Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts

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Abstract Phycobiliproteins are light harvesting pigments in cryptophytes, cyanobacteria, and rhodophytes that allow these organisms to absorb light in the green and orange regions of the electromagnetic spectrum. Unlike chlorophylls and carotenoids, however, phycobilins are rarely quantified as part of routine photobiological studies because they require different extraction protocols. The objectives of this study were (1) to compare 10 existing methods to determine that with the highest extraction efficiency and (2) to determine the maximum time limit for the storage of phycobilins before degradation. Cells of the cryptophyte *Rhodomonas salina* and the cyanobacterium *Synechococcus bacillaris* were harvested either by centrifugation or filtration and then subjected to lyophilization, mechanical disruption, or freeze–thaw techniques. The extraction efficiency for pigments in cells collected on glass fiber filters was always <32±5% and thus always significantly lower than in samples harvested by centrifugation, which had extraction efficiencies of 53±6–98±11%. Disruption of cells by freezing–thawing and sonication both resulted in significantly higher (ANOVA, $p<0.01$) extraction efficiencies than disruption with a tissue grinder. Storage of samples at −80°C showed no significant pigment degradation over the course of 24 weeks.

Keywords Cryptophyte · Cyanobacteria · Phycocyanin · Phycoerythrin · Pigment analysis

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Introduction

Phycobiliproteins are large water-soluble pigment-protein complexes that are unique to cyanobacteria, cryptophytes and rhodophytes (Rowan 1989; Kirk 1994). They are an important component of the light harvesting complexes in these taxa and may comprise up to 60% of their total soluble protein content (Bogorad 1975). Based on absorption properties, phycobiliproteins are divided into three classes: phycoerythrins, which absorb light at 495 and 540–570 nm, phycocyanins, with absorption maxima at 610–620 nm, and allophycocyanins, which absorb in the 650–655 nm range (Rowan 1989). Phycobiliproteins allow phytoplankton to harvest wavelengths that are not absorbed by chlorophylls and carotenoids. Like the content of chlorophylls and carotenoids, the relative phycobilin content of phytoplankton often varies (Kamiya and Miachi 1984; Hauschild et al. 1991; Lewitus and Caron 1991; Kana et al. 1992; Sciandra et al. 2000). Thus, routine quantification of these pigments may provide useful information about photoacclimation in these organisms.

Because phycobiliproteins are found in only a small number of phytoplankton classes, they may also be used as reliable biomass proxies for these taxa. Detection of cyanobacteria (including harmful cyanobacterial blooms) is one such field application (Lee et al. 1994; Leboulanger et al. 2002; Gregor et al. 2007) where in situ fluorometric (Yentsch and Phinney 1985; Wataras and Baker 1988, Richardson et al. 2010) and extractive techniques (e.g., Moreth and Yentsch 1970; Stewart and Farmer 1984) have been used for the quantification of these taxa.

Because phycobilins are water-soluble, they require extraction in a phosphate buffer, and thus, they cannot be quantified together with chlorophylls and carotenoids, which are usually extracted in organic solvents (Jeffrey et al. 2007).

A number of protocols for the quantification of phycobilins from phytoplankton have been published in the past, but they all differ considerably. Some authors collected their samples on filters (Moreth and Yentsch 1970; Stewart and Farmer 1984; Viskari and Colyer 2003), whereas others harvested cells by centrifugation (Glazer et al. 1971; Wedemayer et al. 1991; Gupta and Sainis 2010). Some protocols require only a phosphate buffer, while others recommend addition of enzymes to digest tough cell walls and enhance cell disruption and, hence, extraction. However, others prefer mechanical disruption of cells by grinding, sonication, or by use of a French pressure cell (e.g., Alberte et al. 1984). Studies also differ in the degree to which the resulting crude extracts are further purified. In most cases, information on the effectiveness, i.e., the efficiency, of the extraction procedure is lacking from the publication. Hence, there is little common agreement among phytoplankton ecologists about how to best extract and quantify phycobilins.

Ideally, the protocol of choice should be rapid, reliable, and efficient with a high sample through-put at a minimum cost, so that it can be used routinely to analyze samples collected from cultures and the field. The extraction procedure should also work equally well on samples that contain cells that are easily disrupted, such a cryptophytes, and that are notoriously difficult to disrupt, such as cyanobacteria. Often, cells can be rapidly harvested, but if they cannot be processed immediately upon collection, the storage of samples for several days to months may be unavoidable (e.g., in ship-based field studies). Storage at −20°C or −80°C is the conventional method for pigment samples used for the extraction of chlorophylls and carotenoids. Under these conditions, samples can be preserved for many months without chlorophyll or carotenoid degradation (Mantoura et al. 1997). Whether the same holds true for phycobiliproteins is, to our knowledge, unknown.

Thus, the objectives of the present study were (1) to find a rapid, reliable, cost-effective, and efficient method that can be used for extraction of phycobiliproteins from two different types of phytoplankton (cryptophytes and cyanobacteria) and (2) to determine how long phycobilin samples can be stored before pigment degradation becomes evident. To address these objectives, phycobilins from phytoplankton cultures were harvested and extracted according to published protocols to compare the resulting phycobiliprotein concentrations between the different treatments. The most suitable protocol, i.e., that which resulted in complete extraction of the phycobilins, was then used to determine if pigment degradation occurred over the course of 6 months.

Materials and methods

Cultures of the phycoerythrin-containing cryptophyte *Rhodomonas salina* (CCMP 1319) and the phycocyanin-containing cyanobacterium *Synechococcus bacillaris* (CCMP 1333), obtained from the Provasoli-Guillard National Center for the Culture of Marine Phytoplankton (Bigelow Laboratory, Boothbay Harbor, ME, USA), were grown in 2 L glass bottles at 24 ± 1°C under nutrient replete, semi-continuous conditions in seawater-based f/2 (cryptophyte) and L1 medium (cyanobacterium) (Guillard and Ryther 1962; Guillard and Hargraves 1993). A light intensity of ~70 μmol photons $m^{-2}s^{-1}$ was provided by fluorescent natural day light lamps on a 14:10 h light/dark cycle.

Comparison of extraction methods While samples for chlorophyll and carotenoid analyses are usually collected on glass fiber filters, extraction protocols for phycobilins often use centrifugation to harvest the cells. Thus, for this study, both techniques were used on a subset of samples. Because pigments from cyanobacteria are often hard to extract (Rowan 1989), all extraction techniques were first tested on the cryptophyte. Based on the results obtained, five protocols were then tested on the cyanobacterium.

Filtration A total of 15 cryptophyte culture aliquots (10 mL each) were filtered onto Whatman GF/F glass fiber filters (Fig. 1, treatments a and b) using a gentle vacuum of 20 kPa. Filters were transferred into 20 mL scintillation vials and immediately frozen at −80°C until further processing the next day. Ten of these filters were subjected to one freeze–thaw cycle (treatment a); that is, they were immersed in 5 mL of 0.1 mol L^{-1} phosphate buffer (pH=6) and stored at −20°C until all samples were completely frozen (~2 h), after which they were thawed and left to extract at 5°C for 24 h. The remaining five filters were lyophilized at −50°C for 5 h (treatment b). After addition of 5 mL of phosphate buffer, samples were extracted at 5°C for 24 h.

For the cyanobacterium, 15 culture aliquots (10 mL each) were harvested onto GF/F filters and subjected to one freeze–thaw cycle (without buffer) as described above. The batch was then divided into three subsets of five samples each. One set was not subjected to mechanical disruption (freeze–thaw only; treatment a), whereas filters of the other two sets were either ground (treatment h) or sonicated on ice for 30 s using brief (~5 s) 8 W pulses (treatment i). Grinding was done with an ice-cooled mortar and pestle until the filter had completely disintegrated. During grinding, 0.5 mL of phosphate buffer was added to each sample, and the resulting slurry was washed into a centrifuge tube using up to 3 mL of buffer. The final volume was brought to 4.5 mL. To remove all filter particles, the slurry was first filtered through a clean GF/F filter and then through a syringe filter with a 0.45-μm

Fig. 1 Overview of the different extraction techniques (labeled *a–j*) for samples harvested by filtration onto GF/F filters (treatments a, b and h, i) and by centrifugation (treatments c through g and j). *Solid lines* represent phycoerythrin samples collected from the cryptophyte culture (*Rhodomonas salina*), and *dashed lines* are phycocyanin samples collected from the cyanobacteria culture (*Synechococcus bacillaris*). Samples subjected to the freeze–thaw protocol were frozen for 2 h at −20°C and then thawed and left to extract at 5°C for 24 h.
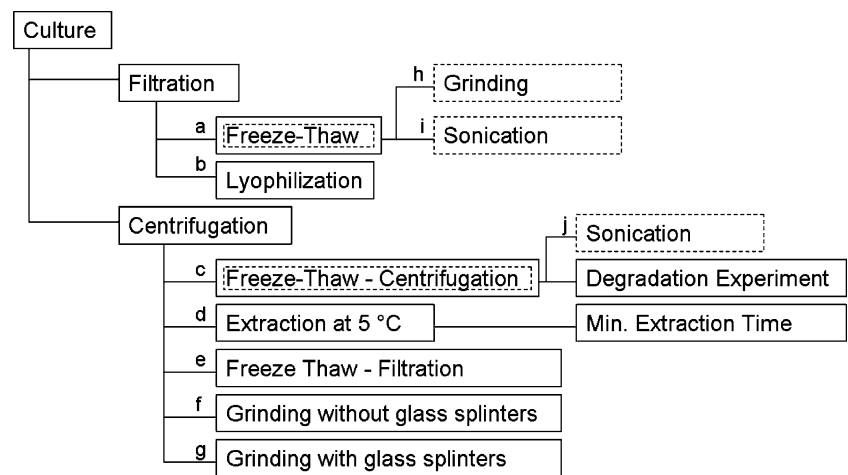
cellulose acetate membrane filter. Extracts that were not sonicated or ground, including all samples in the treatments below, were filtered through the cellulose acetate membrane filter only.

Centrifugation A total of 80 cryptophyte samples (40–50 mL each) were concentrated by centrifugation for 10 min at $2{,}054{\times}g$ (treatments c–g and j). The supernatant was decanted, the pellet re-suspended in 5 mL phosphate buffer, and the slurry homogenized on a vortexer. A subset of 50 samples was extracted at 5°C (treatment d), and in 10 of these samples, phycoerythrin concentrations were measured after 24 h following the protocols described below. The other 40 samples were divided into eight batches of five samples each, and their phycoerythrin concentrations were measured after 2, 3, 5, 8, 12, 24, and 48 h to determine the minimum time needed for complete extraction.

To remove cell debris from the extracts, two methods are commonly used: centrifugation and filtration. Thus, after subjecting 25 samples to one freeze–thaw cycle and extracting them at 5°C for 24 h, the batch was divided into two subsets. In one set, 2 mL aliquots were centrifuged at $10{,}870{\times}g$ for 5 min (treatment c), whereas replicates of the other set were filtered through a 0.45-µm cellulose acetate membrane (treatment e).

In two additional treatments, cryptophyte cells were disrupted in a Konte Duall® glass tissue grinder (treatments f and g). For this purpose, pellets were re-suspended in 1 mL of buffer, homogenized, and washed into the tissue grinder, where the slurry was ground for 60 s on ice (treatment f). To determine if addition of glass splinters resulted in a greater extraction efficiency, a spatula-tip full of finely crushed glass was added to the homogenizer (treatment g). Carefully rinsing the tissue grinder, the slurry was washed into a clean plastic tube, and the final volume was brought to 5 mL. An aliquot of

2 mL was centrifuged at $10{,}870{\times}g$ for 5 min, and phycoerythrin concentrations in the supernatant were measured as described below.

Because treatment c (freeze–thaw and purification of the extract by centrifugation) yielded the highest measurable phycoerythrin concentration in the cryptophyte, this protocol was also tested on the cyanobacterium, but in addition to the freeze–thaw cycle, one subset was sonicated (treatment j) as described above. In the cryptophytes, the phycoerythrin extraction was complete after 24 h, that is, repeated re-suspension of the cell debris in phosphate buffer for an additional 24 h resulted in no additional extraction of phycoerythrin. Samples of the cyanobacterium, however, required longer extraction times. These samples were left at 5°C for 48 h, after which phycocyanin in the supernatant was determined as described below. The pellet was re-suspended once more in fresh buffer and left to extract for another 48 h. Phycocyanin concentrations after 48 and 96 h were added up to yield a final concentration. Extracting the debris for a third time did not result in any detectable amounts of phycocyanin (data not shown).

Spectrophotometric quantification Absorbance (A) of purified extracts was measured from 400 to 750 nm in a UV-VIS 2450 Shimadzu dual beam spectrophotometer using a 1-cm quartz glass cuvette against phosphate buffer as a blank. Absorbance values were scatter-corrected by subtracting the absorbance at 750 nm from the absorbance maximum of the phycobilin peak (545 nm for phycoerythrin and 620 nm for phycocyanin). Phycobilin concentrations (c) (in µg L^{-1}) were calculated as

$$c = \frac{A}{\varepsilon d} \times \mathrm{MW} \times \frac{V_{\text{sample}}}{V_{\text{buffer}}} \times 10^6$$

where ε and MW are the molar extinction coefficients (phycoerythrin, $2.41{\times}10^6$ L mol^{-1}cm^{-1}; phycocyanin, $1.9{\times}10^6$ L mol^{-1}cm^{-1}) and molecular weight of the phycobilins

(phycoerythrin, 240,000 gmol^{-1}; phycocyanin, 264,000 g mol^{-1}), and where d is the path length of the cuvette, and V_{sample} and V_{buffer} are the volume of the sample and buffer, respectively. The extinction coefficients were taken from the product information provided by AnaSpec Inc. (Fremont, CA, USA), the company from which we purchased phycobilin standards with the same absorption properties as the phycobilins extracted from taxa used in the present study.

Extraction efficiency In the cryptophyte, the protocol yielding the highest phycoerythrin concentration was treatment c (harvesting of cells by centrifugation, one freeze–thaw cycle and purification of the slurry by centrifugation). Repeated resuspension of the pellet in buffer did not lead to any detectable phycoerythrin concentrations, and measuring the absorbance of the re-suspended cell debris did not result in any detectable peaks in the regions where phycoerythrin absorb. Thus, we are confident that the extraction was complete and set the extraction efficiency of treatment c to 100%. The concentrations from treatment c were then used to calculate the extraction efficiency for the other treatments according to

$$\text{Extraction efficiency} = \frac{[\text{PE}_\text{c}]}{[\text{PE}_\text{z}]} \times 100$$

where [PE$_\text{c}$] is the phycoerythrin concentration in treatment c and [PE$_\text{z}$] is the phycoerythrin concentration in any other treatment (z). For samples from the cyanobacterium, [PE$_\text{c}$] was replaced with phycocyanin concentrations from treatment j as it resulted in the greatest phycocyanin concentrations.

Pigment degradation during sample storage Cryptophyte cells in a total of 80 samples (30 mL each) were harvested by centrifugation as described above. The pellets were re-suspended in 5 mL phosphate buffer, and samples were frozen at −80°C for up to 24 weeks. Phycoerythrin concentrations in subsets of eight samples were processed after 24 h, 3, 7, and 10 days and after 3, 4, 6, 8, and 24 weeks as described for treatment c.

Statistical analyses The efficiencies of the different extraction techniques were compared with a one-way analysis of variance (ANOVA) using untransformed data and a p value of 0.01. Because the error variances were not homogeneous, Dunnett-T3 pair-wise comparisons were used to identify homogeneous subsets. In most cases, data for each treatment were normally distributed. All residuals were normally distributed and had a mean of zero. To determine if extraction time in buffer and sample storage time had an effect on pigment concentrations, linear regressions between extraction time and pigment concentrations and

between storage time and pigment concentration were conducted. If the slope of the regression line was significantly different from zero ($p<0.01$), time was considered to have an effect on the pigment content. Furthermore, due to departures in normality, small sample sizes, and deviations in homogenous variances, mean phycoerythrin concentrations at the different time points were compared using a non-parametric Kruskal–Wallis test ($p=0.01$) to provided better protection against type 1 errors. All statistical analyses were conducted in SPSS 16.0 GP (SPPS, Inc.).

Results

Comparison of extraction protocols

For both the cryptophyte and the cyanobacterium, collection of cells by filtration resulted in significantly lower extraction efficiencies compared to centrifuged samples (ANOVA; $p<0.01$). In the cryptophyte, the extraction efficiency of filtered samples was always <14±5%, whereas the efficiency of centrifuged samples was always >53±5% (treatment g), relative to treatment c which was defined as 100% (Fig. 2a). Similar observations were made for the cyanobacterium where the extraction efficiency varied between 1±3% and 32±6% in filtered samples, while centrifugation resulted in 89±11% to the defined 100% extraction efficiency with treatment j (Fig. 2b).

In the cyanobacterium, disruption of freeze–thawed filtered samples by grinding with a mortar and pestle (treatment h) was far less effective than sonication of cells (treatment i), and resulted in the lowest extraction efficiency of all protocols. After freeze–thawing (treatment a), the extraction efficiency was 23±3%, and additional disruption by sonication (treatment i) raised it to 32±6%, although the increase was not significant. Subjecting cyanobacteria that were harvested by centrifugation to a freeze–thaw cycle (treatment c) and subsequent sonication (treatment j) resulted in extraction efficiencies of >98%, given that the extraction time was long enough (see below). Both of these protocols worked equally well as indicated by the lack of a significant difference between treatment c and j (ANOVA, $p>0.01$).

Freeze–thawing of cells harvested by centrifugation (treatments c and e) was also the most effective method to extract phycoerythrin from the cryptophyte. Purifications of the crude extract by centrifugation (treatment c) or filtration (treatment e) are both suitable methods to remove cell debris from the samples and resulted in no significant differences in extraction efficiency (ANOVA, $p>0.01$).

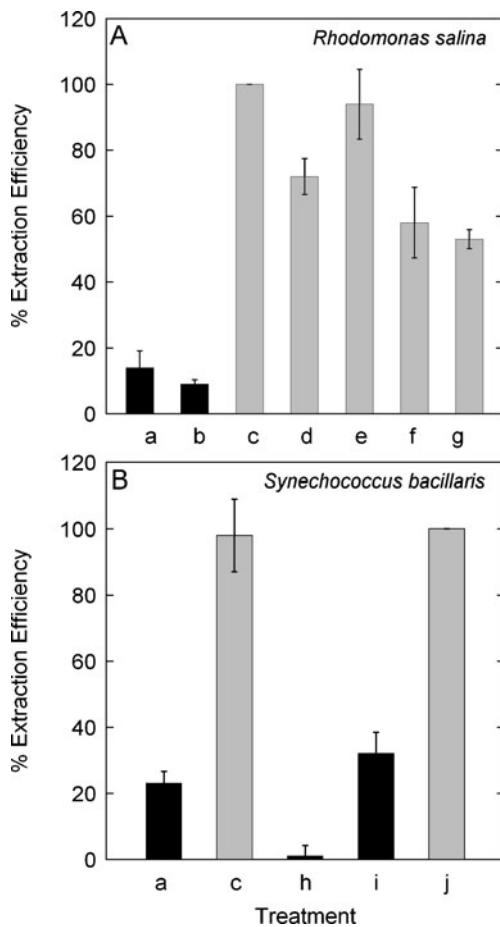Extracting samples in the fridge (treatment d) and grinding of cells (treatments f and g) resulted in signifi-

Fig. 2 Mean extraction efficiency (in %) (±standard deviation, n=5–10) of the different extraction techniques for phycoerythrin from the cryptophyte *Rhodomonas salina* (**a**) and phycocyanin from the cyanobacterium *Synechococcus bacillaris* (**b**). See Fig. 1 and "Materials and methods" for a detailed description of the different treatments. *Solid and gray bars* are samples collected by filtration and centrifugation, respectively
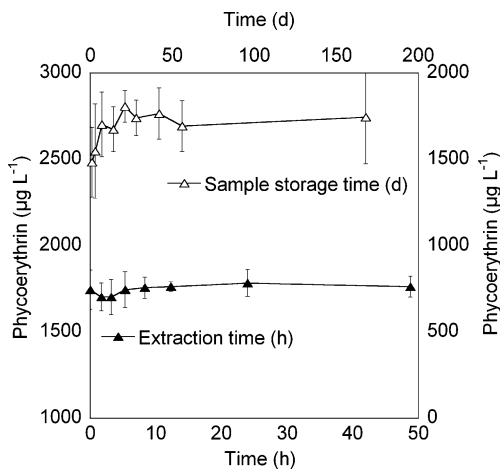

Fig. 3 Dependence of pigment degradation on extraction time (x_1y_1) and sample storage time (x_2y_2). *Error bars* are standard deviations with n=10 for extraction time and n=8 for sample storage time

Table 1 Slopes, p values and adjusted R^2 values of linear regressions between phycoerythrin concentrations and extraction time at 5°C or sample storage time at −80°C, respectively

	Slope	p value	Adjusted R^2
Extraction time	1.087	0.23	0.013
Storage time	0.816	0.03	0.027

cantly lower values. Samples extracted in the fridge (treatment d) had an efficiency of 72±5%, whereas the extraction efficiency of samples disrupted in a tissue grinder with and without glass splinters was 58±11% and 53±3%, respectively (treatments f and g). Lyophilization (treatment b) did not improve extraction and resulted in the lowest extraction percentages in the cryptophyte (Fig. 2).

Overall, harvesting of cryptophyte biomass by centrifugation and subjecting samples to one freeze–thaw cycle (treatment c) was the most efficient way to extract phycobiliproteins.

Minimum extraction time

Over an initial extraction time of 4 h, phycoerythrin concentrations in cryptophyte samples increased slightly but not significantly (Kruskal–Wallis test, p>0.01) (Fig. 3). Afterwards, it remained constant, and linear regression between phycoerythrin concentrations and time showed that the slope was not significantly different from one, indicating that phycoerythrin concentrations did not change significantly for the remaining 44 h (Table 1). Thus, extraction for 4 h at 5°C (after one freeze–thaw cycle) is sufficient to ensure maximum extraction of the pigment in cryptophytes. In the cyanobacterium, however, the extraction was not complete until after 96 h (Fig. 4). After 48 h, only 49% of the phycocyanin was extracted from cells harvested by centrifugation and subjected to one freeze–thaw cycle (treatment c), whereas 87% were recovered from cells that were also sonicated (treatment j).

Storage of phycobilin samples Storage of phycoerythrin samples for up to 24 weeks (6 weeks) resulted in no detectable pigment degradation. Note that the slope of the regression line between phycoerythrin concentrations and time was not significantly different from zero (p>0.01) but had a positive sign (Table 1).

Discussion

Detection and quantification of phycobilins with methods like those used for chlorophylls and carotenoids can
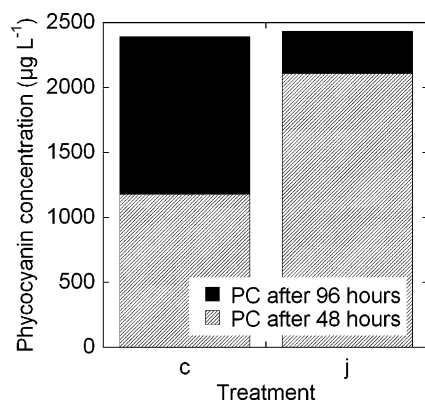
Fig. 4 Change in phycocyanin concentrations after 48 and 96 h from *Synechococcus bacillaris* cells that were harvested by centrifugation and subjected to one freeze–thaw cycle (treatment c) and subsequent sonication (treatment j)

provide additional insights into photobiological responses of phytoplankton to changes in their environment and may be used to survey shifts in phytoplankton community composition in natural habitats. In order for these methods to be used routinely on board ships and in laboratories, sample collection and extraction should be quick, reliable, and efficient.

Harvesting of cells on a filter is a standard approach in pigment analysis and has the advantage that large volumes of water can be cleared of phytoplankton within a relatively short period of time. Such practical considerations are especially important in oligotrophic open ocean waters where often several liters of water have to be filtered to collect enough biomass for photopigment analysis. However, based on our results, cells should not be collected on glass fiber filters. Even with a gentle vacuum of 20 kPa, which is less disruptive to cells and is usually used for samples intended for quantification of non-water-soluble pigment, filtration will result in considerably lower extraction efficiencies relative to samples collected by centrifugation. Our results show that, even when filters are ground or sonicated, only a small proportion of the phycobilins could be recovered. Thus, centrifugation does appear to be the best approach for collection of samples for phycobiliprotein analysis.

Of the different disruption methods, freeze–thawing and sonication proved to be most effective for both the cryptophyte and the cyanobacterium. For cyanobacteria, however, the disruption and extraction efficiencies reported in the literature are much more variable. In five chroococoid cyanobacteria and a *Synechococcus* strain, Stewart and Farmer (1984), were able to disrupt 92% and 88% of the cells, respectively. However, when Viskari and Colyer (2003) repeated the experiments by Stewart and Farmer, they were able to reach an extraction efficiency of only 24% in a different *Synechococcus* strain. They achieved highest extraction efficiencies of 85–88% after subjecting *Synechococcus* samples to a Chaps/asolectin/N_2 cavitation method.

In our experiment, any grinding of filters or cell pellets was insufficient to disrupt cells, and examination of the homogenate by microscopy confirmed that a large number of cells were still intact. This is contradictory to results by Viskari and Colyer (2003) who reported that grinding of frozen cyanobacteria samples with a pestle and a liquid nitrogen-frozen mortar would be much more effective than repeated freeze–thaw cycles. Unfortunately, they did not provide a detailed description of these methods in their publication and; thus, we cannot be certain that their grinding and freeze–thaw approach was identical to the one of the present study. Thus, the observed differences may simply result from discrepancies in methodology. Another, and not mutually exclusive, explanation for the difference in success rates may be that the efficiency of the different extraction protocol varies with the species in the sample, which is something that requires further study.

One thing that stood out, however, was that extraction times differed greatly between studies: anywhere from 0.5 to 24 h (Stewart and Farmer 1984; Viskari and Colyer 2003; Sampath-Wiley and Neefus 2007; Gupta and Sainis 2010). For taxa that are easy to extract, this may not be problematic, but it could introduce considerable error in quantifying cyanobacterial phycobilin concentrations. Stewart and Farmer (1984) found that in various cryptophytes, the extraction was complete after only 2 h in buffer. Using a different method, we were able to extract all phycobilin within 4 h. Because we anticipated that phycobilins would be more difficult to extract from cyanobacteria, we let these samples extract for 48 h, after which 49–87% of the total phycobilin were extracted. Repeated washing and extraction of the pellet in fresh buffer for up to another 48 h were necessary to recover the rest. Thus, by extending the extraction times, one can completely extract cyanobacterial phycobilins with simple methods, such as freeze–thawing and sonication.

In the present study, we also compared techniques to purify the crude extract, a step that is necessary to separate the phycobilin-containing extract from the cell debris, which would interfere with the absorbance measurements. According to our mean extraction efficiencies, centrifugation of the crude extract was slightly more effective than filtration through a cellulose acetate membrane. However, the error was minimal given that the difference between the two treatments was not significant. Filtration is also a method of choice of other groups who recommend (surfactant-free) cellulose filters with a pore size of 0.2–5 μm (Stewart and Farmer 1984; Viskari and Colyer 2003). Centrifugation, on the other hand, has the advantage that the pellet is preserved and can be subjected to additional extraction steps, if needed.

We are not aware of other studies that address the long-term storage of phycobilin samples, but, based on our results, such samples can be kept at −80°C for at least 6 months without pigment degradation, given that they are frozen immediately after centrifugation and addition of the buffer.

Based on our observations and on a careful evaluation of published works, we recommend the following practices for the extraction and preservation of phytoplankton samples intended for the extraction of phycobilins:

1. Cells should be harvested by centrifugation.
2. Samples can be stored up to 6 months at −80°C without detectable pigment degradation.
3. After the addition of phosphate buffer, cells should be disrupted by subjecting the samples to freeze–thawing and subsequent sonication.
4. For cryptophyte samples, an extraction time of 4 h is sufficient to completely recover all phycobilins. For cyanobacteria we recommend extraction for up to 96 h.

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