# Optimising the storage and extraction of chlorophyll samples

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**Abstract**

The effect of different methodological steps on the analysis of chlorophyll *a* (chl *a*) was tested with algae cultures and Baltic Sea water. Selected experiments were tested with ANOVAs for significant differences between treatments. The results of the experiments led to the following recommendations: the sample volume should be low so that filtration takes no longer than 10 min. Extracts rather than filters should be stored if storage of the samples at −20°C is required. However, quick-freezing in liquid nitrogen is recommended. The extraction efficiency was much better in 96% ethanol than in 90% acetone – extraction in the latter solvent requires the filters to be homogenised. The extraction time has no significant influence within a range of 3 to 24 hours if the recommended 96% ethanol is used.

## 1. Introduction

Chlorophyll *a* (chl *a*) is the primary photosynthetic pigment, which occurs only in autotrophic (or mixotrophic) phytoplankton (i.e. algae,

cyanobacteria and prochlorophytes). Since the amount of chl *a* in the cell is related to the biomass, the chl *a* concentration has become a widely used proxy for the phytoplankton biomass. A variety of methods for determining chlorophyll are in use. For example, the trichromatic method for the spectrophotometric measurement of chl $a, b$ and $c$ is now routine in sea water analysis, following the publication of instruction manuals by Strickland & Parsons (1960) and UNESCO (1966). One of its advantages is that it eliminates the interfering effects of chl *b* and *c* in the spectrophotometric measurement of chl *a*. Another method, a fluorometric technique, was developed by Welschmeyer (1994) for determining chl *a* in the presence of chl *b* with the use of special filters at the excitation and emission positions (436 and 680 nm) of the spectrum. Drawbacks of these two techniques include the distortion of results by the natural degradation products (phaeopigments), because their spectra overlap those of chl *a*, and their inability to distinguish chlorophyllide *a* from chl *a* (Mantoura et al. 1997a).

Acidification can be used to correct for phaeopigment in both the fluorometric (Yentsch & Menzel 1963) and the spectrophotometric methods (Lorenzen 1967). Advanced chromatographic methods even permit the quantification of the whole range of photosynthetic pigments, but are nonetheless difficult to apply in routine work (Latasa et al. 1996). For a recent compilation of methods for pigment analysis in phytoplankton, we recommend the manual by Jeffrey et al. (1997).

Measuring the chl *a* concentration requires different methodological steps, each of which may be a source of error and inaccuracy; standardisation is therefore necessary if results are to be comparable. At present, however, such a standardisation on a worldwide scale is not feasible: different strategies are applied, each of which is capable of providing good results. In the present paper, we shall focus on the methods that have recently been applied in marine monitoring programmes, such as the projects organised by HELCOM (Baltic Sea) and ICES (North Atlantic), and the JGOFS (Joint Global Ocean Flux Study) (Edler (ed.) 1979, HELCOM 1988, UNESCO 1994, Aminot & Rey 2001). Within the framework of these projects, intercalibrations between the different contributors are necessary for the verification of data homogeneity, so that only quality-assured data are entered into the data banks (e.g. Larsson et al. 1978, Nusch 1984, HELCOM 1991, ICES/HELCOM 1996, Latasa et al. 1996, QUASIMEME 2000).

Here is a summary of the most critical methodological steps:

1. The actual sampling of the natural phytoplankton can ruin the results if the samples are not representative. Since, however, the

sampling procedure does not belong to the method itself, it will not be mentioned any further.

2. In general, algae are separated from the water by filtration. An appropriate filter pore size and a moderate suction pressure are required to obtain the desired, undisrupted fraction.
3. If not analysed immediately, the filters are stored, but the conditions and duration of storage may have a deleterious effect on the results.
4. The appropriate solvent (e.g. acetone, ethanol, methanol) for extracting the chlorophyll has been a matter of discussion for a long time (Wright et al. 1997). 90% acetone has been traditionally used in marine research (Wright & Mantoura 1997, Aminot & Rey 2001). On the other hand, 96% ethanol is prescribed by HELCOM (2001). In fresh water, 90% ethanol is the required solvent (DIN 38412 1985, ISO 10260 1992).
5. The cells sometimes have to be homogenised in order to improve the extraction efficiency.
6. The appropriate extraction time and conditions have to be chosen.
7. Occasionally, storage of the extract instead of the filter is recommended.
8. In principle, there are two ways of estimating the chlorophyll in the solvent:
   a) by spectrophotometrically measuring the light absorption by the pigment at the wavelength of maximum absorption, or
   b) by fluorometrically measuring the fluorescence.
9. The results depend on the measurement and calculation principle adopted:
   a) the trichromatic method corrects for the influence of chl *b* and *c*, which interfere with the measurement of chl *a* (calculation according to Jeffrey & Humphrey 1975),
   b) the acidification method corrects for phaeopigment (calculation according to Lorenzen 1967). The strength of acidification may influence the results (Wasmund 1984),
   c) sometimes, a simple formula without corrections is also applied (recommended by HELCOM 1988).
10. Light destroys chlorophyll (Moreth & Yentsch 1970, Wasmund 1984), especially in the presence of oxygen (Kowalewska & Szymczak 2001).

Therefore, all work with chlorophyll extracts should be carried out in subdued light. However, we did not regard this condition as a special methodological step and therefore did not investigate it.

In the literature one finds a wide variety of recommendations, some of them contradictory. We tested the sensitive steps from filtration to the end of the extraction process, including sample storage (Table 1), in order to find the most suitable combination of the different methodological steps for a simple routine method. Our recommendations should support the process of the ongoing improvement of practical guidelines for routine measurements of chl *a*.

**Table 1.** Test scheme in the 3 experiments

| | Experiment | | |
|---|---|---|---|
| | July 1996 | March 2002 | March 2003 |
| Storage strategy (instant analysis vs extracts vs filters) | × | | |
| Solvent (acetone vs ethanol) | × | × | × |
| Homogenisation | × | | |
| Storage temperature (−20°C vs −196°C) | | × | × |
| Storage time | | × | × |
| Extraction time | × | | |
| Filtration volume | | × | × |

## 2. Method

### 2.1. Sampling

Natural surface water was taken from station 042 (15 km north of Warnemünde) in the western Baltic Sea on 2 July 1996. Dominated by dinoflagellates (*Glenodinium* spp., *Gymnodinium* spp.), the water was typical of early summer. In parallel, algal cultures grown at the Baltic Sea Research Institute Warnemünde (IOW) were analysed: the diatoms *Phaeodactylum tricornutum* and *Thalassiosira weissflogii*, as well as the cyanobacterium *Microcystis aeruginosa*.

In 2002 and 2003, natural surface water was taken from station 011 in the Fehmarn Belt (28 March 2002), station 012 in the Mecklenburg Bight (22 March 2003), stations 160 and 162 in the Pomeranian Bight (1 April 2002 and 24 March 2003, respectively) and station 113 in the central Arkona Sea (6 April 2002 and 24 March 2003). The three stations sampled in 2002 were characterised by a diatom bloom of *Skeletonema costatum*. In 2003, post-bloom conditions with low biomasses were already obtaining at stations

012 and 113 (athecate dinoflagellates and *Teleaulax* sp. at station 012, and athecate dinoflagellates, *Chaetoceros wighamii* and *Mesodinium rubrum* at station 113); there was a diatom bloom (*Pauliella taeniata*) at station 162.

### 2.2. Filtration

The water samples were passed through Whatman GF/F filters (25 mm diameter) with a suction pressure not exceeding 0.3 atm ($\sim 0.3\ 10^5$ N m$^{-2}$). The filters were folded with the algae inside, blotted with absorbent paper to remove most of the adhering water and extracted immediately or stored deep-frozen as specified in the following section. 80–120 replicate filters were produced per species in 1996 and 24 replicate filters per station in 2002 and 2003. The filters were split into groups of five (1996) or two (2002 and 2003) filters. Each group was treated in a different way as specified below.

The influence of the filtration volume on the chl *a* yield was tested in 2002 and 2003. One set of samples (16 filters per station) was gained by filtering the standard volumes of 200–300 cm$^3$ water during 10 minutes. Much more water (400–600 cm$^3$) was forced through a further set of 8 filters per station: the duration of filtration was much more than 10 minutes and depended on the seston concentration. These filters were stored at −20°C.

### 2.3. Storage and extraction

Replicate filters were treated in different ways in order to investigate the effect of storage conditions on them – for example, the storage of extracts vs filters, storage time and temperature:

a) extraction immediately after filtration, followed by measurement (1996 only),

b) extraction immediately after filtration; extracts centrifuged (SORVALL RC 28S cooling centrifuge for 20 min at 10 000 rpm), decanted, and stored at −20°C for 11–13 weeks (treated as '3 months') prior to measurements (1996 only),

c) filters stored in dark Eppendorf vials at −20°C for 2 weeks (2003), 3 weeks (2002), 2 months (2003) and 11–13 weeks (treated as '3 months'; 1996 and 2002),

d) filters quick-frozen in liquid nitrogen (−196°C), then stored at −80°C for 2 weeks (2003), 3 weeks (2002), 2 months (2003) and 3 months (2002).

Contact with oxygen was not prevented, except for the samples stored in liquid nitrogen. All samples were stored in the dark. Each extraction was performed with 10 cm$^3$ of either 96% ethanol (the usual azeotrope) or 90%

acetone (produced by dilution with water) in the dark at room temperature for 3–24 hours. The centrifuge tube containing the filter and the extract was carefully sealed to prevent evaporation. The effect of the extraction time was checked in 1996 by parallel extractions of 3 and 24 hours' duration.

### 2.4. Homogenisation

The effect of homogenising the filters was tested in 1996. For each of the different treatments described above, 5 replicates were extracted with or without homogenisation. When applied, homogenisation was carried out as follows:

- The filters were cut into small pieces and transferred to a glass homogeniser, which was then placed in a beaker containing crushed ice;
- 4 $cm^3$ solvent (acetone or ethanol) were added;
- homogenisation was carried out by a motor-driven Teflon pestle rotating in the homogeniser tube for 1–2 min at 3000 rpm;
- the Teflon pestle was removed and rinsed with 2 $cm^3$ solvent flowing into the homogeniser;
- the homogeniser was emptied into a centrifuge tube and rinsed twice with 2 $cm^3$ of solvent.

In 2002, all samples were homogenised and centrifuged. In 2003, the filters were generally not homogenised, which rendered centrifugation and correction for evaporation unnecessary (see section 2.5).

### 2.5. Measurement and calculation

The clear extract was decanted into a clean centrifuge tube from where it was transferred into the measuring cuvette of a TURNER 10-AU-005 fluorometer. Measurements were always done against a blank (reference) cuvette. The fluorometer used a wide excitation band around 450 nm and measured at 670 nm. For correcting for phaeopigment, the extract was acidified after the measurement with 10 $\mu$l 1 M HCl per $cm^3$ extract and measured again 1–3 min after acidification. The corrected chl *a* concentration ('chl *a* corr') was calculated according to Holm-Hansen et al. (1965) and UNESCO (1994):

$$\text{chl}\,a\ \text{corr}\ [\text{mg m}^{-3}] = F_m\,(F_m - 1)^{-1}\,(F_0 - F_a)\,K_x\,V_E\,V_S^{-1}. \qquad (1)$$

In parallel, the uncorrected chl *a* (which we call 'chl *a* total') was calculated by a simple equation without correction for phaeopigment, as suggested by Edler (ed.) (1979) and HELCOM (1988), and later applied by Welschmeyer (1994):

$$\text{chl}\,a\ \text{total}\ [\text{mg m}^{-3}] = F_0\ K_x\ V_E\ V_S^{-1}, \qquad (2)$$

where
$F_m$ = acidification coefficient,
$F_0$ = relative fluorescence before acidification,
$F_a$ = relative fluorescence after acidification,
$K_x$ = linear calibration factor [$\mu$g chl $a$ dm$^{-3}$ per fluorescence unit],
$V_E$ = volume of the extract [cm$^3$],
$V_S$ = volume of the filtered sample [cm$^3$].

In order to calculate $K_x$ and $F_m$, the fluorometer was calibrated on the basis of spectrophotometric measurements of a dilution series of pure chl $a$ from *Anacystis nidulans* (Sigma Chemical Company), as described by UNESCO (1994).

All work with the chlorophyll extract was carried out in subdued light. Evaporation of solvent during the homogenisation and centrifugation procedures was measured by weighing. It accounted for $(3.5 \pm 1.1)\%$ with 90% acetone and for $(1.0 \pm 0.6)\%$ with 96% ethanol. In order to avoid additional manipulation of the samples, the evaporated volume was not replaced. The mean evaporated volume was subtracted from the initial extract volume for calculation. Evaporation during the 3 months of storage at $-20$°C was insignificant so long as the tubes had been sealed properly.

### 2.6. Statistical analysis

Selected factorial experiments were tested with analysis of variance (ANOVA) for significant differences between treatments in each of the four sets of the 1996 experiment (natural plankton from station 042, *Microcystis*, *Phaeodactylum* and *Thalassiosira* cultures). If necessary, data were transformed to obtain homogeneity of variances. Heterogeneity of variances was acceptable for experiments showing no significant differences between treatments; only Type 1 errors were affected by this violation. A small heterogeneity of variances ($0.5 > p > 0.1$) was acceptable in those experiments demonstrating significant differences at the $p < 0.01$ level (Underwood 1981).

A two-factorial ANOVA was carried out to test whether (a) solvents under different storage conditions, and (b) homogenisation, affect chl $a$ measurements. The following solvents and conditions were used in the test: (a) ethanol immediately, (b) acetone immediately, (c) ethanol extract stored, (d) acetone extract stored, (e) ethanol filter stored and (f) acetone filter stored. Homogeneity of variances was tested with Cochran's test (1951) ($p$ of untransformed data = 0.22). Post hoc comparisons for significant results were performed with Tukey's (HSD) test (Underwood 1997). Additionally, we tested whether the storage time at room temperature for 3 or 24 hours

affected the estimated chl *a* concentration. Again, different extraction agents were used for these approaches.

The data from 2002 and 2003 were compared using three different ANOVA settings:

- A three-factorial ANOVA design with the factors 'station, storage temperature and extraction agent' (acetone vs ethanol), n=2. Homogeneity of variances was tested with Cochran's test ($p$ of untransformed data = 0.53). Post hoc comparisons for significant results were performed with Tukey's (HSD) test.
- A two-factorial ANOVA to analyse significant differences in chl *a* concentrations at various sampling stations and the subsequent storage of filters at −20°C vs −196°C (data sets: March 2002 and March 2003). Only data from 200–300 $cm^3$ water samples and 96% ethanol were used in this analysis.
- A two-factorial ANOVA to test the influence of filtration volume, with material collected at different stations in 2002 and 2003, and extracted with 96% ethanol.

## 3. Results

### 3.1. Influence of correction for phaeopigment

All chl *a* data were calculated with both the simple formula (without any correction, as recommended e.g. by HELCOM 1988) and the formula of

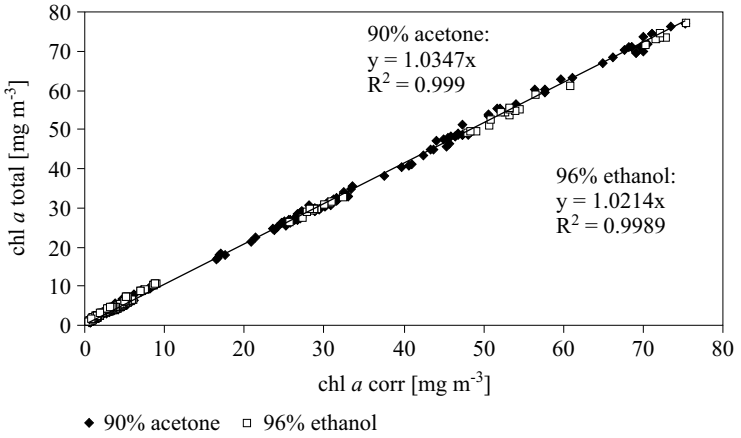


**Fig. 1.** Comparison of uncorrected chl *a* data ('chl *a* total') with the respective chl *a* data corrected for phaeo *a* ('chl *a* corr'), separated for 90% acetone and 96% ethanol

Lorenzen (1967), yielding values of 'chl *a* total' and 'chl *a* corr' respectively. Plotting the pairs of these differently calculated chl *a* values reveals a strong linearity for the two solvents (Fig. 1). The linearity between these two kinds of chl *a* data allowed us to concentrate on only the 'chl *a* total' in the following considerations, where it is simply referred to as 'chl *a*'.

The results of the separate experiments from 1996, 2002 and 2003 are shown in Figs 2–4. Each column in Fig. 2 is based on 5 replicates. As the measured values of the replicates were very close in general, we reduced their number to two replicates per treatment in 2002 and 2003.

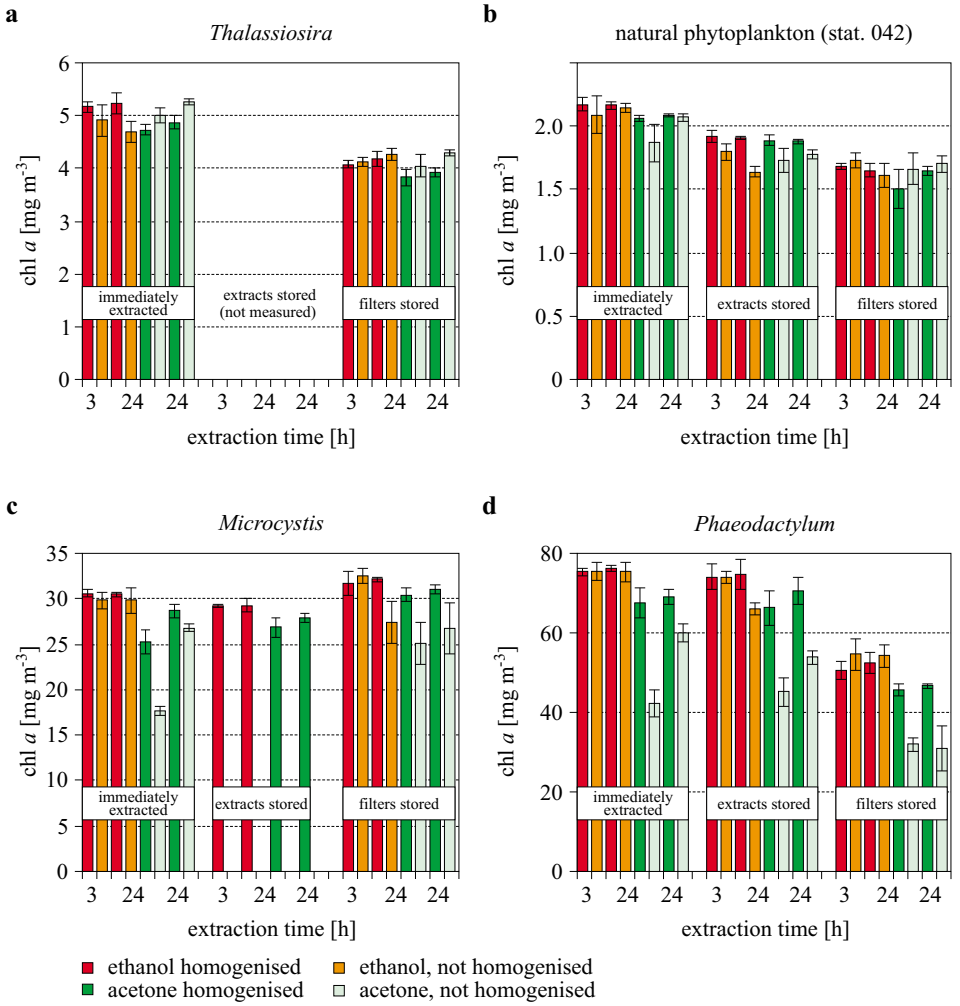


**Fig. 2.** Results of the July 1996 experiment, showing the mean chl *a* concentrations and standard deviations for different treatments (stored medium, solvent, homogenisation, extraction time) in 4 different sample sets (a)–(d)

### 3.2. Storage strategy (instant measurement vs storage, extracts vs filters)

In the July 1996 experiment, two combinations of factors were tested at the same time: (1) immediate extraction and measurement vs storage, and (2) storage of the extracts vs the storage of filters for the same length of time (3 months) and at the same temperature (−20°C). Storage of the extracts was not carried out with *Thalassiosira* (Fig. 2a). In the natural sample (Fig. 2b), the chl *a* yield was insignificantly reduced after storage of the extracts ($F = 0.019$, $df = 1$, $p = 0.89$) but strongly reduced in stored filters in comparison with immediately measured data ($F = 37.01$, $df = 4$, $p < 0.001$). Also, the pure cultures of *Microcystis* (Fig. 2c) and *Phaeodactylum* (Fig. 2d) displayed no significant difference between immediate measurements and long-term storage of extracts at −20°C. If filters were stored at −20°C, the chl *a* yield decreased significantly in diatoms and the natural community (Figs 2a, b, d) but rose in *Microcystis* (Fig. 2c).

### 3.3. Solvent (acetone vs ethanol)

In general, the chl *a* concentration measured in the acetone extracts is lower than in the ethanol extracts, especially in *Phaeodactylum* ($F = 234.93$, $df = 5$, $p < 0.001$; Fig. 2d) and *Microcystis* ($F = 154.33$, $df = 3$, $p < 0.001$; Fig. 2c). This is modified by homogenisation, as shown in section 3.4.

### 3.4. Homogenisation

Homogenisation leads to improved extraction efficiency as a result of cell wall disruption. This process appears to be necessary especially in the acetone extracts of *Microcystis* and *Phaeodactylum* (Figs 2c, d), since non-homogenised filters released significantly less chl *a* ($F = 72.478$, $df = 1$, $p < 0.001$ for *Microcystis* and $F = 159.44$, $df = 1$, $p < 0.001$ for *Phaeodactylum*). In ethanol extracts, homogenisation has no significant effects.

### 3.5. Storage temperature (−20°C vs −196°C)

As shown in Figs 3 and 4, quick-freezing improved the recovery of chl *a* in comparison with freezing at −20°C ($F = 109.14$, $df = 1$, $p < 0.001$).

### 3.6. Storage time

In the March 2002 and March 2003 experiments, the filters were stored for some weeks or months. Chl *a* concentrations decreased slightly

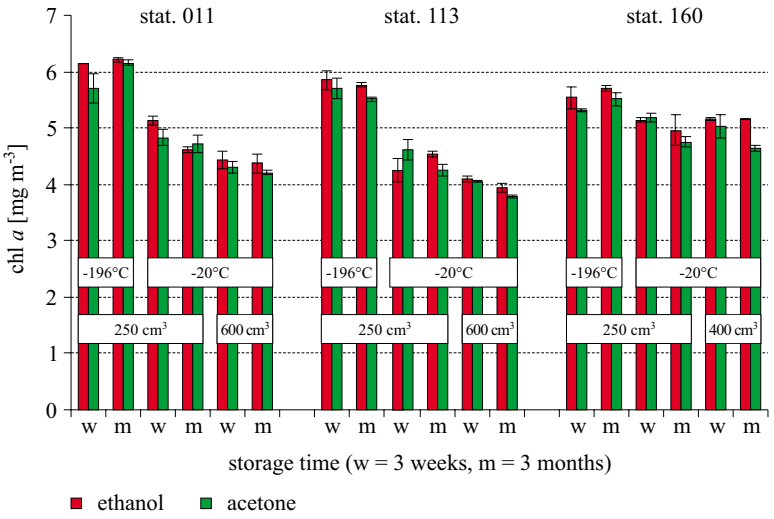


**Fig. 3.** Results of the March 2002 experiment, showing the mean concentrations and standard deviations of chl *a* for different treatments (filtration volume, solvent, storage temperature, storage time) in the surface water at 3 stations

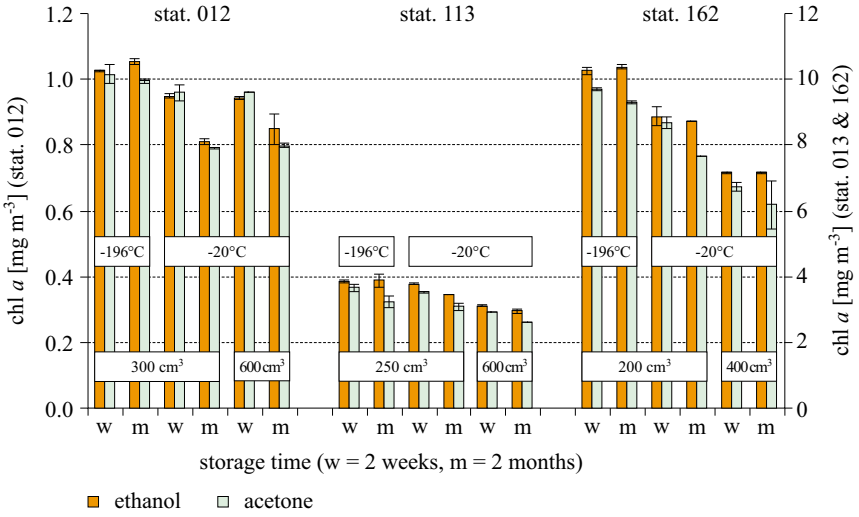


**Fig. 4.** Results of the March 2003 experiment, showing the mean concentrations and standard deviations of chl *a* for different treatments (filtration volume, solvent, storage temperature, storage time) in the surface water at 3 stations. Note the different axes for the stations

during the period between the respective measurements (6–9 weeks) ($F = 4.729$, $df = 1$, $p = 0.503$), primarily in the acetone extracts kept at −20°C (Figs 3 and 4), but the differences were not significant.

### 3.7. Extraction time

All the extracts of the 1996 experiment were measured after 3 and 24 hours. The different sets within this experiment were analysed separately owing to the different heterogeneity of variances. Only one experiment (station 042) displayed a small heterogeneity of variances (Cochran's test, $p = 0.028$), but in this case there were no significant differences in extraction time for 3 and 24 hours ($F = 0.019$, $df = 1$, $p = 0.89$). Because only Type 1 errors are affected by a violation of the homogeneity of variances, the outcome of this treatment is reliable. However, the experiments with *Phaeodactylum* and *Microcystis* showed that the extraction time can influence the chl *a* concentrations ($F = 13.17$, $df = 1$, $p < 0.01$ and $F = 5.75$, $df = 1$, $p = 0.022$ respectively). Extraction without homogenisation for 3 hours was too short in some cases, especially when samples were extracted in acetone (Figs 2c, d). This supports our statement that ethanol is a more efficient extraction agent than acetone.

### 3.8. Filtration volume

The data from March 2002 and March 2003 (Figs 3, 4) show that a larger filtration volume causes chl *a* concentrations to be underestimated ($F = 142.57$, $df = 1$, $p < 0.001$). 200–300 $cm^3$ of Baltic Sea water was sufficient for the sensitive fluorometric method, but increasing the sample volume to 400–600 $cm^3$ led to filtration times in excess of 10 min, and in consequence, greater mechanical stress, which resulted in pigment loss.

## 4. Discussion

### 4.1. Effect of the correction for phaeopigment

For the purpose of our work it was sufficient to concentrate on the fluorometric method and the calculation according to formula (2). Mantoura et al. (1997a) found good agreement between the fluorometric (Holm-Hansen et al. 1965) and spectrophotometric (Lorenzen 1967) methods. The two calculation methods (formulas (1) and (2)) gave similar results (Fig. 1).

The strong correlation between chl *a* data corrected for phaeopigment *a* ('chl *a* corr', calculation according to Lorenzen 1967) and chl *a* corrected for chl *b* and chl *c* ('chl *a* J&H', calculation according to Jeffrey & Humphrey 1975) was already demonstrated by Wasmund (1984) in the formula:

$$\text{chl}\,a\ \text{J\&H} = \text{chl}\,a\ \text{corr} + 0.6\ \text{phaeo}\,a. \quad (3)$$

In a recent test with 33 participating laboratories, QUASIMEME (2003) found very good agreement among chl *a* data measured with the methods of both Lorenzen (1967) and Jeffrey & Humphrey (1975).

### 4.2. Storage strategy (instant measurement vs storage, extracts vs filters)

Generally speaking, immediate extraction and measurement generates the most reliable results. If storage is unavoidable, it is the filters that are usually stored; under normal circumstances, extracts are not stored for very long periods. Nusch (1999) showed that 30-day storage of chl *a* filters in ethanol at −25°C did not lead to any significant chl *a* degradation, but the chl *a* content did decrease by about 25% if the filters were stored without solvent. In an intercalibration exercise (HELCOM 1991) the results after freezing at −25°C for 1 month were significantly better if extracts instead of filters had been stored. The ensuing recommendation to store extracts instead of filters was, however, not followed up by most laboratories, presumably out of habit. We confirmed that extract storage is superior to filter storage if a deep-freeze is used. For *Microcystis* and *Phaeodactylum*, there was no significant difference between samples extracted and measured immediately, and samples whose extracts had been stored for 3 months. Filters stored at −20°C displayed significantly lower chl *a* concentrations in diatoms and natural phytoplankton. Only in the case of the cyanobacterium *Microcystis* did the storage of filters improve the yield slightly. Freezing and defrosting the damp filters may have effectively destroyed the mucilage and the cell wall of the cyanobacteria.

### 4.3. Solvent (acetone vs ethanol)

We tested the extraction efficiency of acetone and ethanol, which are the least harmful of the solvents commonly used for pigment extractions. Our data suggest that acetone is inferior to ethanol (cf. also Nusch & Palme 1975, HELCOM 1991). Wright et al. (1997) recommended methanol rather than acetone for routine marine samples. Nusch & Palme (1975), Moed & Hallegraeff (1978), and Jespersen & Christoffersen (1987) showed that ethanol is as efficient as methanol. We found that the use of denatured rather than pure ethanol had no effect on the data (cf. Nusch & Palme 1975). The extraction efficiency of the various solvents seems to depend on other factors, such as the taxonomic composition of the algal community. In our experiments, we obtained significantly more chl *a* when *Phaeodactylum* and *Microcystis* were extracted in ethanol as opposed to acetone. This was also the case with natural populations dominated by diatoms (Fig. 3 and station 162 in Fig. 4).

### 4.4. Homogenisation

The extraction efficiency of certain solvents can be improved if the cells are first disrupted. Some algal groups, like cyanobacteria and Chlorophyceae, are rather resistant to extraction and may require mechanical disruption (Marker 1972, Sand-Jensen 1976, Holm-Hansen & Riemann 1978). We compared simple soaking with a homogenisation method (grinding). Generally, there was no significant difference between homogenised and non-homogenised samples if they had been extracted in 96% ethanol. In 90% acetone, however, the chl *a* yield was on occasion significantly reduced if the samples had not been homogenised, especially in the case of *Microcystis* and *Phaeodactylum*. Wright et al. (1997) determined a high percentage of chl *a* degradation products in frozen *Phaedactylum* after grinding in 90% acetone. Although they did obtain an improved yield in 90% acetone after grinding, they eventually recommended methanol in combination with sonication.

Homogenisation introduces additional errors and variability owing to the additional exposure of the extracts to light, heat, and irreproducible manipulation. At the very least, it can lead to evaporation, which has to be compensated for by replenishing the solvent or by some correction to the calculation formula. Moreover, the extract becomes turbid during this process, so it then has to be cleared by centrifugation, an additional step causing variability. Homogenisation should therefore be avoided – this is possible with ethanol extraction. In this case, solvent evaporation is insignificant and needs no correcting for.

### 4.5. Storage temperature ($-20^{\circ}$C vs $-196^{\circ}$C)

As high temperatures accelerate chlorophyll degradation (Holm-Hansen & Riemann 1978, Wasmund 1984), the filters or extracts have to be stored at a low temperature. Simple deep-freezes ($-20^{\circ}$C) are the most popular because they are readily available in the laboratory and recommended in the standard manuals (UNESCO 1966, Parsons et al. 1984, HELCOM 1988). Nevertheless, Jeffrey & Hallegraeff (1980) found that 5–10% of chl *a* kept at $-20^{\circ}$C degraded within six weeks. The strong degradation of chl *a* in filters stored at $-20^{\circ}$C could be due to enzymatic reactions taking place during the slow freezing of the damp filters (Moreth & Yentsch 1970). Both long-term storage at $-20^{\circ}$C and freeze-drying procedures are rejected (Mantoura et al. 1997b). Our findings also revealed that storage at $-20^{\circ}$C cannot be recommended for damp filters. The remaining water still enables some biochemical activity during slow freezing. Freezing dried filters may improve this situation, but there is still some chance of metabolic activity during drying, which is a slow process. Drying the filters in a stream of argon could be an alternative (Kowalewska & Szymczak 2001), but one that

we did not test in our studies because it has not been applied in routine work to date. Since extraction with water-poor solvents stops enzymatic chlorophyll degradation immediately (Barrett & Jeffrey 1971), the storage of extracts may be another alternative, provided that deep-freezes (−20°C) are available (cf. section 4.2.). Damp filters have to be quick-frozen in liquid nitrogen to suppress enzymatic activity. Afterwards, they can be transferred to ultracold freezers (−80°C). Freezing in liquid nitrogen at −196°C has become accepted in HPLC studies (Wright et al. 1991, Barlow et al. 1993, Goericke & Repeta 1993, Bidigare & Ondrusek 1996) and should become standard practice in pigment storage (Mantoura et al. 1997b). One of our main aims was to discourage the practice of freezing filters at −20°C for storage, as still recommended by Aminot & Rey (2001) and HELCOM (2001).

### 4.6. Storage time

The storage time depends mainly on the stored objects (filters or extracts) and the temperature. As shown above, extracts can be stored for 3 months at −20°C without significant loss of chl *a* (Fig. 2). Storage of filters for 2 months (Fig. 4) or 3 months (Fig. 3) is only advisable in liquid nitrogen (−196°C) or in an ultracold freezer (−80°C) after quick-freezing in liquid nitrogen. Mantoura et al. (1997b) recovered 98% and 83% of the original chl *a* concentration in mixed microalgae after storage at −196°C for 60 days and 328 days respectively. Chl *a* recovery may also depend on the solvent. In some cases (e.g. station 162 in Fig. 4), the chl *a* concentration is fairly stable between weeks 2 and 8 in ethanol, in contrast to that in acetone at −20°C.

### 4.7. Extraction time

Non-homogenised filters require a longer extraction time than homogenised ones. It became evident that, in some cases, extraction in 90% acetone was still incomplete after 3 hours. In *Phaeodactylum*, the extraction may be incomplete even after 24 hours. Better results were achieved with 96% ethanol. The extraction time has to be increased if the temperature is lower than the recommended room temperature. Stauffer et al. (1979) extracted at 4°C for one and a half days and afterwards had to bring the extract to room temperature. Such a prolonged extraction time should not have any adverse effect if enzymatic degradation processes are suppressed by water-poor solvents. Extraction in hot ethanol (complete after 6 hours) could also stop enzymatic processes (Nusch & Palme 1975).

### 4.8. Filtration volume

The lower detection limit of chl *a* by fluorescence was found to lie at 0.01 $\mu$g (Holm-Hansen et al. 1965), which is about 5% of that required for a spectrophotometric determination. Thus, the sample volume can be reduced significantly if fluorometry is applied. The disadvantage of a smaller sample, however, is that it is less representative, especially if it has been taken from natural waters, which are liable to be patchy. On the other hand, high filtration volumes, as required for the spectrophotometric method, may lead to the chl *a* concentration being underestimated because the prolonged filtration time or filtration pressure may cause greater mechanical stress and cell disruption. The filtration time also depends on the filter type. GF/F filters allow a much higher filtration volume to pass through than 0.2 $\mu$m membrane filters, but are not inferior as far as the retention properties are concerned (Chavez et al. 1995), except in the case of oligotrophic oceanic water (Dickson & Wheeler 1995). Because of their undeniable advantages, like their high filtration capacity, GF/F filters are highly recommended (Mantoura et al. 1997b, Aminot & Rey 2001), and were used in our work without further testing.

### 4.9. Recommendations

- The filtration volume should not be too large, so that the filtration process takes no longer than 10 min at a suction pressure of 0.3 atm ($\sim 0.3\ 10^5$ N m$^{-2}$). If it is not possible to obtain enough material for a reliable spectrophotometric reading within 10 min of filtration, basic changes to the method, such as larger filters (50 mm diameter instead of 25 mm), reduced extraction volumes, and longer measuring cuvettes (50 mm instead of 10 mm) should be considered. Alternatively, fluorometry can be tried.

- If instant measurement is not possible, the filters can be stored in liquid nitrogen. After quick-freezing, they can be transferred to an ultracold freezer ($-80$°C), where they can be kept for 3 months without significant loss of chl *a*. If liquid nitrogen is not available, the extracts can be stored at $-20$°C for 3 months. It is not recommended to freeze damp filters at $-20$°C before storage.

- 96% ethanol is preferable as solvent. Extraction in 90% acetone requires the filters to be homogenised.

- The extraction time has no significant influence within the 3–24 hour range if the recommended 96% ethanol is applied.

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