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# Spectrophotometric analysis of chlorophyll a in freshwater phytoplankton

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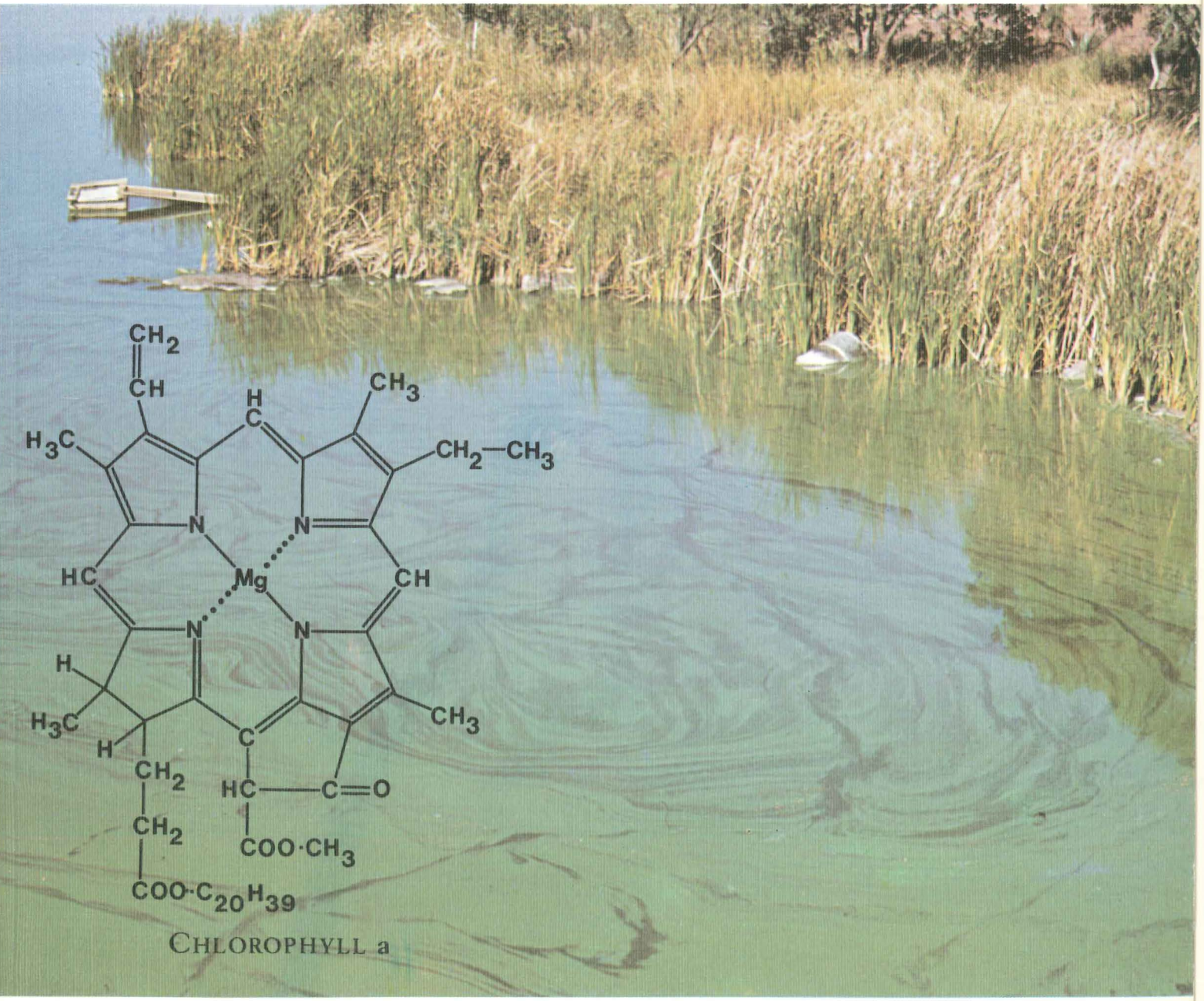


CHLOROPHYLL a

**DEPARTMENT OF WATER AFFAIRS**
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# SPECTROPHOTOMETRIC ANALYSIS OF CHLOROPHYLL *a* IN FRESHWATER PHYTOPLANKTON

by David P. Sartory
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## AUTHOR'S NOTE

Throughout this work the concentrations of the solvents used for the extraction of photosynthetic pigments from freshwater phytoplankton have been described in terms of %(v/v) aquaeous solutions. Although it is incorrect in terms of the S.I. system to refer to concentrations such as 90% acetone, 95% ethanol, etc. these terms are well established and readily recognised in their usage, particularly by those involved in research employing the extraction and analysis of photosynthetic pigments from algae.

A table is, however, presented below by which %(v/v) solvent concentrations cited in this study are related to mol $\ell^{-1}$.

| Solvent | Solution (%) | Concentration (mol $\ell^{-1}$) |
|---|---|---|
| Acetone | 80 | 10,9 |
| | 90 | 12,3 |
| Ethanol | 80 | 13,7 |
| | 90 | 15,4 |
| | 95 | 16,3 |
| Methanol | 90 | 22,4 |
| | 95 | 23,6 |
| | 100 | 24,8 |

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# CHAPTER 1 LITERATURE REVIEW

## 1.1 INTRODUCTION

Water supply in South Africa is becoming increasingly limited, and by the turn of the century (or soon after) demand may outstrip the supply (Commission of Enquiry into Water Matters, 1970; Du Plessis and Van Robbroeck, 1978). Eutrophication, the influx of plant nutrients into a water body normally resulting in excessive algal and/or macrophytic growth, is already a major water quality problem in certain areas of South Africa, (Toerien, 1975, 1977). Algal biomass is most commonly estimated indirectly by the determination of chlorophyll a (e.g. Walmsley, Toerien and Steyn, 1978; Pieterse and Toerien, 1978), which constitutes 1 to 2% of the dry weight of organic material in phytoplankton (Standard Methods, 1976). Much work is now being conducted on the prediction of algal biomass in terms of chlorophyll a as a function of a nutrient, usually phosphate (e.g. Pieterse and Toerien, 1978; Nicholls and Dillon, 1978; Walmsley and Butty, 1979).

The most commonly used methods for chlorophyll and phaeopigment determinations involve spectrophotometric analysis at selected wavelengths of an eluant of the algal photosynthetic pigments using a variety of solvents (Richards and Thompson, 1952; Parsons and Strickland, 1963; Lorenzen , 1967; Moss, 1967a,

1967b; Marker, 1972; Strickland and Parsons, 1972; Riemann, 1976; Shoaf and Lium, 1976; Holm-Hansen and Riemann, 1978; Nusch 1980). Other methods described involve fluorometry (Yentsch and Menzel, 1963; Holm-Hansen, Lorenzen, Holmes and Strickland, 1965; Loftus and Carpenter, 1971; Slovacek and Hannan, 1977), paper chromatography (Jensen and Liaaen-Jensen, 1959; Jensen and Sakshaug, 1973), thin-layer chromatography (Jeffrey, 1968; Garside and Riley, 1969; Daley, Gray and Brown, 1973) and reflectance (Bergmann and Peters, 1980).

A wide variety of spectrophotometric methods are used by researchers in the field of limnology, varying mainly in procedures for pigment extraction and method of calculation. The various solvents employed in extracting chlorophyll have marked differences as regards efficiency of extraction of the photosynthetic pigments from different groups of algae (Steemann Nielsen, 1971; Marker, 1972; Riemann, 1976). It is, therefore, difficult to compare results obtained by various workers. There is a real need for a reliable and efficient standard method for chlorophyll a determination.

In this chapter the literature pertaining to the spectrophotometric determination of chlorophyll a in phytoplankton is reviewed. It was decided to investigate the spectrophotometric procedures for chlorophyll a determination as this method is most commonly employed due to its simplicity, cheapness to

conduct and since most laboratories own a spectrophotometer. Evidence presented here shows that there are many problems associated with the "standard procedures" which have been employed since their inception in the early 1950's.

## 1.2 SPECTROPHOTOMETRIC METHODS OF CHLOROPHYLL a DETERMINATION

The spectrophotometric methods for the determination of chlorophyll a all have the following three basic steps:-

1) Concentration of the algae by filtration, aided by gentle suction, of a measured volume of sample (normally between 0,5 and 2,5 litres depending on the density of the algae) onto a glass fibre or membrane filter. This step is sometimes accompanied by the addition of a small quantity of magnesium carbonate to aid retention and prevent acidity.

2) Extraction of the photosynthetic pigments by placing the filter and its entrapped plankton into a known volume of a suitable solvent. In most cases either acetone or methanol (each with varying percentages of water) are used, cold or boiling. Extraction times vary from 10 minutes to 24 hours. The filters are sometimes homogenised or ground to aid extraction. After extraction the extract is clarified by centrifugation.

3) Spectrophotometric analysis of the extracts for their optical density at one or three wavelengths in the red spectral region (600 - 700 nm). An optical density reading at 750 nm is subtracted from these readings to correct for "background" interferences. The concentration of chlorophyll *a* in $\mu g\ \ell^{-1}$ ($mg\ m^{-3}$) is then calculated using one of the many published formulae.

### 1.2.1 FILTRATION

Both membrane filters and glass fibre filters are commonly used although Holm-Hansen and Riemann (1978) suggest that some workers are reluctant to use glass fibre filters due to the lack of an advertised "pore size", and thus question their ability to retain small cells. The use of the membrane filter (Millipore type AA) was first recommended by Creitz and Richards (1955) to replace the plankton centrifuge, and later glass fibre filters were proposed by Yentsch and Menzel (1963).

Long and Cooke (1971) compared chlorophyll *a* values in acetone extracts obtained when using Millipore HA membrane filters and Whatman GF/C and GF/A glass fibre filters. They found that absorbance readings were 6 to 18% higher when glass fibre filters were used. Filtering time with the Whatman filters was cut by a factor of 10 and the cost of materials reduced by a factor of four. Sheldon (1972)

examined the particle size retention properties of, amongst others, Millipore HA membrane filters, Whatman GF/C and GF/A glass fibre filters and Reeve Angel 984H micro-fine glass fibre filters, and found that the glass fibre filters had retention characteristics similar to those of the membrane filters. Holm-Hansen and Riemann (1978) found that the amount of chlorophyll recovered from glass fibre filters (Whatman GF/C) was equal to or greater than that recovered from membrane filters (0,22 and 0,45 µm pore size). The Whatman GF/C also gave comparable results to those obtained with the micro-fine Reeve Angel 984H glass fibre filter. In contrast Lenz and Fritsche (1980) reported that, when using samples of brackish water, 0,45 µm membrane filters had an average of 10% higher retention than glass-fibre filters.

Millipore HA membrane filters have been recommended by Greeson, Ehlke, Irwin, Lium and Slack (1977) but Creitz and Richards (1955) reported that these filters, which dissolve when placed in 90% acetone, showed absorbancy at the wavelengths used for chlorophyll determinations. Daley, Grey and Brown (1973) found that the use of cellulose ester filters caused interference during chromatographic separation of pigments. Jensen and Sakshaug (1973) also reported interference problems from dissolution of Millipore HA filters, which had to be removed by precipitation with diethyl ether prior to chromatography.

Thus, glass fibre filters have several advantages over membrane filters:-

1) Samples are filtered more rapidly through glass fibre filters than through membrane filters (Long and Cooke, 1971; Talling, 1974).

2) Glass fibre filters are much cheaper than membrane filters. In South Africa a 47 mm diameter glass fibre filter costs approximately 15 cents while 47 mm diameter membrane filters (with 0,45 $\mu$m pore size) cost in the region of 50 cents each (1981 prices).

3) There are no absorbancy problems associated with the use of glass fibre filters.

4) Where grinding or homogenisation of the sample is used, glass fibre filters provide excellent abrasive material to aid the process (Holm-Hansen and Riemann, 1978).

The addition of a small quantity of magnesium carbonate to the filter or the sample during the filtration process is often recommended (Richards and Thompson, 1952; Creitz and Richards, 1955; Holm-Hansen et al, 1965; Strickland and Parsons, 1972; Talling, 1974; Standard Methods, 1976;

Jensen, 1978). The main reason for this is to prevent acidity which would cause the breakdown of chlorophyll to phaeophytin (Richards and Thompson, 1952; Strickland and Parsons, 1972). The addition of $MgCO_3$ to the filter before the sample is filtered has been reported to result in more effective retention of the algae (Humphrey and Wootten, 1966; Garside and Riley, 1969; Sand-Jensen, 1976). If, however, the $MgCO_3$ is added to the sample during filtration then this property is not apparent during the initial phase of filtration (Sand-Jensen, 1976).

Rai (1973), Riemann (1976) and Holm-Hansen and Riemann (1978) found that the omission of $MgCO_3$ did not result in the lowering of chlorophyll values. Reduction of phaeophytin _a_ and other degradation products due to the addition of $MgCO_3$, however, has been reported (Daley, Grey and Brown, 1973; Riemann, 1976; Holm-Hansen and Riemann, 1978). Daley _et al_ (1973) found a small reduction in phaeophytin _a_ concentrations, and losses of chlorophyllide _a_ and phaeophorbide _a_ of up to 20% and 70% respectively. They also reported no improvement of retention efficiency when $MgCO_3$ was added to the filters. Riemann (1976) found an 8% reduction in phaeophytin _a_ values when $MgCO_3$ was added to July samples from Lake Mossø. Using an alternative to $MgCO_3$, dimethylaniline, Patterson and Parsons (1963) also found that phaeophytin _a_ values were reduced. It therefore appears that the presence of $MgCO_3$

results in a significant decrease in these degradation products, probably due to their adsorption by $MgCO_3$ (Riemann, 1976; Holm-Hansen and Riemann, 1978). Sand-Jensen (1976) found a decrease in chlorophyll a values when filters containing $MgCO_3$ were stored. He attributes this to the formation of aggregates of algae and $MgCO_3$ which were very difficult to dissolve. Holm-Hansen and Riemann (1978) feel that this phenomenon may be species specific. They found, during their studies on marine and freshwater algae, no reduction in chlorophyll values during storage associated with the addition of $MgCO_3$.

Thus the benefits of addition of $MgCO_3$ appear to be outweighed by the problems associated with its use, particularly when chlorophyll degradation products are to be studied. Despite reports of faster and more effective retention when a thin layer of $MgCO_3$ is added to the filter (Humphrey and Wootten, 1966; Sand-Jensen, 1976) it appears that the use of this procedure should only be considered when very small algal cells constitute a significant portion of the phytoplankton and then only after consideration of the potential error associated with the use of $MgCO_3$.

### 1.2.2 STORAGE OF FILTERED SAMPLES

After filtration the filters should be immediately placed in the extraction solvent (Strickland and Parsons, 1972;

Talling, 1974; Greeson et al, 1977). Should extraction be delayed, however, the dry filters may be stored frozen (-20°C) in a desiccator in the dark to prevent photochemical chlorophyll breakdown (Strickland and Parsons, 1972; Weber, 1973; Standard Methods, 1976; Greeson et al, 1977). This procedure, however, results in lower values and increased difficulty in extraction of chlorophyll (Strickland and Parsons, 1972; Talling, 1974). Glooschenko (personal communication in Talling, 1974) found no loss of pigments when samples were freeze-dried at ~15°C. Lenz and Fritsche (1980) reported, however, that storage of freeze dried filters in the dark at room temperature resulted in a 34% reduction in the chlorophyll a content. Holden (1976) states that freeze-dried plant material can be stored for several months at -20°C in the dark without degradation of chlorophyll, provided the tissue was not acid to start with. Holm-Hansen and Riemann (1978) found no loss of chlorophyll or change in chlorophyll:phaeopigment ratios when filters were stored in the wet state at -20°C for up to two weeks and then extracted with 90% acetone. There were also no losses of chlorophyll when filters were extracted in 100% methanol after storage at -20°C for three weeks. Lenz and Fritsche (1980) found that deep-frozen filters yielded reliable results even after a 6 month storage period.

Sand-Jensen (1976) found a marked drop in chlorophyll concentrations when filters with either Scenedesmus quadricauda or Nitzschia palea were stored in a desiccator at

4°C for three days and subsequently extracted with either methanol or 90% acetone. The loss was more pronounced in the methanol extracts (38,9% and 48,9% for Scenedesmus quadricauda and 14,1% and 25,2% for Nitzschia palea) than in the 90% acetone extracts (17,6% and 18,7% for Scenedesmus quadricauda and 13,7% and 16,8% for Nitzschia palea). Using a natural plankton from the Frederiksborg Slotssø (consisting mainly of Stephanodiscus and Asterionella) the reduction was 30,5% in methanol and 1,6% in 90% acetone. A similar reduction (20,0%) was also found with Scenedesmus quadricauda stored at -15°C for three days and then extracted with methanol.

From these results it is apparent that storage of filters should be avoided whenever possible. If, however, extraction cannot be begun immediately (e.g. on field trips) then storage of the filters at -20°C may be considered. Freeze-drying is impractical on such occasions.

### 1.2.3 EXTRACTION OF CHLOROPHYLL a AND PHAEOPHYTIN a

The chlorophyll and phaeopigments of phytoplankton are normally extracted in either acetone (usually 90%) or methanol (90% or 100%) (Richards and Thompson, 1952; Yentsch and Menzel, 1963; Strickland and Parsons, 1972; Talling, 1974; Tett, Kelly and Hornberger, 1975; Riemann, 1976; Standard Methods, 1976; Holm-Hansen and Riemann, 1978). Recently 90% and 95% ethanol

has been employed (Nusch and Palme, 1975; Bergmann and Peters, 1980; Nusch, 1980) to extract algal pigments, although ethanol has been used to extract leaf chlorophylls (Wickliff and Aronoff, 1962).

A method for the extraction of chlorophyll using chromatographic columns packed with XAD-1 resin has been developed by Wun, Rho, Walker and Litsky (1979a, 1979b).

### 1.2.3.1 EXTRACTION WITH ACETONE OR WITH METHANOL

For many years 90% acetone has been employed for extraction of chlorophylls and their degradation products (Richards and Thompson, 1952; Parsons and Strickland, 1963; Strickland and Parsons, 1972; Weber, 1973; Standard Methods, 1976; Greeson et al, 1977). Vernon (1960) used 80% acetone to extract plant pigments. Acetone containing 10 to 20% water has been used in preference to anhydrous acetone as:

(a) it eliminates the necessity of drying the sample, and

(b) chlorophyll had proven difficult to extract with 100% acetone (Marker, 1972).

Acetone was selected originally as it is readily miscible with water thus facilitating extraction from fresh tissues (Marker, 1972).

The major attraction for its use today is the wealth of data on the spectral characteristics of chlorophyll and phaeophytin in acetone solutions, and thus an abundance of equations for the calculation of chlorophyll concentrations (Richards and Thompson, 1952; Parsons and Strickland, 1963; Talling and Driver, 1963; UNESCO, 1966; Jeffrey and Humphrey, 1975).

More recently, however, methanol has been gaining popularity. This is mainly because of its proven superiority as an extraction agent, particularly in regard to members of the Chlorophyceae and Cyanophyceae (Steemann Nielsen, 1961; Marker, 1972; Rai, 1973; Riemann, 1976; Sand-Jensen, 1976; Holm-Hansen and Riemann, 1978; Riemann, 1980). Steemann Nielsen (1961) reported that complete extraction from Chlorella vulgaris only occurred with anhydrous methanol. He found that chlorophyll was not fully extracted when the cells were boiled in 90% acetone for one minute. Marker (1972) also reported that the threadform algae Cladophora and Vaucheria could only be completely extracted with absolute methanol. Marker (1972) found that 90% acetone extracted only 14% of the chlorophyll from a three week old culture of Chlorella pyrenoidosa and 80% from Spirogyra. In both cases the remaining chlorophyll was extracted with 100% methanol. Using Scenedesmus quadricauda, Rai (1973) found methanol was a better extraction agent than a 1 : 1 (v/v) acetone - methanol mixture, which in turn was more efficient than 90% acetone. Sand-Jensen (1978) found that absolute methanol extracted about 30% more chlorophyll a from

Scenedesmus quadricauda cells than did 90% acetone with homogenisation. The two solvents were nearly equal in the amounts of chlorophyll a extracted from the diatom Nitzschia palea, although 2,4 to 4,6% higher for 90% acetone than methanol. Riemann (1976) and Holm-Hansen and Riemann (1978) found that methanol not only extracted more chlorophyll from blue-green algae, but an extraction time of only one hour was required. Twenty hour acetone extraction of a natural diatom population from Lake Mossø was equivalent to a 1 hour extraction period with absolute methanol (Table 1). With a blue-green algae population dominated by Aphanizomonon flos aquae, 90% acetone extraction for 20 hours resulted in chlorophyll values of 88% when compared to 1 hour extraction in absolute methanol (Table 1). Riemann (1976) also found that 1 hour was the optimum time for extraction with methanol. When extraction time was increased to 20 hours there was a significant decrease in chlorophyll a values (from 158 $\mu g\ \ell^{-1}$ to 123 $\mu g\ \ell^{-1}$) and an accompanying increase in phaeopigment a values (from 29 $\mu g\ \ell^{-1}$ to 58 $\mu g\ \ell^{-1}$).

This change in chlorophyll and phaeopigment concentrations may be avoided when a reducing agent is added to the methanol. The addition of $H_2S$ to methanol was used by Jensen and Sakshaug (1973) who found that when chlorophyll was extracted with a 50% aqueous methanol solution for 15 minutes, most of the chlorophyll a was converted to chlorophyllide a. This did not occur when absolute methanol containing $H_2S$ was employed. Jensen (1978)

TABLE 1: Efficiency of 90% acetone and 100% methanol for the extraction of chlorophyll from natural diatom and blue-green algae populations from Lake Mossø. (From Riemann (1976) and Holm-Hansen and Riemann (1978)).

| Dominant phytoplankton | Extraction time (h) | µg chlorophyll a per sample using | |
|---|---|---|---|
| | | 90% acetone* | methanol* |
| Diatoms | 1 | 34,2 ± 0,4 | 37,0 ± 0,6 |
| | 6 | 35,1 ± 0,8 | |
| | 12 | 36,2 ± 0,7 | |
| | 20 | 36,8 ± 0,6 | |
| Blue-green algae | 1 | 28,6 ± 0,2 | 34,1 ± 0,4 |
| | 6 | 29,4 ± 0,4 | |
| | 12 | 29,8 ± 0,8 | |
| | 20 | 30,0 ± 0,5 | |

* mean and standard error of five replicates.

states that planktonic algae are best extracted with methanol containing $H_2S$. The presence of $H_2S$ inhibits allomerisation (auto-oxidation in alcohols) of chlorophylls, the process being associated with an uptake of oxygen equimolar with the chlorophyll present (Riemann, 1976). Allomerisation is brought about by chlorophyllase activity (Marker, 1972) which is still active in

high concentrations of alcohols and acetone (Weast and Mackinney, 1940, as cited by Riemann, 1976).

Riemann (1976) experimented with extraction from natural phytoplankton dominated by Melosira, Tabellaria and Aphanizomonon using methanol containing 0%, 10% and 20% water. The result was a substantial decrease in the concentrations of chlorophyll a in the two aqueous methanol extractions (Figure 1). Riemann attributes this to decreased extraction efficiency rather than chemical change such as allomorisation. The extraction of phaeophytin a was unaffected by the water content in the methanol.

In contrast to the results above showing improved extraction from blue-green algae using methanol, Talling and Driver (1963) found that 90% acetone and 90% methanol gave the same results when used to extract chlorophyll a from Oscillatoria agardi var. isothrix, and a natural phytoplankton population from Lake Victoria. Youngman (1978) reported better extraction with 90% acetone at 4°C than with 90% methanol at 4°C on Spring diatom and Autumn blue-green algae populations from Farmoor Reservoir. The reverse was true during the bloom of green algae during the summer. Both those workers, however, used 90% methanol which Riemann (1976) and Marker (1980) have shown is not as efficient as absolute methanol (Figure 1).

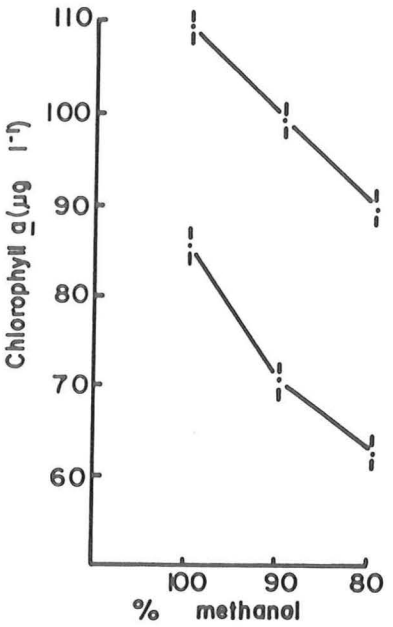


FIGURE 1: Amount of chlorophyll a extracted from natural lake plankton (see text) using methanol containing varying amounts of water. The two curves represent different levels of concentration. 95 % confidence intervals are indicated (n = 4). From Riemann (1976).

According to Talling (1974), boiling in methanol for a short period (e.g. 30 seconds) greatly accelerates extraction, which can often be completed within 10 minutes. Tett et al (1975) used boiling methanol at 70°C for 2 minutes to extract chlorophyll a and phaeophytin a from

benthic microalgae. Youngman (1978) found that boiling 90% methanol produced similar results to those of cold acetone when used on Winter and Spring Farmoor Reservoir populations (mainly diatoms). In Summer, when the population was mainly green algae, boiling 90% methanol extracted pigment concentrations were 24% higher than those from cold acetone. Increased pigment extraction with boiling 90% methanol was also noted when Microcystis aeruginosa dominated the phytoplankton. These results indicate that boiling methanol may provide an adequately rapid method of chlorophyll extraction. Riemann (1976) however, found that prolonged boiling (3 minutes or more) in 100% methanol resulted in a large decrease in chlorophyll a and an increase in phaeophytin a. Using a phytoplankton population from Lake Mossø dominated by blue-green algae, he found that boiling in a water bath at 100°C for $\frac{1}{2}$, 1 or 2 minutes resulted in no improvement over unboiled extracts. Boiling for three minutes or more resulted in a significant loss in chlorophyll a. Repeating the experiment, but boiling in a water bath at 70°C instead, he found a minor increase after 1 minute and a limited decrease in chlorophyll a concentration after prolonged boiling. In both cases the concentration of phaeophytin a increased. Riemann (1976) attributed the large loss with the 100°C treatment to the violent nature of the boiling, when extracts were splashed onto the side of the flask, and two rinses with methanol did not remove all the

chlorophyll. Although the presence of $H_2S$ may prevent oxidation, it will not, however, prevent loss of magnesium from chlorophyll producing phaeophytins (Riemann, 1976).

As discussed above many studies on the relative extraction efficiencies of acetone and methanol have been conducted and finally, following the workshop on the measurement of photosynthetic pigments in freshwater held in 1978 at the Max Planck Institute, Plön, West Germany, it has been recommended that "acetone should no longer be used because of poor extraction of pigments from green and blue-green algae" (Marker, Nusch, Rai and Riemann, 1980).

Recently a modification of the 90% acetone extraction method employing dimethyl sulphoxide (DMSO) has been proposed (Shoaf and Lium, 1976; Burnison, 1980). This involves initial extraction with DMSO (with grinding) for three minutes and then doubling the volume with 90% acetone resulting in a 1 : 1 (v/v) mixture of DMSO and 90% acetone. As the absorption spectrum of chlorophyll a was the same in the acetone-DMSO mixture as in the 90% acetone, the formulae used for calculation of the chlorophyll a concentration in 90% acetone can also be used for this method. Using the acetone-DMSO method the authors found equal extraction to 90% acetone for diatoms and blue-green algae and a very much improved extraction with green algae.

To avail himself of the spectral characteristics of chlorophylls and phaeopigments in acetone after having extracted in methanol, Marker (1972, 1976) transfered the pigments to 90% acetone. This was done by evaporating a small volume of the methanol extract to dryness in a rotary evaporator with a high vacuum at a temperature of 35°C in the dark. When evaporation was completed the pigments were then redissolved in 90% acetone. Using Melosira varians and a young growth of Cladophora, Marker (1972) reported that the transfer procedure did not result in a degradation of pigments.

### 1.2.3.2 ETHANOL AS EXTRACTION SOLVENT

More recently ethanol has been employed as the extraction solvent (Nusch and Palme, 1975; Bergmann and Peters, 1980; Nusch, 1980). Ethanol as an 80% aqueous solution has been used to extract leaf chlorophylls (Wickliff and Arønoff, 1962), and Wintermans and de Mots (1965) have published spectrophotometric characteristics of chlorophylls a and b in 96% ethanol, and of phaeophytins a and b in acidified 80% ethanol. Nusch and Palme (1975) and Nusch (1980) employed 90% ethanol, while the method used by Bergmann and Peters (1980), developed by M. Ostrovsky (Biology Department, Alleghany College, Meadville, Penn., U.S.A.), uses 95% ethanol.

Nusch and Palme (1975) and Nusch (1980) reported that, when extracting from a culture of Scenedesmus, ethanol and methanol

extracted equally efficiently and both were considerably more efficient than acetone. This was particularly so where the algae used were in the stationary growth phase. The greater yields provided by ethanol and methanol over acetone were not so conspicuous when algae in the log growth phase were used.

The extraction efficiency of all three solvents was improved when they were heated although hot acetone did not yield the efficiency of the alcoholic extractions (Nusch and Palme, 1975; Nusch, 1980). The best results obtained by Nusch (1980) were when boiling 90% ethanol (at 78°C) was poured over damp filters, and an extraction time of six hours was allowed. Nusch (1980) recommends the use of ethanol in preference to methanol as it is less of a health hazard and presented fewer problems during extract acidification for spectrophotometric analysis.

The method used by Bergmann and Peters (1980) involved extraction of the pigments in 90% ethanol overnight in a refrigerator. The samples were shaken on addition of the ethanol and again after one hour. According to Bergmann and Peters (1980), Ostrovsky has found that this method offers similar advantages to methanol over using acetone, due to ease of, and better, extraction.

The use of ethanol as the pigment extraction agent, however, has yet to be widely tested over a range of physiological and ecological conditions (Marker, Nusch et al, 1980).

1.2.3.3 XAD-1 COLUMN METHOD

Wun, Rho, Walker and Litsky (1979a, 1979b) have recently developed an extraction process employing XAD-1 resin. The method as described by Wun *et al*, (1979b) is as follows:- plankton is collected on a Whatman GF/F filter paper on top of a high pressure chromatographic column (18 cm x 1,5 cm internal diameter) packed with XAD-1 (60 x 120 mesh size) resin. The pigments are extracted with 50 mℓ methanol containing 7% ammonium hydroxide which is passed through the column at 5 mℓ $min^{-1}$. The addition of 7% ammonium hydroxide to the methanol was found to increase the adsorption of chlorophylls onto the resin without impairing the efficiency of the benzene elution step. The chlorophylls are adsorbed onto the resin while a large proportion of the yellowish pigments pass through. The chlorophylls are then eluted with 30 - 40 mℓ benzene. The benzene eluant is evaporated to dryness in a rotary evaporator under reduced pressure and the residues redissolved in 90% acetone for spectrophotometric analysis.

Using the chlorophytes *Chlorococcum hypnosporum*, *Chlamydomonas moewussi* and the cyanophyte *Oscillatoria tenuis*, Wun *et al*, (1979b) found that the extraction efficiency of this method compared favourably with that of the traditional 90% acetone method.

### 1.2.4 DISRUPTION OF THE CELLS AS AN AID TO EXTRACTION

Grinding of the filters in the appropriate solvent with a tissue homogeniser or with a glass rod is often recommended as an aid to chlorophyll extraction (Lorenzen, 1967; Weber, 1973; Standard Methods, 1976; Greeson *et al*, 1977; Jensen, 1978). Improved extraction has been reported with sonication (Nelson, 1960; Garside and Riley, 1969) and mechanical grinding (Yentsch and Menzel, 1963; Humphrey and Wooton, 1966). Youngman (1978) reported that grinding of filters with a pestle and mortar resulted in an average increase of 24% in chlorophyll values with 90% cold acetone extraction, and an average of 26% with 90% cold methanol extraction. He concluded, however, that the extra effort involved for such increase was not worthwhile for routine monitoring purposes. Rai (1973) found that when using a "Vibrogen-Zellmuhle" with glass beads on *Scenedesmus quadricauda* in 90% acetone, extraction was much more rapid (approx. 10 min) and complete than 20 hour cold extraction. Nusch (1980) reported improved ethanolic extraction when filters were homogenised in brown glass extraction vessels for about 2 minutes. Both sonification and mechanical grinding, however, have been shown to lead to degradation of chlorophyll *a* (Kerr and Subba Rao, 1966; Daley *et al*, 1973). Daley *et al* (1973) reported that the use of a sonic probe for five minutes resulted in complete extraction without formation of degradation products.

Riemann (1976) and Holm-Hansen and Riemann (1978) found no improvement in chlorophyll extraction when diatom and blue-green algae samples were ground in a homogeniser (for up to 3 min) after either 24 hour extraction in 90% acetone, or 1 hour extraction in absolute methanol. They concluded that, although they found no improvement with grinding, individual researchers should investigate its use for whatever algae are being studied.

### 1.2.5 DETERMINATION OF PHAEOPHYTIN a

Phaeophytin a is a natural degredation product of chlorophyll a in which the magnesium molecule is lost from the porphyrin ring. If the chlorophyll a molecule loses its phytyl chain (through the action of the enzyme, chlorophyllase) the resulting compound is chlorophyllide a. Loss of the magnesium molecule from the porphyrin ring of this compound results in the formation of phaeophorbide a which has basically the same spectral properties as phaeophytin a (Brown, 1968). Throughout this study phaeophytin a should be taken to include that contribution from phaeophorbide a.

Phaeophytin a concentration is determined either by acidification of the extract, thus degrading the chlorophyll to phaeophytin and observing the changes in the spectral properties at 665 nm (Lorenzen, 1967), or by its proportion in relation to total pigments as indicated by the ratio of absorbance at 430 nm to

absorbance at 410 nm (Moss, 1967a). However both methods are dependant on the nature of the extraction solvent, the nature of the pigments, the amount of contact time of the acid used for acidification of chlorophylls (Holm-Hansen and Riemann, 1978). Both procedures are based on the following assumptions (Riemann, 1976, 1978a):-

1. Treatment of the extract with acid leads to rapid conversion of the chlorophyll to phaeophytin.

2. No further changes occur in the extract, including no degradation of the formed phaeophytin.

3. Phaeophytin is the only degradation product in the original extract.

4. No other chlorophylls or phaeopigments interfere with the measurement of chlorophyll a and phaeophytin a.

The first assumption appears to be an established fact (Riemann, 1976, 1978a). Assumption 2, however, appears to be dependent on the concentration of the acid used (Holm-Hansen and Riemann, 1978; Riemann, 1978a). Assumption 3 is invalid as both chlorophyllides and phaeophorbides occur as normal degradation products, neither of which are corrected for by any of the proposed methods (Riemann, 1976). Unless great care is taken in the acidification process, a large number of products formed from carotenoids will interfere in the 600 - 750 nm region,

thus invalidating assumption 4 (Riemann, 1978a).

The method of Lorenzen (1967) involves reading the absorbance of the extract at 665 nm, acidification of the extract, and rereading the absorbance at 665 nm. Lorenzen (1967) acidified acetone extracts with two drops of a 1 mol $\ell^{-1}$ HCl solution, resulting in a final concentration in the cuvette of about $3 \times 10^{-2}$ mol $\ell^{-1}$ (Riemann, 1978a). The Standard Methods (1976) version of Lorenzen's method results in a final HCl concentration of $8 \times 10^{-2}$ mol $\ell^{-1}$. Tett et al (1975) acidified methanol extracts with 2 drops of 2 mol $\ell^{-1}$ HCl which gives a final cuvette concentration of about $6 \times 10^{-2}$ mol $\ell^{-1}$ (Riemann, 1978a). Holm-Hansen and Riemann (1978), however, reported that high concentrations of acid (greater than $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl) in methanol extracts resulted in a lowering of readings at 665 nm due to a shift in the absorption spectrum. This shift has been reported before by Livingstone, Pariser, Thompson and Weller (1953) and Marker (1972). Both Marker (1972) and Holm-Hansen and Riemann (1978) found that neutralisation restored the peak to 665 nm. Acidification in 90% acetone does not result in this large shift in the absorption spectrum (Marker, 1972). Also Riemann (1978a) reported interference from carotenoids (particularly fucoxanthin products) in both 90% acetone and absolute methanol extracts when the concentration of HCl was above $3 \times 10^{-3}$ mol $\ell^{-1}$. Acidification of the extract to

$3 \times 10^{-3}$ mol $\ell^{-1}$ HCl allows complete degradation of chlorophyll *a* to phaeophytin *a* without conversion of carotenoids into interfering compounds (Riemann, 1978a). Both Riemann (1976) and Holm-Hansen and Riemann (1978) also reported that when acidification was carried out with high concentrations of acid, subsequent neutralisation with $MgCO_3$ was extremely slow (e.g. more than 1 hour to neutralise methanol extracts made up to $1,2 \times 10^{-2}$ mol $\ell^{-1}$ with HCl, Holm-Hansen and Riemann, 1978). Holm-Hansen and Riemann (1978) concluded that for spectrophotometric determinations of chlorophyll *a* and phaeophytin *a* the extract should be acidified to about $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl, allowed to react for three minutes, and neutralised with 25 mg $MgCO_3$ per m$\ell$ of extract for ten minutes with slow stirring. Grobbelaar (personal communication) reports adequate neutralisation using an equal volume of an equimolar solution of NaOH to that of the acid used.

The final acid concentrations in ethanolic extracts required for complete phaeophytinization are higher than those for acetone and methanol (Moed and Hallegraeff, 1978; Nusch, 1980). Too high an acid concentration in ethanolic extracts have been shown to lead to interference due to formation of the dicationic form of phaeophytin (Usacheva, 1971). There is little interference from dicationic forms in acetone extracts (Marker, Nusch *et al*, 1980). The high chlorophyll to phaeophytin ratios obtained by Hallegraeff (1976) when he achieved a final very

high HCl concentration of ~0,1 mol $\ell^{-1}$ in acetone extracts is indicative of the formation of the dicationic form. Moed and Hallegraeff (1978) achieved complete degradation in 80% ethanol by adding 0,25 m$\ell$ of a 0,4 mol $\ell^{-1}$ HCl solution to 20 m$\ell$ of extract resulting in a final acid concentration in the extract of 5 x $10^{-3}$ mol $\ell^{-1}$.* Nusch (1980) reported complete phaeophytinization in 90% ethanol was achieved with a final HCl concentration of 6 x $10^{-3}$ mol $\ell^{-1}$. In both cases formation of the dicationic form appears not to have occurred.

The method of Moss (1967a, 1967b) makes use of the different absorption maxima of chlorophyll _a_ and phaeophytin _a_ in the 400 nm band. Chlorophyll _a_ has a maximum at 430 nm while phaeophytin _a_ has a maximum at 410 nm. The ratio of the absorbance at 430 nm to the absorbance at 410 nm would thus be in relation to the percentage of phaeophytin _a_ present to the total _a_ pigments.

Moss (1967a) made two solutions in 90% acetone, one assumed to contain no phaeopigments (i.e. "100% chlorophyll") and its companion, having been acidified and neutralised, being 100% phaeophytin. Portions of these solutions were

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*Quoted incorrectly in the original paper as 4 x $10^{-3}$ mol $\ell^{-1}$.

mixed so as to give extracts with percentage chlorophyll : percentage phaeopigments ratios of 0:100, 20:80, 40:60, 60:40, 80:20, and 100:0. These were then analysed at 430 nm and 410 nm, and the 430:410 ratios plotted against percentage degradation products to give a calibration curve, with a 430:410 ratio of 1,44 being equivalent to 100% chlorophyll *a* and no phaeophytin *a*. A pure phaeopigment extract gives a 430:410 ratio of approximately 0,15. Degraded whole algal extracts, however, give much higher ratios (in the region of 0,7) (Moss, 1967a). Once a calibration curve for a phytoplankton population is drawn up, the amount of degradation products as a percentage of total chlorophylls and degradation products can be read off upon spectrophotometric determination of the 430:410 ratio. This method, however, only gives percentage degradation and a real value would still have to be calculated from absorbance readings at 665 nm using the equation of Moss (1967b). It is also apparent that calibration curves would have to be recalculated should changes occur in the phytoplankton population. Marker (1972) found that the method could not be adapted for methanol as anomalous results occur when chlorophyll *b* and its derivatives are present.

### 1.2.6 SPECTROPHOTOMETRIC DETERMINATION AND CALCULATION OF CHLOROPHYLL a AND PHAEOPHYTIN a CONCENTRATIONS

The majority of spectrophotometric methods employed involve extraction of chlorophyll with 90% acetone, and thus an

abundance of equations based on the spectral characteristics of photosynthetic pigments in this solvent have been published (Richards and Thompson, 1952; Parsons and Strickland, 1963; UNESCO, 1966; Jeffrey and Humphrey, 1975). These equations are termed trichromatic as they are based on absorbance readings at three wavelengths in an attempt to correct for the presence of other chlorophylls. Monochromatic equations, i.e. based on absorbances at only one wavelength, have also been formulated (e.g. Talling and Driver, 1963; Lorenzen, 1967) on the premise that the other chlorophylls do not constitute an important interference in the measurement of chlorophyll a. Lorenzen (1967) and Moss (1967a, 1967b) have published methods and equations for chlorophyll a and phaeopigments a concentrations in 90% acetone extracts. These have recently been modified by Marker (1972).

Spectrophotometric readings are taken after the extracts have been cleared, usually by centrifugation.

#### 1.2.6.1 IN 90% ACETONE

The main equations for the calculation of chlorophyll a and phaeophytin a in 90% acetone after spectrophotometric analysis are presented in Table 2. The optical density (E) of the extract is read at one or three wavelengths in a cuvette of known pathlength, and the values corrected by subtracting a turbidity blank reading at 750 nm (Strickland and Parsons, 1972).

TABLE 2: Equations used for the determination of chlorophyll a (Ca) and phaeophytin a (Phaeo) concentrations (mg $\ell^{-1}$) in 90% acetone extracts. (E = optical density (log $I_o/I$) at indicated wavelengths, corrected for turbidity by subtraction of a 750 nm reading).

| | Equation | Reference |
|---|---|---|
| 1) | $Ca = 15,6\ E_{665} - 2,0\ E_{645} - 0,8\ E_{630}$ | Richards and Thompson, 1952. |
| 2) | $Ca = 14,3\ E_{665}$ | Odum, McConnell and Abbott, 1958. |
| 3) | $Ca = 15,6\ E_{665} - 1,8\ E_{645} - 1,3\ E_{630}$ | Humphrey, 1963. |
| 4) | $Ca = 11,9\ E_{665}$ | Talling and Driver, 1963. |
| 5) | $Ca = 11,6\ E_{665} - 1,31\ E_{645} - 0,14\ E_{630}$ | Parsons and Strickland, 1963. |
| 6) | $Ca = 11,64\ E_{663} - 2,16\ E_{645} + 0,1\ E_{630}$ | UNESCO, 1966 |
| 7) | $Ca = 26,73\ (E_{665}{}^{o} - E_{665}{}^{a})$ | i) Lorenzen, 1967. |
| 8) | $Phaeo = 26,73\ (1,7\ (E_{665}{}^{a}) - E_{665}{}^{o})$ | i) Lorenzen, 1967. |
| 9) | $Ca = \frac{r\ E_{665}}{r\ k_{Ca} + k_{Phaeo}}$ | ii) Moss, 1967b. |
| 10) | $Phaeo = \frac{E_{665}}{r\ k_{Ca} + k_{Phaeo}}$ | ii) Moss, 1967b. |
| 11) | $Ca = 11,85\ E_{664} - 1,54\ E_{647} - 0,08\ E_{630}$ | Jeffrey and Humphrey, 1975. |

TABLE 2 (CONTINUED)

| | Equation | Reference |
|---|---|---|
| 12) | $Ca = \frac{2.43 \times 10^3 (E_{663}{}^{o} - E_{663}{}^{a})}{Kc}$ | iii) Golterman, Clymo and Ohnstad, 1978. |
| 13) | $Phaeo = \frac{10^3 (2.43E_{663}{}^{a} - 1.43E_{663}{}^{a})}{Kp}$ | iii) Golterman, Clymo and Ohnstad, 1978. |

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i) $E_{665}{}^{o}$ = optical density before acidification;

$E_{665}{}^{a}$ = optical density after acidification;

ii) See text.

iii) Kc = Absorption coefficient of chlorophyll *a*
= 91.

Kp = Absorption coefficient of phaeophytin *a*
= 55.

One of the equations is then used to calculate the concentration of pigment in mg $\ell^{-1}$ in the extract. The concentration of chlorophyll *a* in the original sample is then calculated using the following equation (Parsons and Strickland, 1963):

$$\text{Chlorophyll } \underline{a} \text{ in mg } m^{-3} = \frac{Ca \times v}{1 \times V} \quad \ldots\ldots\ldots \quad 1$$

where Ca = concentration of chlorophyll *a* in the extract;

v = volume of extract in millilitres (usually 10 or 15 mℓ);

l = pathlength of cuvette in centimeters, and

V = volume of original sample in litres.

The equations most commonly used are those of Richards and Thompson (1952), its modification by Parsons and Strickland, (1963), and of UNESCO (1966). These equations vary in the results they give from the same samples. Talling and Driver (1963) assessed that determinations of chlorophyll *a* concentrations using the Richards and Thompson (1952) equation are about 25% too high. This is borne out by the work of Rai (1973) who found this equation gave much higher results when compared to those obtained with the Parsons and Strickland (1963) and UNESCO (1966) equations. Hallegraeff (1976) reported that the latter two equations gave similar chlorophyll *a* values on the same samples, while the Richards and Thompson (1952) equation gave values which were 20% higher.

This overestimation of chlorophyll *a* when using the Richards and Thompson (1952) equation is due to the employment of specific absorption coefficients for chlorophyll *a* and *b* (those of Zscheile, 1934) in the development of the equation which are too low (Parsons and Strickland, 1963). The similarity of the results obtained with the Parsons and Strickland (1963) and the UNESCO (1966) equations was also noted by Banse and Anderson (1967). More recently Wartenburg (1978) has produced a series of conversion tables whereby chlorophyll *a*, *b* and *c* data obtained with one set of equations can be recalculated for an alternative set of equations. For this to be applied, however, chlorophyll *a*, *b* and *c* values are required.

The trichromatic equations are an attempt to correct for the possible interference from other chlorophylls (i.e. *b* and *c*). Workers have noted that large discrepancies can arise when errors caused by instrumentation are compounded by the errors in the specific absorption coefficients used for the trichromatic equations (Marker, 1972). By making the assumption that, for phytoplankton, chlorophyll *a* is the only significant pigment absorbing at 665 nm, Odum, McConnell and Abbott (1958) and Talling and Driver (1963) have produced monochromatic equations based on the absorbance at 665 nm. The equation of Odum *et al* (1958), however, is based on the data of Richards and Thompson (1952) and would thus be subject to the same inherent overestimation of chlorophyll *a* concentration. Hallegraeff (1976)

reported that the Talling and Driver (1963) equation gave chlorophyll *a* values about 5% higher than those obtained with the Parsons and Strickland (1963) and UNESCO (1966) equations.

All the above equations fail to take into account the effect of phaeopigment *a* absorbance in the red band of the spectrum on the calculation of chlorophyll *a* values. Should phaeophytin *a* or phaeophorbide *a* constitute a significant portion of the pigments then a large error is introduced into chlorophyll *a* values as calculated by the standard trichromatic equations. Recently Lorenzen (1967) and Moss (1967b) have published paired equations for the simultaneous calculation of chlorophyll *a* and phaeophytin *a* concentrations.

The equations of Lorenzen (1967) rely upon the change in absorption at 665 nm upon acidification of the extract (converting all the chlorophyll *a* to phaeophytin *a*). This reduction in absorbance at 665 nm is the result of phaeophytin *a* absorbing less light per unit weight (i.e. a lower specific absorbance) than chlorophyll *a* (Lorenzen, 1967). The presence of either chlorophyll *b* or *c* or phaeophytin *b* or *c* does not have a marked effect on absorption at 665 nm (Talling and Driver, 1963; Moss, 1967b; Marker, Nusch *et al*, 1980), and thus any changes in absorbance at 665 nm following acidification are assumed to be due solely to the conversion of chlorophyll *a* to phaeophytin *a*. Lorenzen (1967) found that upon acidification of 100% chlorophyll *a* the ratio of the absorbance at 665 nm before acidification to the absorbance at

665 nm after acidification ($E_{665}a$), known as the acid ratio was 1,7. The ratio for a 100% phaeophytin _a_ solution is by definition 1,0. Using this relationship Lorenzen then developed the equations 7 and 8 in Table 2 for the calculation of chlorophyll _a_ and phaeophytin _a_ concentrations. The factor of 26,73 in these equations is the sum of the absorption factor for 100% chlorophyll _a_ (11,0) and a factor (2,43) to equate the reduction in the absorbancy following acidification to the initial chlorophyll _a_ concentration.

Marker (1972) re-evaluated Lorenzen's equations in order to increase accuracy. Employing a specific absorption coefficient for chlorophyll _a_ of 91,1 $\ell\ g^{-1}cm^{-1}$ (Vernon, 1960), an absorption factor of 10,97 is calculated. Marker reduced this by an arbitrary 4,5% (to 10,48) to correct for the presumed presence of other pigments. Inserting this factor the modified equations are (Marker, 1972) :

$$\text{Chlorophyll } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = 25,47^{*}(E_{665}o - E_{665}a) \quad \ldots\ldots \quad 2$$

$$\text{Phaeophytin } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = (E_{665}o - 2,43(E_{665}o - E_{665}a))10,48 \times 1,7 \quad \ldots\ldots \quad 3$$

* 1.e. 10,48 x 2,43

Marker also pointed out that the equation for phaeophytin _a_ is uncorrected for the loss of magnesium (a factor of 2,7%)

To correct for this the value obtained from equation 3 is thus multiplied by 0,973.

The choice of which specific absorption coefficient for chlorophyll *a* to use in acetone has been open to debate. Following the workshop at the Max-Planck Institute, however, it was proposed that the value of 89 $\ell\ g^{-1}\ cm^{-1}$ for chlorophyll *a* in 90% acetone at 665 nm be accepted (Marker, Nusch *et al*, 1980).

The method of Moss (1967b) also utilises absorbance at 665 mm but includes the use of percentage degradation data calculated using the 430 : 410 ratio method of Moss (1967a). The ratio of chlorophyll *a* : phaeophytin *a* (r) is inserted into the equations:-

$$Ca = \frac{r\ E_{665}}{r\ k_{Ca} + k_{Phaeo}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 4$$

$$Phaeo = \frac{E_{665}}{r\ k_{Ca} + k_{Phaeo}} \quad \ldots\ldots\ldots\ldots\ldots\ldots\ldots \quad 5$$

where Ca = concentration of chlorophyll *a* in g $\ell^{-1}$, Phaeo = concentration of phaeophytin *a* in g $\ell^{-1}$, $E_{665}$ = absorbance at 665 nm, $k_{Ca}$ = specific absorption coefficient of chlorophyll *a* (90,8 $\ell\ g^{-1}\ cm^{-1}$), and $k_{Phaeo}$ = specific absorption coefficient of phaeophytin *a* (55,2 $\ell\ g^{-1}\ cm^{-1}$). Inserting the specific absorbance values and varying values of r, a graph may be constructed (Figure 2) from which, knowing the

percentages of chlorophyll _a_ and phaeophytin _a_, factors ($F_c$ and $F_p$) are read off. The absorbance readings ($E_{665}$) are multiplied by these factors to obtain the concentrations of chlorophyll _a_ and phaeophytin _a_ in mg $\ell^{-1}$ extract, viz:-

Chlorophyll _a_ = 95%

Phaeophytin _a_ = 5%

$E_{665}$ = 0,625

From the graph (Figure 2) the factor for 95% chlorophyll _a_ ($F_c$) = 10,7 and for 5% phaeophytin _a_ ($F_p$) = 0,6 thus

Chlorophyll _a_ (mg $\ell^{-1}$ extract) = 0,625 x 10,7 = 6,688

Phaeophytin _a_ (mg $\ell^{-1}$ extract) = 0,625 x 0,6 = 0,375.

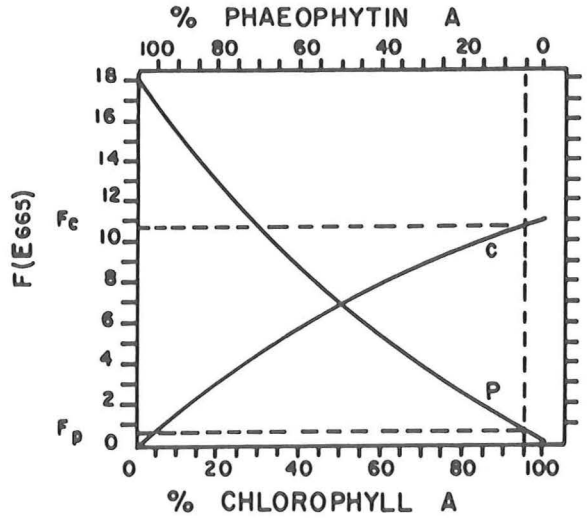


FIGURE 2: The factors by which E665 (F(E665)) must be multiplied to give chlorophyll _a_ and phaeophytin _a_ in mg $\ell^{-1}$, plotted against chlorophyll _a_ and phaeophytin _a_ as percentages of total chlorophyll _a_ + total phaeophytin _a_ in the extract. C — chlorophyll _a_. P — phaeophytin _a_. e.g. 95 % chlorophyll factor = $F_c$, 5% phaeophytin factor = $F_p$ (from Moss, 1967 b)

The equations of Moss (1967b) have also been modified by Marker (1972) to correct for the presumed presence of other pigments. Taking the 4,5% correction factor for chlorophyll *a* and a 7,65% correction factor for phaeophytin *a*, an equation correction factor ($C_f$) is derived thus:-

$$C_f \text{ (\% correction)} = \frac{(4{,}5 \times r) + (7{,}65 \times 1)}{1 + r} \quad \ldots\ldots\ldots \quad 6$$

where r = the ratio chlorophyll *a* : phaeophytin *a*. This factor is inserted into the equations thus:-

$$Ca = \frac{r(E_{655} - ((E_{665} \times C_f)/100))}{r\,k_{Ca} + k_{Phaeo}} \quad \ldots\ldots\ldots\ldots\ldots \quad 7$$

$$Phaeo = \frac{E_{655} - ((E_{665} \times C_f)/100)}{r\,k_{Ca} + k_{Phaeo}} \quad \ldots\ldots\ldots\ldots\ldots \quad 8$$

As with the modification of the equations of Lorenzen (1967) the equation for phaeophytin *a* is multiplied by 0,973 to correct for the loss of magnesium.

The equations of Lorenzen (1967) and Moss (1967a; 1967b) and their modifications by Marker (1972) are more realistic in their estimation of chlorophyll *a* concentrations as they take into account the effect of degradation products, which may constitute a sizeable fraction in extracts from freshwater phytoplankton populations (Moss, 1967a). The presence of phaeophytins is an indication of the physiological condition of the phytoplankton.

1.2.6.2 IN METHANOL

Due to the lack of reliable information about the specific absorption coefficients of chlorophyll a in methanol, few equations for the determination of chlorophyll a and phaeophytin a in this solvent are available. Talling and Driver (1963) produced a monochromatic equation for the determination of chlorophyll a in 90% methanol based on the difference in absorbance of chlorophyll solutions in 90% acetone and in 90% methanol at 665 nm. The 90% acetone values were higher than the 90% methanol values by a factor of 1,17. Using this factor, Talling and Driver (1963) constructed, from their 90% acetone equation, an equation for 90% methanol, viz:

$$\text{Chlorophyll } a \text{ (mg } \ell^{-1} \text{ extract)} = 13{,}9\ E_{665} \quad \ldots\ldots\ldots \quad 9$$

Talling and Driver (1963) found no significant difference in absorbances in 90% and 100% methanol. It is, therefore, assumed that this equation can be used for pigment extracts in 90 - 100% methanol.

The equations of Lorenzen have been modified by Marker for use with methanol extracts. Marker (1972) found that in 95% methanol the maximum acid ratio was 1,5. Using the data of Talling and Driver (1963) Marker calculated a specific absorbance coefficient for chlorophyll a in 90 - 100% methanol at 665 nm of 76,07 $\ell\ g^{-1}\ cm^{-1}$. The absorption factor (the reciprocal of 76,07 (x $10^3$))

is then corrected by 4,5% (for other pigments) and a value of 12,5 obtained. The factor to equate the reduction in absorbancy following acidification to the initial chlorophyll concentration (2,43 in 90% acetone) is calculated to be 3,0. The modified equations are thus (Marker, 1972):-

$$\text{Chlorophyll } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = 37{,}5^{*}(E_{665}{}^{o} - E_{665}{}^{a}) \quad \ldots\ldots \quad 10$$

$$\text{Phaeophytin } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = (E_{665}{}^{o} - 3{,}0(E_{665}{}^{o} - E_{665}{}^{a}))12{,}5 \times 1{,}5 \quad \ldots\ldots \quad 11$$

* i.e. 12,5 x 3,0.

The phaeophytin a concentration is again multiplied by 0,973 to correct for loss of magnesium.

The specific absorbtion coefficient for chlorophyll a in methanol used by Marker (1972) was derived from an acetone value divided by the 1,17 factor of Talling and Driver (1963). Directly obtained coefficients, however, have been published. From the data of Seely and Jensen (1965) a value of 74,5 $\ell\ g^{-1}\ cm^{-1}$ is calculated. This value was also reported by MacKinney (1941). Lenz and Zeitzschel (1968) reported a value of 75,0 $\ell\ g^{-1}\ cm^{-1}$. Taking the value of 74,5 $\ell\ g^{-1}\ cm^{-1}$, an absorbance factor of 12,8 is obtained which can be inserted into the above equations in replacement of Marker's factor.

Riemann (1976) used the Lenz and Zeitzschel (1968) value of 75,0 $\ell\ g^{-1}\ cm^{-1}$ to modify the equation of Moss (1967b) for use with methanol. Using the 1,5 factor of Marker (1972), he calculated the specific absorbance coefficient of phaeophytin a in methanol to be 50,0 $\ell\ g^{-1}\ cm^{-1}$ (75,0/1,5). These values for $k_{Ca}$ and $k_{Phaeo}$ are now inserted into the equations of Moss (1967b) for the calculation of a pigment concentrations.

More recently higher values for the specific absorption coefficient for chlorophyll a in methanol have been put forward. Based on pigments transfered between acetone, methanol and ethanol Riemann (1978b) suggested a value of 77,9 $\ell\ g^{-1}\ cm^{-1}$. Marker, Nusch et al,(1980) have tentatively adopted a value of 77 $\ell\ g^{-1}\ cm^{-1}$. As pointed out by Marker, Nusch et al,(1980) new determinations of specific absorption coefficients for chlorophyll a, particularly for ethanol and methanol, are urgently required.

It must be pointed out here that the 4,5% correction factor for other pigments (Marker, 1972) is purely arbitrary. Should the investigator find that chlorophyll b and c and phaeophytin b and c levels are low or absent, as did Riemann (1976) in his Lake Mossø studies, then this factor may be omitted.

### 1.2.6.3 IN ETHANOL

There are no trichromatic equations for the determination of chlorophyll a in ethanol. The equation used in the method of

Ostrovsky (as reported by Bergmann and Peters, 1980) is that developed by Wintermans and de Mots (1965) for the determination of chlorophylls in plant extracts in 96% ethanol, viz:-

$$\text{chlorophyll } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = 13{,}7E_{665} - 5{,}76E_{649} \quad \ldots\ldots \quad 12$$

As with the trichromatic equations for 90% acetone, this equation does not take into account the effect of phaeopigments on the determination of chlorophyll _a_.

Again, the Lorenzen equations have been modified for use with ethanol. Both Nusch and Palme (1975) and Nusch (1980) reported an acid ratio in 90% ethanol of 1,7. Moed and Hallegraeff (1978) reported the same ratio for chlorophyll extracts in 80% ethanol.

As with methanol there is a great need for new determinations of specific absorption coefficients for chlorophyll in ethanol. Schötz (1962) determined a value of 83 $\ell\ g^{-1}\ cm^{-1}$ and Wintermans and de Mots (1965) a value of 83,4 $\ell\ g^{-1}\ cm^{-1}$ for 96% ethanol. Nusch (1980), having found that chlorophyll extinction in ethanol was 3% lower than in acetone, employed a value of 82 $\ell\ g^{-1}\ cm^{-1}$ (based on a 90% acetone value of 84 $\ell\ g^{-1}\ cm^{-1}$ from Vollenweider, 1970). Using a 90% acetone value of 89 $\ell\ g^{-1}\ cm^{-1}$ and an extinction difference between 90% acetone and 90% ethanol of 2% Marker, Nusch _et al_ (1980) tentatively adopted a value of 87 $\ell\ g^{-1}\ cm^{-1}$ as the specific absorption coefficient for chlorophyll _a_ in 90% ethanol at 665 nm.

## 1.3 CONCLUSIONS

It is evident that a large number of methods and spectrophotometric equations and data are available for the analysis of chlorophyll *a* in freshwaters. Many of the methods are inefficient, particularly when acetone is employed and use of much of the spectrophotometric data and equations will lead to erroneous results.

The use of methanol for the extraction of chlorophyll *a* has become popular despite the problems with its use. The use of ethanol, with its advantages over methanol, demands further investigation. Lastly, the establishment of a simple, effective, standard routine spectrophotometric method is imperative.

It is with these points in mind that this study was carried out.

# CHAPTER 2 EQUIPMENT AND MATERIALS

## 2.1 THE SPECTROPHOTOMETER

The quality of the spectrophotometer used for the determination of photosynthetic pigments will have a marked effect on the final results. The absorbance of chlorophyll extracts is best measured using a spectrophotometer with a grid monochromator (Marker, Nusch *et al*, 1980; Nusch, 1980). Prismatic monochromators should be avoided as they are optically inferior, particularly in the longer wavelengths, and are susceptible to problems such as thermoshifting and vibration (Tolstoy, 1977; Nusch, 1980).

The wavelength setting is critical. A shift of only 1 nm has been reported to cause an error of 15% in the measurement of chlorophyll *b* (Ziegler and Egle, 1965). A variation of 5 nm can lead to errors of 20 to 30% when calculating chlorophyll *a* concentrations in methanol extracts, where acid ratios vary considerably with wavelength (Marker, 1972; Marker, Crowther and Gunn, 1980). Regular checking of wavelength settings by means of the hydrogen line at 656,3 nm is recommended (Marker, Nusch *et al*, 1980).

The spectral purity (i.e. the band pass) of the light at any given wavelength is dependent on the slit width. There is confusion in the literature about the difference between slit width and band pass/width. A slit width of 0,2 nm will allow

a band pass of approximately 1 nm. Many manufacturers, however, calibrate their slit widths in terms of band pass. It is essential, therefore, that researchers ensure whether their equipment is calibrated in terms of actual slit width or band pass. A large band pass (e.g. 20 nm) will cause a plateauing of the absorbance spectrum and a reduction in the absorbance maxima (Brown, Dromgoole and Guest, 1980). For the measurement of chlorophyll a absorbance in the 660 nm range the optimal band pass is 1 to 2 nm (Marker, Nusch et al, 1980).

Prior to spectrophotometric analysis differences in absorbance between cuvettes are corrected by filling the cuvettes with the reference solvent and zeroing. The pigmental absorbance at 665 nm is best measured in the range 0,2 to 0,8 (Tolstoy, 1977; Marker, Nusch et al, 1980). In this range an error of 2% or less is found (Tolstoy, 1977). Absorbance values should preferably not drop below 0,1 (Bruinsma, 1963; Marker, Nusch et al , 1980). This range can be achieved by varying the volume of sample initially filtered and the volume of extraction solvent used, and by choice of cuvette size. A range of cuvette sizes, between 1 cm and 12 cm pathlength, have been employed (Parsons, 1966). Tolstoy (1980), however, reported differences in final results due to differences in cuvette size used. Concentrations calculated from measurements using 4 cm pathlength cuvettes were between 3% and 9% higher than those using 1 cm pathlength cuvettes (Tolstoy, 1977). This error was due to too low absorbances at 750 nm being measured with the 4 cm cuvettes in relation to the 1 cm cuvettes (Tolstoy, 1977).

Throughout this study a Varian Techtron Model 635 D UV-Visible spectrophotometer (Varian Techtron Ltd., Australia) was used. This model is equipped with a grid monochromator and the band pass was set at 1 nm. Absorbance values were read to three decimal places in the range 0,100 to 0,800. The wavelength setting was checked regularly (approximately every two weeks) by means of the hydrogen line of 656,3 nm.

## 2.2 FILTRATION APPARATUS

Throughout this study a Millipore 6-place manifold with stainless steel filter cups (Millipore Corporation, U.S.A.) was used. The filtering of the samples was aided by suction pressure provided by a vacuum/pressure pump. The suction pressure was set at 53 kPa and did not exceed 68 kPa. UNESCO (1966) recommends a suction pressure of 68 kPa and Nusch (1980) recommended that this value should not be exceeded. In this study no chlorophyll was detected in the filtrate after a dense culture of unicellular *Microcystis aeruginosa* had been filtered through a glass fibre filter using a suction pressure of 68 kPa.

Glass fibre filters are generally accepted as being the most suitable for the concentration of algae. They have several advantages over membrane filters (see Chapter 1 page 6 ). Whatman glass fibre filters (especially GF/C) are the most popular and have a proved efficiency against other types of filters (Marker, Nusch *et al*, 1980). Throughout this study, however, 47 mm Sartorius SM 13400 glass fibre filters (Sartorius GmbH, West Germany) were used as they were already available

in large supply. These filters were tested by filtering a culture of unicellular Microcystis aeruginosa through five Sartorius SM 13400 filters, pooling the filtrates and filtering it through one Whatman GF/C filter. No chlorophyll was detected in the pooled filtrates.

Due to the problems associated with the procedure the filters were not coated with $MgCO_3$ (see discussion in Chapter 1, pages 6 to 8).

## 2.3 SOLVENTS AND CHEMICALS

The three principal solvents used in this study were ethanol, methanol and acetone. The methanol and acetone used were Analar grade (B.D.H. Chemicals) and the ethanol used was Aerosol grade absolute alcohol (National Chemical Products, Ltd., South Africa). All other chemicals used in this study were either Analar grade (B.D.H. Chemicals) or Pro Analisi grade (Merck Chemicals). Purified chlorophyll a was obtained from Sigma Chemicals (Code C5753).

## 2.4 ALGAL CULTURES AND NATURAL SAMPLES

### 2.4.1 ALGAL CULTURES

The majority of the experiments carried out during this study used algae obtained from laboratory cultures. A range of green and blue-green algae were used (Table 3). Stock cultures were maintained on BG 11 medium (Allen, 1968), as modified by

TABLE 3: Algal cultures used in this study and their source.

| Alga | Source |
|---|---|
| (a) Green algae | |
| Scenedesmus quadricauda | NIWR* code WR 1003 |
| Selenastrum capricornutum | NIWR code WR 1002 |
| (b) Blue-green algae | |
| Microcystis aeruginosa (unicellular) | UOFS** code UV 007ƒ |
| Anabaena flos-aquae | NIWR code WR 14 |
| Oscillatoria brevis | NIWR code WR 45 |

* National Institute for Water Research, Council for Scientific and Industrial Research, Pretoria, R.S.A.

** University of the Orange Free State, Bloemfontein, R.S.A.

ƒ = Strain 7005 from University of California, Berkeley, U.S.A. (Stanier et al, 1971)

Stanier, Kunisawa, Mandel and Cohen-Bazire (1971), and solidified with 10 g $\ell^{-1}$ Agar-Agar (Merck Chemicals). Prior to experimentation batch cultures were grown up in the modified liquid BG 11 medium in 300 mℓ aliquots. These were pooled to give a 3 ℓ bulk sample from which 25 mℓ to 100 mℓ subsamples were withdrawn for analysis depending on the algal concentration. The bulk sample was continuously agitated during the subsampling to ensure homogeneity. For all experiments involving cultures, the cultures were between late log growth phase and early stationary growth phase. This was checked by daily measurements of cell turbidity in a colorimeter.

### 2.4.2 NATURAL SAMPLES

Natural phytoplankton samples were collected from Roodeplaat Dam epilimnion in 25 ℓ plastic aspirators. Roodeplaat Dam is an eutrophic impoundment 30 km north east of Pretoria, South Africa. Sixty litres of sample was pooled in a large clean plastic dustbin and, depending on algal concentration, between 0,5 ℓ and 1,0 ℓ subsamples were withdrawn for filtration. Throughout the subsampling the main sample was agitated to ensure homogeneity.

Algal population composition was determined by preserving 100 mℓ of sample with Lugols iodine and allowing the algae to settle in a small plastic bottle. The supernatant was then decanted and a 1 mℓ sample withdrawn. The species composition

and relative abundance was estimated using a Sedgewick-Rafter cell under a microscope.

Furthermore, two species of filamentous algae were also used in this study. Fresh samples of Cladophora glomerata were collected from the irrigation canals of Hartbeespoort Dam, Transvaal, and fresh samples of Stigeoclonium lubricum were collected from the Pienaars River, Transvaal, just below the outfall of a sewage works.

## 2.5 OTHER EQUIPMENT

Throughout this study all extracts were centrifuged for 10 to 15 min at 4000 rpm prior to spectrophotometric analysis. For this a MSE Super Minor centrifuge (MSE Scientific Instruments, U.K.) fitted with a 8 x 15 mℓ swing-out head was used.

For the homogenisation experiments a variable speed motor (Heidolph-Elecktro K.G., West Germany) set at 800 rpm and equipped with a teflon pestle (Tri-R Instruments, Inc., U.S.A.) for use with 30 mℓ capacity tubes was used. Sonication experiments were carried out using a DAWE Sonicleaner Type 6442A sonic water bath (Dawe Instruments, U.S.A.).

Boiling experiments were carried out using LSE water baths equipped with contact immersion thermometers (Labotec, Ltd, South Africa).

## 2.6 LABORATORY CONDITIONS

No special laboratory conditions were created for this study. Because of the light sensitive nature of chlorophyll some workers have conducted pigment analyses under dim light or week green light conditions (Jensen, 1978; Tolstoy and Töth, 1980).

During this study the light conditions in the laboratory were measured using a Model QSM-2500 Quantaspectrometer (Techtum Instruments, Sweden). At the working area the normal light intensity in the photosynthetically active range (PAR, 400 nm to 750 nm) was between 20 $\mu E\ m^{-2}\ sec^{-1}$ to 25 $\mu E\ m^{-2}\ sec^{-1}$ and did not exceed 30 $\mu E\ m^{-2}\ sec^{-1}$. The PAR intensity of full sunlight is approximately 2000 $\mu E\ m^{-2}\ sec^{-1}$. To assess the effect of these light conditions on chlorophyll _a_ extracts three ethanolic chlorophyll solutions were left standing on the bench and samples were withdrawn for spectrophotometric analysis at several intervals up to 20 min. The first solution was an extract from _Microcystis aeruginosa_, the second an extract from _Selenastrum capricornutum_ and the third a solution of purified chlorophyll _a_ (Sigma Chemicals). The light intensity during the experiment ranged between 22,3 $\mu E\ m^{-2}\ sec^{-1}$ and 23,1 $\mu E\ m^{-2}\ sec^{-1}$. Over the period of 20 min no increase in degradation products or decrease in chlorophyll _a_ was noted in any of the solutions.

TABLE 4: Effect of laboratory light conditions on chlorophyll *a* values. 20 subsamples of a culture of *Selenastrum capricornutum* were analysed, 10 under normal conditions and 10 under very dim light. Samples were extracted with 90% ethanol.

| | Chlorophyll *a* mg $\ell^{-1}$ extract* | Phaeophytin *a* mg $\ell^{-1}$ extract* |
|---|---|---|
| Normal light conditions | 2,814 ± 0,061 | 0,071 ± 0,021 |
| Very dim light conditions | 2,797 ± 0,070 | 0,068 ± 0,019 |

* Mean and standard deviation (n = 10).

A second experiment was conducted where 10 subsamples of a culture of *Selenastrum capricornutum* were filtered and analysed under normal laboratory conditions and 10 further subsamples analysed under very dim light conditions. There was no difference in the chlorophyll *a* values obtained (Table 4).

# CHAPTER 3 SPECTROPHOTOMETRY METHODS

## 3.1 INTRODUCTION

Following the workshop at the Max-Planck Institute, Plön, West Germany, it was concluded that the use of trichromatic equations to calculate chlorophyll *a* concentrations in freshwater phytoplankton could not be recommended (Marker, Nusch *et al*, 1980). These equations overestimate chlorophyll *a* (Rai, 1973), do not correct for the presence of phaeopigments and falsely indicate the presence of chlorophyll *c* (Töth, 1970; Rai, 1973). When monochromatic determinations of total *a* pigments (i.e. chlorophyll *a* uncorrected for phaeopigments) are compared to trichromatic determinations negligible differences are found (Marker, Nusch *et al*, 1980). This implies that it is unnecessary to correct for chlorophylls *b* and *c*. Phaeophytin *a*, on the other hand, has been found to represent as much as 56,2% of the total *a* pigments in freshwater algae (Riemann, 1978c) and it is, therefore, necessary to employ a technique that corrects for the presence of phaeopigments.

The spectrophotometry procedures and equations used in this study, and recommended by Marker, Nusch *et al* (1980), are those of Lorenzen (1967) modified, however, for use with methanolic and ethanolic chlorophyll extracts. The method relies upon the differing absorbance coefficients of chlorophyll *a* and phaeophytin

a in the 600 nm range. The absorbance of an extract is read at 665 nm (total a pigments). The extract is acidified and chlorophyll a reduced to phaeophytin a and the absorbance is reread. The values are inserted into the following equations from which chlorophyll a and phaeophytin a concentrations are calculated (See Chapter 1 pages 34 to 36).

$$\text{Chlorophyll } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = \frac{(E_{665}{}^{o} - E_{665}{}^{a}) \times (R/R-1) \times k}{L} \quad \ldots\ldots\ldots\ldots\ldots 13$$

$$\text{Phaeophytin } \underline{a} \text{ (mg } \ell^{-1} \text{ extract)} = \frac{(R[E_{665}{}^{a}] - E_{665}{}^{o}) \times (R/R-1) \times k}{L} \quad \ldots\ldots\ldots\ldots\ldots 14$$

where $E_{665}{}^{o}$ = absorbance at 665 nm before acidification

$E_{665}{}^{a}$ = absorbance at 665 nm after acidification

R = acid ratio (maximum ratio of $E_{665}{}^{o}:E_{665}{}^{a}$ i.e. for an extract containing no phaeopigments)

k = Absorbance coefficient of chlorophyll a in chosen solvent (= 1000 x reciprocal of the specific absorption coefficient)

L = pathlength of cuvette.

The values obtained are dependent on the specific absorption coefficient (SAC) and the acid ratio employed and the degree of phaeophytinization achieved upon acidification of the extract. Thus before any studies on relative solvent extraction efficiencies

can be carried out, spectrophotometric procedures must be assessed and deliniated.

## 3.2 SPECIFIC ABSORPTION COEFFICIENTS (SAC)

Throughout this study a specific absorption coefficient (SAC) for chlorophyll *a* in 90% acetone at 665 nm of 89,0 $\ell\ g^{-1}\ cm^{-1}$ (Marker, Nusch *et al* 1980) has been used. This figure has been used as a basis for assessing the SACs of chlorophyll *a* in ethanol and methanol for which a wide variety of values have been published (Table 5). The value for the SAC chosen will have a marked effect upon the final result. Taking the two extreme values for methanolic solutions, those calculated from the data of Ogawa and Shibata (1963) and Stauffer, Lee and Armstrong (1979) (see Table 5), results obtained using these values would vary by 13%. Relatively little data on SACs of chlorophyll *a* in ethanol are available (Table 5).

### 3.2.1 EXPERIMENTAL

The SACs of chlorophyll *a* in 90% acetone, ethanol and methanol were compared by performing transfer experiments using purified chlorophyll *a*. Three concentrations of chlorophyll *a* corresponding to approximately 1 mg $\ell^{-1}$, 2 mg $\ell^{-1}$ and 3 mg $\ell^{-1}$ were made up in 90% acetone. The solutions were then divided into nine 5 m$\ell$ aliquots (in short test tubes) per chlorophyll *a* concentration and evaporated to dryness in a darkened vacuum oven under vacuum at room temperature. The deposits were then redissolved

TABLE 5: Quoted Specific Absorption Coefficients (SAC) for chlorophyll *a* in methanolic and ethanolic solutions.

| Solvent | SAC ($\ell\ g^{-1}\ cm^{-1}$) | Ref. |
|---|---|---|
| Methanol | 73,1 | from the data of Ogawa and Shibata (1963). |
| | 74,5 | MacKinney (1941). |
| | 74,5 | from the data of Seely and Jensen (1965). |
| | 75,0 | Lenz and Zeitzschel (1968). |
| | 76,1 | Marker (1972). |
| | 77,0 | Marker, Nusch *et al* (1980). |
| | 77,9 | Riemann (1978b). |
| | 83,9 | from the data of Stauffer, Lee and Armstrong (1979). |
| 90% Ethanol | 82,0 | Nusch (1980). |
| | 87,0 | Marker, Nusch *et al* (1980). |
| 96% Ethanol | 83,0 | Schötz (1962). |
| | 83,4 | Wintermans and de Mots (1965). |
| Ethanol | 77,7 | from the data of Seely and Jensen (1965). |

in one of three solvents - 90% acetone, 95% ethanol and absolute methanol. This was done by first dissolving the residue in 2 mℓ of the solvent, decanting into a fresh tube and then washing the old tube three times with 1 mℓ aliquots of solvent, adding the washings to the new tube. In this way three sets 5 mℓ aliquots of each chlorophyll a concentration for each solvent was obtained. The absorbances of each solution at 665 nm was then measured (Table 6).

The absorbances of the 95% ethanol chlorophyll a solution were between 94,1% and 95,1% of those in 90% acetone. This gives SAC values between 83,7 $\ell\ g^{-1}\ cm^{-1}$ and 84,6 $\ell\ g^{-1}\ cm^{-1}$ (SAC in 90% acetone = 89,0 $\ell\ g^{-1}\ cm^{-1}$). The methanol solution absorbances were 86,9% and 88,2% of the 90% acetone absorbances equatable to SAC values of 77,3 $\ell\ g^{-1}\ cm^{-1}$ and 78,5 $\ell\ g^{-1}\ cm^{-1}$.

To test the effect of water concentration on the SAC of chlorophyll a in ethanolic solutions the experiment was repeated using 95% ethanol stock solutions of approximately 1 mg $\ell^{-1}$ and 2 mg $\ell^{-1}$ purified chlorophyll a. Three sets of 5 mℓ aliquots for each chlorophyll a concentration and each solvent concentration were made as before and the absorbances read at 665 nm (Table 7).

There was no difference in absorbances at 665 nm of the chlorophyll a solutions in 98%, 95% and 90% ethanol. The absorbances in 80% ethanol were slightly lower than in 95% ethanol but the difference was less than 2%.

TABLE 6: Mean absorbances of three readings of solutions of chlorophyll *a* in 90% acetone, 95% ethanol and 100% methanol at 665 nm (see text for details). Percentages in relation of absorbance in 90% acetone are shown in brackets.

| | Chlorophyll *a* concentration | | |
|---|---|---|---|
| Solvent | ~1 mg $\ell^{-1}$ | ~2 mg $\ell^{-1}$ | ~3 mg $\ell^{-1}$ |
| 90% Acetone | 0,085 (100%) | 0,160 (100%) | 0,287 (100%) |
| 95% Ethanol | 0,080 (94,1%) | 0,151 (94,4%) | 0,273 (95,1%) |
| 100 Methanol | 0,075 (88,2%) | 0,139 (86,9%) | 0,253 (88,2%) |

TABLE 7: Absorbances of solutions of chlorophyll *a* in various ethanol solutions at 665 nm (see text for details). Mean of three readings. Percentages in relation to absorbance in 95% ethanol are shown in brackets.

| | Chlorophyll *a* concentration | |
|---|---|---|
| Solvent | ~1 mg $\ell^{-1}$ | ~2 mg $\ell^{-1}$ |
| 98% Ethanol | 0,104 (100%) | 0,256 (100%) |
| 95% Ethanol | 0,104 (100%) | 0,256 (100%) |
| 90% Ethanol | 0,104 (100%) | 0,255 (99,6%) |
| 80% Ethanol | 0,102 (98,1%) | 0,252 (98,6%) |

### 3.2.2 DISCUSSION

Marker, Nusch *et al* (1980) tentatively adopted chlorophyll *a* SAC values of 87,0 $\ell\ g^{-1}\ cm^{-1}$ in 90% ethanol and 77,0 $\ell\ g^{-1}\ cm^{-1}$ in methanol. Nusch (1980) reported that the extinction in 90% ethanol was 3% lower than in 90% acetone. He used this to calculate a SAC value of 82,0 $\ell\ g^{-1}\ cm^{-1}$ (based on a 90% acetone SAC value of 84,0 $\ell\ g^{-1}\ cm^{-1}$ from Vollenweider, 1970). Using the accepted 90% acetone SAC value of 89,0 $\ell\ g^{-1}\ cm^{-1}$, a value of 86,3 $\ell\ g^{-1}\ cm^{-1}$ in 90% ethanol is obtained. In this study extinction values in 95% ethanol were 4,9 to 5,9% lower than in 90% acetone (Table 6) and there was no difference in extinctions in 98%, 95% and 90% ethanol. This gives SAC values for ethanolic chlorophyll *a* solutions between 83,7 $g\ \ell^{-1}\ cm^{-1}$ and 84,6 $g\ \ell^{-1}\ cm^{-1}$. As these values are closer the directly obtained value of 83,4 $g\ \ell^{-1}\ cm^{-1}$ of Wintermans and de Mots (1965) than the 87,0 $g\ \ell^{-1}\ cm^{-1}$ value of Marker, Nusch *et al* (1980) the former value is adopted as the SAC for chlorophyll *a* in 90 to 98% ethanol.

The extinctions in methanol were 11,8 to 13,1% lower than those in 90% acetone (Table 6). This gives SAC values in methanol of 77,3 $g\ \ell^{-1}\ cm^{-1}$ and 78,5 $\ell\ g^{-1}\ cm^{-1}$. These are close to the values of 77,0 $\ell\ g^{-1}\ cm^{-1}$ adopted by Marker, Nusch *et al* (1980) and 77,9 $\ell\ g^{-1}\ cm^{-1}$ of Riemann (1978b) (based on pigment transfers between methanol and acetone or ethanol), and higher than the commonly used value of 75,0 $\ell\ g^{-1}\ cm^{-1}$ of Lenz and Zeitzschel (1968).

The value of 83,9 $\ell\ g^{-1}\ cm^{-1}$ of Stauffer *et al* (1979) appears to be unrealistically high. It was decided for the purpose of this study to adopt the Riemann (1978b) value, based on similar experiments, of 77,9 $\ell\ g^{-1}\ cm^{-1}$ as the SAC for chlorophyll *a* in methanol.

It is apparent, however, that there is a desperate need for new directly obtained specific absorption coefficients for chlorophyll *a* in ethanolic and methanolic solutions.

## 3.3 ACIDIFICATION PROCEDURES

Riemann (1978a) clearly showed that overacidification of extracts results in interferences caused by other pigments, particularly fucoxanthin products. Too weak acid results in incomplete phaeophytinization. In both cases the ascertainment of the acid ratio is affected and during routine analyses will cause underestimation of chlorophyll *a* and overestimation of phaeophytin *a*.

### 3.3.1 ACID CONCENTRATION

Riemann (1978a) reported optimum phaeophytinization in 90% acetone and methanol extracts with a final HCl concentration of $3 \times 10^{-3}$ mol $\ell^{-1}$. This allowed complete conversion of chlorophyll *a* to phaeophytin *a* without the formation of interfering compounds (see Chapter 1, pages 23 to 27). With methanol,

however, neutralization is required as the phaeophytin a absorbance peak is pH sensitive (Livingstone et al, 1953). In ethanolic solutions, however, higher acid concentrations have been used. Moed and Hallegraeff (1978) used a final HCl concentration in 80% ethanol of 5 x $10^{-3}$ mol $\ell^{-1}$ and Nusch (1980) a final concentration of 6 x $10^{-3}$ mol $\ell^{-1}$ HCl in 90% ethanol. Too high acid concentrations in ethanolic extracts results in interferences caused by the formation of the dicationic form of phaeophytin a (Usacheva, 1971).

3.3.1.1 EXPERIMENTAL

Solutions of purified chlorophyll a were made up in 90% acetone, 100% methanol and 95% ethanol. These were acidified to varying concentrations of HCl ranging from 1,5 x $10^{-3}$ mol $\ell^{-1}$ to 1,5 x $10^{-2}$ mol $\ell^{-1}$, and the rate of phaeophytinization followed in the spectrophotometer. The acid was added directly to 4 m$\ell$ of extract in a cuvette using a 100 $\mu\ell$ microsyringe (Hamilton Bonadaz A.G., Switzerland). The methanolic solutions were not neutralised and phaeophytinization was followed at the phaeophytin peak. Phaeophytinization in the 90% acetone and 95% ethanol solutions was monitored at 665 nm.

In the 90% acetone and 100% methanol solutions complete phaeophytinization was achieved within 4 mins after acidification to 3 x $10^{-3}$ mol $\ell^{-1}$ HCl (Figures 3 and 4). Acidification

to 6 x $10^{-3}$ mol $\ell^{-1}$ HCl in both these solvents led to higher absorbance readings, presumably due to formation of interference products.

In 95% ethanol phaeophytinization was completed within 4 to 5 min with an HCl concentration of 6 x $10^{-3}$ mol $\ell^{-1}$ and within 2 min with 9 x $10^{-3}$ mol $\ell^{-1}$ HCl (Figure 5). With a final concentration of 3 x $10^{-3}$ mol $\ell^{-1}$ HCl, phaeophytinization was incomplete after 15 min. With acidification up to 9 x $10^{-3}$ mol $\ell^{-1}$ HCl there was no evidence of formation of interference compounds or formation dicationic phaeophytin a. When the experiment was repeated using a 90% ethanol solution of chlorophyll a similar results were obtained.

With all three solvents there was a shift in the absorbance peak upon acidification, but the shift in 90% acetone and 95% ethanol was minor and neutralization was not required. In 95% ethanol the shift was to between 665,0 nm and 666,5 nm and in 90% acetone the shift was less than 1 nm. In methanol, however, the shift was pronounced but subsequent neutralization with an equal volume of an equimolar solution of NaOH restored the peak to 665 nm $\pm$ 1 nm.

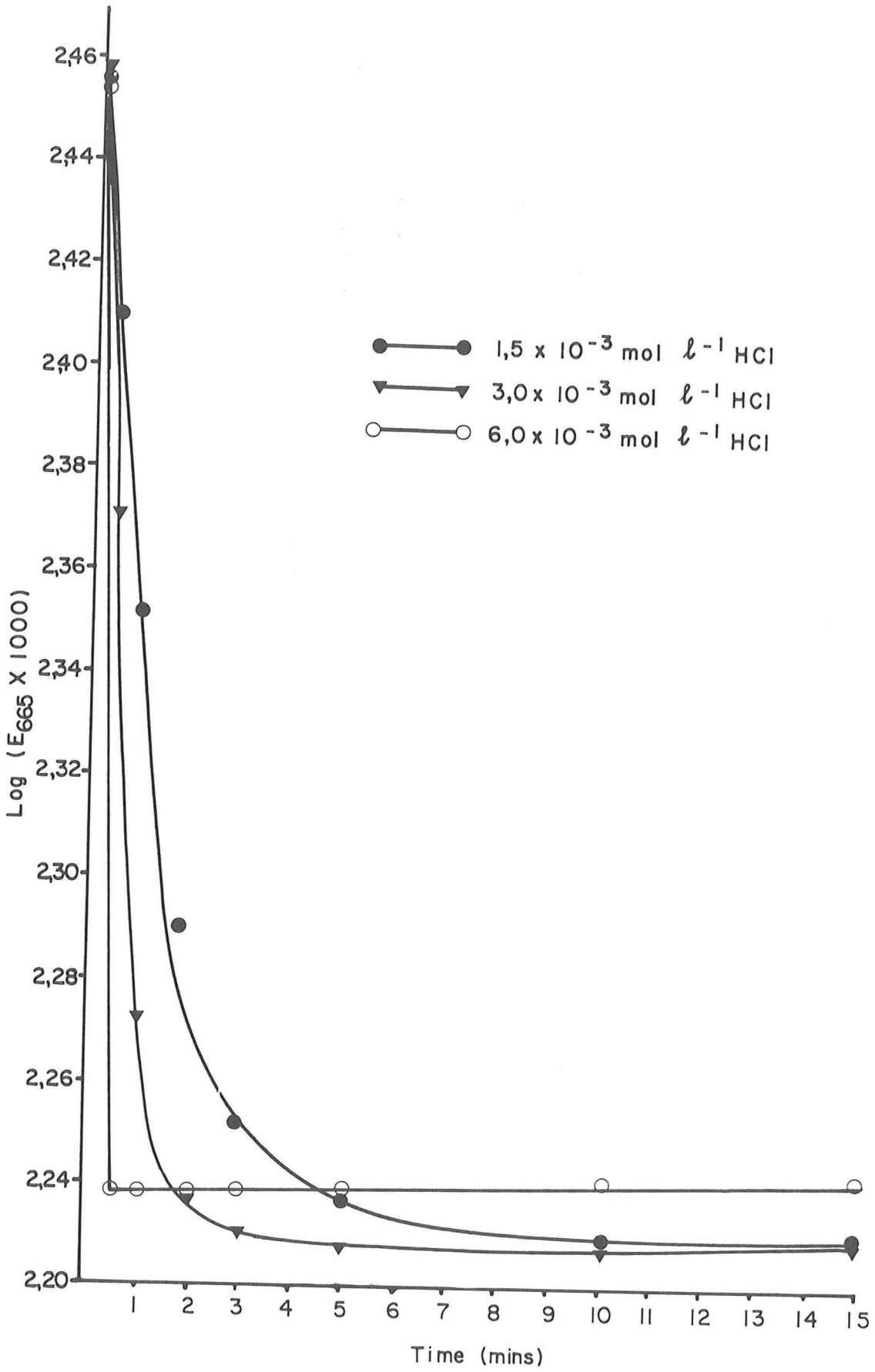


FIGURE 3. The effect of final acid concentration on the rate of phaeophytinization of chlorophyll a in 90 % acetone.

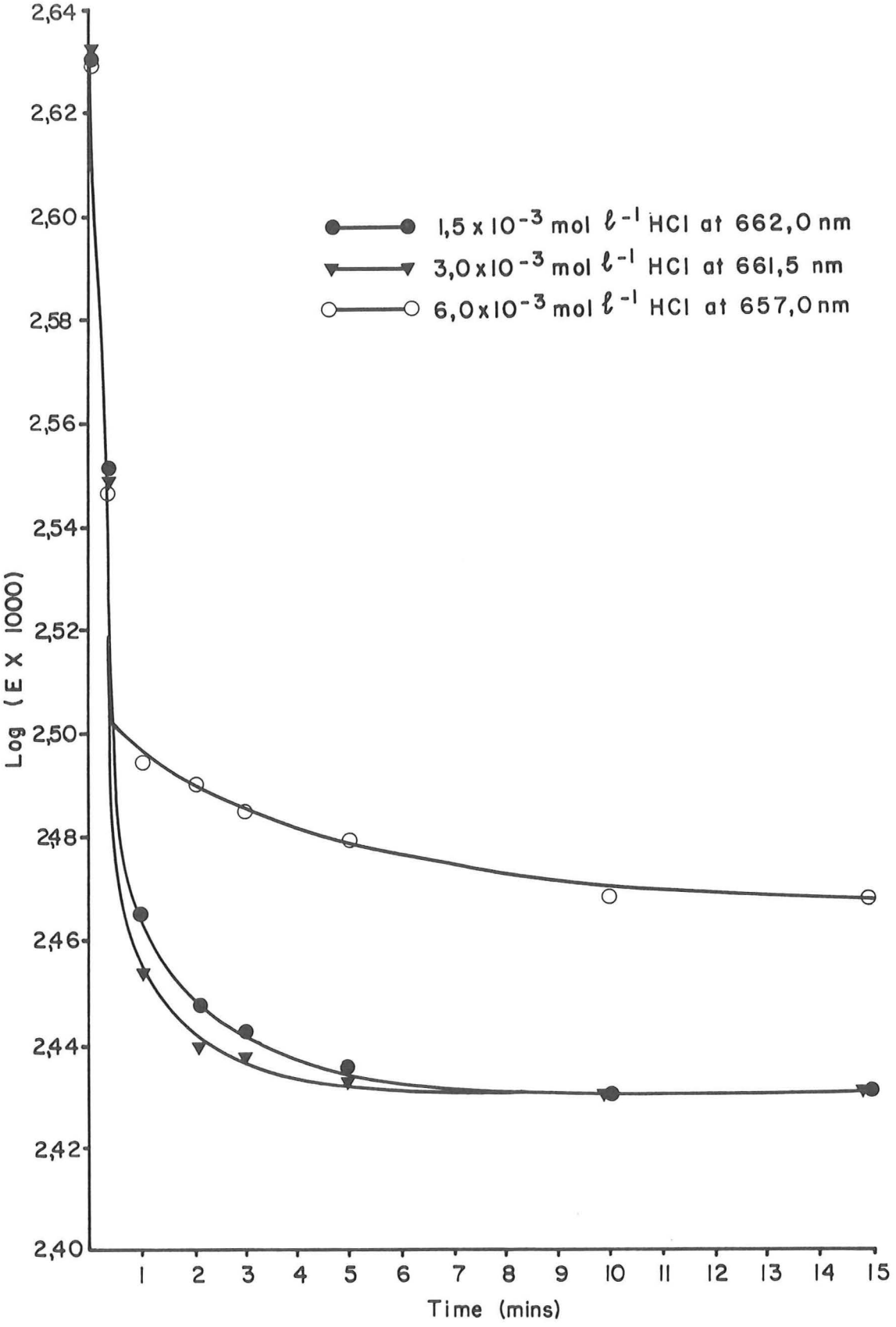


FIGURE 4. The effect of final acid concentration on the rate of phaeophytinization of chlorophyll a in 100% methanol.

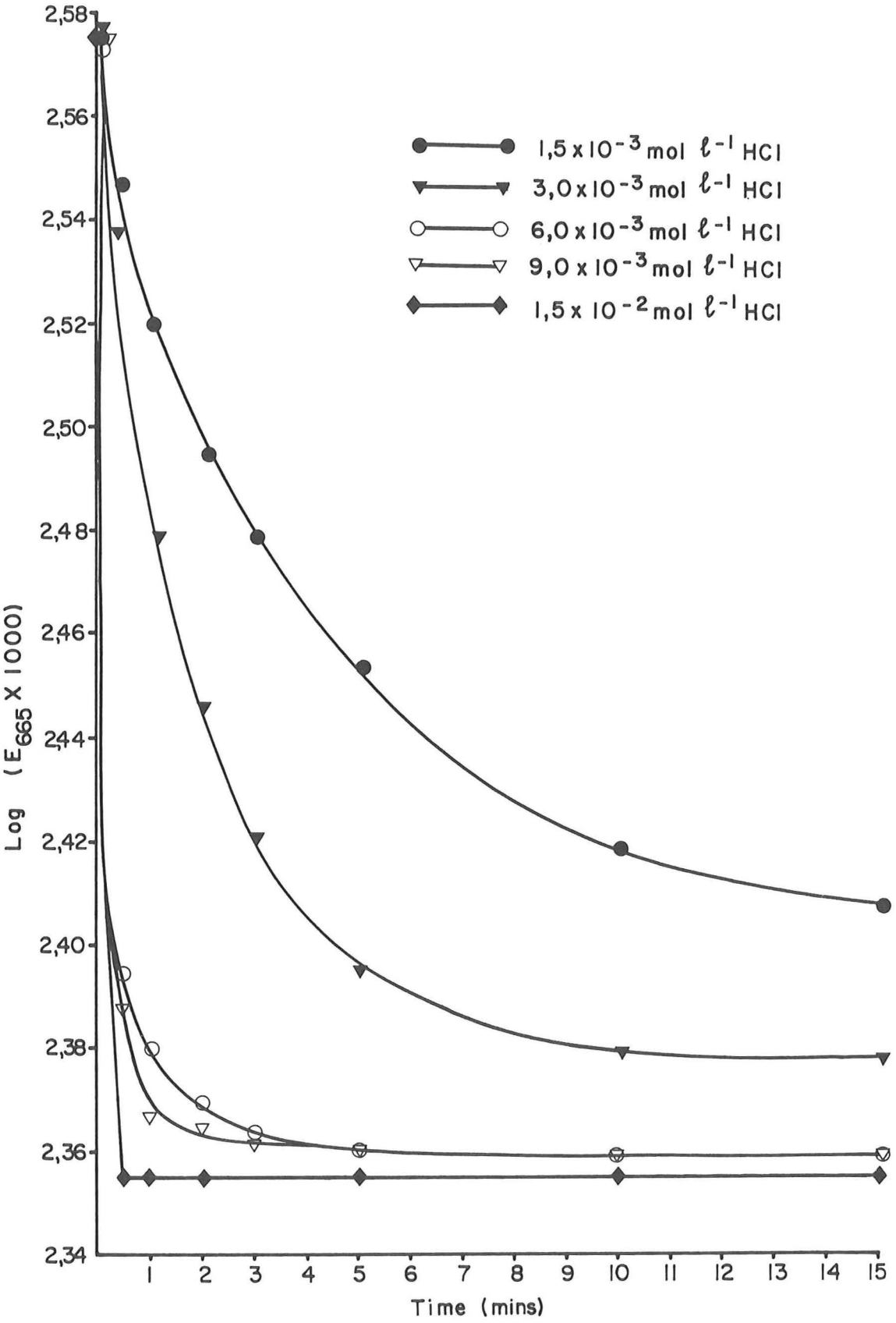


FIGURE 5. The effect of final acid concentration on the rate of phaeophytinization of chlorophyll a in 95% ethanol.

3.3.1.2 DISCUSSION

The final acid concentration of $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl recommended by Riemann (1978a) was adequate to ensure complete phaeophytinization of chlorophyll *a* in 90% acetone and neutralization was not required as the shift due to acidification was neglibible. The post-acidification absorbance reading, therefore, may also be taken at 665 nm.

With methanol a final acid concentration of $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl was also adequate to cause complete phaeophytinization without the formation of degradation products. Neutralization, however, is required as the shift of the phaeophytin *a* peak is marked (Livingstone *et al*, 1953). This may be satisfactorily done using NaOH and the post-acidification reading made by scanning for the peak between 664 nm and 666 nm.

Ethanolic extracts, however, required a higher final acid concentration to ensure complete phaeophytinization within a reasonable time. The concentration of $5 \times 10^{-3}$ mol $\ell^{-1}$ HCl as used by Moed and Hallegraeff (1978) for 80% ethanol extracts would lead to an extended phaeophytinization period. A final acid concentration of between 6,0 and 9,0 mol $\ell^{-1}$ HCl proved adequate to ensure complete degradation of chlorophyll *a* within a reasonable time period and without the formation of undesirable byproducts. Consequently a final acid concentration of $7,5 \times 10^{-3}$ mol $\ell^{-1}$ HCl (added as 100 $\mu\ell$ of a 0,3 mol $\ell^{-1}$ HCl solution to

4 mℓ of extract in the cuvette) was employed throughout this study. In both 90% and 95% ethanol extracts this concentration brought about complete phaeophytinization within 2 min. Neutralization of the extract after acidification is not necessary as the shift of the phaeophytin a peak is small and can be picked up by scanning between 665 nm and 666,5 nm.

### 3.3.2 ACID RATIOS

The acid ratio is the maximum ratio between absorbance before acidification and the absorbance following acidification (and neutralization if required). Employment of a too low acid ratio will result in underestimation of phaeophytin a and the occurrence of negative results. Too high an acid ratio will result in overestimation of the degradation products. The quoted acid ratios are 1,7 for 90% acetone extracts (Lorenzen, 1967), 1,5 for methanolic extracts (Marker, 1972) and 1,7 for 90% ethanol (Nusch, 1980).

#### 3.3.2.1 EXPERIMENTAL

Purified chlorophyll a solutions and extracts from cultured algae, natural filamentous algae and a natural phytoplankton population dominated by Microcystis aeruginosa were prepared in 90% acetone, 100% methanol and 95% ethanol. The acid ratios of the samples in each solvent were ascertained as follows:

In 90% acetone:- The absorbance at 665 nm was read and the extract acidified to 3 x $10^{-3}$ mol $\ell^{-1}$ HCl, shaken and left for 4 min. The absorbance was reread at 665 nm.

In 100% methanol:- The absorbance at 665 nm was read and the extract acidified to 3 x $10^{-3}$ mol $\ell^{-1}$ HCl, shaken and left for 4 min. The extract was neutralized with an equal volume of an equimolar solution of NaOH to that of the acid used (120 $\mu\ell$ of a 0,1 mol $\ell^{-1}$ solution to 4 m$\ell$ of extract) and left for 4 min. The absorbance peak between 664 nm and 666 nm was read.

In 95% ethanol:- The absorbance at 665 nm was read and the extract acidified to 7,5 x $10^{-3}$ mol $\ell^{-1}$ HCl, shaken and left for 2 min. The absorbance peak was reread between 665 nm and 666,5 nm.

The pre-acidification reading is $E_{665}o$ and the second reading is $E_{665}a$. The acid ratio is thus $E_{665}o/E_{665}a$. In most cases the acidification was done on 10 subsamples for each solvent (Table 8).

Young exponentially growing cultures were used to ensure minimal phaeophytin content. The natural phytoplankton sample and the older cultures could be expected to have a higher content of degradation products.

TABLE 8: Acid ratios of purified chlorophyll *a* and algal chlorophyll *a* extracts in 90% acetone, 100% methanol and 95% ethanol. * Mean and standard deviation.

| Chlorophyll *a* Source | No. determinations | Acid Ratios in 90% Acetone* | 100% Methanol* | 95% Ethanol* |
|---|---|---|---|---|
| 1. Purified chlorophyll *a* (Sigma Chemicals) | 6 | 1,69 ± 0,02 | 1,53 ± 0,01 | 1,72 ± 0,01 |
| 2. Blue-green algae: | | | | |
| Microcystis aeruginosa 7 day culture | 10 | 1,70 ± 0,01 | 1,54 ± 0,02 | 1,71 ± 0,01 |
| Microcystis aeruginosa 14 day culture | 10 | 1,69 ± 0,02 | 1,53 ± 0,02 | - |
| Anabaena flos-aquae 7 day culture | 10 | - | - | 1,70 ± 0,02 |
| Oscillatoria brevis | 3 | 1,70 ± 0,02 | 1,52 ± 0,02 | 1,72 ± 0,01 |
| 3. Green algae: | | | | |
| Scenedesmus quadricauda 7 day culture | 10 | 1,68 ± 0,01 | 1,48 ± 0,03 | 1,68 ± 0,04 |
| Scenedesmus quadricauda 14 day culture | 10 | - | - | 1,66 ± 0,02 |
| Selenastrum capricornutum 7 day culture | 10 | 1,69 ± 0,01 | 1,49 ± 0,02 | 1,70 ± 0,02 |
| Selenastrum capricornutum 14 day culture | 10 | 1,70 ± 0,01 | 1,51 ± 0,01 | 1,67 ± 0,01 |
| 4. Filamentous algae: | | | | |
| Cladophora glomerata | 10 | - | - | 1,64 ± 0,02 |
| Stigeoclonium lubricum | 10 | - | - | 1,62 ± 0,03 |
| 5. Natural phytoplankton population dominated by Microcystis aeruginosa (98+%). | 10 | 1,68 ± 0,01 | 1,52 ± 0,02 | 1,70 ± 0,02 |

The acid ratios for purified chlorophyll *a* were 1,69 in 90% acetone, 1,53 in 100% methanol and 1,72 in 95% ethanol (mean of six determinations for each solvent, Table 8). For the 90% acetone algal pigment extracts the mean acid ratio ranged between 1,68 and 1,70. For the 100% methanol extracts the mean acid ratios ranged from 1,48 to 1,54. The ratios higher than 1,5 mostly occurred with the extracts from the blue-green algae from which ratios of 1,55 and 1,56 were not uncommon. The mean ratios in 95% ethanol extracts ranged from 1,62 to 1,72 with a maximum acid ratio of 1,73 being very rarely recorded. Three low mean ratios of 1,62, 1,64 and 1,66 were recorded with the natural samples of *Cladophora glomerata*, *Stigeocloniun lubricun* and a 14 day old culture of *Selenastrum capricornutum*. These low ratios are presumably due to a significant amount of phaeophytin *a* being present in the cells. The remaining values were 1,68 or higher.

### 3.3.2.2 DISCUSSION

The acid ratio of 1,7 for chlorophyll *a* extracts in 90% acetone (Lorenzen, 1967) is generally accepted (Marker, Nusch *et al*, 1980). The results shown here are in good agreement with this value and thus it is retained.

The values for acid factors in 100% methanol are on the whole higher than the normally used value of 1,5 (Marker, 1972; Marker,

Nusch *et al*, 1980). Marker (1972), using 95% methanol extracts from a set of laboratory cultures and a natural population of *Bactrachospermum* sp., quoted values ranging from 1,48 to 1,61 with a mean of 1,53. He finally, however, chose a value of 1,5 for the Lorenzen (1967) equations and this value has been used since. In these experiments values greater than 1,5 were very much the norm and values of 1,55 not uncommon. Subsequently to this study values of 1,52 to 1,55 have been found to commonly occur during routine analyses, particularly when blue-green algae dominated the populations. Since using 1,5 would lead to negative phaeophytin results and an overestimation of chlorophyll *a* in these situations the higher value of 1,55 is chosen as the acid ratio for 100% methanol extracts.

The acid ratios obtained in the 95% ethanol extracts are generally slightly higher than those obtained in 90% acetone extracts. Nusch (1980) used a value 1,7 and reported that on more than 5000 determinations the highest ratios did not exceed 1,7 by much. In these experiments the ratios from purified chlorophyll *a* solutions and extracts from healthy algal cultures were between 1,68 and 1,72. The average mean for the young cultures and purified chlorophyll *a* was 1,71. An acid ratio of 1,72, however, commonly occurred and to avoid the possibility of overestimating chlorophyll *a* and inducing negative phaeophytin *a* results the value of 1,72 is adopted as the acid ratio for ethanolic pigment extracts.

## 3.4 RECOMMENDED PROCEDURES

Based on these experiments and the works of Riemann (1978a), Marker, Nusch et al (1980) and Nusch (1980) the following spectrophotometry procedures are recommended for the analysis of chlorophyll a from freshwater phytoplankton.

(a) In 90% acetone:- Four millilitres of extract is decanted into a 1 cm pathlength cuvette and the absorbance read at 665 nm. The sample is then acidified to $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl by adding 120 $\mu\ell$ of a 0,1 mol $\ell^{-1}$ HCl solution. A small volume of acid is added to avoid significant changes in sample volume (Marker, Nusch et al, 1980). Very small volumes of a high acid concentration, however, should also be avoided as this presents a chance for interference products to be locally formed when the acid is initially added. The sample is shaken and allowed to stand for 4 min after which the absorbance is reread at 665 nm. An acid ratio of 1,7 and a specific absorption coefficient of 89,0 $\ell\ g^{-1}\ cm^{-1}$ should be used in the Lorenzen (1967) equations.

(b) In methanolic solutions:- Four millilitres of extract is decanted into a 1 cm pathlength cuvette and the absorbance read at 665 nm. The sample is then acidified in the cuvette to $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl, shaken and left to stand for 4 min.

The extract is then neutralized by the addition of an equal volume of an equimolar solution of NaOH to that of HCl used, reshaken and left to stand for 4 min. The absorbance is reread by scanning for the peak between 664 nm and 666 nm. For the Lorenzen (1967) equations an acid ratio of 1,55 and a specific absorption coefficient of 77,9 $\ell\ g^{-1}\ cm^{-1}$ are recommended.

(c) In ethanolic solutions:- Decant 4 mℓ of sample into a 1 cm pathlength cuvette and read the absorbance at 665 nm. Acidify the extract to $7,5 \times 10^{-3}$ mol $\ell^{-1}$ HCl, shake and leave to stand for 2 min. Reread the absorbance by scanning for the peak between 665 nm and 666,5 nm. For the Lorenzen (1967) equations an acid ratio of 1,72 and a specific absorption coefficient of 83,4 $\ell\ g^{-1}\ cm^{-1}$ are recommended.

All absorbance readings are corrected for turbidity by the subtraction of a reading of 750 nm (Strickland and Parsons, 1972).

# CHAPTER 4 EXTRACTION SOLVENTS AND PROCEDURES

## 4.1 INTRODUCTION

As discussed in Chapter 1 there is great variation in the reported efficiencies of the solvents used to extract the photosynthetic pigments from phytoplankton. The traditional solvent, 90% acetone, has lost favour due to its inefficient extraction of pigments from the green and blue-green algae and it has been recommended that this solvent is no longer used (Marker, Nusch et al, 1980). Of the remaining solvents methanol is the most popular with ethanol gaining favour. These two solvents are the principal subjects of this study. Their efficacy of extraction is compared to that of 90% acetone and dimethyl sulphoxide (DMSO). Aids to the extraction process such as homogenisation, sonication and boiling are also investigated.

## 4.2 EXTRACTION EFFICIENCY AND HOMOGENISATION

The efficiency of extraction of the solvent employed in chlorophyll a analyses is the crux of the method used. A solvent which, though an efficient pigment extractor with some groups of algae, does extract all the chlorophyll or a constant percentage of the chlorophyll from all groups of algae is undesirable for the purposes to which chlorophyll a results are used. Ideally a solvent should, for the spectrophotometric method being used

in this study, extract all the a pigments present in the algal cells without causing any change in their ratios. The inefficiency of 90% acetone is well documented (see Chapter 1 pages 11 to 13). and the use of alcohols provides a good chance of complete extraction from all groups of phytoplankton.

Of the aids to extraction employed, grinding or homogenisation are the most popular. There is, however disagreement with regard to its efficacy. Some workers (e.g. Rai, 1973; Tolstoy, 1977; Youngman, 1978; Nusch, 1980) report improved extraction with homogenisation while others (e.g. Riemann, 1976; Holm-Hansen and Riemann, 1978) report no improvement.

### 4.2.1 EXPERIMENTAL

Equivolume subsamples from cultures of two green algae (Selenastrum capricornutum and Scenedesmus quadricauda), one blue-green alga (unicellular Microcystis aeruginosa) and a natural phytoplankton population dominated by Microcystis aeruginosa (95%) were filtered and extracted in 10 mℓ of one of three solvents:- 90% acetone, 100% methanol and 95% ethanol. In three cases twenty sets of subsamples were prepared for each solvent. With Scenedesmus quadricauda ten sets for each solvent were prepared. With all four algae half the sets for each solvent were homogenised for 3 min. Those that were homogenised were done so in 5 mℓ of solvent and the slurry decanted into the extraction tube. The homogenisation tube was then washed twice, first with 3 mℓ of solvent and then with 2 mℓ, the washings being added to the extraction tube. Homogenisation was not carried beyond 3 min

as the tubes began to get warm after that time and it was feared that the increase in temperature would induce pigment changes and formation of degradation products, particularly with the methanolic extracts. The samples were then left in the dark in the refrigerator (5 to 8°C) for 20 h before spectrophotometric analysis. The other half of each set was left in the dark at 5 to 8°C for 20 h before analysis. Prior to analysis the pads were squeezed using a pair of forceps and the extract shaken to ensure a homogeneous solution. The chlorophyll a concentrations were measured and calculated as outlined in Chapter 3. The results were analysed statistically using Snedecor's F-test for analyses of variance (multiway classifications) (Snedecor and Cochran, 1967) and Tukey's $\omega$ procedure for least significant difference (Steel and Torrie, 1960) to determine statistically significant differences between means.

The results are shown in Tables 9 to 12. In all cases 90% acetone without homogenisation was the poorest extractor of algal pigments. With Scenedesmus quadricauda only 68,4% of the pigments (relative to extraction with 95% ethanol) was extracted using 90% acetone and with Selenastrum capricornutum only 27,6% was extracted. Homogenisation significantly ($p = 0,05$) improved extraction from Selenastrum capricornutum but not to the levels obtained with 100% methanol and 95% ethanol. Nusch (1980) reported that 90% acetone extracted only 25% of chlorophyll from Scenedesmus when compared to alcoholic extractions. It is not clear whether he used a young or old culture but he does state that "the inefficient extraction with acetone is less conspicuous when the algae are taken from a fast growing culture in the logphase" (Nusch, 1980).

TABLE 9: The relative efficiencies of 100% methanol, 95% ethanol and 90% acetone to extract chlorophyll a from Scenedesmus quadricauda, with and without homogenisation. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ACETONE | |
| EXTRACTION TIME | 20 h | 20 h | 20 h | 20 h | 20 h | 20 h |
| HOMOGENISATION | NO | YES | NO | YES | NO | YES |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 2,820 | 2,484 | 2,883 | 2,682 | 1,972 | 2,043 |
| STANDARD DEVIATION (mg) | 0,128 | 0,257 | 0,219 | 0,119 | 0,345 | 0,301 |
| COEFFICIENT OF VARIATION (%) | 4,5 | 10,4 | 7,6 | 4,5 | 17,5 | 14,7 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,173 | 0,144 | 0,072 | 0,091 | 0,093 | 0,079 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 1 | 4 | 2 | 6 | 5 |
| 11,64* | 0,511 | 2,883 | 2,820 | 2,682 | 2,484 | 2,043 | 1,972 |

TABLE 10: The relative efficiencies of 100% methanol, 95% ethanol and 90% acetone to extract chlorophyll a from Selenastrum capricornutum, with and without homogenisation. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ACETONE | |
| EXTRACTION TIME | 20 h | 20 h | 20 h | 20 h | 20 h | 20 h |
| HOMOGENISATION | NO | YES | NO | YES | NO | YES |
| SAMPLE SIZE (n) | 10 | 10 | 10 | 10 | 10 | 10 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,941 | 0,954 | 1,139 | 1,144 | 0,314 | 0,706 |
| STANDARD DEVIATION (mg) | 0,059 | 0,083 | 0,048 | 0,074 | 0,049 | 0,126 |
| COEFFICIENT OF VARIATION (%) | 6,3 | 8,7 | 4,2 | 6,5 | 15,6 | 17,9 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,051 | 0,072 | 0,071 | 0,068 | 0,031 | 0,028 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | ω | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4 | 3 | 2 | 1 | 6 | 5 |
| 16,55* | 0,104 | 1,144 | 1,139 | 0,954 | 0,941 | 0,706 | 0,314 |

TABLE 11: The relative efficiencies of 100% methanol, 95% ethanol and 90% acetone to extract chlorophyll *a* from unicellular *Microcystis aeruginosa*, with and without homogenisation. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ACETONE | |
| EXTRACTION TIME | 20 h | 20 h | 20 h | 20 h | 20 h | 20 h |
| HOMOGENISATION | NO | YES | NO | YES | NO | YES |
| SAMPLE SIZE (n) | 10 | 10 | 10 | 10 | 10 | 10 |
| CHLOROPHYLL *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 1,465 | 1,506 | 1,470 | 1,493 | 1,297 | 1,489 |
| STANDARD DEVIATION (mg) | 0,042 | 0,056 | 0,041 | 0,053 | 0,060 | 0,101 |
| COEFFICIENT OF VARIATION (%) | 2,9 | 3,7 | 2,8 | 3,6 | 4,6 | 6,8 |
| PHAEOPHYTIN *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,036 | 0,052 | 0,043 | 0,034 | 0,048 | 0,070 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 2 | 4 | 6 | 3 | 1 | 5 |
| 17,82* | 0,078 | 1,506 | 1,493 | 1,489 | 1,470 | 1,465 | 1,297 |

TABLE 12: The relative efficiencies of 100% methanol, 95% ethanol and 90% acetone to extract chlorophyll *a* from a natural phytoplankton population dominated by *Microcystis aeruginosa* (95%).(See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ACETONE | |
| EXTRACTION TIME | 20 h | 20 h | 20 h | 20 h | 20 h | 20 h |
| HOMOGENISATION | NO | YES | NO | YES | NO | YES |
| SAMPLE SIZE (n) | 10 | 10 | 10 | 10 | 10 | 10 |
| CHLOROPHYLL *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 3,164 | 3,008 | 3,210 | 3,214 | 2,011 | 2,141 |
| STANDARD DEVIATION (mg) | 0,286 | 0,311 | 0,294 | 0,361 | 0,401 | 0,386 |
| COEFFICIENT OF VARIATION (%) | 9,0 | 10,3 | 9,2 | 11,2 | 19,9 | 18,0 |
| PHAEOPHYTIN *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,341 | 0,299 | 0,301 | 0,298 | 0,236 | 0,258 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4 | 3 | 1 | 2 | 6 | 5 |
| 26,34* | 0,478 | 3,214 | 3,210 | 3,164 | 3,008 | 2,141 | 2,011 |

With the unicellular Microcystis aeruginosa 90% acetone extracted 88,2% of the chlorophyll a (relative to extraction with 95% ethanol) while with the natural Microcystis aeruginosa population it extracted only 62,7%. It, therefore, appears that the gelatinous coating of the natural Microcystis aeruginosa impairs pigment extraction by 90% acetone.

The extraction efficiencies of 100% methanol and 95% ethanol, with and without homogenisation, on both Microcystis aeruginosa populations and the Scenedesmus quadricauda culture did not differ significantly ($p = 0,05$). In all cases there were no signs of chlorophyll remaining on the pads after extraction. On the other hand Selenastrum capricornutum proved to be the most difficult to extract chlorophyll a from. The most efficient pigment extractor from this alga was 95% ethanol, with or without homogenisation, which was significantly ($p = 0,05$) better than 100% methanol, with or without homogenisation (Table 10). With all three solvents, however, incomplete extraction was indicated by much pigment still remaining on the filters. Because of this Selenastrum capricornutum was chosen as the main test alga in place of Scenedesmus quadricauda, which is recommended by UNESCO (1966).

Homogenisation of the filters and algae did not improve pigment extraction with 100% methanol nor 95% ethanol. With Scenedesmus quadricauda a decrease in chlorophyll a concentrations (of up to 11,9% with 100% methanol) was noted with homogenised samples (Table 9). With 90% acetone homogenisation did improve

chlorophyll *a* extraction with the cultured algae, but only with the unicellular *Microcystis aeruginosa* did the chlorophyll *a* levels approach the levels obtained with 95% ethanol (Table 11).

### 4.2.2 DISCUSSION

From these results it is clear that, for the green and blue-green algae, 90% acetone is an inefficient extractor of photosynthetic pigments. This is in agreement with the generally held opinion (Marker, Nusch *et al*, 1980). Absolute methanol and 95% ethanol proved to be more efficient extractors of algal pigments, achieving complete extraction from two populations of the blue-green *Microcystis aeruginosa* and the UNESCO (1966) test alga, *Scenedesmus quadricauda*. With *Selenastrum capricornutum*, pigment extraction was incomplete, although 95% ethanol proved to be a more efficient extractor from this alga than 100% methanol, both of which were far superior to 90% acetone, with or without homogenisation.

Homogenisation of the filters for 3 min did not result in improved extraction with the alcoholic solvents, but with acetone did result in improved extraction, although not to the levels achieved with 95% ethanol. Whether extended homogenisation would improve extraction further is open to question. In these experiments, however, excessive heating was noted after 3 min and it was feared that formation of degradation products would result if homogenisation was continued. Extended mechanical grinding has been shown to lead to formation of

degradation products and artifacts (Kerr and Subba Rao, 1966; Daley *et al*, 1973) and this coupled with the poor returns for the effort expended nulifies the potential of homogenisation as an aid to extraction.

Further experiments were thus conducted to improve pigment extraction from *Selenastrum capricornutum* using 95% ethanol and 100% methanol.

## 4.3 EXTRACTION TIME AND TEMPERATURE

The first experiments conducted to improve pigment extraction were to ascertain whether extending the time of standing yielded more complete extraction and whether extraction efficiency was effected by storage temperature.

### 4.3.1 EXPERIMENTAL

Equivolume subsamples of a culture of *Selenastrum capricornutum* were filtered and extracted in 10 mℓ aliquots of either 100% methanol or 95% ethanol for 24 h, 48 h and 72 h in the refrigerator, after which chlorophyll *a* concentrations were measured as before. The results, analysed statistically as before, are shown in Table 13.

Extending the extraction period from 24 h to 72 h did result in a small increase in chlorophyll *a* in the 95% ethanol extracts but the increase was not significant ($p = 0,05$) (Table 13).

TABLE 13: The effect of extended extraction time on the extraction of chlorophyll a from Selenastrum capricornutum using 100% methanol and 95% ethanol. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | | 95% ETHANOL | | |
| EXTRACTION TIME | 24 h | 48 h | 72 h | 24 h | 48 h | 72 h |
| EXTRACTION TEMP. (°C) | 5 - 8 | 5 - 8 | 5 - 8 | 5 - 8 | 5 - 8 | 5 - 8 |
| SAMPLE SIZE (n) | 3 | 3 | 3 | 3 | 3 | 3 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 1,197 | 1,380 | 1,490 | 1,930 | 2,159 | 2,207 |
| STANDARD DEVIATION (mg) | 0,021 | 0,092 | 0,113 | 0,230 | 0,288 | 0,303 |
| COEFFICIENT OF VARIATION (%) | 1,8 | 6,7 | 7,6 | 10,7 | 13,3 | 13,7 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE | 0,046 | 0,039 | 0,045 | 0,046 | 0,083 | 0,070 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 5 | 4 | 3 | 2 | 1 |
| 11,20* | 0,628 | 2,207 | 2,159 | 1,930 | 1,490 | 1,380 | 1,197 |

With the methanolic extracts there was also an improvement in chlorophyll *a* extraction, but the maximum levels did not reach those obtained with 95% ethanol. There was no increase in phaeophytin *a* percentage in either solvent over the extended extraction period. Even after 72 h, however, large smounts of pigment were still found on the filters showing incomplete extraction.

To ascertain the effect of extraction temperature on chlorophyll *a* extraction by 95% ethanol and 100% methanol three cultures of algae were used. Equivolumes of subsamples from cultures of *Anabaena flos-aquae* and *Scenedesmus quadricauda* were filtered and extracted in 95% ethanol in the dark at either refrigerator temperature (5 to 8°C) or room temperature (22 to 24°C). The experiment was repeated using a culture of unicellular *Microcystis aeruginosa* and extracting with 100% methanol. The extraction periods were 12 h, 24 h and 48 h and the results and the statistical analyses are presented in Tables 14 to 16.

There was no significant difference ($p = 0,01$) between refrigerator stored and room temperature stored extractions for either of the ethanolic extractions (Tables 14 and 15) or the methanolic extraction (Table 16). There was also no increase in phaeophytin *a* in the room temperature stored extractions as against those stored in the refrigerator.

### 4.3.2 DISCUSSION

From these results it is clear that by simply extending extraction time chlorophyll *a* extraction is not improved. Extending

TABLE 14: The effect of extraction temperature on the extraction of chlorophyll *a* from *Anabaena flos-aquae* by 95% ethanol. (See text for details).

| METHODS | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 95% ETHANOL | | | | | |
| EXTRACTION TIME | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h |
| EXTRACTION TEMP.(°C) | 5 - 8 | 5 - 8 | 5 - 8 | 22 - 24 | 22 - 24 | 22 - 24 |
| SAMPLE SIZE (n) | 3 | 3 | 3 | 3 | 3 | 3 |
| CHLOROPHYLL *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 1,471 | 1,471 | 1,471 | 1,471 | 1,462 | 1,481 |
| STANDARD DEVIATION (mg) | 0,016 | 0,044 | 0,033 | 0,044 | 0,029 | 0,033 |
| COEFFICIENT OF VARIATION (%) | 1,1 | 3,0 | 2,2 | 3,0 | 2,0 | 2,2 |
| PHAEOPHYTIN *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,028 | 0,028 | 0,035 | 0,035 | 0,031 | 0,033 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 1 | 2 | 3 | 4 | 5 |
| 0,86 | - | 1,481 | 1,471 | 1,471 | 1,471 | 1,471 | 1,462 |

TABLE 15: The effect of extraction temperature on the extraction of chlorophyll a from Scenedesmus quadricauda by 95% ethanol. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | | | 95% ETHANOL | | | |
| EXTRACTION TIME | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h |
| EXTRACTION TEMP. (°C) | 5 - 8 | 5 - 8 | 5 - 8 | 22 - 24 | 22 - 24 | 22 - 24 |
| SAMPLE SIZE (n) | 3 | 3 | 3 | 3 | 3 | 3 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 1,462 | 1,481 | 1,538 | 1,462 | 1,490 | 1,491 |
| STANDARD DEVIATION (mg) | 0,058 | 0,158 | 0,083 | 0,058 | 0,125 | 0,131 |
| COEFFICIENT OF VARIATION (%) | 4,0 | 10,7 | 5,4 | 4,0 | 8,4 | 8,8 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,031 | 0,039 | 0,058 | 0,031 | 0,030 | 0,119 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | ω | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 3 | 6 | 5 | 2 | 1 | 4 |
| 0,19 | - | 1,538 | 1,491 | 1,490 | 1,481 | 1,462 | 1,462 |

TABLE 16: The effect of extraction temperature on the extraction of chlorophyll a from unicellular Microcystis aeruginosa by 100% methanol. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | | | | |
| EXTRACTION TIME | 12 h | 24 h | 48 h | 12 h | 24 h | 48 h |
| EXTRACTION TEMP. (°C) | 5 - 8 | 5 - 8 | 5 - 8 | 22 - 24 | 22 - 24 | 22 - 24 |
| SAMPLE SIZE (n) | 3 | 3 | 3 | 3 | 3 | 3 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 2,159 | 2,159 | 2,130 | 2,140 | 2,092 | 2,159 |
| STANDARD DEVIATION (mg) | 0,060 | 0,073 | 0,044 | 0,120 | 0,050 | 0,033 |
| COEFFICIENT OF VARIATION (%) | 2,8 | 3,4 | 2,1 | 5,6 | 2,4 | 1,5 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,083 | 0,063 | 0,030 | 0,034 | 0,040 | 0,042 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 1 | 2 | 6 | 4 | 3 | 5 |
| 0,44 | - | 2,159 | 2,159 | 2,159 | 2,140 | 2,130 | 2,092 |

extraction time beyond three days is undesirable for routine purposes.

Extraction is most commonly carried out in the dark in a refrigerator (sometimes called, euphemistically, 4°C) (e.g. Strickland and Parsons, 1972; Weber, 1973; Talling, 1974; Standard Methods, 1976; Youngman, 1978). Some manuals recommended extraction in the dark at room temperature (e.g. UNESCO, 1966; Greeson et al, 1977). The results reported here show that there is no difference between extractions carried out of refrigerator temperatures (5 to 8°C) and those carried out at room temperatures (22 to 24°C). There is no real evidence of an increase in phaeophytins after 48 h at room temperature. Higher room temperatures such as those experienced in the tropics or in non-air conditioned environments, however, may have an effect and individual workers would have to assess the risk if they are not using refrigerated extractions.

## 4.4 SONICATION AS AN AID TO EXTRACTION

Treatment of algal pigment extractions in an ultrasonic bath has been reported to improve chlorophyll a extraction (Nelson, 1960; Garside and Riley, 1969). Nelson (1960) reported a 30% improvement in extraction with 90% acetone after sonicating extractions for 9 mins followed by 2 to 4 h standing. Garside and Riley (1969) reported complete extraction with methanol without the formation of degradation products after 15 min agitation in an ultrasonic bath. On the other hand, however, Daley et al (1973) reported incomplete recovery of chlorophylls after 30 min in an ultrasonic bath.

They also found an increase in degradation products. Using a sonic probe for 5 min, Daley *et al* (1973) achieved complete extraction without concomitant formation of degradation products.

### 4.4.1 EXPERIMENTAL

Twenty-five equivolume subsamples of a culture of *Selenastrum capricornutum* were filtered and divided in five groups of five. To each set was added 10 mℓ of 95% ethanol. The first set was extracted in the dark at room temperature for 24 h. The other sets were placed in an ultrasonic bath for 1 min, 2 min, 5 min and 10 min and then left to extract in the dark at room temperature for 24 h. Chlorophyll *a* and phaeophytin *a* concentrations were analysed as outlined in Chapter 3. The results and statistical analyses are shown in Table 17.

Sonication of up to 10 min did not result in improved chlorophyll *a* extraction ($p = 0,01$) from *Selenastrum capricornutum* and large amounts of pigment still remained in the cells. There was also no increase in phaeophytins, even after 10 min sonication.

### 4.4.2 DISCUSSION

Despite reports of improved extraction with sonication of 90% acetone and methanol extractions (Nelson, 1960; Garside and Riley, 1969) this experiment did not show an improvement in chlorophyll *a* extraction using 95% ethanol and sonication for

TABLE 17: The effect of treatment of extractions in an ultrasonic bath on extraction of chlorophyll *a* from *Selenastrum capricornutum* by 95% ethanol. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 |
|---|---|---|---|---|---|
| SOLVENT | 95% ETHANOL | | | | |
| EXTRACTION TIME | 24 h | 24 h | 24 h | 24 h | 24 h |
| SONICATION TIME | 0 min | 1 min | 2 min | 5 min | 10 min |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL *a* | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 1,599 | 1,530 | 1,542 | 1,651 | 1,536 |
| STANDARD DEVIATION (mg) | 0,286 | 0,207 | 0,375 | 0,242 | 0,249 |
| COEFFICIENT OF VARIATION (%) | 17,9 | 13,5 | 24,3 | 14,7 | 16,2 |
| PHAEOPHYTIN *a* | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,052 | 0,042 | 0,035 | 0,037 | 0,049 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | ω | Methods in decreasing order of means** | | | | |
|---|---|---|---|---|---|---|
| | | 4 | 1 | 3 | 5 | 2 |
| 0,53 | - | 1,651 | 1,599 | 1,542 | 1,536 | 1,530 |

up to 10 min. Also there was no increase in phaeophytin concentrations after 10 min sonication. Garside and Riley (1969) also found no formation of phaeophytins after 15 min sonication of methanol extracts, while Daley *et al* (1973) report formation of degradation products after sonication for 30 min. It, therefore, appears that duration of sonication affects chlorophyll *a* breakdown, limiting sonication time to 10 to 15 min if degradation is to be avoided. This short sonication time, however, does not improve the extraction efficiency of 95% ethanol and thus sonication is discarded as an aid to pigment extraction.

## 4.5 BOILING AS AN AID TO EXTRACTION

### 4.5.1 INTRODUCTION

The potential of using hot or boiling solvents to extract photosynthetic pigments from phytoplankton has not been properly investigated. Youngman (1978) reported that heating methanolic extractions in a water bath at 75 to 90°C for 2 to 3 min resulted in a 24% increase in extracted total pigments (over cold acetone extraction) when used on summer populations from Farmoor Reservoir dominated by green and blue-green algae. With winter and spring populations from Farmoor Reservoir and diatom populations from the River Thames the two procedures were equally efficatious. Riemann (1976), however, reported a decrease in chlorophyll *a* concentrations

and an increase in phaeophytin a when methanolic extracts were placed in a water bath for more than 3 min at 100°C. He attributed this to the violent nature of the boiling and loss of chlorophyll by adherence of pigment to the side of the vessel which could not be easily eluted. At 70°C the effects were much less pronounced (Riemann, 1976). Nusch (1980) reported optimum extraction using "hot" 90% ethanol and grinding. The ethanol is heated to boiling point (78°C) and poured over the filter. The solvent is allowed to cool. The filter is then ground, rinsed and time allowed for complete extraction by standing for 6 to 24 h. The use of "hot" 90% ethanol yielded higher values than did "hot" methanol. The use of "hot" acetone improved yields over "cold" acetone, but not, however, to the levels achieved with alcohols. A major advantage of using "hot" or boiling solvents is that heat treatment destroys chlorophyllase (Bacon and Holden, 1967), an enzyme responsible for the conversion of chlorophyll to chlorophyllide (Marker, 1972). This enzyme remains active in the alcohols (Marker, 1972) and may lead to allomerisation. Riemann (1980) showed that the instability of chlorophyll a in methanol could be avoided by reducing the water content and using the shortest possible extraction time.

In this section the effect of water content on the extraction efficiency of ethanol is also investigated. Nusch (1980) states that a water content of ~10% aids extraction and Moed and Hallegraeff (1978) report more controlled acidi-

fication during the spectrometry procedures with a 10 - 20% water content. The addition of water to methanol, on the other hand, has been shown to lead to much reduced extraction efficiency (Riemann, 1976).

### 4.5.2 EFFECT OF BOILING ON CHLOROPHYLL a

#### 4.5.1.2 EXPERIMENTAL

Solutions of purified chlorophyll a were made up in 95% ethanol and 100% methanol and boiled under reflux in a water bath set at either the solvent boiling point (78°C for 95% ethanol and 65°C for methanol) or 100°C for 30 min, during which samples were withdrawn, cooled and examined spectrophotometrically as outlined in Chapter 3. The results are presented in Table 18.

For both solvents boiling in a water bath at 100°C resulted in the formation of phaeophytin a after a short time (with 30 s in 95% ethanol and 2 min with absolute methanol). The boiling action was very vigorous and chlorophyll was seen to adhere to the sides of the boiling flask where it splashed, particularly with methanol. This chlorophyll was not removed by simple elution with fresh solvent.

At the solvents' boiling points, however, there was no formation of phaeophytin a after 20 min. It is not clear why phaeophytin a values were obtained at 3 min and 10 min in methanol and 5 min in ethanol, but it is most likely due to spectrophotometric error. It must be borne in mind that these are single measurements and are therefore subject to random error.

TABLE 18: The effect of water bath temperature on the formation of degradation products (increase in phaeophytin a) in boiling ethanolic and methanolic purified chlorophyll a solutions.

| Time (min) | % degradation products in 95% Ethanol at 78°C | 95% Ethanol at 100°C | 100% Methanol at 65°C | 100% Methanol at 100°C |
|---|---|---|---|---|
| 0 | 0,0 | 0,0 | 0,0 | 0,0 |
| 0,5 | 0,0 | 0,4 | 0,0 | 0,0 |
| 1 | 0,0 | 0,5 | 0,0 | 0,0 |
| 2 | 0,0 | 0,6 | 0,0 | 1,7 |
| 3 | 0,0 | 1,5 | 1,8 | 2,1 |
| 5 | 1,2 | 3,2 | 0,0 | 4,1 |
| 10 | 0,0 | 2,9 | 0,0 | 4,1 |
| 15 | 0,0 | 2,8 | 0,5 | 8,3 |
| 20 | 0,0 | 3,5 | 0,0 | 9,5 |
| 30 | 3,1 | 5,4 | 2,7 | 14,5 |

4.5.2.2 DISCUSSION

From this experiment it is clear that boiling in a water bath of 100°C results in the formation of degradation products in either solvent tested. Chlorophyll a was also lost through adherence of splashes to the walls of the boiling flask, a problem also noted by Riemann (1976). Using chlorophyll from

barley leaves, Bacon and Holden (1967) also found an increase in phaeophytins after heating in distilled water at 100°C for 5 min. At the solvents' boiling points, however, no formation of phaeophytin was found before 20 min. Riemann (1976), boiling methanol extracts in a water bath at 70°C, found a small increase in chlorophyll a extraction after 1 min (as against cold extraction) and a limited decrease after prolonged boiling.

From this experiment, coupled with the beneficial effect of destruction of chlorophyllase, boiling was considered not to be detrimental to chlorophyll a determinations provided it is done at the solvent's boiling point.

### 4.5.3 EFFICACY OF EXTRACTION BY BOILING

#### 4.5.3.1 EXPERIMENTAL

Equal volumes of subsamples of a culture of Selenastrum capricornutum were filtered and extracted with 95% ethanol. Six sets of 5 were prepared. The first set was extracted in the dark at room temperature for 24 h before being examined spectrophotometrically. The other 5 sets were boiled in a water bath set at 78°C for 0,5 to 5 min, allowed to cool and analysed as set out in Chapter 3. The results and their statistical analysis (as before) are presented in Table 19.

Boiling the extractions for 0,5 min, 1 min and 2 min did not result in an improvement of chlorophyll a extraction and all three were significantly lower ($p = 0,05$) than the 24 h extraction.

TABLE 19: The effect of boiling time on the extraction of chlorophyll *a* from *Selenastrum capricornutum* by 95% ethanol at 78°C. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 95% ETHANOL | | | | | |
| EXTRACTION TIME | 24 h | - | - | - | - | - |
| BOILING TIME | - | 0,5 min | 1 min | 2 min | 3 min | 5 min |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 6,093 | 4,523 | 4,712 | 4,597 | 7,630 | 7,784 |
| STANDARD DEVIATION (mg) | 0,403 | 0,855 | 0,350 | 0,522 | 0,260 | 0,389 |
| COEFFICIENT OF VARIATION (%) | 6,6 | 18,9 | 7,4 | 11,4 | 3,4 | 5,0 |
| PHAEOPHYTIN *a* | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,399 | 0,257 | 0,328 | 0,318 | 0,423 | 0,441 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 5 | 1 | 3 | 4 | 2 |
| 40,59* | 1,063 | 7,784 | 7,630 | 6,093 | 4,712 | 4,597 | 4,523 |

Boiling for 3 min or 5 min, however, resulted in a significant improvement ($p = 0,05$) over 24 h extraction of 25,2% and 27,8% respectively. There was also no increase in phaeophytin *a* in the boiled extracts as against the unboiled extracts. There were, however, traces of pigment left on the filters which slowly disappeared on standing.

A second experiment was conducted to test the influence of standing after boiling extraction whereby 20 equivolume subsamples of a culture of *Selenastrum capricornutum* were filtered and extracted in 95% ethanol. Five were extracted in the dark at room temperature for 24 h, while the remaining 15 were boiled for 5 min in a water bath at 78°C. Of these 5 were allowed to cool for a few minutes after boiling before analysis, 5 were left to stand in the dark at room temperature for 1 h before analysis and the last 5 treated the same for 24 h. The results are shown in Table 20.

Although not significantly different ($p = 0,05$) the boiled extracts which were left to stand for 1 h and 24 h yielded 7,0% and 10,1% higher amounts of chlorophyll *a* over the boiled extracts, all of which were significantly better than the unboiled extracts ($p = 0,05$). Also no trace of pigment could be seen on the filters from those extracts which had been boiled and left to stand. There was, again, no increase in phaeophytins.

TABLE 20: The effect of post boiling standing time on the extraction of chlorophyll *a* from *Selenastrum capricornutum* by boiling 95% ethanol. (See text for details).

| METHOD | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| SOLVENT | | 95% ETHANOL | | |
| BOILING TIME | - | 5 min | 5 min | 5 min |
| STANDING TIME | 24 h | - | 1 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 |
| CHLOROPHYLL *a* | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 6,254 | 8,334 | 8,913 | 9,177 |
| STANDARD DEVIATION (mg) | 0,854 | 0,629 | 0,219 | 0,314 |
| COEFFICIENT OF | 13,7[a)] | 7,6 | 2,5 | 3,4 |
| PHAEOPHYTIN *a* | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,461 | 0,749 | 0,686 | 0,757 |

Statistical analysis of chlorophyll *a* concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | ω | Methods in decreasing order of means** | | | |
|---|---|---|---|---|---|
| | | 4 | 3 | 2 | 1 |
| 24,34* | 1,276 | 9,177 | 8,913 | 8,334 | 6,254 |

a) High coefficient of variation due to inclusion of two low values. Values ranged from 5,188 to 6,964 mg $\ell^{-1}$ extract.

#### 4.5.3.2 DISCUSSION

These experiments clearly show that the boiling of extractions resulted in a very much improved chlorophyll *a* yield over unboiled extracts. Concomitant with this there was no increase in phaeophytins in any of the boiled extracts over the level found in unboiled extracts. The improvement on *Selenastrum capricornutum* is greater than that indicated by Nusch (1980) for *Scenedesmus quadricauda* (see Fig. 4 in Nusch, 1980), but as shown before *Selenastrum* proved to be more resistant to extraction than *Scenedesmus*. Complete extraction of pigments was achieved by leaving the extracts to stand for 1 to 24 h after boiling to ensure extraction of the last traces of pigment.

### 4.5.4 EFFECT OF WATER CONTENT ON EXTRACTION EFFICIENCY

So far in this study 95% ethanol has been used as the ethanolic extraction agent. Nusch (1980), however, recommends 90% as the increased water content aids extraction. A 10 to 20% water content is also recommended by Moed and Hallegraeff (1978) for a more controlled acidification step during spectrophotometric analysis as the initial pH drop is not so great in 80 to 90% ethanol as it is in 95% ethanol, thus reducing the chances of causing chlorophyll degradation.

#### 4.5.4.1 EXPERIMENTAL

Equivolume samples from a mixed green algae population, consisting of 70% cultured *Selenastrum capricornutum*, and 20% *Golenkinia*

radiata and 10% Scenedesmus quadricauda from a natural source, were filtered and extracted in one of three solvents, viz. 80% ethanol, 90% ethanol and 95% ethanol. Ten samples for each solvent were prepared. One half of the samples for each set were allowed to extract in the dark at room temperature for 24 h. The other half were boiled in a water bath at 78°C for 5 min and then left to stand in the dark at room temperature for 24 h. After 24 h the extracts were analysed spectrophotometrically as outlined in Chapter 3. The results are shown in Table 21.

Of the unboiled extractions, 80% ethanol was the poorest pigment extractor, yielding only 60% of the chlorophyll a present. All the unboiled extracts were significantly lower ($p = 0,05$) in chlorophyll a than the boiled extracts. The highest yield was achieved with 90% ethanol, although there was no significant difference between the boiled extractions ($p = 0,05$). Although 95% ethanol was the best solvent in unboiled extractions it was the poorest in the boiled extractions. There was an increase in phaeophytin a in the 80% ethanol extractions, but the levels were not significantly different ($p = 0,05$) from those in 90% and 95% ethanol.

#### 4.5.4.2 DISCUSSION

The increasing of water content in ethanolic extractions from 5% to 10% did not result in a significant ($p = 0,05$) improvement in chlorophyll a extraction with the unboiled extracts. A 20% water content resulted in a significant ($p = 0,05$) decrease

TABLE 21: The effect of percentage of water of ethanolic solution on the extraction of chlorophyll a from a mixed green algae population. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 80% ETHANOL | | 90% ETHANOL | | 95% ETHANOL | |
| BOILING TIME | - | 5 min | - | 5 min | - | 5 min |
| STANDING TIME | 24 h | 24 h | 24 h | 24 h | 24 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 3,714 | 5,996 | 4,563 | 6,231 | 4,964 | 5,864 |
| STANDARD DEVIATION (mg) | 0,116 | 0,077 | 0,082 | 0,148 | 0,151 | 0,169 |
| COEFFICIENT OF VARIATION (%) | 3,1 | 1,3 | 1,8 | 2,4 | 3,0 | 2,9 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,359 | 0,488 | 0,262 | 0,373 | 0,286 | 0,252 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 4 | 2 | 6 | 5 | 3 | 1 |
| 857,54* | 0,471 | 6,231 | 5,996 | 5,864 | 4,964 | 4,563 | 3,714 |

in pigment extraction. Of the boiled extracts there was no significant difference between the solvents although chlorophyll *a* values in 90% ethanol were an average 5,9% higher than those in 95% ethanol. Also there was no difference in phaeophytin *a* content in the 90% and the 95% ethanol extracts (Table 21).

From these results it is seen that boiling 80% and 90% ethanol are as effective extraction agents as boiling 95% ethanol. The advantages of the 10 to 20% water content discussed by Moed and Hallegraeff (1978) were, however, not apparent in this experiment. Taking the attitude that is best to avoid all possible sources of error, the use of 90% ethanol may thus be preferable to 95% ethanol.

### 4.5.5 COMPARATIVE EXTRACTIONS

#### 4.5.5.1 EXPERIMENTAL

Three algal populations were used to compare the extraction efficiency of boiling extractions using 100% methanol (boiled at 65°C) and 90% and 95% ethanol (boiled at 78°C). The populations were:-

(a) A culture of *Selenastrum capricornutum*.

(b) A natural phytoplankton population from an evaporation pan at Roodeplaat Dam comprising of approximately 60% *Scenedesmus quadricauda*, 30% *Golenkinia radiata* and 10% *Pediastrum* spp.

(c) A natural phytoplankton population from Roodeplaat Dam comprising of approximately 60% Microcystis aeruginosa, 10% Anabaena circinalis, 15% diatoms (mainly Melosira granulata) and 5% Cryptomonas sp.

Thirty equivolume samples from each population were filtered and ten each extracted in each of the three solvents. For each solvent and algal population 5 samples were boiled for 5 min allowed to stand in the dark at room temperature for 1 h, while the other 5 were boiled for 5 min and allowed to stand for 24 h. Spectrophotometric analyses were carried out as outlined in Chapter 3. The results of these experiments and their statistical analysis are presented in Tables 22 - 24.

For all three algal populations the highest mean chlorophyll a values were obtained with 90% ethanol, boiled for 5 min and allowed to stand for 24 h. With the Selenastrum capricornutum culture the poorest solvent was boiling 100% methanol and standing for 1 h, which was significantly poorer than the boiling 90% ethanol / 24 h extraction (Table 22). The coefficients of variation for all six treatments were low with a maximum of 2,2% being recorded for both methanolic extractions. There was no significant difference ($p = 0,05$) between any of the ethanolic extraction procedures.

The poorest extractor from the natural green algae population was boiling 95% ethanol followed by 1 h extraction which, although only 4,5% lower than the 90% ethanol / 24 h extraction,

TABLE 22: Efficacy of 90% ethanol, 95% ethanol and 100% methanol, boiled at solvent boiling point, for the extraction of chlorophyll a from Selenastrum capricornutum. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ETHANOL | |
| BOILING TIME | 5 min | 5 min | 5 min | 5 min | 5 min | 5 min |
| STANDING TIME | 1 h | 24 h | 1 h | 24 h | 1 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 3,465 | 3,561 | 3,536 | 3,577 | 3,577 | 3,605 |
| STANDARD DEVIATION (mg) | 0,076 | 0,079 | 0,033 | 0,032 | 0,037 | 0,031 |
| COEFFICIENT OF VARIATION (%) | 2,2 | 2,2 | 0,9 | 0,9 | 1,0 | 0,9 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,061 | 0,062 | 0,050 | 0,051 | 0,047 | 0,055 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,05 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 4 | 5 | 2 | 3 | 1 |
| 3,71* | 0,113 | 3,605 | 3,577 | 3,577 | 3,561 | 3,536 | 3,465 |

TABLE 23: Efficacy of 90% ethanol, 95% ethanol and 100% methanol, boiled at solvent boiling points, for the extraction of chlorophyll a from a natural phytoplankton population dominated by green algae. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ETHANOL | |
| BOILING TIME | 5 min | 5 min | 5 min | 5 min | 5 min | 5 min |
| STANDING TIME | 1 h | 24 h | 1 h | 24 h | 1 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 4,542 | 4,579 | 4,408 | 4,597 | 4,557 | 4,614 |
| STANDARD DEVIATION (mg) | 0,058 | 0,069 | 0,062 | 0,038 | 0,117 | 0,061 |
| COEFFICIENT OF VARIATION (%) | 1,3 | 1,5 | 1,4 | 0,8 | 2,6 | 1,3 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,265 | 0,226 | 0,277 | 0,215 | 0,210 | 0,214 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 4 | 2 | 5 | 1 | 3 |
| 6,39* | 0,131 | 4,614 | 4,597 | 4,579 | 4,557 | 4,542 | 4,408 |

TABLE 24: Efficacy of 90% ethanol, 95% ethanol and 100% methanol, boiled at solvent boiling points, for the extraction of chlorophyll a from a natural phytoplankton population dominated by blue-green algae. (See text for details).

| METHOD | 1 | 2 | 3 | 4 | 5 | 6 |
|---|---|---|---|---|---|---|
| SOLVENT | 100% METHANOL | | 95% ETHANOL | | 90% ETHANOL | |
| BOILING TIME | 5 min | 5 min | 5 min | 5 min | 5 min | 5 min |
| STANDING TIME | 1 h | 24 h | 1 h | 24 h | 1 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 | 5 | 5 |
| CHLOROPHYLL a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 3,583 | 3,568 | 3,669 | 3,651 | 3,697 | 3,715 |
| STANDARD DEVIATION (mg) | 0,108 | 0,084 | 0,064 | 0,052 | 0,029 | 0,052 |
| COEFFICIENT OF VARIATION (%) | 3,0 | 2,4 | 1,7 | 1,4 | 0,8 | 1,4 |
| PHAEOPHYTIN a | | | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,060 | 0,062 | 0,046 | 0,059 | 0,055 | 0,054 |

Statistical analysis of chlorophyll a concentrations for the above treatments.* Value significant at the p = 0,05 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | ω | Methods in decreasing order of means** | | | | | |
|---|---|---|---|---|---|---|---|
| | | 6 | 5 | 3 | 4 | 1 | 2 |
| 3,46* | 0,144 | 3,715 | 3,697 | 3,669 | 3,651 | 3,583 | 3,568 |

was significantly lower (p = 0,05) than all the other procedures (Table 23). There was no significant difference (p = 0,05) between the remaining extractions. Again the coefficients of variation were low, ranging from 0,8% to 2,6%

The poorest solvents from the natural blue-green dominated phytoplankton population were the methanolic procedures extracting 96,5% and 96,0% of the chlorophyll a as compared to 90% ethanol / 24 h extraction. There was no significant difference (p = 0,05) between the ethanolic extractions and again the coefficients of variation were low with a maximum of 3,0 being recorded for the 100% methanol / 1 h extraction.

4.5.5.2 DISCUSSION

From these experiments 90% ethanol, boiled for 5 min and allowed to stand for 24 h yielded the highest chlorophyll a results. The coefficients of variation for this procedure ranged from 0,8% to 2,6%. The lower chlorophyll a values tended to be recorded from the methanolic extractions, although there was not much difference between all three solvents, particularly when left for 24 h to complete extraction.

Thus all three solvents are suitable for extraction of photosynthetic pigments from phytoplankton when boiled at solvent boiling point for 5 min and left to stand in the dark at room temperature for 24 h, with a preference, however, for 90% ethanol.

## 4.6 DMSO AS THE EXTRACTION AGENT

### 4.6.1 INTRODUCTION

The use of dimethyl sulphoxide (DMSO) as the extraction agent for pigments from phytoplankton was first put forward by Shoaf and Lium (1976) who reported that a DMSO : 90% acetone mixture of 1 : 1 (v/v) was more efficient than 90% acetone when extracting pigments from green algae and equally efficient when extracting from diatoms and blue-green algae. Stauffer *et al* (1979), using an extended cold extraction time of 40 h, reported improved chlorophyll *a* extraction from some blue-green algae by the DMSO : 90% acetone mixture over extraction with 90% acetone. Burnison (1980) developed a DMSO method which was more efficient than the grinding method of Shoaf and Lium (1976). He found that by boiling the filters in 4 mℓ DMSO at 65°C for 10 mins, filtering and bringing up to 10 mℓ with 90% acetone gave higher chlorophyll *a* values from cultures of *Scenedesmus basiliensis* and *Selenastrum capricornutum* than the two previously described DMSO methods (Shoaf and Lium, 1976; Stauffer *et al*, 1979) and 90% acetone.

Shoaf and Lium (1976) found that the spectral characteristics of chlorophyll *a* in the DMSO : 90% acetone mixture were the same as in 90% acetone. This was confirmed by Stauffer *et al* (1979), who also reported that on acidification the absorbance peak shifted 2 nm to the longer wavelengths.

Shoaf and Lium (1976) also reported the acid ratio in the DMSO : 90% acetone mixture was the same as that in 90% acetone (i.e. 1,7) while Burnison (1980) reported a decrease of this ratio in the mixture of 3,1% when compared to 90% acetone.

### 4.6.2 SPECTROPHOTOMETRY PROCEDURES

#### 4.6.2.1 EXPERIMENTAL

The absorbances of chlorophyll *a* in 1 : 1 (v/v) DMSO : 90% acetone, 100% DMSO and 90% acetone were compared using the same procedure as used in Chapter 3 (part 2 page 55). Using two sets of three concentrations of purified chlorophyll *a* for each solvent the absorbances in the DMSO : 90% acetone mixture were found to be between 0,8% and 2,5% ($\bar{x}$ = 1,5%) lower than in 90% acetone. The absorbances in 100% DMSO were between 3,5% and 4,9% ($\bar{x}$ = 4,1%) lower than in 90% acetone.

To check the acid ratio in the DMSO : 90% acetone mixture, solutions of purified chlorophyll *a* and extracts from a healthy exponentially growing culture of *Selenastrum capricornutum* were acidified to $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl, absorbances being read before and after acidification. With the DMSO : 90% acetone solutions and extracts stabilization of the reading often did not occur until 12 - 15 min after addition of the acid. Once stabilization had occurred, however, readings did not alter for up to 15 mins, even after addition of a further $3 \times 10^{-3}$

mol $\ell^{-1}$HCl. From this it was assumed that acidification to 3 x $10^{-3}$ mol $\ell^{-1}$HCl brought about complete, although prolonged, phaeophytinization. The acid ratio in the DMSO : 90% acetone mixture was found to be 1,70 $\pm$ 0,01 (n = 5). On acidification the absorption peak shifted for 665 nm to between 666 nm and 667,5 nm and had to be scanned for. When the acidification experiment was repeated using DMSO alone, spectrophotometric readings became highly erratic following acidification and did not settle even after 1 h. Due to this the idea of using DMSO alone in the determination of chlorophyll a in phytoplankton was discarded.

4.6.2.2 DISCUSSION

Shoaf and Lium (1976) state that the absorption spectra of chlorophyll a in 90% acetone and in DMSO : 90% acetone were the same and consequently the same specific absorption coefficient could be used for both. The work of Stauffer et al (1979) confirmed this view. In these experiments the absorbances in the DMSO : 90% acetone mixture were on average 1,5% lower than in 90% acetone. As this is negligible the specific absorption coefficient for 90% acetone of 89,0 $\ell$ $g^{-1}$ $cm^{-1}$ is also adopted for DMSO : 90% acetone mixtures.

The acid ratio in the DMSO : 90% acetone mixture determined in these experiments was 1,7, which is the same as that in

90% acetone and is in agreement with the assertion of Shoaf and Lium (1976) that there was no difference in acid ratios for the two solvents. Burnison (1980) reported that acid ratios were 3,1% lower in the mixture than in 90% acetone (equivalent to a ratio of 1,65) but considered the difference to be insignificant. The amount of acid required for phaeophytinization was the same in the DMSO : 90% acetone mixture as is used for 90% acetone and 100% methanol, but the phaeophytinization period was often prolonged to 15 min. Scanning for the absorbance peak following acidification is essential as shifts of up to 2,5 nm were observed.

Thus, for this study the following procedure was adopted for the analyses of chlorophyll a in DMSO : 90% acetone mixtures:-

Four millilitres of extract was decanted into a 1 cm pathlength cuvette and the absorbance read at 665 nm. The sample was then acidified to $3 \times 10^{-3}$ mol $\ell^{-1}$ HCl by the addition of 120 $\mu\ell$ of a 0,1 mol $\ell^{-1}$ HCl solution to the cuvette. The contents were mixed and allowed to stand for 15 min in the dark at room temperature, after which the absorbance peak was reread by scanning between 665 and 668 nm. An acid ratio of 1,7 and a specific absorption coefficient for chlorophyll a of 89,0 $\ell\ g^{-1}\ cm^{-1}$ were used in the Lorenzen (1967) equations.

### 4.6.3 EXTRACTION EFFICIENCY OF DMSO

#### 4.6.3.1 EXPERIMENTAL

Twenty equivolume subsamples from a culture of *Selenastrum capricornutum* were filtered and divided into 4 sets of 5 and treated as follows:-

Set 1 was extracted in 10 mℓ 90% ethanol in the dark at room temperature for 24 h before spectrophotometric analysis.

Set 2 was extracted in 10 mℓ 90% ethanol, boiled for 5 min at 78°C and left to stand in the dark at room temperature for 24 h before spectrophotometric analysis.

Set 3 was extracted in 10 mℓ of a DMSO : 90% acetone mixture (1 : 1 v/v) (Shoaf and Lium, 1976) in the dark at room temperature for 24 h before analysis.

Set 4 was placed in 4 mℓ of DMSO in screw-capped test tubes which were placed in a water bath at 65°C and the solvent allowed to boil for 10 mın (Burnison, 1980). After this the extracts were allowed to cool and 6 mℓ of 90% acetone added to each. These were left to stand in the dark at room temperature for 24 h prior to spectrophotometric analysis.

The results are presented in Table 25.

TABLE 25: Pigment extraction efficiencies of 90% ethanol and DMSO/90% acetone mixtures using *Selenastrum capricornutum*. (See text for details).

| METHOD | 1 | 2 | 3 | 4 |
|---|---|---|---|---|
| SOLVENT | 90% ETHANOL | | DMSO/ACETONE | |
| BOILING TIME | - | 5 min | - | 10 min |
| STANDING TIME | 24 h | 24 h | 24 h | 24 h |
| SAMPLE SIZE (n) | 5 | 5 | 5 | 5 |
| CHLOROPHYLL *a* | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 7,371 | 8,328 | 6,767 | 7,112 |
| STANDARD DEVIATION (mg) | 0,326 | 0,159 | 0,241 | 0,152 |
| COEFFICIENT OF VARIATION (%) | 4,4 | 1,9 | 3,6 | 2,1 |
| PHAEOPHYTIN *a* | | | | |
| MEAN VALUE (mg $\ell^{-1}$ extract) | 0,569 | 0,532 | 0,661 | 1,774 |

Statistical analysis of chlorophyll *a* concentration for the above treatments.* Value significant at the p = 0,01 level.** Methods connected by a line do not differ significantly at the p = 0,05 level.

| F-value | $\omega$ | Methods in decreasing order of means** | | | |
|---|---|---|---|---|---|
| | | 2 | 1 | 4 | 3 |
| 45,0* | 0,420 | 8,328 | 7,371 | 7,112 | 6,767 |

Chlorophyll *a* concentrations in the boiled 90% ethanol extracts were significantly higher ($p = 0,01$) than in the other three solvents/procedures. The mean total *a* pigments in the boiled DMSO : 90% acetone extracts (Method 4) was the same as that in boiled 90% ethanol extracts (Method 2) (8,886 and 8,860 mg $\ell^{-1}$ extract respectively). The phaeophytin *a* content in the boiled DMSO : 90% acetone, however, was considerably higher (20% of the pigments as against 6% in the boiled 90% ethanol extracts). This implies that, although boiling DMSO : 90% acetone mixtures may be efficient pigment solvents, substantial degradation of chlorophyll *a* occurs. With the DMSO : 90% acetone (1 : 1 v/v)/ 24 h extraction (Method 3) no large increase in phaeophytins was noted. Thus the degradation of chlorophyll was due to the boiling DMSO step. The DMSO : 90% acetone (1 : 1 v/v) mixture was the poorest of the solvents tested. With both Method 1 (90% ethanol standing for 24 h) and Method 3 (DMSO : 90% acetone standing for 24 h) large amounts of pigment were found on the filters following extraction.

#### 4.6.3.2 DISCUSSION

Shoaf and Lium (1976) and Stauffer *et al* (1979) showed that a 1 : 1 (v/v) mixture of DMSO and 90% acetone was superior to 90% acetone in extracting photosynthetic pigments from green and blue-green algae. Stauffer *et al* (1979) also reported that the DMSO : 90% acetone mixture is as an efficient pigment extractor from blue-green algae as methanol. A modified DMSO

method, whereby extracts are boiled, has been shown to be superior to the original method of Shoaf and Lium (1976) and 90% acetone (Burnison, 1980).

The results of these experiments show that simple 24 h extraction with a 1 : 1 (v/v) DMSO : 90% acetone mixture was vastly inferior to 90% ethanol extractions and also Burnison's (1980) developed method. The method of Burnison (1980) did result in good pigment extraction (when compared on a total a pigment basis to boiled 90% ethanol) but the process resulted in a significant increase in phaeophytin a and concomitant loss of chlorophyll a. Shoaf and Lium (1977) claimed that pigment extraction using DMSO did not degrade chlorophyllous pigments. In these experiments the use of simple 24 h extraction with 1 : 1 DMSO : 90% acetone did not result in an increase in phaeophytins and thus the degradation is assumed to be due to the boiling action in DMSO. Burnison (1980), however, makes no mention of phaeophytin contents in his description of the method.

The use of boiling DMSO should, therefore, be avoided and "cold" DMSO : 90% acetone is here shown to be inferior to 90% ethanol, and by token also inferior to methanol.

## 6.7 CONCLUSIONS AND PROCEDURES

From these results the following conclusions are made:-

(1) The alcohols, methanol and ethanol, are superior to acetone and dimethyl suphoxide (DMSO) in the extraction of photosynthetic pigments from green and blue-green algae.

(2) Boiling of filters in either ethanolic solutions or methanol for 5 min at the solvent's boiling points followed by 24 h extraction results in complete pigment extraction without degradation of chlorophyll a.

(3) Homogenisation and sonication do not improve pigment extraction.

(4) Extractions can be carried out at room temperature without loss of chlorophyll a.

(5) Extension of extraction time beyond 24 h does not result in an increase in extract chlorophyll a concentrations.

Consequently it is recommended that the extraction of photosynthetic pigments from freshwater phytoplankton is carried out using a boiling ethanol or methanol method.

The procedure adopted should be:-

Entrappment of algae upon a suitable glass fibre filter;

Placing of filter into a suitable tube, either screw capped or fitted with a cooling bulb;

Addition of a known volume of solvent, either 90% or 95% ethanol or 100% methanol;

Placing of tube in a water bath at the boiling point of the solvent used (78°C for ethanol and 65°C for methanol);

Allowing the solvent to boil for 5 min;

Removing the tube and placing in a dark cupboard at room temperature for 24 h prior to centrifugation and spectrophotometric analysis.

CHAPTER 5

# STORAGE OF SAMPLES

## 5.1 INTRODUCTION

It is not always possible to analyse samples the same day as they are collected and thus storage of the sample may be necessary. Samples are usually stored in the filtered state and it is generally recommended in analytical manuals that the filters are stored dry in a dessicator, in the dark at -20°C (Strickland and Parsons, 1972; Weber, 1973; Standard Methods, 1976; Greeson et al, 1977). Such storage, however, may result in lower chlorophyll a values and increased difficulty in extraction (Strickland and Parsons, 1972; Talling, 1974). Holm-Hansen and Riemann (1978) stored filtered samples in the wet state at -20°C for up to three weeks with no loss of chlorophyll upon subsequent extraction with methanol. Lenz and Fritsche (1980) reported that wet filters deep frozen (at -18°C) for 6 months yielded reliable results. Storing filtered Scenedesmus quadricauda in a dessicator at -15°C, Sand-Jensen (1976) found a 20% loss of chlorophyll after three days storage followed by extraction with methanol. When samples were stored at 4°C losses of up to 48,9% were reported (Sand-Jensen, 1976).

Storage of samples in the freeze-dried state has been recommended for algae and plant extracts (Glooschenko, personal communication

in Talling, 1974; Holden, 1976) but Lenz and Fritsche (1980) reported a 34% loss of chlorophyll from phytoplankton samples freeze-dried and stored at room temperature.

Storage of whole samples appears not to be generally practiced. Processing of samples with the minimum of delay is generally advocated. Strickland and Parsons (1972) state that samples may be stored in a cool dark place for up to 8 h.

Tolstoy (1977) found that samples of "less eutrophic" waters may be stored for 1 d at room temperature, or more preferably at 5°C. More eutrophic waters tended to show a loss of chlorophyll upon storage of the whole sample. One sample consisting mainly of Oscillatoria agardhii was stored for 14 d without loss of chlorophyll. This was explained by the fact that this algae has been observed to live in environmental conditions of 6 to 10°C with no light for photosynthesis and thus would survive in refrigerated samples (Tolstoy, 1977). Tolstoy (1977) also tested freezing of the whole sample but found a decrease of chlorophyll in all frozen samples, the decrease being greater with green algae than with blue-green algae.

The aim of the work in this section was to assess the effects of storage of whole samples on chlorophyll concentrations, to investigate the use of preservatives and to evaluate the effect of storage of filtered samples.

## 5.2 STORAGE OF WHOLE SAMPLES

### 5.2.1 EXPERIMENTAL

Sixty litres of a natural phytoplankton population from eutrophic Roodeplaat Dam were collected in a large plastic bin. The algal composition of the population was approximately 80% Microcystis aeruginosa, 8% Pandorina sp., 7% diatoms and 5% Cryptomonas sp. One litre subsamples were taken and stored in plastic bottles. Three sets were prepared. The first set were stored in the refrigerator (5 to 8°C) for up to 20 d. The other two sets were preserved, one with 20 mg mercury (II) $\ell^{-1}$ sample and the other with 4% (v/v) formaldehyde. Mercury (II) at 20 mg $\ell^{-1}$ sample is used to preserve chemical samples (Grobler, Bruwer and van Vliet, 1978) and was added in the form of a solution of mercury (II) chloride. The formaldehyde was not neutralized (pH of 40% v/v stock solution was approximately 2,5) and was added as 110 m$\ell$ of 40% v/v stock solution to 1 $\ell$ of sample. Chlorophyll a and phaeophytin a concentrations before and after preservation and after storage at 5 to 8°C were measured after extraction with boiling 90% ethanol. The results are presented in Table 26.

The initial chlorophyll a concentration was 69,71 $\mu$g $\ell^{-1}$ and the phaeophytin a concentration 2,30 $\mu$g $\ell^{-1}$. The degradation products thus represented 3,2% of the total a pigments.

The samples stored unpreserved in the refrigerator at 5 to 8°C showed no increase in degradation products after storage for 3 d (Table 26). There was also no decrease in chlorophyll *a* concentrations. There was a small increase in degradation products to 5,7% after 7 d with a further increase to 12,3% after 10 d.

Both preservation methods led to an immediate increase in degradation products upon addition of the preservative, the effect being greater with the addition of $HgCl_2$ than with formaldehyde (Table 26). With the mercury (II) preserved sample the deleterious effect continued during refrigerated storage up to 48 h. The initial increase in degradation products upon addition of formaldehyde was to 14,1% and this level did not increase upon storage at 5 to 8°C for 72 h. The addition of 4% v/v formaldehyde to natural phytoplankton samples from Roodeplaat Dam resulted in a reduction in pH, after mixing, from 8,6 to approximately 7,0. The increase in degradation products due to the preservative may be ascribed to local drastic lowering of pH on addition of 110 mℓ of 40% v/v formaldehyde (pH 2,5) to the sample.

### 5.2.2 DISCUSSION

From these results it is clear that preservation of samples with either mercury (II) or formaldehyde is impracticable due to the formation of degradation products.

TABLE 26: The effect of storage and preservation with either 20 mg $\ell^{-1}$ Hg (II) or 4% (v/v) formaldehyde on natural phytoplankton samples. (See text for details). Samples stored at 5 to 8°C.

| Time | Chlorophyll a concentrations (mg $\ell^{-1}$ extract) and % degradation products (in brackets) in | | |
|---|---|---|---|
| | (a) Unpreserved | (b) Preserved with Hg (II) | (c) Preserved with formaldehyde |
| 0 h | 6,97 (3,2) | | |
| After initial preservation | | 5,47 (21,5) | 5,70 (14,1) |
| 24 h | 6,89 (2,9) | 3,44 (53,3) | 4,97 (17,2) |
| 48 h | 7,15 (3,6) | 5,01 (32,7) | 6,31 (15,1) |
| 72 h | 7,03 (3,3) | ND | 5,20 (12,4) |
| 7 d | 6,54 (5,7) | ND | ND |
| 10 d | 6,05 (12,3) | ND | ND |
| 15 d | 5,99 (10,4) | ND | ND |
| 20 d | 5,79 (12,1) | ND | ND |

ND = Not determined.

Storage of the whole sample at 5 to 8°C for up to 3 d appears not to result in a loss of chlorophyll a or an increase in degradation products. The samples used in these experiments were obtained

from Roodeplaat Dam, a highly eutrophic impoundment (Walmsley, Toerien and Stëyn, 1978), and contained a moderately high concentration of algae (chlorophyll *a* = 69,71 $\mu g\ \ell^{-1}$). Tolstoy (1977) reported that samples from "less eutrophic" waters may be stored for 1 d at 5°C, but storage of "more eutrophic" waters led to loss of chlorophyll *a*. The type of population present, however, appears to influence storage potential. Natural samples dominated by *Oscillatoria agardhii* could be stored up to 7 d at 5°C without changes in pigment content (Tolstoy, 1977). The samples used in these studies were also dominated by a blue-green alga (*Microcystis aeruginosa*) and storage of samples at 5°C for up to 3 d did not result in a change in pigment composition. It, therefore, appears that limited storage of blue-green algal populations at 5°C in the dark is feasible, but each worker should assess the effects of storage on his own populations.

## 5.3 STORAGE OF FILTERED SAMPLES

### 5.3.1 EXPERIMENTAL

Three algal populations were used to assess the effect of storage of filtered samples on chlorophyll *a* concentrations. These were:

(1) A culture of *Selenastrum capricornutum*.

(2) A culture of unicellular *Microcystis aeruginosa*.

(3) A natural phytoplankton population from Roodeplaat Dam consisting of approximately 80% Microcystis aeruginosa, 8% Pandorina sp., 7% diatoms and 5% Cryptomonas sp.

Subsamples were filtered through Sartorius SM 13400 47 mm diameter glass-fibre filters, which were then placed in 47 mm diameter plastic petri dishes with tight fitting lids (Millipore Corporation, U.S.A.). The dishes and subsamples were stored in the dark at either room temperature (22 to 24°C), in the refrigerator (5 to 8°C) or in a deep-freeze (~ -18°C) for up to ninety days. At time intervals of 1, 2, 3, 4, 7, 10, 15, 21, 30, 45, 63 and 90 d three samples from each population for each storage condition were analysed for chlorophyll a and phaeophytin a after extraction with boiling 90% ethanol for 5 min followed by standing for 24 h. The Microcystis aeruginosa samples were only analysed on days 1, 2, 3, 7, 15, 21 and 45. The results (means and standard deviations; n = 3) are depicted in Figures 6 to 14.

For the Selenastrum capricornutum culture storage at 5 to 8°C for up to 21 d did not result in loss of pigment or degradation of chlorophyll a (Figure 6). After 21 d there was a gradual loss of total a pigments to 92,8% of the initial concentration after 30 d and 89,3% after 90 d. Concomitant with this there was a gradual loss of chlorophyll a which was accentuated after

63 d due to an increase in phaeophytins from a percentage contribution of between 10,8% and 13,4% to 20,8% after 63 d and 34,2% after 90 d.

Storage of Selenastrum capricornutum at -18°C resulted in a rapid loss of total a pigments coupled with a slow increase in percentage degradation products (Figure 7). The loss of pigments was due to incomplete extraction from samples frozen for 7 d or more by the method employed. After seven days storage 82,1% of the initial concentration of total a pigments was extracted. This fell to 71,0% after 21 d and to 61,7% after 45 d. The percentage contribution of phaeophytin a rose from an initial 10,8% to 20,3% after 15 d, 23,4% after 63 d and 40,7% after 90 d.

When the Selenastrum capricornutum samples were stored in the dark at room temperature (22 to 24°C) there was no loss in total a pigments (Figure 8) despite the filters and entrapped algae becoming very dried out. There was, however, a gradual decline in chlorophyll a concentrations (to 79,0% of the initial concentration after 90 d) and a gradual increase in phaeophytin a content.

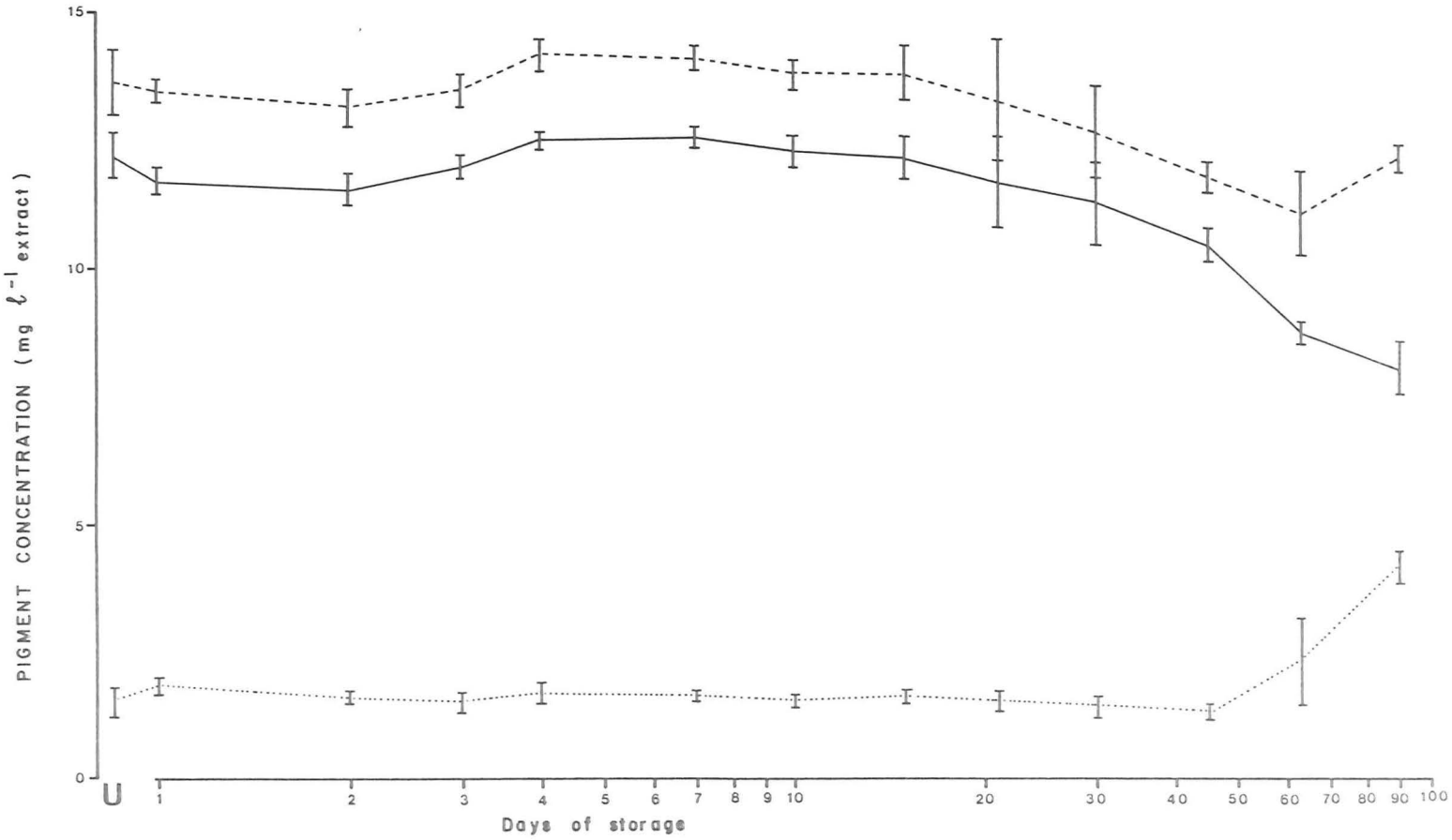


FIGURE 6. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF Selenastrum capricornutum AT 5 - 8 °C. ----- Total a pigments, —— chlorophyll a, ····· phaeophytin a. MEANS AND STANDARD DEVIATIONS (n = 3). U = UNSTORED

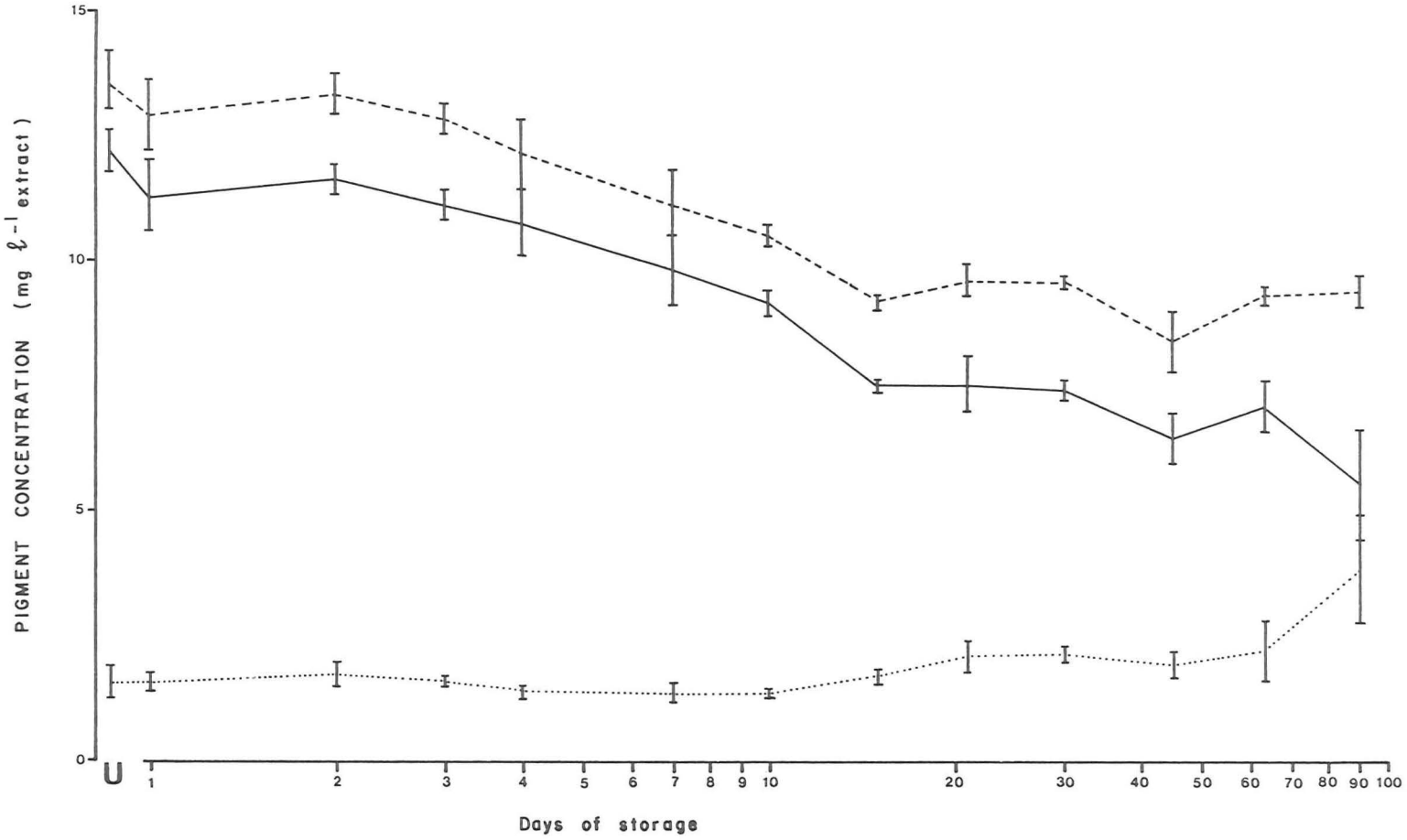


FIGURE 7. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF Selenastrum capricornutum AT -18 °C. ---- Total a pigments, —— chlorophyll a, ····· phaeophytin a. MEANS AND STANDARD DEVIATIONS ( n = 3 ). U = UNSTORED

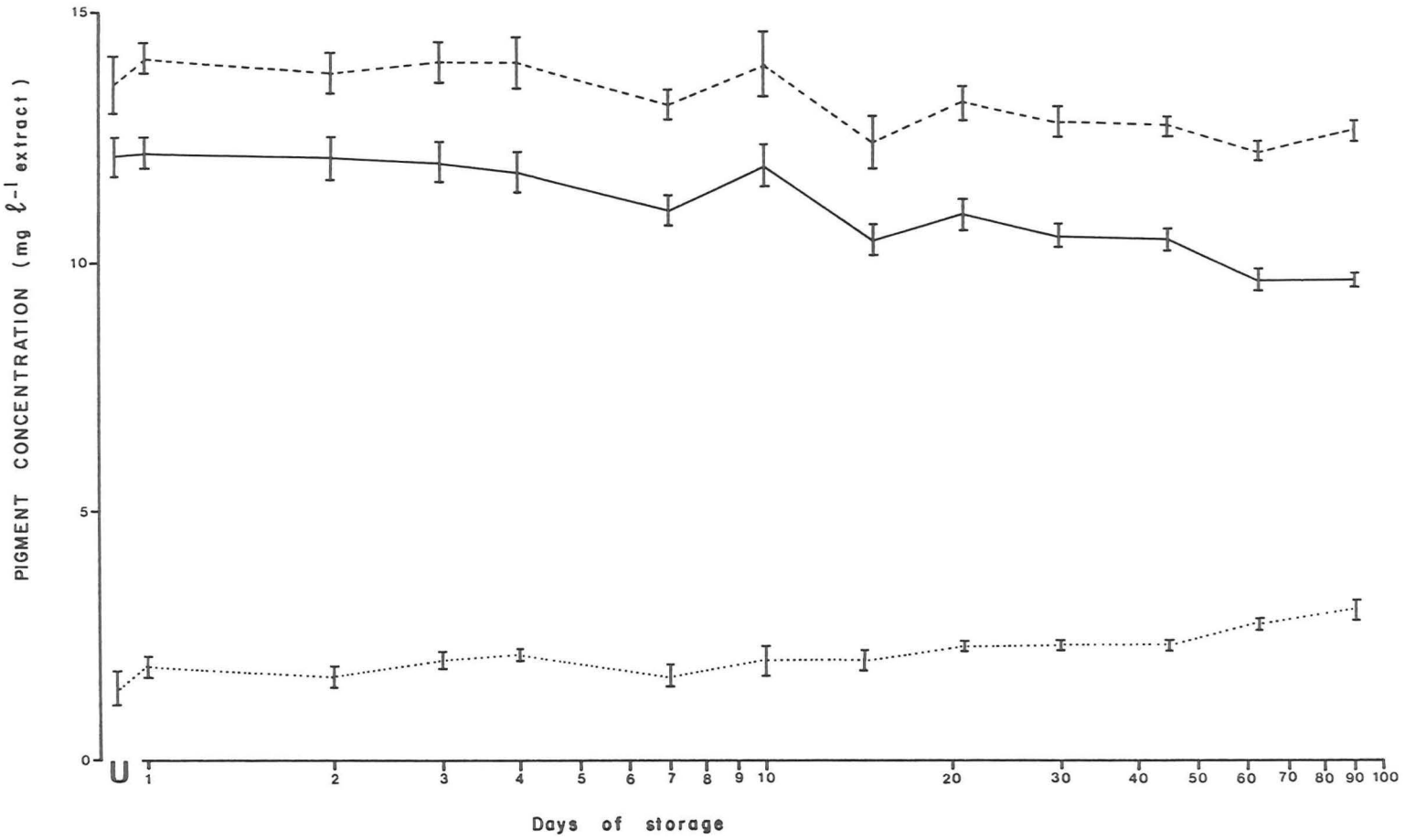


FIGURE 8. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF Selenastrum capricornutum AT 22 - 24 °C. ----- Total a pigments, —— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS ( n = 3 ). U = UNSTORED

The storage of Microcystis aeruginosa on filters at each of the three conditions for up to 21 d did not result in any major changes (Figures 9 to 11) although there was a gradual, but not significant ($p = 0,05$), decrease in total a pigments at 22 to 24°C (Figure 11) and a small increase in phaeophytin a content in samples stored at -18°C (Figure 10). After 45 d, however, there was an increase in phaeophytin a percentage, and a concomitant decrease in chlorophyll a concentrations, in the samples stored frozen and at room temperature (chlorophyll a = 84,0% and 89,4% of the original concentrations respectively) (Figures 10 and 11). With the frozen samples after 45 d there was also a loss of pigments due to incomplete extraction (Figure 10) resulting in only 89,0% of the a pigments being extracted.

The natural phytoplankton samples showed a much greater instability upon filtered storage than did the cultured algae. There was no significant ($p = 0,05$) change in total a pigments in samples stored at 5 to 8°C but there was a gradual decline in chlorophyll a values to 95,4% of the initial concentration after 7 d, 92,8% after 30 d and 80,9% after 90 d (Figure 12). Storage beyond 45 d resulted in a large increase in phaeophytin a which constituted 24,0% of the pigments after 90 d storage.

The samples stored at -18°C showed a small decrease in total a pigments after 21 d storage (Figure 13) and again chlorophyll a values steadily declined during storage reaching 80,9% of the initial concentration after 21 d and 71,1% after 63 d.

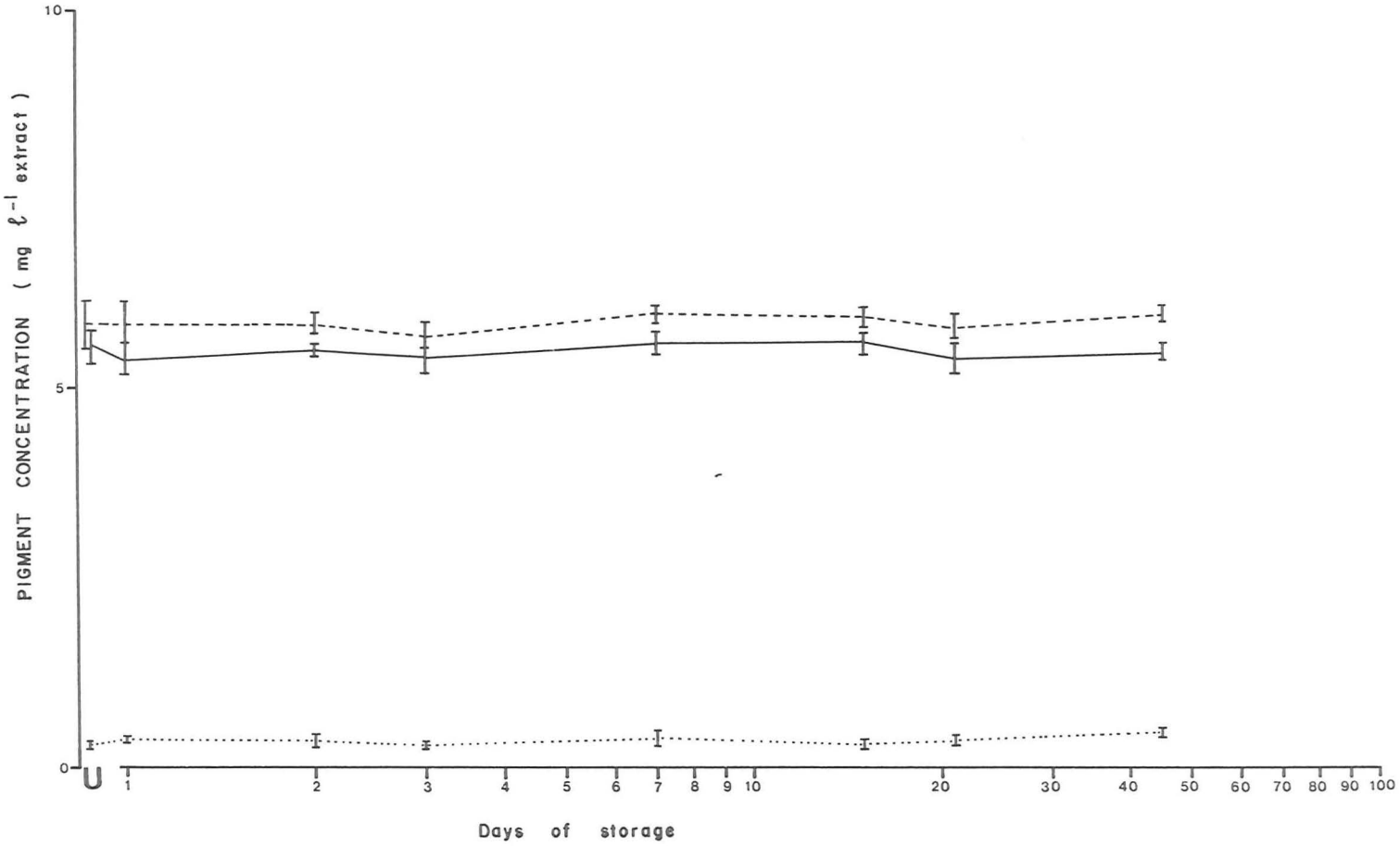


FIGURE 9. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF UNICELLULAR Microcystis aeruginosa AT 5 - 8° C. ----- Total a pigments, —— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS (n = 3). U= UNSTORED

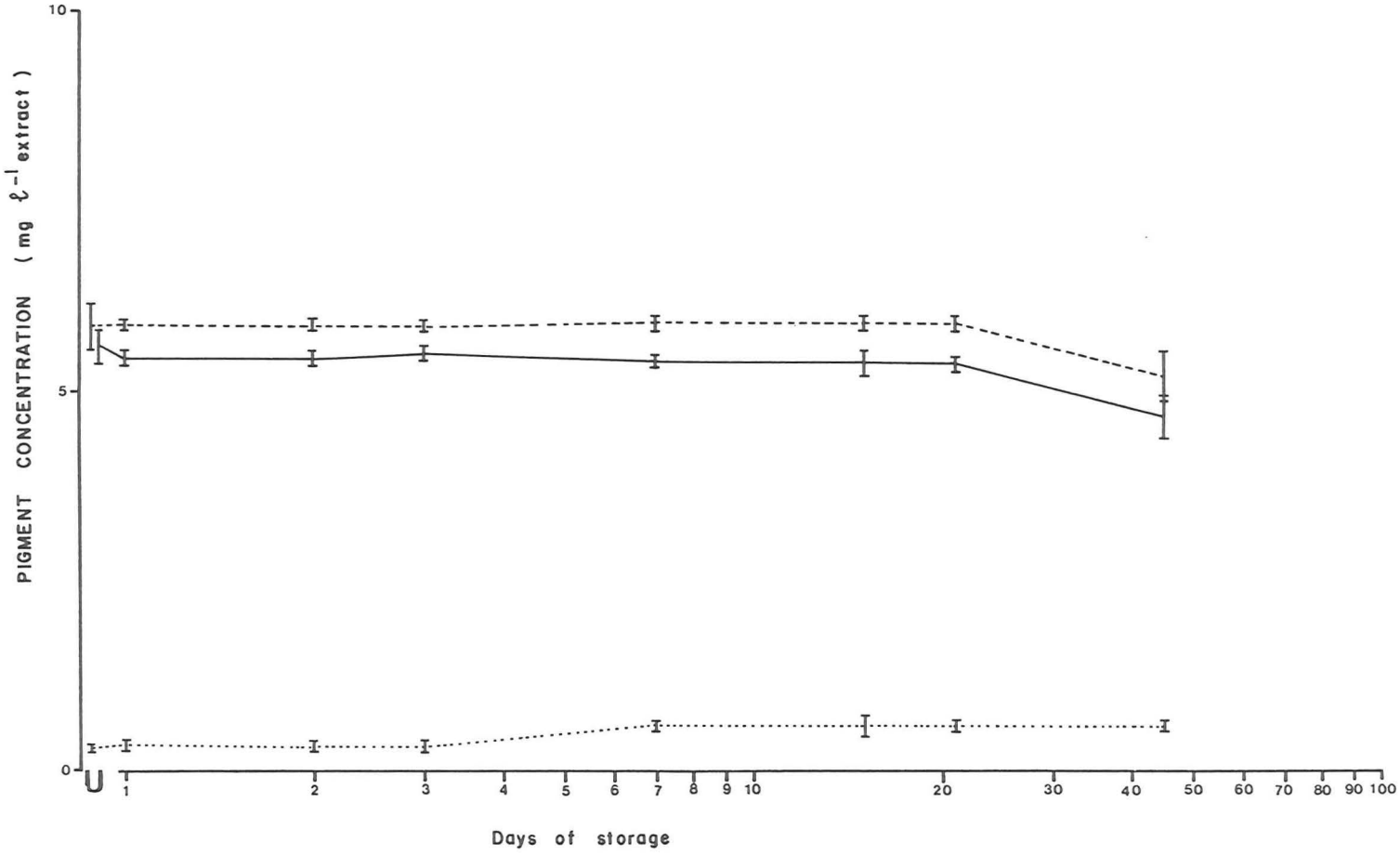


FIGURE 10. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF UNICELLULAR Microcystis aeruginosa AT − 18° C. ---- Total a pigments, —— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS ( n = 3 ). U = UNSTORED

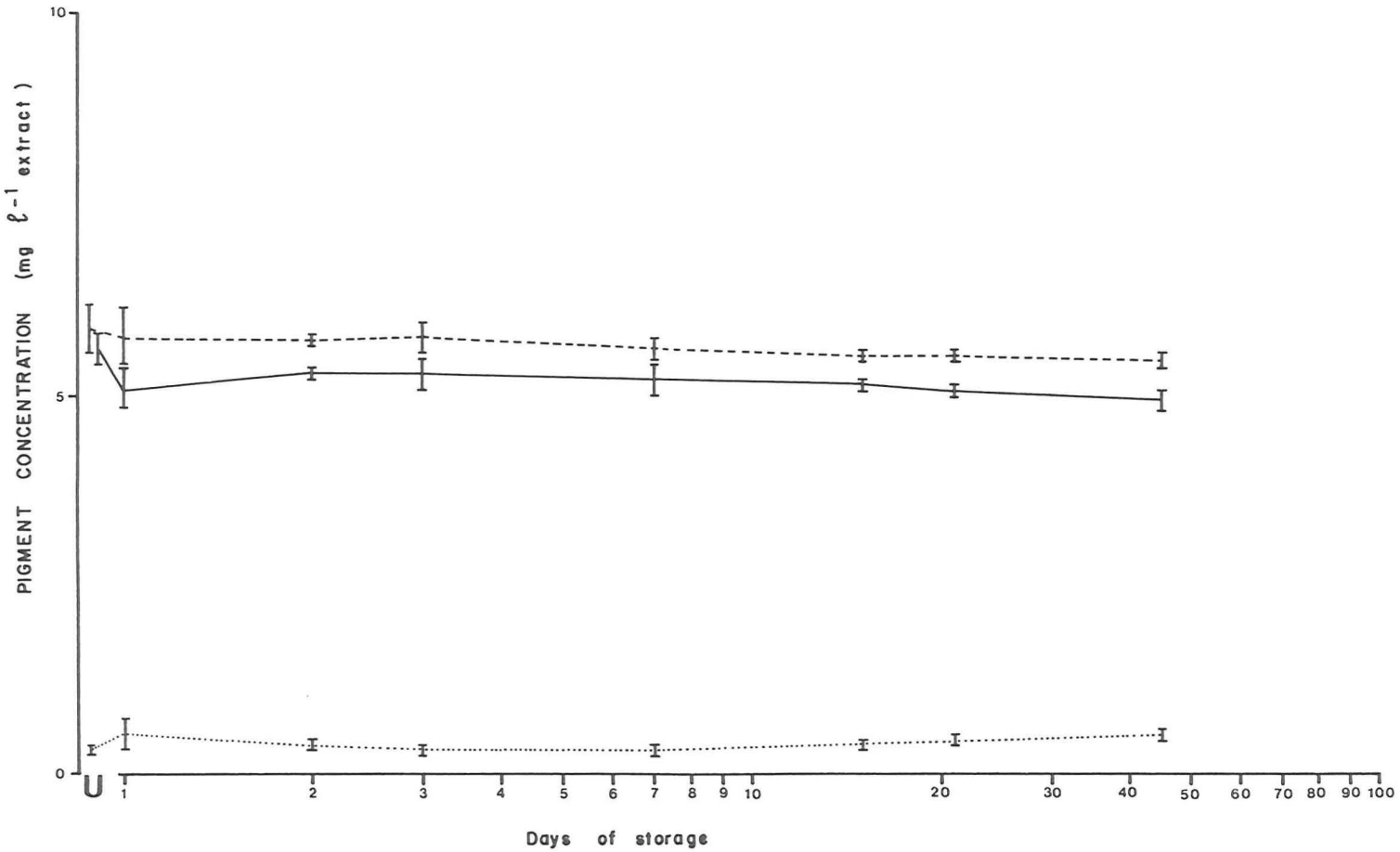


FIGURE 11. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF UNICELLULAR Microcystis aeruginosa AT 22 - 24 °C. ---- Total a pigments, —— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS (n=3). U = UNSTORED

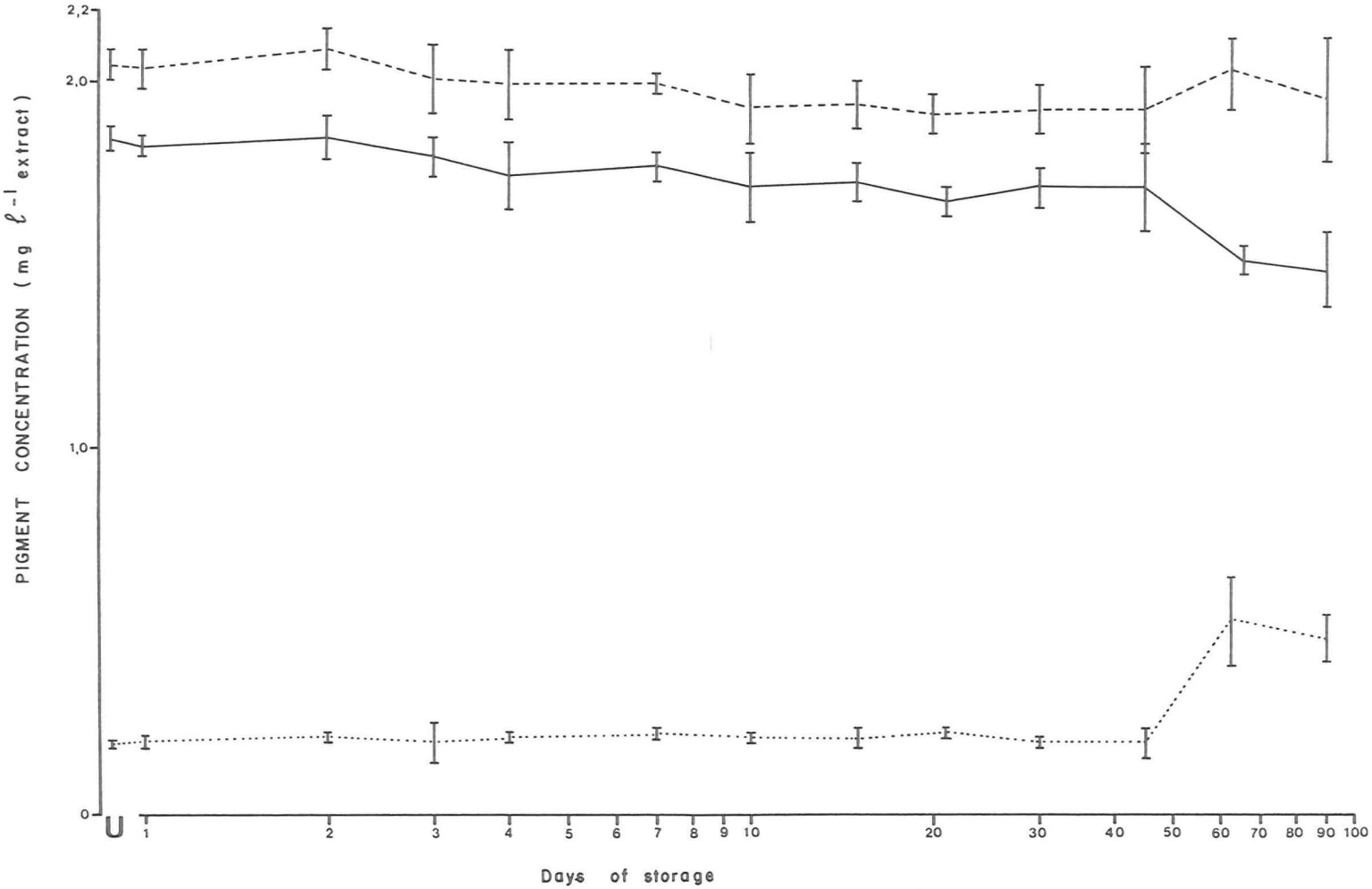


FIGURE 12. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF A NATURAL PHYTOPLANKTON POPULATION DOMINATED BY Microcystis aeruginosa AT 5 - 8 °C. -----Total a pigments , —— chlorophyll a , ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS ( n = 3) U = UNSTORED

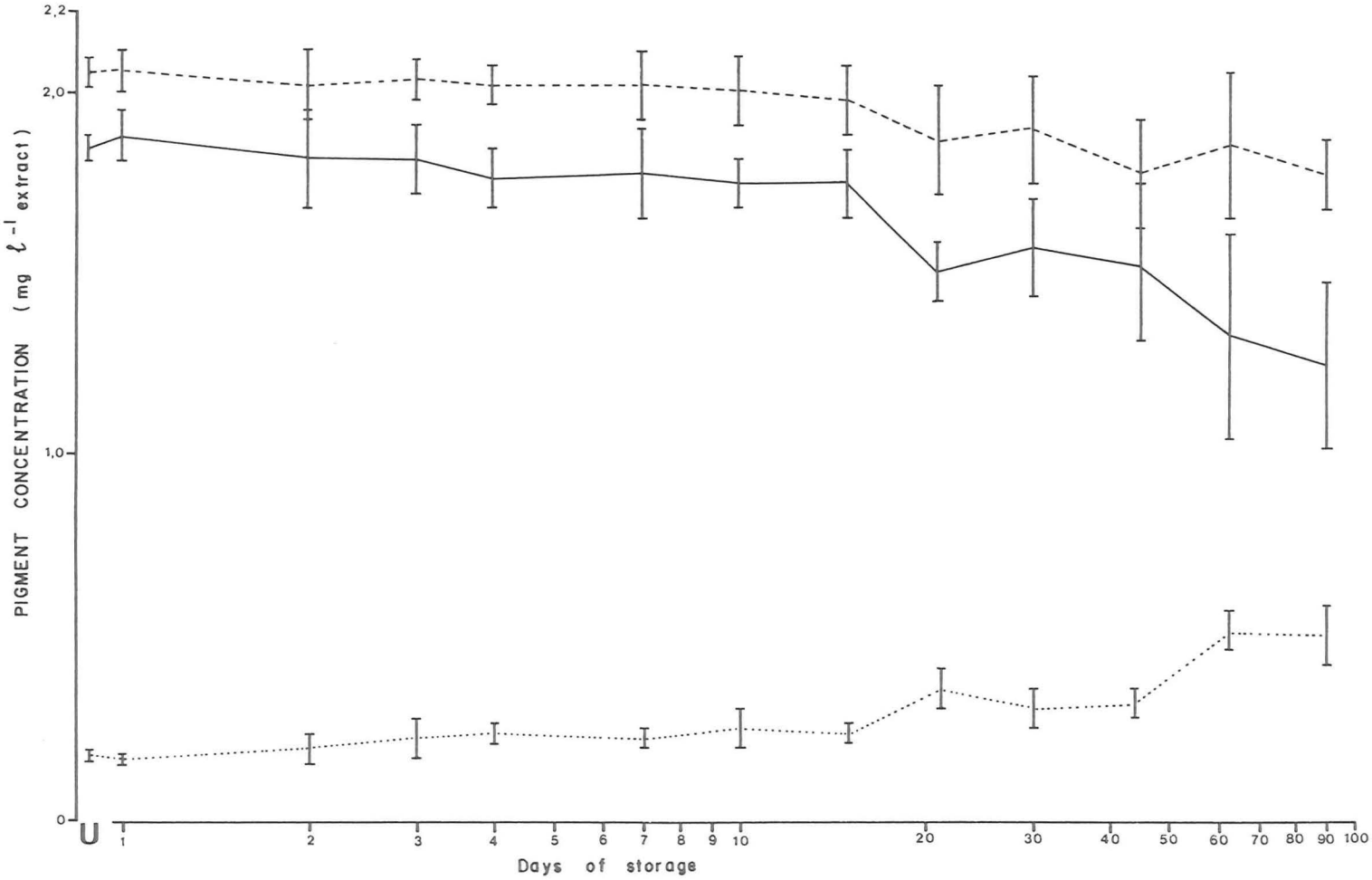


FIGURE 13. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF A NATURAL PHYTOPLANKTON POPULATION DOMINATED BY Microcystis aeruginosa AT − 18° C. ---- Total a pigments, ——— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS (n = 3)
U = UNSTORED

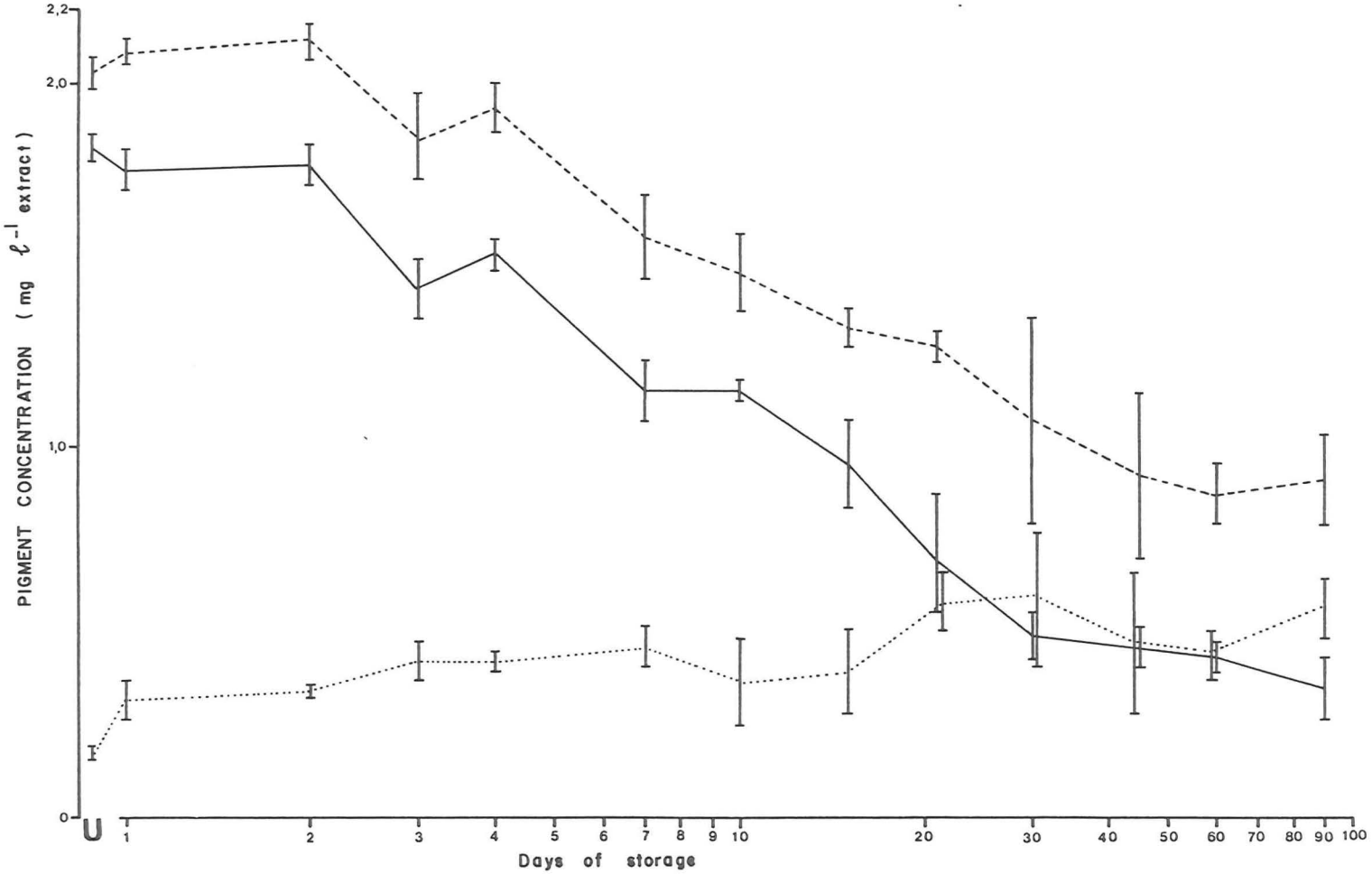


FIGURE 14. THE EFFECT OF STORAGE OF FILTERED SAMPLES OF A NATURAL PHYTOPLANKTON POPULATION DOMINATED BY Microcystis aeruginosa AT 22 - 24 °C. -----Total a pigments, ——— chlorophyll a, ..... phaeophytin a. MEANS AND STANDARD DEVIATIONS (n = 3)
U = UNSTORED