Annex II

Analytical techniques

1. CHLOROPHYLL *A*

The methanol extract technique described in Pearson, Mara and Bartone (1987) should be used.

Materials and equipment

(a) 1% (w/v) aqueous suspension of $MgCO_3$;
(b) 90% (v/v) aqueous methanol'
(c) 25 mm glass fibre filter papers (e.g. Whatman GF/C);
(d) compatible filtration system (e.g. Whatman 1960 032 with a 250-1000 ml filter flask) and vacuum source;
(e) simple spectrophotometer (663 and 750 nm);
(f) small bench centrifuge (500*g*)

Different sized filter papers may be used, and if glass fibre filter papers are not available a good quality general purpose paper (e.g. Whatman grade 2) may be used. The centrifuge is not essential, but improves the spectrophotometry by removing any turbidity present.

Method

(a) Filter 2.5 ml of the $MgCO_3$ suspension (this aids retention of the algae and maintains alkaline conditions to prevent denaturation of the chlorophyll during extraction).
(b) Filter a known volume (at least 10 ml and preferably close to 50 ml) of well-stirred pond column subsample.
(c) Place the filter paper in a glass test tube and add 10 ml of 90% methanol. Boil for 2 minutes to extract the chlorophyll (the solvent boils at around 65ºC, so it can be boiled in a hot water bath). The filter paper should become white; if it does not, macerate it with a glass rod to aid extraction.
(d) If a centrifuge is available, centrifuge the extract at 500 *g* for 10 minutes. Otherwise leave the extract for 15 minutes in the dark to allow most of the debris to settle out.
(e) Make up the extract volume to exactly 10 ml by adding 90% methanol, and transfer a portion of the extract to a 1 cm cuvette.
(f) Set the wavelength on the spectrophotometer to 663 nm (or 665 nm if calibrated in 5 nm divisions). Zero with 90% methanol, and read the absorbency of the chlorophyll extract (the absorbency should be between 0.2 and 0.8; if it is less, re-extract using a larger sample volume; if more, dilute with a known volume of methanol). Set the wavelength to 750 nm,

re-zero and read the absorbency of the extract (this corrects for turbidity by measuring non-specific absorbency).

(g) Calculate the concentration of chlorophyll *a* from:

$$\text{Chl}a\ (\mu g/l) = [OD_{663} - OD_{750})/77][V/S] \times 10^6$$

where OD_{663} and OD_{750} are the absorbency readings at 663 and 750 nm, and V and S are respectively the solvent extract volume and original sample volume, both in ml. The figure of 77 is the extinction coefficient for chlorophyll *a* in 90% methanol in l/g cm. If the path length of the cuvette used is not 1 cm, then the absorbency difference should be divided by the path length in cm.

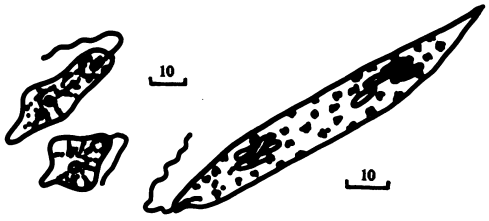
(h) Pond samples should not be stored prior to analysis for longer than 6 hours. In the field the best stage for storage is after filtration. The filter papers should be dried in the dark and at as low a temperature as possible (preferably 4°C). If they are then kept in the dark (e.g. wrapped in foil), they may be stored for several weeks prior to spectrophotometric analysis with a maximum absorbency loss of only 10%.

2. ALGAL IDENTIFICATION

Microscopic examination should first be carried out using a magnification of × 100, (usually × 10 objective and × 10 eyepiece), which will enable the detection of large algal cells such as *Euglena*. However smaller cells, such as *Chlorella*, and certain cell constituents, such as the spines of *Micractinium* or *Scenedesmus* and the eyespot of *Euglena*), can only be observed using a magnification of × 400 or greater. Cell sizes can be determined using an eyepiece graticule that has been calibrated using a stage micrometer (this is a microscope slide on which a 1 mm line, divided into 100 equal divisions has been etched).

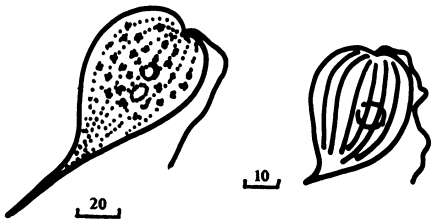
If it is not possible to examine the samples immediately, then they can be preserved with either 4% formalin or 0.7% Lugol's iodine. Ideally two subsamples should be taken and on preserved with each. Formalin preservation results in a more natural colouration, while iodine has the advantage of acting as a cytological stain for starch granules and aoligosaccharides, the location of which within the cell can aid identification. Iodine preservation also results in increasing the density of the cells, which can aid in the concentration and sedimentation of algal cells.

In the figures below, the bar dimensions are micrometres.



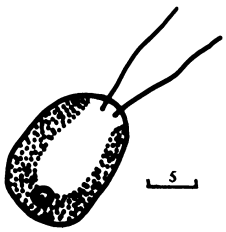
Euglena

Probably the most commonly occurring waste stabilization pond alga. Often present as a surface-stratified layer, especially in facultative ponds. Large cells (up to 150 μm long). Can be elongated and highly motile or amoeboid and slow moving, changing shape continuously, and often rounding up and remaining motionless on microscope slides. Very green with a usually conspicuous red eyespot.



Phacus

Sometimes confused with *Euglena*, but possesses conspicuous tapering tail and striated body. Also cells often with pronounced dorsoventral flattening (leaf-like in shape), often with some part of cell twisted. Changes shape like *Euglena*.

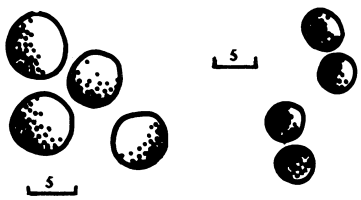


Chlamydomanas

May occur as a surface film in anaerobic ponds, but very common in highly loaded facultative ponds. Very small, ovoid with anterior end rounded. 5-10 μm diameter. 10-20 μm broad. Highly motile. Two flagella but not usually visible.

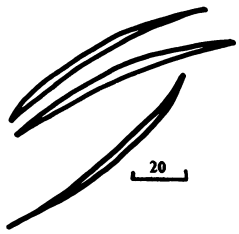
Pandorina

Common in highly loaded ponds. Cells pear shaped and embedded in a spherical mass of mucilage with 8 or 16 (rarely 32) cells per colony. Cells 8-15 μm broad. Colonies 20-50 μm diameter. Most distinctive feature is tumbling motion of colonies through the water.



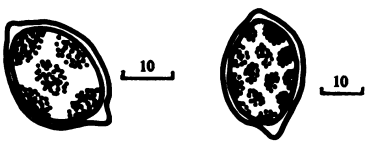
Chlorella

Very common in all types of aerobic ponds.
Unicellular. Spherical. Very small.
Has a cup-shaped chloroplast
C.pyrenoidosa : 3-5 μm diameter
C.vulgaris : 5-10 μm diameter.



Ankistrodesmus

Sometimes found in maturation ponds.
Cells needle-like (25-100 μm long by 2-6 μm broad).

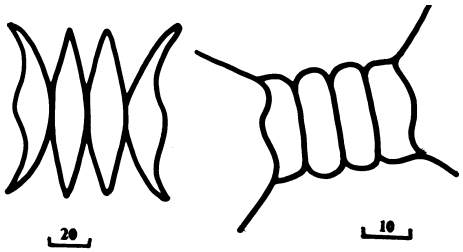


Oocystis

Common in all types of aerobic ponds. Solitary or in groups still enclosed in mother cell wall. Up to 8 cells per colony. Polar nodules on individual cells. Most common pond species is *O.crassa*, approximately 10-20 μm broad, 14-26 μm long.

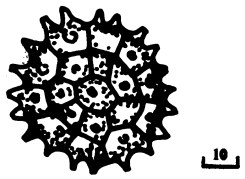
Scenedesmus

Very common in maturation ponds. Can exist as unicells or colonies of up to 16 cells. Spines often (but not always) present. Most common pond species is *S.quadricauda* with 4 cells (colony approximately 7 × 16 μm) and two spines on each terminal cell.



Pediastrum

Sometimes found in maturation ponds. Colonial. Cells on outer edge have two blunt projections. Individual cell approximately 15μm diameter. Most common pond species is *P.boryanum* with 36 cells per colony.

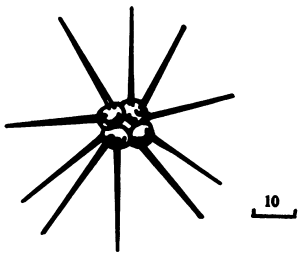


Selenastrum

Sometimes found in maturation ponds. Cells lunate to arcuate (strongly curved). Often in aggregates of 4, 8 or 16 cells. 2-8 μm broad, 7-38 μm long.

Micractinium

Very common in maturation ponds. Colonial. 4-16 cells in pyramid or square. Outside cells have fine tapering spines or setae (1-5 in number) 10-35μm long. Individual cells spherical, approximately 3-7 μm diameter.



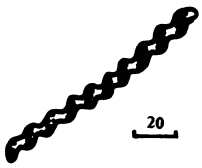
Diatoms

Diatoms are also common in waste stabilization ponds on some occasions. Their occurrence is partly determined by the silicon content of the water as this element is a necessary constituent of the cell wall. They are characterised by a thick cell wall ('frustole') which is in two halves; the shape and patterning of this silica frustole are characteristic of the diatom genera.

Cyanobacteria

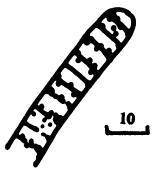
Cyanobacteria, also called blue-green algae, occur in maturation ponds receiving a very light organic loading. They are thus characteristic of 'clean' ponds. Three common genera are:

Spirulina



Regularly spirally coiled cylinder in which individual cells are not obvious. 1-15 μm broad. Common in still waters and can be strongly dominant in ponds having long retention times and whose water is rich in dissolved solids due to concentration by evaporation.

Oscillatoria



Filamentous, individual cells, truncate. Approximately 10 μm diameter and 3-5 μm long. Able to move actively across surfaces through a gliding oscillatory motion.

Anabaena

Filamentous. Cells spherical to cylindrical, 3-5 μm long. Able to move actively through oscillatory motion. Often possess two types of cell: normal vegetative cells; and larger less opaque cells, called heterocysts, which have thickened cell walls. Heterocysts are either terminal (at the end of a filament) or intercalary (located within the filament). At high ammonia or nitrate concentrations heterocysts are absent.

3. SULPHIDE

Sulphide analysis should be carried out using the following procedure:

Reagents

a) Phenylenediamine: 0.2% *w/v* N, N-dimethyl-p-phenylenediamine sulphate in 20% (v/v) H_2SO_4. Dissolve 2 g compound in 200 ml distilled water and add 200 ml of conc. H_2SO_4. Allow to cool and dilute with distilled water to 1000 ml. **Caution**: *this is very poisonous by skin absorption.*

b) Ferric reagent: 10% w/v ammonium ferric sulphate in 2% (v/v) H_2SO_4. To 10 g Fe_3 (NH_4) $(SO_4)_2$. $12H_2O$ add 2 ml conc. H_2SO_4. Dilute to 100 ml with distilled water. Heating will be required to dissolve the compound.

Method

1) Sulphide is rapidly oxidised to sulphate when oxygen is present. Once samples have been taken, it is therefore essential to fix the sulphide immediately. This can be done by adding the first reagent to the samples. 10 ml volumes of reagent (a) should be dispensed into 100 ml volumetric flasks and these taken to the sampling points. 10 ml volumes of sample should then be dispensed into these volumetric flasks immediately after the samples have been taken. The sulphide fixed in this way will be stable for at least one hour, but stability beyond this time has not yet been evaluated.
2) Add 2 ml of reagent (b) and leave for ten minutes. A pink colour will develop initially but this should only be transitory. The presence of sulphide will then be indicated by the development of a deep blue colour.
3) Dilute samples to 100 ml and read absorbence at 670 nm. Blanks should consist of 10 ml of sample in 90 ml of distilled water. Readings can be converted into sulphide concentrations using a standard curve.

Preparation of standard curve

Dissolve 0.75606 g of $Na_2S.9H_2O$ in distilled water and make up to 100 ml in a volumetric flask. This stock solution will contain 100 µg total sulphide per ml. Using this stock solution, make up the following solutions in 100 ml volumetric flasks:

Stock solution (ml)	Distilled water (ml)	Reagent (a) ml	Reagent (b) ml
0.1	87.9	10	2
0.2	87.8	10	2
0.3	87.7	10	2
0.4	87.6	10	2
0.5	87.5	10	2

Reagent (b) should be added last of all. This will produce a standard curve which is linear up to an absorbence of about 0.7, i.e. within a total sulphide range of 0-50 µg per 100 ml final volume. This procedure should be carried out as quickly as possible so as to avoid oxidation of the sulphide in the stock solution.