3- Preservation, storage and concentration of phytoplankton sample

It is best to examine samples as early as possible after collection, while the algae remain alive because many features helpful in identifying algae are more clearly seen in live specimens. If you have to wait a few hours after collection to examine a sample it would be desirable to keep the sample cold on ice or in portable refrigerator. However, in some instances, particularly for counting purposes, it is necessary to stain them and preserve the algae. If possible, therefore, identify the phytoplankton in your sample first, before staining them for counting.

How do we preserve phytoplankton for analysis?

Don’t preserve; count fresh

Advantages
  a. Don’t lose color, a good diagnostic for division level identification
  b. Don’t lose motility – can also help with identification

Disadvantages
  a. Need to do right away or algae will die or grow, so counts will be inaccurate

Sample Preservation

To preserve the samples for later analyses, several procedures must be applied directly after the collection to prevent the adverse effects of light, temperature and microorganisms which might cause rapid decay of organisms.

Fixatives/preservatives

Preservatives have to meet the following requirements:
  • The effect of the agent on the loss of organisms by chemical shock or otherwise must be known beforehand.
  • The preservative must effectively prevent the microbial degradation of organic matter at least during the storage period of the samples.
  • The preservative must guarantee a good recognition of taxa at least during the storage period of the samples.

The more common fixatives currently employed are:

1. Formaldehyde-based solutions

Formaldehyde (HCHO) is a gas produced by the oxidation of methyl alcohol, whereas 100% Formalin is a saturated solution of this gas in water. The concentration provided by the manufacturer is typically a 40% solution (aqueous Formaldehyde). Formalin is generally the preferred fluid for fixation and is widely used. To make a solution of 10% Formalin, nine parts of water are added to one part of 40% (aqueous) Formaldehyde. Therefore, a 10% solution of Formalin is the equivalent of a 4% solution of Formaldehyde.

Formaldehyde

Hazardous fumes avoid inhaling the formaldehyde (harmful!) and keep sample bottles closed if not in use, distorts many cells, bleaches color, added at 2-5% as formalin (40% formaldehyde).

Neutralized Formaldehyde

200 ml of 20% Formaldehyde solutions (100 ml dist. water and 100 ml of 40% Formaldehyde solutions) + 20 gm of Hexamethylene tetramine.
It is a general preservative for all phytoplankton. The fixing agent have to be fresh-prepared some days before the samples collection, kept at 5-6 °C and put in the dark glass bottles. 100 ml of water samples are fixed with 2 ml of neutralized formaldehyde. Water samples should be kept in the dark for long term storage.

**Acidified Formaldehyde**  
Prepared by combining equal volumes of 20% formaldehyde solution and 50% glacial acetic acid solution (CH₃ COOH). It is a good preservative for all phytoplankton especially diatoms but not for "naked" flagellates and coccolithophorids as the acid may dissolve coccoliths (Add 2 ml for 100 ml of sample).

**Advantages and disadvantages of formaldehyde**  
**Advantages**  
- Good fixing and preserving agent for algae with a more rigid cell wall.  
- Cell wall structures and other characteristics like eye spots remain visible.  
- When stored properly in appropriate bottles samples will stay in good condition for many years without attention.  
- Auto-fluorescence of chlorophyll $\text{a}$, though decaying, remains intact for at least several days if the samples are stored in the dark continuously.

**Disadvantages**  
- Formaldehyde is irritating at very low concentrations in the air and may lead to allergic reactions. Some laboratories will not analyse samples preserved with substances such as formaldehyde, as these are carcinogenic and represent an occupational health and safety hazard.  
- Some algal species can be distorted or cannot be recovered in the sample at all.  
- Organisms may shrink resulting in lower cell volumes and calculated biomasses.

2. Lugol’s solution and its adaptations  
**Neutral Lugol's Solution**  
20 gm potassium iodide (KI) dissolved in 200 ml of distilled water + 10 gm I₂ (Crystalline iodine)

**Acidic Lugol's Solution**  
20 gm potassium iodide (KI) dissolved in 200 ml of distilled water + 10 gm I₂ (Crystalline iodine) + 20 ml of glacial acetic acid (CH₃ COOH).  
The solution can be made up a few days ahead and stored in a dark bottle for convenience.  
Lugol’s solution is added in a ratio of 0.3-1 part to 100 parts of the seawater sample.  
This preservative is good for all phytoplankton but not for coccolithophorids as the acid may dissolve the coccoliths. If coccolithophorids need to be preserved with the coccoliths intact, a parallel sub-sample should be fixed with 0.5-1.0% of alkaline Lugol's solution.

**Alkaline Lugol's solution** Replace the acetic acid of the acid solution by 50 g sodium acetate (CH₃COONa). Use a small part of the water to dissolve the acetate. Lugol’s solution can be stored in a dark bottle at room temperature for at least 1 year.

**Advantages and disadvantages of Lugol’s**  
**Advantages**  
- Better for accurately quantifying than many aldehyde-based fixatives.
• Lugol's stains cells a dark brown colour, making counting easier.
• Lugol's solution is relatively harmless (not very toxic) compared to aldehyde-based or other more toxic fixatives.
• Adds weight to cells and helps phytoplankton cells sink to bottom of settling chamber

Disadvantages
• Breaks down in sunlight – need to keep samples and stock solution in dark and cool.
• Samples preserved in Lugol's fixative do not have a long shelf life. Samples stored for more than one year are of little use. Lugol’s loses preservative power over time-must re-add every 6 months,
• Lugol’s masks chlorophyll fluorescence, which may be needed to recognize mixotrophic species.
• Lugol’s dissolves hard structures such as coccoliths and diatom frustules and, therefore, is not ideal for long-term storage of many plankton taxa
• Lugol's stains cells a dark brown colour, which obscures some of the characteristic features of ciliates (e.g. macronucleus), making it harder to identify them to division (no color cues). Darkly stained specimens however, is not a problem with algal cultures, where you know what algae are there and are just enumerating, so is good for that use.
• Lugol’s does not necessarily preserve the cell shape and size of live specimens - can cause 30-40% shrinkage from live biovolume.

3. Glutaraldehyde and its adaptations

Glutaraldehyde, added at 1-5%

Buffered glutaraldehyde. For specific organism groups, advanced research methods and further identification with an electron microscope, material can be preserved in buffered glutaraldehyde fixative, (2% GTA with sodium cacodylate of barate/borax, a combined buffered glutaraldehyde/osmium tetraoxide cold fixative (GTA/OsO4) has been strongly recommended.

Advantages and disadvantages of glutaraldehyde

Advantages
• Preserves much of the color and fluorescence
• limited cell distortion

Disadvantages
• Is quite toxic with hazardous fumes

Sample Storage

Living samples
Living samples for preliminary analysis should be kept in the dark at a temperature of between 4 and 10 °C. They can be kept unpreserved for up to 24 h in polypropylene or polyethylene bottles.

NOTE. In samples with a very high density of organisms, such as blooms, depletion of oxygen should be prevented by diluting the sample, by leaving a large amount of space for air in the bottle or by exposing it to a small amount of light. The last option requires practical experience in determining the right exposure without inducing growth.
Preserved samples
Samples preserved with Lugol’s solution (or aldehydes) should always be stored in the dark and cooled to 4 °C (not higher than 10°C), unless they are analysed within a week. In that case they can be stored in the dark at room temperature. The only reason for cooled storage is to slow down the rate of physical and chemical processes that lead to a reduction in the quality of the sample. Storage in the dark is always necessary to prevent photo-oxidation and, in formaldehyde samples, preserve auto-fluorescence of pigments. If oxidation is prevented the maximum storage time of Lugol preserved samples in the dark and below 5° C is 6 months. Preservation and storage for periods of years is only possible after addition of formaldehyde.

Quality Control Samples that are preserved in the field after sampling should be checked after a couple of days for oxidation of the Lugol. The sample must have a ‘tea’-like colour. If not, Lugol’s solution should be added until the sample has regained this colour.

Sample Concentration Methods
The water collected through the different water samplers is concentrated to get a higher density for counting. Concentration by settling, centrifugation and filtration through fine mesh nylon or filter papers are the most used methods.

Reverse Filtration
When phytoplankters are required in a live and undamaged condition, they may be concentrated gently by removal of water that flows upward through a filter. The reverse concentrator consists essentially of: (a) a vessel for containing the sample: and (b) an insert of smaller circumference having some type of filter on the lower end and open at the upper end. The water seeps upward through the filter and is drawn off, leaving the organisms more concentrated in the sample vessel. This method was developed to overcome the damages caused, to certain groups of phytoplankton by vacuum filtration, centrifugation.

Figure  Schematic drawing of a reverse-filtration device.
**Centrifugation**
With the help of an electrical centrifuge 5-20 ml of water sample is centrifuged for about 10-20 mins at 1500-2000 rpm. The supernatant water is removed by decanting. The plankton is precipitated by adding a few drops of 1% Potassium aluminium sulphate or fixed weak neutralized formalin or Lugol’s solution. Centrifugation is inappropriate for fragile taxa and colonial species, due to disintegration and cell destruction. Centrifugation is not recommended for counting, disrupts flagellates, hard to make it quantitative.

**Phytoplankton Observation**
Many dinoflagellates and naked flagellates are damaged or severely deformed by formalin and Lugol’s solutions, so an inspection of live material is desirable. The living cells must also not be subjected to the heat from a microscope lamp for more that a few minutes due to their extreme sensitivity.
Observe prepared microscope slides and live samples for phytoplankton. To observe the live sample you must first prepare a “live mount” as follows:
- obtain and clean a depression slide
- obtain a slide cover slip.
- place one to two drops of plankton containing water into the depression
- hold the cover slip over the filled depression
- allow one edge of the slip to touch the slide
- allow the cover slip to drop over the water

Place the slide onto the stage of the microscope and view. You should sketch several of the organisms that you see.

**Sample preparation for chlorophyll analysis lab.**
From your unpreserved water sample, measure 500 ml in a cylinder and filter on 25 mm diameter Whatman GF/F glass fiber filter. Fill approximately 150 ml into the filtration funnel. The vacuum on the pump gauge shall not read more than –300 mbar to prevent cell damage during filtration (which can lead to underestimation of phytoplankton chlorophyll).
Refill the funnel before the filter runs dry. Let the filter run dry at the end of filtration. Under applied vacuum, lift the GF/F filter carefully with forceps and place filter on the bottom of a 20 ml glass scintillation vial with the filtered sample up. Close vial and store in the freezer at –20°C until chlorophyll extraction during next class. Make sure again that all your vials and tubes are properly labeled, using labeling tape and ball pen.