 636	286	15.05
20×	118 125	1 181	30.50
32×	472 500	4 725	61.00
63×	2 953 125	29 531	152.00

Calculation of concentrations using the conversion table:
cells ml^{-1} = (cells subsampling $unit^{-1} \times$ CF)/volume of chamber.
Calculating concentrations:

Example 1:
Volume of sample concentrated on the filter = 100 ml
Counts (entire filter area) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50/100) = 0.5 cells ml^{-1} = 500 cells l^{-1}

Example 2:
Volume of sample concentrated on filter = 100 ml
Counts (dwi 10×) = 50 *Dinophysis acuminata*
Calculating concentrations:
(50 × 15.05)/100 = 7.5 cells ml^{-1} = 7,500 cells l^{-1}

The suspension containing the epiphytic species can then be investigated using light, epifluorescence or electron microscopy as described below. Cells can be 'extracted' from other substrates either by washing with filtered seawater, brushing or scraping. If the material fits under a microscope the surface can be investigated directly using epifluorescence microscopy or SEM.

A rough estimate of the abundance of epiphytic species can be obtained using counting cells, the Utermöhl method or epifluorescence microscopy according to Andersen and Kristensen (1995), see section 4.6.4. The abundance of epiphytic species can be presented as cells cm^{-2} or cells g^{-1} dw of substrate.

4.7.2 Benthic microalgae from sandy/muddy substrate

The substrate is sampled and either fixed in the field, using Lugol's or formaldehyde, or brought unfixed to the laboratory for extraction of live cells. Live samples must be kept in the dark, at *in situ* temperature and for as short a time as possible. Qualitative analysis can be carried out on live cells or cells fixed in Lugol's or formaldehyde using the same procedures as for plankton samples. The samples can be obtained using several different approaches. Species can be extracted from unfixed samples of sandy sediment samples using the Uhlig method (Uhlig, 1964), previously used for ciliates, for example (applying ice on top of the sediment kept in a cylinder with a mesh of 200 µm in the bottom which is in contact with water, e.g. in a Petri dish). In the case of muddy sediments, the mud can be spread out in a Petri dish and cover slips placed on the surface of the sediment – some species will then attach to the cover slip and can be investigated using light, epifluorescence or electron microscopy.

Finally the sediment can be spread in a thin layer on a slide and investigated directly. In the case of thecate dinoflagellates, the fluorochrome Calco Fluor (works only at pH 7; samples can be fixed in neutral Lugol's) can be added to the live samples or samples fixed in neutral Lugol's for investigation using epifluorescence microscopy.

Estimates of the abundance of benthic species can be obtained using counting cells, the Utermöhl method or epifluorescence microscopy according to Andersen and Kristensen (1995), see section 4.6.4. The abundance of benthic species can be presented as cells/cm^2 or cells/g dw of sediment.

Rough estimates of the abundance of species that can be cultured can be obtained from dilution series of sediment slurries using the most probable number (MPN) technique (see Section 4.12).

4.8 Statistics – how many cells to count

Counting microalgae can be a rather time-consuming process so it is preferable not to count too many cells to obtain a good estimate of the concentration in a sample. Under the assumption that the cells are randomly distributed in a sample, that is in the counting chamber or on the filter, the 95% confidence limits (Fig. 4.5) can be calculated according to the Poisson distribution:

$$= \pm(2 \times \sqrt{n} \times 100\%)/n = \pm 200\%/\sqrt{n}.$$

The more cells counted, the more precision gained (Table 4.8 and Fig. 4.5). At the beginning of the count, the precision is greatly increased each time you add

another cell. On the other hand, if you have already counted many cells only a small increase in precision will be achieved by adding more cells to the count. To obtain an estimate of the cell concentration with a precision of ±10%, you need to count approximately 400 cells. In many cases, where the species of interest occur in rather low concentrations (a few hundred cells l^{-1}), you may have to accept a precision of approximately 15–30%, corresponding to counts of 200 and 50 cells, respectively. In the case of colonial species, the input for the calculation of confidence limits must be the number of colonies counted and not the actual number of cells counted in the colonies. Note that the condition for the calculation of confidence limits, that the cells are randomly distributed, is not always met. In such cases the confidence limits calculated are too narrow (see Venrick, 1978, for further discussion of counting statistics).

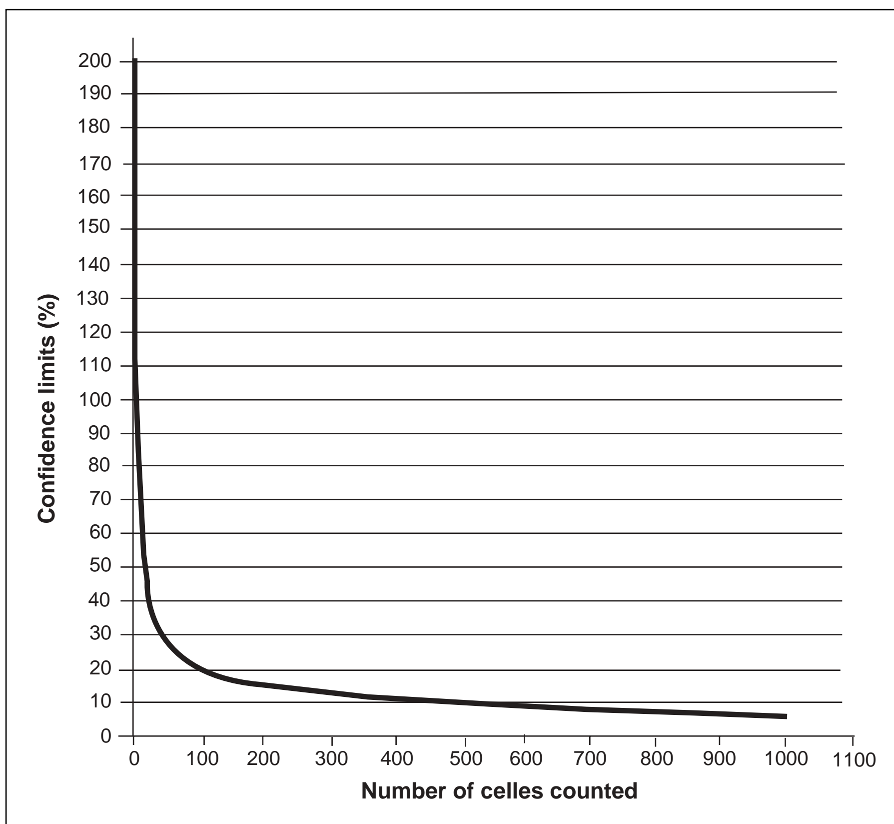

Figure 4.5
Relationship between number of cells counted and confidence limits (at 95% significance level).

TABLE 4.8 Relationship between number of cells counted and confidence limits (at 95% significance level)

Counts	Confidence limits ± (%)
1	200
2	140
4	100
5	90
10	63
20	45
40	32
50	28
100	20
200	14
400	10
500	9
1000	6

Example:
Sample volume = 100 ml
Counts = 50 *Dinophysis acuminata*
Concentration of Dinophysis:
50/100 = 0.5 cells ml^{-1} = 500 cells l^{-1}
Calculating statistics:
Relative limits of expectations:
$\pm 200\% / \sqrt{50} = 28\%$
Absolute limits of expectations:
28% of 500 cells l^{-1} = $(500/100) \times 28$ = 140 cells l^{-1}
Final result:
Concentration = 500 ± 140 *Dinophysis acuminata* l^{-1}

4.9 ESTIMATING BIOMASS FROM MICROSCOPE OBSERVATIONS

Estimates of the biomass of microalgae can be very useful in many cases, such as to

- calculate the total phytoplankton biomass;
- calculate the biomass of taxonomical groups such as diatoms, dinoflagellates, etc.;
- calculate the biomass of functional groups such as auto-, mixo- or heterotrophs;
- compare the abundance of different species and their contribution to the total biomass of phytoplankton;
- evaluate to which extent one or several HAB species contribute to the pool of available food for filtering molluscs.

The biomass can be expressed as biovolume (mm^3 l^{-1}) which is approximately equal to the wet weight (mg l^{-1}) or as carbon biomass (µg Cl^{-1}). Biovolume can be calculated from measurements of linear dimensions of cells measured under the microscope using appropriate geometric formulae (Edler, 1979). Carbon biomass can be estimated from the biovolume using conversion factors from plasma volume

to carbon biomass. Edler (1979) suggests a conversion factor from plasma volume to carbon biomass of 0.11 for all phytoplankton and ciliates except thecate dinoflagellates. For thecate dinoflagellates a conversion factor of 0.13 is suggested.

4.10 Storage of raw data – data forms; spreadsheets; databases

All raw data should be filled into standard forms with information on

- position of sampling site;
- sampling depth;
- date;
- volume of sample used for quantitative analysis;
- method used for quantitative analysis;
- ID of person responsible for analysis.

Data should be stored as documentation for the calculated concentrations and biomass. Data can be stored in paper files, but preferably they should be stored in electronic form, either in spreadsheets or in a database. If raw data are properly stored it is possible to document the exact observations and to go back and recalculate concentrations and biomass of species if necessary. Furthermore, storage of data in databases facilitates data presentation/analysis including the plotting of population dynamics of selected species from week to week, or comparison of the abundance of microalgae between stations as well as between different seasons and years. Routines can be developed that make it very easy to present data stored in a database on the Internet , for example using Geographical Information System (GIS) technology. If samples of marine microalgae are routinely analysed, it is extremely helpful to use a database for storage of raw data as well as calculation of concentrations and biomass. Furthermore, the use of a database with a well-documented species list and basic information on how concentrations and biomass are calculated ensures that data are comparable for analysis and presentation. Use of such databases is implemented by several monitoring agencies, research departments and consulting companies worldwide.

4.11 Quality assurance

A detailed description of all procedures including the following should be produced:

- field sampling;
- handling of samples;
- qualitative analysis in the laboratory;
- quantitative analysis in the laboratory; and
- calculation of concentrations and biomasses.

Before the data are distributed throughout the monitoring system, they should be properly checked using a specified routine by at least one person who did not perform the quantitative analysis. Raw data should be kept on a long-term basis, in files for later documentation, examination or investigation.

4.12 Serial dilution culture (SDC) method

The present method (as introduced for phytoplankton by Knight-Jones, 1951, and later modified by Throndsen, 1978) has proved convenient, especially for the isolation of bloom-forming species. The principle of the method is to dilute the sample until only one specimen is left in the subsample (which is used as inoculum for cultures). Keeping track of the dilution, such as by proceeding stepwise, will also offer the opportunity to make estimates of the original cell number. By using three to ten parallel series of dilutions and applying statistics to the obtained pattern of presence and absence in each dilution, the probability of the estimate may be calculated. Presence and absence is revealed by growth or no growth in the tubes. To facilitate growth, the whole dilution series is made with a suitable medium (see Chapter 3). For toxic algal blooms, the quantitative estimates are often more easily made by direct counts but the SDC method will provide a convenient means for bringing the species into culture for further studies. The method has been applied with success to establish cultures of, for example, *Prymnesium*, *Chrysochromulina*, *Heterosigma*, *Aureococcus*, *Nannochloropsis* and single-celled stages of *Phaeocystis*. The SDC method can easily be combined with different media to find the most suitable one for a particular species under the defined laboratory conditions.

The dilution series could be achieved in two ways; the standard (pipette) dilution method based on separate dilution series set up for each parallel in the series, or the syringe method based on batch dilutions with subsamples inoculated into the parallels. The precision of both techniques will depend on the skill of the operator as well as the accuracy of the equipment. If the MPN is important, a test from algal material precounted, for example by haemacytometer (blood-counting cell), Coulter counter or flow cytometry, may be carried out to reveal deficiencies in the set-up or equipment (see concluding remarks). The pipette method requires one 10 ml graduated pipette for each parallel dilution series and culture tubes prefilled with exactly 9 ml of growth medium. The syringe method uses one 10 ml graduated syringe only, and the culture tubes may contain whatever amount of medium is convenient for the growth of the cultures. The syringe SDC technique may easily be adapted for inoculation into larger volumes of media and/or an increased number of tubes at each step (up to eight with the 10 ml syringe used as standard). Changing the volumes of the inocula or the size of the syringe used for the dilution will offer a variety of dilution possibilities. However, the mixing achieved in each type of syringe used has to be checked.

The following procedures for making a dilution culture series are carried out with simple equipment, and the initial steps (inoculation) can be performed in 5–10 min, on location. When on location take care to avoid direct sunlight and temperature shocks. The important precautions to be taken are to use clean equipment for culturing, to keep temperature and salinity well within the tolerance limits of the species, and to choose the appropriate medium. The fulfilment of the latter condition may be a matter of trial and error, but some media are more universal than others (e.g. Erd-Schreiber). Before deciding on the media to be used, consult Chapter 3. Another important measure to provide optimal culturing conditions is to prepare the medium from water sampled together with the inoculum. The rationale is that the organisms in question are adapted to this milieu; that possible hostile fellow organisms will be killed during the heating of the medium; and that nutrient depletion, which may be substantial at least under late bloom conditions, will be compensated

by the addition of nutrients to the growth medium. Filtration may not be a feasible method for sterilization of the seawater for the medium as viruses passing the filter may harm the cultures. Material to be used for starting SDC should be collected with a non-toxic water sampler and handled carefully to avoid temperature, salinity and light shocks. Before starting inoculation, ensure that the temperature and salinity conditions are the same in the sample and the medium, and that all equipment and accessories needed are at hand to facilitate rapid completion of the procedure. Tubes, pipettes and syringes which are presterilized at purchase ought to be tested for toxicity with sensitive algal species (e.g. *Chrysochromulina* sp.). Materials tolerated by the human organism may prove fatal to many algal cells.

4.12.1 Set-up for the syringe method

For a standard SDC series use a test tube rack that holds 30 (or more conveniently 40 to 50) test tubes in rows of five. Fill 25 test tubes with 9–10 ml of growth medium each, and mark the tubes for five dilution steps of five parallels, for example 1I, 1II, 1III (meaning first dilution step, parallel one, two and three), 2I, 2II, 2III, 3I, –, to 5I, –, at which step the inoculum will be 0.1 µl. Five tubes for performing the dilutions (Fig. 4.6D) are filled completely (15–20 ml). A disposable 10 ml sterile syringe, preferably with exocentric opening, and graduated into divisions of 1 ml, is also necessary. The amount of medium required for each SDC series is approximately 300 ml, but it may be wise to prepare extra in order to have a suitable medium for possible subculturing of interesting species that grow in the SDC.

Procedure. Start by rinsing the syringe with sample water, then (Fig. 4.6):

A, draw 10 ml of the water sample into the syringe;

B, dispense 1 ml into five of the first tubes (parallels I-V);

C, dispose of all but the last 1 ml from the syringe;

D, draw 9 ml of medium from one of the full tubes;

E, dispose of 1 ml.

Repeat steps B to E (Figs. 4.6F, 4.6G) for the next four dilution steps.

4.12.2 Set-up for the pipette method

For the pipette method, arrange 25 marked tubes in a rack, each filled with exactly 9 ml of culture medium. Have six culture-clean 10 ml graduated pipettes at hand. Total amount of medium required is 225 ml.

Procedure. Start with one graduated pipette (Fig. 4.7):

A, draw at least 6 ml of sample into the pipette;

B, dispense 1 ml into each of the five parallel tubes of the first step in the series;

C, with a new pipette, thoroughly mix the content of the first tube by sucking and ejecting;

D, draw 1 ml of suspension into the pipette;

E, add the 1 ml to the next tube in the dilution series;

F, with the same pipette, repeat mixing and transfer (C to E) for the remaining dilution steps.

Then, using each of the tubes filled in step B as basis, repeat the dilution procedure (C to F) for each parallel in the series.

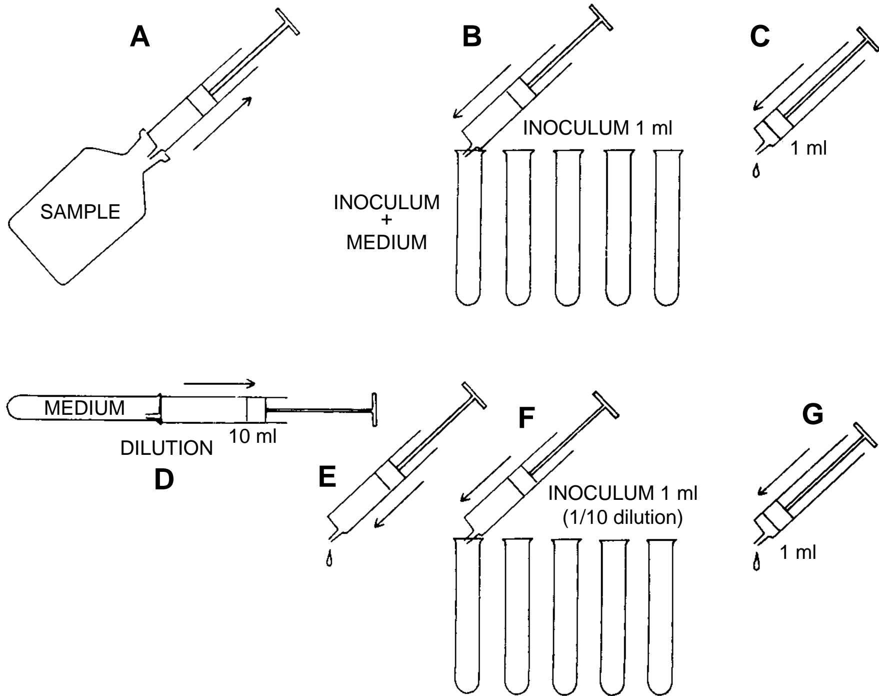

Figure 4.6
Serial dilution culture method, dilutions by syringe. A, subsampling from original water sample; B, inoculation of 1 ml into five test tubes/ growth tubes each with 9–10 ml of medium; C, expelling all but 1 ml to prepare for the next dilution; D, making 1/10 dilution by sucking in fresh medium from a test tube filled with medium; E, expelling 1 ml to ensure that the first inoculum is equal to the rest in F, inoculation of 1 ml (diluted sample) into five test tubes/growth tubes each with 9–10 ml of medium; G, expelling all but 1 ml to prepare for the next dilution. Repeat steps D to G for as many dilution steps as required.
Source: adapted from Throndsen (1978).

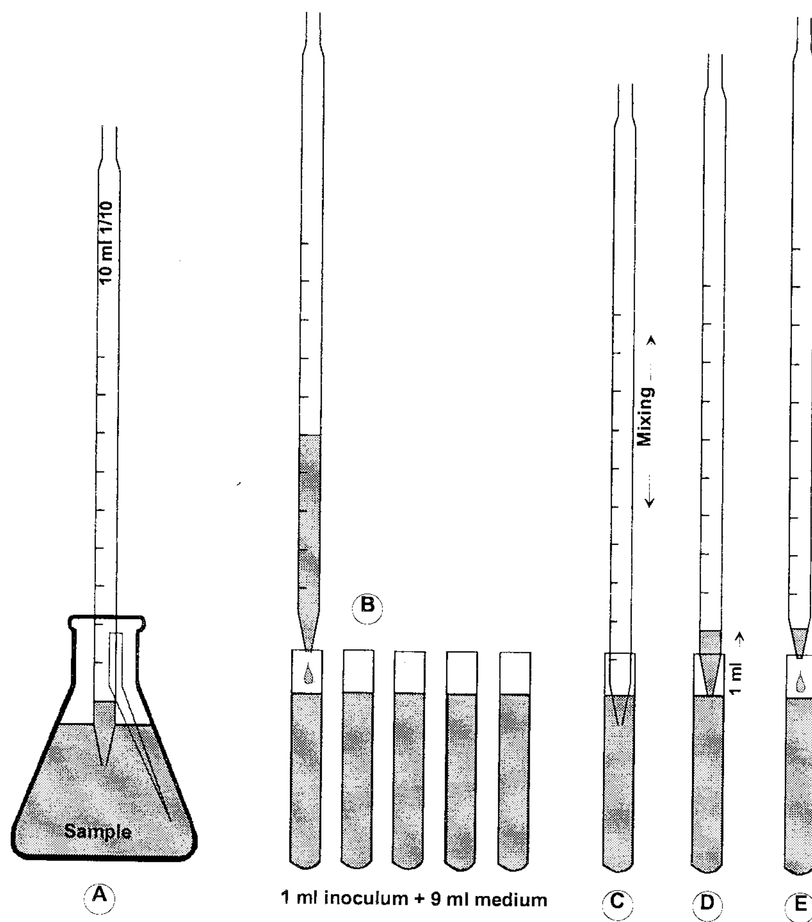

Figure 4.7
Serial dilutions set up with the pipette method. A, subsampling from original water sample; B, adding 1 ml of sample to 9 ml of medium (in five tubes to produce the basic parallels of the series); C, mixing thoroughly; D, withdrawing 1 ml of the suspension into the pipette; E, adding the 1 ml to the next tube with 9 ml medium. Repeat steps C to E to provide for the next dilution steps. (With a new pipette repeat the procedure, starting with the second tube in B, then the third.)

4.12.3 Incubation

The culture series should be placed under fluorescent tubes or daylight at an irradiance of about 10% of full daylight; lower light intensities may be better for deep-sea plankton or benthic species. More detailed information on incubation light intensities can be found in Chapter 3. Take care to keep the temperature variation within the tolerance limits of the phytoplankton species under consideration. Examination (by optical microscope) of the cultures after four and six weeks will often be sufficient for routine work in temperate areas. For microalgae from tropical and subtropical areas, the first examination ought to take place after two weeks, whereas Arctic and Antarctic dilution cultures may need up to two months (at 2–3°C) before cell densities sufficient for further examination under the microscope are established.

4.13 Results

The expected result of a dilution culture series is growth of a variety of the species present in the original sample, with the most abundant species established as unialgal cultures at higher dilutions within the series. Also, in tubes with more than one species present, a particular species may dominate, and by further dilution it can give rise to unialgal cultures. For qualitative and quantitative purposes, the presence or absence of the different species is noted for each of the tubes, starting with the most diluted. These presence and absence data can then be referred to tables for the most probable number (Table 4.9). When estimating cell numbers from Table 4.9, use the set of three successive dilution steps that gives the highest MPN, but make sure that growth has been recorded in at least two of the steps. Note that the SDC method only records cells viable under the culture conditions offered, thus MPN will be minimum values, however, with a standard deviation of ±20–50% of the mean estimate.

Table 4.9 Most probable number (MPN) in original sample. To use the table, compare the presence and absence of the species in question with the values below. Choose the three most diluted steps in which growths occur for the estimate (also see text for advice)

Growth in inoculum			**MPN cells ml^{-1}**		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
5	5	5	>24 000	>2 400	>240
5	5	4	16 000	1 600	160
5	5	3	9 200	920	92
5	5	2	5 400	540	54
5	5	1	3 500	350	35
5	5	0	2 400	240	24
5	4	5	4 300	430	43

.../...

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
5	4	4	3 500	350	35
5	4	3	2 800	280	28
5	4	2	2 200	220	22
5	4	1	1 700	170	17
5	4	0	1 300	130	13
5	3	5	2 500	250	25
5	3	4	2 100	210	21
5	3	3	1 800	180	18
5	3	2	1 400	140	14
5	3	1	1 100	110	11
5	3	0	790	79	7.9
5	2	5	1 800	180	18
5	2	4	1 500	150	15
5	2	3	1 200	120	12
5	2	2	950	95	9.5
5	2	1	700	70	7
5	2	0	490	49	4.9
5	1	5	1 300	130	13
5	1	4	1 100	110	11
5	1	3	840	84	8.4
5	1	2	640	64	6.4
5	1	1	460	46	4.6
5	1	0	330	33	3.3
5	0	5	950	95	9.5
5	0	4	760	76	7.6
5	0	3	580	58	5.8
5	0	2	430	43	4.3
5	0	1	310	31	3.1
5	0	0	230	23	2.3
4	5	5	810	81	8.1

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
4	5	4	720	72	7.2
4	5	3	640	64	6.4
4	5	2	560	56	5.6
4	5	1	480	48	4.8
4	5	0	410	41	4.1
4	4	5	690	69	6.9
4	4	4	620	62	6.2
4	4	3	540	54	5.4
4	4	2	470	47	4.7
4	4	1	400	40	4.0
4	4	0	340	34	3.4
4	3	5	590	59	5.9
4	3	4	520	52	5.2
4	3	3	450	45	4.5
4	3	2	390	39	3.9
4	3	1	330	33	3.3
4	3	0	270	27	2.7
4	2	5	500	50	5.0
4	2	4	440	44	4.4
4	2	3	380	38	3.8
4	2	2	320	32	3.2
4	2	1	260	26	2.6
4	2	0	220	22	2.2
4	1	5	420	42	4.2
4	1	4	360	36	3.6
4	1	3	310	31	3.1
4	1	2	260	26	2.6
4	1	1	210	21	2.1
4	1	0	170	17	1.7
4	0	5	360	36	3.6

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
4	0	4	300	30	3.0
4	0	3	250	25	2.5
4	0	2	210	21	2.1
4	0	1	170	17	1.7
4	0	0	130	13	1.3
3	5	5	450	45	4.50
3	5	4	410	41	4.10
3	5	3	370	37	3.70
3	5	2	320	32	3.20
3	5	1	290	29	2.90
3	5	0	250	25	2.50
3	4	5	400	40	4
3	4	4	360	36	3.60
3	4	3	320	32	3.20
3	4	2	280	28	2.80
3	4	1	240	24	2.40
3	4	0	210	21	2.10
3	3	5	350	35	3.50
3	3	4	310	31	3.10
3	3	3	280	28	2.80
3	3	2	240	24	2.40
3	3	1	210	21	2.10
3	3	0	170	17	1.70
3	2	5	310	31	3.10
3	2	4	270	27	2.70
3	2	3	240	24	2.40
3	2	2	200	20	2
3	2	1	170	17	1.70
3	2	0	140	14	1.40
3	1	5	270	27	2.70

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
3	1	4	230	23	2.30
3	1	3	200	20	2
3	1	2	170	17	1.70
3	1	1	140	14	1.40
3	1	0	110	11	1.10
3	0	5	230	23	2.30
3	0	4	200	20	2
3	0	3	160	16	1.60
3	0	2	130	13	1.30
3	0	1	110	11	1.10
3	0	0	78	7.80	0.78
2	5	5	320	32	3.20
2	5	4	290	29	2.90
2	5	3	260	26	2.60
2	5	2	230	23	2.30
2	5	1	200	20	2
2	5	0	170	17	1.70
2	4	5	280	28	2.80
2	4	4	250	25	2.50
2	4	3	230	23	2.30
2	4	2	200	20	2
2	4	1	170	17	1.70
2	4	0	150	15	1.50
2	3	5	250	25	2.50
2	3	4	220	22	2.20
2	3	3	200	20	2
2	3	2	170	17	1.70
2	3	1	140	14	1.40
2	3	0	120	12	1.20
2	2	5	220	22	2.20

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
2	2	4	190	19	1.90
2	2	3	170	17	1.70
2	2	2	140	14	1.40
2	2	1	120	12	1.20
2	2	0	93	9.3	0.93
2	1	5	190	19	1.90
2	1	4	170	17	1.70
2	1	3	140	14	1.40
2	1	2	120	12	1.20
2	1	1	92	9.20	0.92
2	1	0	68	6.80	0.68
2	0	5	160	16	1.60
2	0	4	140	14	1.40
2	0	3	120	12	1.20
2	0	2	91	9.10	0.91
2	0	1	68	6.80	0.68
2	0	0	45	4.50	0.45
1	5	5	240	24	2.40
1	5	4	220	22	2.20
1	5	3	190	19	1.90
1	5	2	170	17	1.70
1	5	1	150	15	1.50
1	5	0	130	13	1.30
1	4	5	220	22	2.20
1	4	4	190	19	1.90
1	4	3	170	17	1.70
1	4	2	150	15	1.50
1	4	1	130	13	1.30
1	4	0	110	11	1.10
1	3	5	190	19	1.90

…/…

Growth in inoculum			MPN cells ml^{-1}		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
1	3	4	170	17	1.70
1	3	3	150	15	1.50
1	3	2	130	13	1.30
1	3	1	100	10	1
1	3	0	83	8.30	0.83
1	2	5	170	17	1.70
1	2	4	150	15	1.50
1	2	3	120	12	1.20
1	2	2	100	10	1
1	2	1	82	8.20	0.82
1	2	0	61	6.10	0.61
1	1	5	140	14	1.40
1	1	4	120	12	1.20
1	1	3	100	10	1
1	1	2	81	8.10	0.81
1	1	1	61	6.10	0.61
1	1	0	40	4	0.40
1	0	5	120	12	1.20
1	0	4	100	10	1
1	0	3	80	8	0.80
1	0	2	60	6	0.60
1	0	1	40	4	0.40
1	0	0	20	2	0.20
0	5	5	190	19	1.90
0	5	4	170	17	1.70
0	5	3	150	15	1.50
0	5	2	130	13	1.30
0	5	1	110	11	1.10
0	5	0	94	9.40	0.94
0	4	5	170	17	1.70

…/…

Growth in inoculum			**MPN cells ml^{-1}**		
1.0 ml	100 µl	10 µl			
100 µl	10 µl	1 µl			
10 µl	1 µl	100 nl			
0	4	4	150	15	1.50
0	4	3	130	13	1.30
0	4	2	110	11	1.10
0	4	1	94	9.40	0.94
0	4	0	75	7.50	0.75
0	3	5	150	15	1.50
0	3	4	130	13	1.30
0	3	3	110	11	1.10
0	3	2	93	9.30	0.93
0	3	1	74	7.40	0.74
0	3	0	56	5.60	0.56
0	2	5	130	13	1.30
0	2	4	110	11	1.10
0	2	3	92	9.20	0.92
0	2	2	74	7.40	0.74
0	2	1	55	5.50	0.55
0	2	0	37	3.70	0.37
0	1	5	110	11	1.10
0	1	4	91	9.10	0.91
0	1	3	73	7.30	0.73
0	1	2	55	5.50	0.55
0	1	1	36	3.60	0.36
0	1	0	18	1.80	0.18
0	0	5	90	9	0.90
0	0	4	72	7.20	0.72
0	0	3	54	5.40	0.54
0	0	2	36	3.60	0.36
0	0	1	18	1.80	0.18
0	0	0	0	0	0

Source: based on data from American Public Health Association (1955).

4.14 Comments

The procedure may be adopted for different purposes and the set-up can be varied, see above. When cell concentrations are high (>10^6 cells l^{-1}) more dilution steps are added in order to obtain unialgal cultures. If the aim is to clean up or isolate an organism already in culture, the original cell density will determine which dilution steps are critical; dilute so that the inoculum contains one or two cells, use many parallel dilutions, and add one or two further dilution steps. Provided growth occurs only in some of the tubes, the most diluted culture is likely to be clonal, unless cells in the inoculum are clumping.

The quality of the inoculum, the type of medium and the growth conditions offered will determine the success of the method. This selectivity may be used deliberately by choosing a medium which facilitates the growth of particular organisms. For general purposes, a modified Erd-Schreiber medium (Throndsen 1978, 1997) appears to be suitable for coastal waters, whereas a diluted Guillard f medium (Guillard and Ryther, 1962) as (f/2–f/50) may prove better for more oligotrophic areas.

The advantage of the SDC method is that a dominant species may easily be brought into culture provided that it accepts the conditions offered for growth. Not all harmful species fulfil this, for example no *Dinophysis* species has been brought into permanent culture so far. Numerous other taxa, such as *Prymnesium parvum* and several *Chrysochromulina* species, have however been grown through this method. The disadvantage of the SDC method is that less-common species will show up in mixed cultures only. But, drawing upon differences in growth strategies, further SDC series may produce unialgal cultures for the less common species also. As for the MPN estimates, the obvious selectivity and dependence on growth conditions is an important disadvantage, but for many of the species in question the SDC method is the best way to obtain numerical information about cell concentrations and community structure. In the cultures as in nature, not all cells will be typical, but as the quantification is determined by observing the presence or absence of a species, it is not critical that every single specimen in the sample examined be identified. Note, however, that presence or absence of taxa at higher levels has to be deduced from the species (level) in order to obtain a reliable estimate.

Three conditions are particularly important when estimating MPN in mixed cultures:

(a) The level of extinction by dilution may be obscured by competition between different species and/or the effect of algal viruses. Both factors are likely to be inversely related to the dilution.

(b) With growth in all tubes at the highest dilution, the estimate will always be too low and the MPN value should be given a prefix > to indicate that the value is assumed to be less than the real number in the sample surveyed. If possible, this problem could be avoided by adding a sixth dilution step with 1 µl inoculum.

(c) In almost every SDC series there is a serious lack of information on the least-common species encountered in the series.

4.15 Conclusions

At present the SDC is the only simple method of cultivating dominant phytoplankton species, and for the unarmoured nanoplankton taxa it is the easiest way to estimate the number of viable cells. More sophisticated methods such as flow cytometry may

be more precise as regards cell numbers, but demand fairly high operating skills and limited possibilities for species identification. The success or failure of the method depends on (a) the cleanliness of the equipment; (b) the suitability of the growth medium; and (b) the external culture conditions (temperature and light). Experience has shown that in coastal waters the species present are fairly halotolerant, but for some reason SDC prepared with water from the sampling site has proven to have a higher species diversity than those prepared with standard pre-made growth medium.

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