Eur. J. Phycol. (2013), 48(3): 278–286 European Journal of Phycology

Extraction methods of phycocyanin determination in freshwater algae and its application in Lake Balaton

Hajnalka Horváth[1], Attila W. Kovács[1], Caitlin Riddick[2], Mátyás Présing[1]

[1]Centre for Ecological Research, Hungarian Academy of Sciences, Balaton Limnological Institute, H-8237 Tihany

[2]University of Stirling, Department of Biological & Environmental Sciences, School of Natural Sciences , Stirling , UK

Correspondence to: H. Horváth. e-mail: horvath.hajnalka@okologia.mta.hu.

Abstract

Phycocyanin (PC) is one of the water-soluble accessory pigments of cyanobacteria species, and its concentration is used to estimate the presence and relative abundance of blue-green algae. In laboratory experiments PC content and PC/chl-*a* ratio of four N_2-fixing filamentous cyanobacteria strains (*Cylindrospermopsis raciborskii*, *Anabaena spiroides*, *Aphanizomenon flos-aquae* and *Aphanizomenon issatschenkoi*) were determined using Sarada's freeze-thaw method. However, a strong linear correlation was found between the extracted PC and chl-*a* concentrations (almost stable PC/chl-*a* ratio in 20−100 μg l^{-1} chl-*a*) for all strains, but the determination at lower concentrations (under 20 μg l^{-1} chl-*a*) proved to be uncertain (standard deviation exceeded 10−15%). The effectiveness of four selected extraction methods (repeated freeze-thaw method, homogenization with mortar and pestle, Ultrasonic and Polytron homogenizer) for PC with *C. raciborskii* was compared. It was found that the extraction efficiency of phycocyanin was the highest (of the methods compared) when a single freezing-thawing cycle was followed by sonication (25% more yield was extracted than with freezing-thawing method alone). Applying this combined method to surface water of Lake Balaton, there was a good correlation between PC concentration and cyanobacterial biomass (r = 0.9714), while the repeated freezing-thawing method found no detectable PC content. It has been shown that the sonication method is suitable to measure cyanobacteria PC content and to estimate cyanobacteria contribution to total biomass for the characterization of natural waters.

Keywords: *Cylindrospermopsis raciborskii*, extraction methods, freshwater, N_2-fixing cyanobacteria, phycocyanin, pigment analyses

Introduction

Phycobiliproteins are pigment-protein, amino-acid storage complexes of cyanobacteria, rodophyta and some cryptophytes species, including mainly allophycocyanin (APC), phycocyanin (PC) and in some case phycoerythrin (PE) (Williams *et al.*, 1980). Phycobiliproteins are located in the supramolecular phycobilisomes on the external surface of the thylakoid membrane (Sidler, 1994), and act as major photosynthetic accessory pigments.

Because phycobiliproteins are unique to cyanobacteria and a few eukaryotic algae classes, they can be used for the detection of these taxa. Assessing cyanobacteria presence or dominance with phycocyanin detection is well known; in situ fluorometric field application (e.g. Seppälä *et al.*, 2007), sensitive fluorometric technique (Downes & Hall, 1998), remote sensing (e.g. Simis *et al.*, 2005) or in vitro extraction methods have been used for cyanobacteria quantification (Sarada, 1999). A number of studies have been published on phycocyanin extraction methods and their comparative analyses (Zhu *et al.*, 2007, Lawrenz *et al.*, 2011, Zimba, 2012), but there is no standard protocol for the maximum extraction of phycocyanin from cyanobacteria cells. Repeated freeze-thaw and sonication methods are generally used in centrifuged (Patel *et al.*, 2005) or filtered samples (Viskari & Colyer, 2003), but homogenization of lyophilized or frozen samples with a vortexer (Lawrenz *et al.*, 2011), mortar and pestle (Viskari & Colyer, 2003) or glass bead (Eisele *et al.*, 2000) are also used. The extraction solution most frequently used for extraction is a phosphate buffer in a relatively large concentration (0.001 M to 0.05 M) (Furuki *et al.*, 2003), but in some case 90% acetone (Zimba, 2012) or lyzozime (Boussiba & Richmond, 1979) is also suitable for cell wall rupturing. In these studies, the extraction protocols were followed by spectrophotometric (e.g. Sarada

et al., 1999), liquid chromatographic, capillary (Viskari & Colyer, 2003) and gel electrophoresis (Soni *et al.*, 2006) or single step chromatographic analyses. Most studies have dealt with species originating from tropical/subtropical freshwater alkaline lakes (*Spirulina platensis*, Silveira *et al.*, 2007, Furuki *et al.*, 2003), salt water bodies (*Oscillatoria qudripunctulata*, Soni *et al.*, 2006) or marine environments (*Synechococcus sp*., 6301 Yamanaka & Glazer, 1980, *Lyngbya sp*., Patel *et al.*, 2005), but there has been little investigation into PC extraction methods in freshwater.

The objective of this study was to investigate an adequate, rapid, reproducible and cheap protocol for phycocyanin determination in freshwaters and hypertrophic reservoirs in order to estimate the relative cyanobacterial biomass.

Materials and methods

Algae culture and growth conditions

Cylindrospermopsis raciborskii (Wolosz.) Seenayya et Subba Raju ACT 9605, *Anabaena spiroides* (Kleb.) ACT 9505, *Aphanizomenon flos-aquae* (L.) Ralfs ACT 9607 and *Aphanizomenon issatschenkoi* (Ussatzew.) Proschkina-Lawrenko) ACT 9603 were grown in batch cultures under 24°C and a 14-10 h light-dark regime. The applied light intensity was 40 $\mu M\ m^{-2}\ s^{-1}$.

A modified BG-11 medium (Fe-citrate instead of $Fe(NH_4)$ citrate) without $NaNO_3$ was used to grow the cyanobacteria species. As the four selected cyanobacteria species are able to fix nitrogen, there was no need for an additional nitrogen source in the medium.

Volume of extraction solution

Different volumes of phosphate buffer (11 ml and 20 ml) were applied to determine the optimal volume for the exact phycocyanin measurements. *C. raciborskii* and *A. spiroides* were used in relatively large concentrations for this study (~ 4000 µg l^{-1} and ~ 500µg l^{-1} chl-*a*, respectively).

Extraction methods for phycocyanin determination in laboratory experiments

Pigment samples were extracted from the same culture of *C. raciborskii*. The cell walls were ruptured using the following methods with 15 ml of 0.05 M phosphate buffer (pH = 6.8; containing Na_2HPO_4 and KH_2PO_4):

A. Freezing-thawing method (Sarada *et al.*, 1999): biomass was harvested by filtration on GF/C (Whatman) filters and pigment was extracted by repeated (5 cycles) freeze-thaw cycles. Samples were frozen at − 20 °C and thawed at 9 ± 1°C in a water bath (NESLAB RTE 17 Thermo Electron Corporation).

B. Homogenization with mortar and pestle: the centrifuged (BHG HERMLE Z320 centrifuge with 4000 rpm for 10 min.) biomass was homogenized with an ice-cooled mortar and pestle in five periods. After homogenization, the extract was centrifuged. The supernatant containing phycocyanin was measured and the pellet was re-extracted with further 5 ml phosphate buffer. Extraction periods were repeated when no phycocyanin was detected. The result was obtained after five periods.

C. Homogenization with an Ultrasonic Homogenizer (Cole Parmer Instrument Ultrasonic Homogenizer 4710) with a normal sonication probe, output 5 and duty cycle 50%: samples were concentrated with filtration on GF/C (Whatman) filters and the cell walls were ruptured by sonication for different times (0; 15; 30; 45; 60; 90 and 120 s) in three replicates.

D. Homogenization with Polytron Homogenizer (PT 10-35; 220 V, 50Hz, 710 W): cells were harvested with centrifugation (4000 rpm for 10 min., with the same type of centrifuge) and the collected biomass was homogenized with Polytron for different times (0; 15; 30; 45; 60; 90 and 120 s).

E. Freezing-thawing method combined with Ultrasonic Homogenizer (the same device was used as mentioned in method 'C'): biomass was collected with filtration on GF/C (Whatman) filters, frozen at − 20°C and thawed at 9 ± 1°C in the same water bath as in method 'A'. Phycocyanin was extracted by sonication for different times (0; 15; 30; 45; 60; 90 and 120 s) in three replicates.

To purify the extracts from the cell walls, filtration (Whatman GF/C filter) was used after digestion (except in method 'B'). Samples were iced and kept in the dark to avoid pigment degradation during extraction methods. A Shimadzu UV-1601 spectrophotometer was used for PC measurements and the concentration was calculated using the equation by Siegelman & Kycia (1978):

C-phycocyanin (PC) = $(A_{615} - 0.474 * A_{652}) / 5.34$. (Equation 1)

where: - A_{615}: measured absorbance at 615 nm;

- A_{652}: measured absorbance at 652 nm.

Pigment analysis of algae cultures and field samples

Algae culture

To determine the PC content of the four selected species, Sarada's freezing-thawing method was applied as the extraction protocol. A series of different concentrations of the four investigated cyanobacteria species were prepared by dilution. The chlorophyll-*a* concentrations in each series were approximately: 6, 12, 25, 50 and 100 μg l^{-1}. Cell walls were ruptured in five freeze-thaw cycles in three replicates. After each freeze-

thaw period, the extract was filtered on GF/C (Whatman) filters and PC content of the filtrate was measured with the same spectrophotometer and calculated with Equation 1 (Siegelman & Kycia, 1978).

Field samples

Water samples were taken from the surface of Lake Balaton in 2010. After collection, water samples were filtered within 1 hour in five replicates and put into 15 ml of 0.05 M phosphate buffer (pH = 6.8), then frozen at − 20°C. Phycocyanin concentrations of three of the five samples were determined immediately by Sarada *et al.* (1999). Two replicates were stored at −80°C for later pigment determination. Phycocyanin content of these two samples was extracted using 15 second sonication after thawing and concentrations were calculated with the same equation (Siegelman & Kycia 1978).

Chlorophyll-*a* concentrations were measured as in Iwamura *et al.* (1970) on the same day of sampling. A Shimadzu UV-1601 spectrophotometer was used for the chlorophyll-*a* and phycocyanin measurements.

Counting of biomass/microscopical identification

Phytoplankton samples were preserved in Lugol's solution. Algal species were enumerated with an inverted plankton microscope (Utermöhl 1958). The wet weight of each species was calculated from cell volumes (Németh & Vörös 1986). At least 25 cells (or filaments) of each species were measured to determine biomass and at least 400 were counted.

Results

Volume of extraction solution

C. raciborskii and *A. spiroides* cultures were used to determine the optimal volume of phosphate buffer required for accurate phycocyanin measurements. It was necessary to know how much buffer volume is required for complete pigment extraction, particularly in the higher chl-*a* concentration range (~4000 μg l^{-1} and ~500μg l^{-1} chl-*a*, respectively). The applied volumes were 11 ml and 20 ml (Fig. 1). No significant differences were found in the maximum extracted pigment concentrations, thus, in further extraction experiments the applied volume was 15 ml. This volume is enough for use with the 4 cm cuvette used in spectrophotometric determination.

Extraction with freeze-thaw (A) method

A/1 Number of cycles

Four filamentous cyanobacteria species (*C. raciborskii*, *A. spiroides*, *Aph. flos-aquae* and *Aph. issatschenkoi)* were used to determine the optimal or sufficient number of freeze-thaw cycles over a 0−100 μg l^{-1} chl-*a* concentration range (Fig.2). The extracted PC concentrations reached their maxima after the second freeze-thaw cycle for three species (Fig 2a−c) in the range of 20−100 μg l^{-1} chl-*a*, and decreased significantly with further freeze-thaw cycles. Usually one cycle was enough for the maximum pigment extraction under this concentration range for all species. The phycocyanin concentration of *Aph. issatschenkoi* decreased continuously at all concentrations after one cycle. The maximum extracted pigment content was achieved after one freeze-thaw cycle, and the PC concentration decreased in each subsequent cycle by 25−30%. At similar environmental conditions, *C. raciborskii* had the largest PC concentration (420 μg l^{-1}) of the species examined, which is 1.4, 2.95 and 3.69 times larger than *A. spiroides*, *Aph.*

flos-aquae and *Aph. issatschenkoi* in the same chl-*a* concentration range (~ 100 μg l^{-1}), respectively.

A/2 Algae biomass

To assure the stability and reliability of the freeze-thaw method, changes of PC concentration in response to chl-*a* concentrations were compared. Because PC concentration changed with the number of freeze-thaw cycles (Fig. 2), maximum PC concentration in Fig. 3 was chosen to correspond with the ideal number of cycles. PC/chl-*a* ratios were almost constant above 10 μg l^{-1} chl-*a* for *C. raciborskii* and *A. spiroides*, but at lower chl-*a* concentrations the ratios changed with dilution. The degree of decrease in the pigment ratio was 6.3% and 9.3% for *C. raciborskii* and *A. spiroides*, respectively (Fig 3 a, b). Continuous decreasing tendency was found in PC/chl-*a* for *Aph. flos-aquae* and *Aph. issatschenkoi*; over the whole concentration range the decrease was 50% and 100%, respectively (Fig 3 c, d). At the smallest chl-*a* concentration (6 μg l^{-1}), PC content of *Aph. issatschenkoi* was undetectable. When comparing PC/chl-*a* ratios for the four selected species (Fig. 3), *A. spiroides* had slightly less pigment ratio (3.5−4) than measured in *C. raciborskii*, but *Aphanizomenon* species had much lower values. Under these well-defined conditions *Aph. flos-aquae* and *Aph. issatschenkoi* synthesized approximately equal content of chl-*a* and PC (R = 1.4 and 1.2, respectively).

Increasing standard deviation (SD%, Table 1) was observed at most dilution series with decreasing chl-*a* concentration. This tendency was negligible in *C. raciborskii* above 20 μg l^{-1} chl-*a* concentration, but below this concentration SD% of pigment determination sometimes exceeded 10%. *A. spiroides* had a similar extraction period for achieving the maximum extracted pigment content and had similar determination efficiency to *C.*

raciborskii; standard deviation of the freeze-thaw method was almost under 10% above 25 μg l^{-1} chl-*a* concentration. Pigment extraction of *Aphanizomenon* species was reliable at > 50μg l^{-1} chl-*a* concentration, but below this the determination became uncertain. There was no detectable PC content in several replicates of *Aph. flos-aquae* and *Aph. issatschenkoi* under 50 μg l^{-1} chl-*a* concentration and with repeated extraction cycles. Generally, standard deviation increased with increasing number of freeze-thaw cycles and decreasing chl-*a* concentration.

Other extraction methods (B, C, D)

All methods were tested from the same culture and biomass of *C. raciborskii* (chl-*a* 575 μg l^{-1}). Our experience suggested the unreliability of the traditional freezing-thawing method, so the efficiencies of three different extraction methods (B, C and D) were compared using cultures of *C. raciborskii* (Fig. 4). PC concentrations differed greatly by different extraction methods as well as by extraction periods. The lowest extraction efficiency resulted when samples were ground by the Polytron Homogenizer (Fig. 4d). The maximum pigment concentration was less than 300 μg l^{-1}, which was far less (< 10%) than the other methods. The effectiveness of freeze-thaw and sonication methods (Fig 4 a, c) was much better. At the beginning of the disruptions, increasing concentrations with increasing extraction time was observed. Although, concentration of extracted PC by freeze-thaw method continuously decreased after the second cycle, but significant decreasing was started following only the forth cycle. Extracted PC concentration by sonication started to reduce after 90 seconds and yielded approximately 25% more pigment content than in the maximum extraction of the freeze-thaw method alone. The highest PC concentration was measured (Fig. 4b) after the extractions by mortar and pestle, but significant differences were observed between

the four replicates. The standard deviation for the mortar and pestle method ranged from 5−94% at the different extraction periods. Apart from this digestion, sonication gave the highest pigment concentrations, and had the lowest extraction time (less than 90 s) and standard deviation (2.7%), therefore this method is the best possible within the compared methods.

Combined methods (E)

On the basis of our observations, the extracted PC content was not reduced greatly using only one freeze-thaw cycle as a pre-extraction method. Thus, the freezing-thawing method combined with sonication (method E) and extraction of fresh samples with only sonication (method C) was compared for the same diazotrophic cyanobacterium species (Fig. 5). Chl-*a* concentrations of *C. raciborskii*, *A. spiroides*, *Aph. flos-aquae* and *Aph. issatschenkoi* were 970 μg l^{-1}, 490 μg l^{-1}, 790 μg l^{-1} and 1450 μg l^{-1}, respectively. Almost the same phycocyanin concentration was measured for three species (Fig 6 a−c) in both methods (differences were between 0.10−9.95%), and half the extraction time was required for those samples which were frozen at −20°C before disruption. *Aph. issatschenkoi* was the only exception, where the PC content of frozen samples were 10% lower than those of sonicated fresh samples, and the pigment concentration slightly decreased after 15 seconds of sonication with either technique.

Field samples

Sarada's method and the above described combined method were compared in water samples from Lake Balaton (5−28 μg l^{-1} chl-*a*) (Fig. 6). Significant differences were found between the results of the two methods that correspond to results of laboratory experiments. There was no detectable pigment content with freezing-thawing method after the third cycle, while with the freezing-thawing combined with sonication, PC

content could be measured in all samples, and a strong correlation (R^2 = 0.9436) was obtained between PC and cyanobacterial biomass. In the summer in Lake Balaton, phytoplankton biomass is characterized by the presence of cyanobacteria species, including *Aph. flos-aquae, Anabaena aphanizomenoides, Aph. issatchenkoi* and *C. raciborskii.* The contribution of these algae to the total phytoplankton biomass was generally greater than 70%. Usually *Aph. issatchenkoi* was the dominant cyanobacteria species of these, with its contribution exceeding 40% in the considerable part of samples (2/3).

Discussion

During the investigation of PC extraction methods, a volume of buffer was determined for *C. raciborskii* and *A. spiroides*. According to Simis *et al.* (2005), 6 ml phosphate buffer was enough to achieve maximum extraction in the concentration range of 2−50 μg l^{-1}. However, this study aimed to clarify whether this method was useful not only in oligotrophic lakes, but in hypertrophic, blue-green algae dominated waters. The lower value of the selected concentration range in this study (500 μg l^{-1}) is typical in hypertrophic reservoirs. It was found that there was no significant difference in the extracted PC yield (3.68% and 4.03% for *C. raciborskii* and for *A. spiroides*, respectively) when using either 11 ml or 20 ml buffer volume, therefore 15 ml buffer was applied to further extractions. This volume was also enough for spectrophotometric determination.

In contrast to Simis *et al.* (2005), experiments with *C. raciborskii* indicated that there is no need for 5−9 freeze-thaw cycles for the complete extraction of phycocyanin. Our study agrees with the observation of Sarada *et al.* (1999) and Zhu *et al.* (2007),

which suggested that 3−5 cycles were enough to achieve the highest extraction efficiency. In the experiments of this study, this number of cycle was suitable when chl-*a* concentrations of *C. raciborskii* and *A. spiroides* cultures were more than 500 μg l^{-1}. This chl-*a* concentration is typical in fish-ponds or hypertrophic reservoirs, but under this concentration, less than five cycles were required to reach the highest extracted PC content (Fig. 2). Because PC content of cyanobacteria cells depends on the species and algae biomass, PC concentrations of the four most common species of blue-green algae in Hungary (especially Lake Balaton) were determined. Comparative analyses were conducted to determine PC concentration of these cyanobacteria species in response to chl-*a* (Fig. 3). It was found that phycocyanin content depends on species characteristics. Under the same conditions, *C. raciborskii* and *A. spiroides* synthesized 4.35 and 3.5 times larger content of phycocyanin than chl-*a* (Fig. 3), while *Aph. flos-aquae*, and *Aph. issatschenkoi* built up near equal PC and chl-*a* content (pigment ratios were 1.4 and 1.2, respectively). Usually in the 20−50 μg l^{-1} chl-*a* concentration range PC yield can be determined using only one freeze-thaw cycle for all cyanobacteria species in this study. Based on standard deviations (Table 1) of determination, it was observed that the repeated freeze-thaw method was reliable above 20 μg l^{-1} chl-*a* concentration of cultures. According to PC/chl-*a* ratios, *Aphanizomenon* species was more sensitive for mechanical disruption than other examined species, their pigment ratios continuously reduced with increasing number of freeze-thaw cycles and decreasing chl-*a* concentration. This observation demonstrates the limited applicability of repeated freeze-thaw method in lower pigment concentration range.

Several unique extraction protocols have been described in literature; Sarada *et al.* (1999) reported that the total extracted PC concentration of *Spirulina platensis* was

obtained by 3-5 freeze-thaw cycles, depending on whether acid treated or untreated samples were examined. Viskari & Colyer (2003) suggested the most effective method for PC determination was for cyanobacteria species that were frozen and ground with a pestle in a liquid nitrogen frozen mortar. Lawrenz *et al.* (2011) demonstrated freezing-thawing and a subsequent sonication proved to be the most efficient extraction method. Our experiments resulted in a similar observation as Lawrenz *et al.* (2011). In this study, cell wall rupturing with the Polytron Homogenizer was found to be the least effective of all extraction protocols of those compared. The highest PC concentration was resulted when cells were homogenized using mortar and pestle, but this method was more complicated and time-consuming, and its standard deviation varied greatly (between 5−94%). However, the freeze-thaw method proved to be an accurate (SD = 2.94%), simple and cost-effective method that does not require a special device or extraction solution. However, this method is laborious and the number of freeze-thaw cycles varied greatly depending on the species and trophic state of the water. Otherwise, according to Fig. 3 the freeze-thaw method underestimated the real values, particularly in lower concentration range. This was supported by the higher extraction efficiency of the sonication method; it was 25% more effective than the repeated freeze-thaw method (Fig. 4). Furthermore, sonication is the least time-consuming method; 15−90 seconds of sonication time was enough to obtain the highest extraction efficiency. This is an advantage when it is needed to rapidly establish the relative content of cyanobacteria in the biomass. Downes & Hall (1998) described that the extraction efficiency depends on sonication time and power output at the sonication tip, but the required sonication time was also dependent upon species characteristics and biomass. In the case of *C. raciborskii* (~1000 μg l^{-1} chl-*a*), 1.5 minutes were needed to achieve the maximum

extraction efficiency, while *Aph. flos-aquae* (~500 μg l^{-1} chl-*a*), *A. spiroides* (~800 μg l^{-1} chl-*a*) and *Aph. issatschenkoi* required less than 60 seconds. The 15 second sonication time for *Aph. issatschenkoi* (where biomass was close to 1500 μg l^{-1}) also indicated that this algae has a high sensitivity for mechanical disruptions. Because phycocyanin content did not decrease during the freezing at −20°C (see Fig. 5), one freeze-thaw cycle provides a more efficient and rapid extraction, and determination can be realized several months after the sampling occurred (6 months at −80°C, as in Lawrenz *et al.* 2011).

In a comparison of the freeze-thaw and the freeze-thaw combined with sonication methods in Lake Balaton, a similar conclusion was found to laboratory experiments (Fig. 6). No detectable pigment content was found in the field samples with the freeze-thaw method after the third cycle, but PC concentration could be determined with the combined method. Despite the fact that *Aph. issatchenkoi* was the dominant species in the samples and proved to be the most sensitive to sonication, PC concentration was strongly correlated (R^2 = 0.9436) with cyanobacterial biomass. This correlation also supported the reliability and sensitivity of the freeze-thaw method combined with sonication at low pigment concentrations.

According to our observations:

- Sample freezing allows for later determination of PC concentration,
- provides a more efficient and rapid extraction,
- gives a chance to conduct preliminary experiments to determine the sufficient extraction time, which depends on species composition and algae biomass, which is almost impossible to assess in advance.

Acknowledgement

Authors are highly grateful to George Borbély for helpful discussions during the initial stages of our research. We are pleased to thank to Lajos Vörös for counting of phytoplankton biomass. We are also thankful to Eszter Zsigmond for her help in the phycocyanin determination and Caitlin Riddick for correcting the manuscript's style.

References

Boussiba, S. & Richmond, A.E. (1979). Isolation and characterization of phycocyanins from the blue-green algae Spirulina platensis. *Archives of Microbiology*, **120** : 155–159.

Downes, M. T. & Hall, J.A. (1998). A sensitive fluorometric technique for the measurement of phycobilin pigments and its application to the study of marine and freshwater picophytoplankton in oligotrophic environments. *Journal of Applied Phycology*, **10** : 357–363.

Eisele, L.E., Bakhru, S.H., Liu, X., MacColl, R. & Edwards, M.R. (2000). Studies on C-phycocyanin from Cyanidium caldarium, a eukaryote at the extremes of habitat. *Biochimica et Biophysica Acta*, **1456** : 99–107.

Furuki, T., Maeda, S., Imajo, S., Hiroi, T., Amaya, T., Hirokawa, T., Ito, K. & Nozawa, H. (2003). Rapid and selective extraction of phycocyanin from Spirulina platensis with ultrasonic cell disruption. *Journal of Applied Phycology*, **15** : 319–324.

Iwamura, T., Nagai, H. & Ishimura, S. (1970). Improved methods for determining contents of chlorophyll, protein, ribonucleic and desoxyribonucleic acid in planktonic populations. *International Review of Hydrobiology*, **55** : 131–147.

Lawrenz, E., Fedewa, J.E. & Richardson, T.L. (2011). Extraction protocols for the quantification of phycobilins in aqueous phytoplankton extracts. *Journal of Applied Phycology*, **23** : 865–871.

Németh, J. & Vörös, L. (1986). Koncepció és módszertan felszíni vizek algológiai monitoringjához. *OKTH, Budapest*, 135 pp. (in Hungarian).

Patel, A., Mishra, S., Pawar, R. & Ghosh, P.K. (2005). Purification and characterization of C-Phycocyanin from cyanobacterial species of marine and freshwater habitat. *Protein Expression and Purification*, **40** : 248–255.

Sarada, R., Pillai, M.G. & Ravishankar, G.A. (1999). Phycocyanin from Spirulina sp: influence of processing of biomass on phycocyanin yield, analysis of efficacy of extraction methods and stability studies on phycocyanin. *Process Biochemistry*, **34** : 795–801.

Seppälä, J., Ylöstalo, P., Kaitala, S., Hällfors, S., Raateoja, M. & Maunula, P. (2007). Ship-of-opportunity based phycocyanin fluorescence monitoring of the filamentous cyanobacteria bloom dynamics in the Baltic Sea. *Estuarine, Coastal and Shelf Science*, **73** : 489–500.

Sidler, W.A. (1994). Phycobilisome and phycobiliprotein structures. In: *The Molecular Biology of Cyanobacteria* (Bryant D.A. editor), 139–216. Kluwer Academic Publisher, Dordrecht.

Siegelman, H. & Kycia, J.H. (1978). Alga biliproteins. In: *Handbook of phycological methods*: physiological and biochemical methods. (Hellebust, J.A. & Craigie, J.S., editors), 72–78. Cambridge University Press, Cambridge.

Silveira, S.T., Burkert, J.F.M., Costa, J.A.V., Burkert, C.A.V. & Kalil, S.J. (2007). Optimization of phycocyanin extraction from Spirulina platensis using factorial design. *Bioresource Technology*, **98** : 1629–1634.

Simis, S.G.H., Peters, S.W.M. & Gons, H.J. (2005). Remote sensing of the cyanobacterial pigment phycocyanin in turbid inland water. *Limnology and Oceanography*, **50** : 237–245.

Soni, B., Kalavadia, B., Trivedi, U. & Madamwar, D. (2006). Extraction, purification and characterization of phycocyanin from Oscillatoria quadripunctulata—Isolated from the rocky shores of Bet-Dwarka, Gujarat, India. *Process Biochemistry*, **41** : 2017–2023.

Utermöhl, H. (1958). Zur Vervollkommnung der quantitative Phytoplankton-Methodik. *Mitteilungen Internationale Vereinigung für Theoretische und Angewandte Limnologie*, **9** : 1–38.

Viskari, P.J. & Colyer, C.L. (2003). Rapid extraction of phycobiliproteins from cultured cyanobacteria samples. *Analytical Biochemistry*, **319** : 263–271.

Williams, R.C., Gingrich, J.C. & Glazer, A.N. (1980). Cyanobacterial phycobilisomes. *Journal of Cell Biology*, **85** : 558–566.

Yamanaka, G. & Glazer, N.A. (1980). Dynamic apects of phycobilisome structure. *Archives of Microbiology*, **124** : 39–47.

Zhu, Y., Chen, X.B., Wang, K.B., Li, Y.X., Bai, K.Z., Kuang, T.Y. & Ji, H.B. (2007). A simple method for extracting C-phycocyanin from Spirulina platensis using Klebsiella pneumonia. *Applied Microbiology and Biotechnology*, **74** : 244–248.

Zimba, P.V. (2012). An improved phycobilin extraction method. *Harmful Algae*, **17** : 35–39.

Table 1. Standard deviation (%) of freeze-thaw method of four cyanobacteria species (*C. raciborskii*, *A. spiroides*, *Aph. flos-aquae* and *Aph. issatschenkoi*)

Species	chl-*a* ($\mu g\ L^{-1}$)	SD% of freeze-thaw method in increasing cycles					
		1	2	3	4	5	mean
Cylindrospermopsis raciborskii	*~100*	1.99	2.32	7.42	4.76	3.87	4.07
	~50	1.32	4.06	5.76	6.97	3.72	4.37
	~25	5.47	4.81	2.48	2.33	4.55	3.93
	~10	1.80	17.41	1.73	11.68	7.30	7.98
	~5	0.93	13.84	4.97	12.64	12.32	8.94
Anabaena spiroides	*~100*	1.82	1.75	1.39	8.99	5.43	3.87
	~50	4.64	4.21	4.73	11.83	10.83	7.25
	~25	10.56	9.19[a]	5.63	0.88	6.80	6.61
	~10	14.26	4.73	19.32	4.75	16.49	11.91
	~5	2.86	14.28	10.26	69.08[a]	14.65	22.23
Aphanizomenon flos-aquae	*~100*	6.57	3.97	12.39	23.44[a]	20.82[a]	13.44
	~50	11.84	17.55	11.35	4.11[a]	-	11.21
	~25	6.14	16.18[a]	4.76[a]	-	-	9.03
	~10	20.21	17.47[a]	-	-	-	18.84
	~5	14.27	-	-	-	-	-
Aphanizomenon issatschenkoi	*~100*	5.57	5.66	11.02	60.95	-	20.80
	~50	2.62	2.36[a]	1.17	43.43	-	13.32
	~25	14.79	-	b	-	-	18.12
	~10	8.85[a]	-	-	-	-	-
	~5	-	-	-	-	-	-

[a]: Standard deviation (SD) was calculated from two replicates;

b: PC content was measured in one of three replicates;

[a], b: PC content in the second and/or third replicates was under the limit of detection;

-: No detectable PC content found.

Fig. 1: Efficiency of the extraction and PC concentration in different buffer volume in *C. raciborskii* (A) and *A. spiroides* (B). Circle with black line represents 11 ml buffer solution and diamond with dotted line marks 20 ml buffer solution. Error bars are standard deviations n=3.

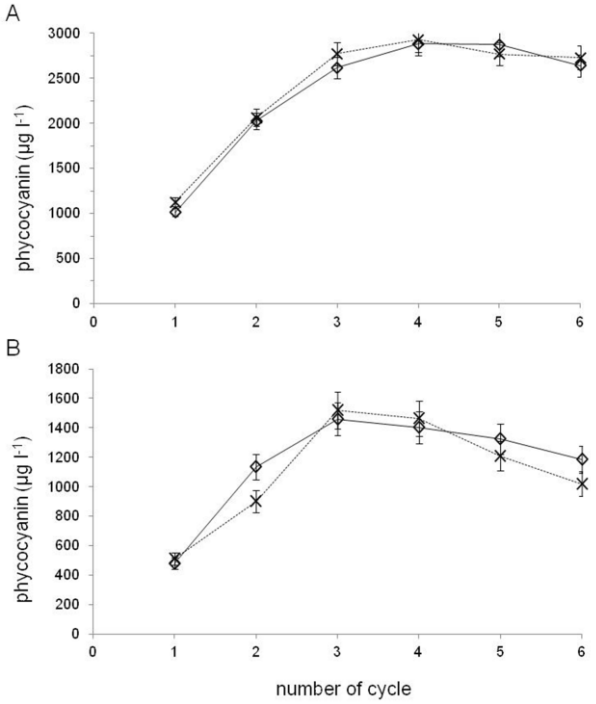

Fig. 2: Changes of phycocyanin concentration in response to increasing number of freeze-thaw cycles at different chl-*a* concentrations (A: *C. raciborskii*, B: *A. spiroides*, C: *Aph. flos-aquae*, D: *Aph. issatschenkoi*). Different marks represent different chl-*a* concentrations. Error bars are standard deviations with n=3.

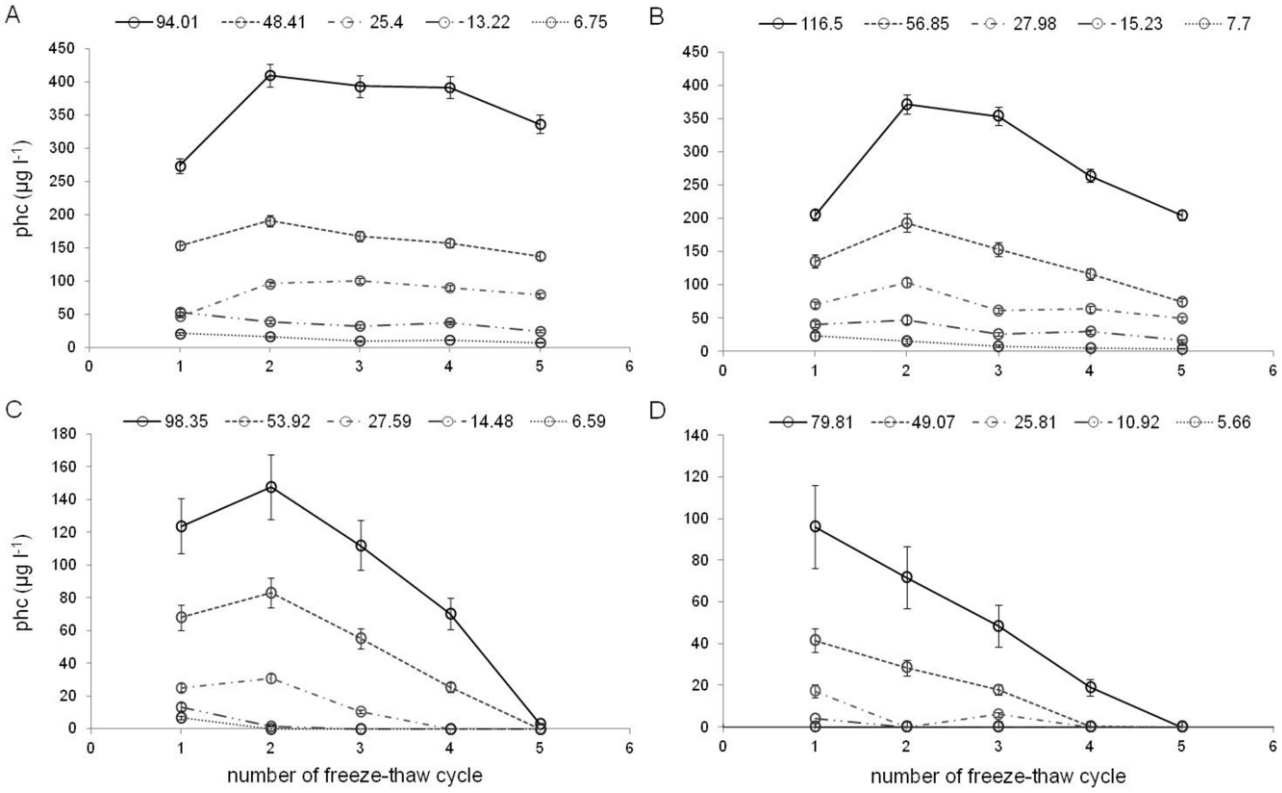

Fig. 3: Changes of phycocyanin (PC) and chlorophyll-a (chl-*a*) concentration and their ratios of PC/chl-a (R) in different algae species (A: *C. raciborskii*, B: *A. spiroides*, C: *Aph. flos-aquae*, D: *Aph. issatschenkoi*). Dotted line represents the linear regression of PC/chl-*a*.

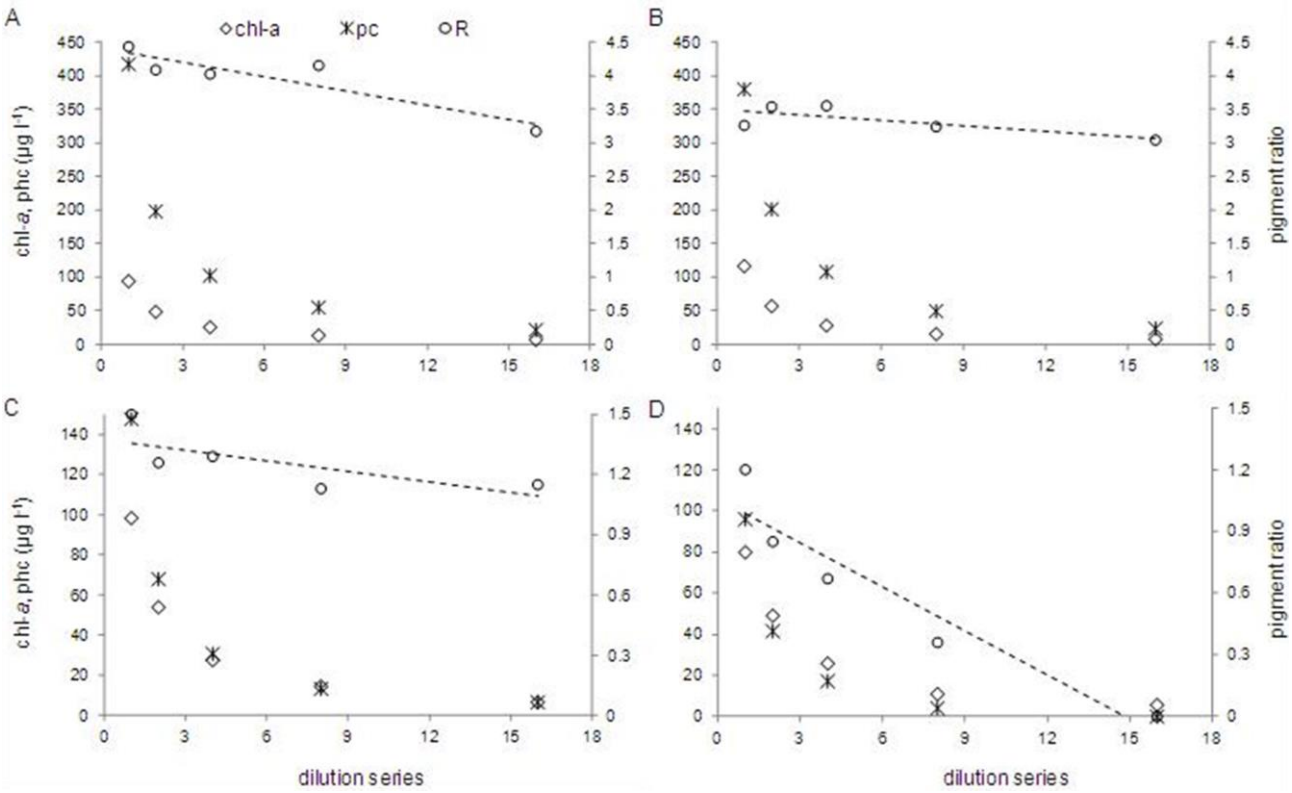

Fig. 4: Comparison of the different extraction methods of phycocyanin in *C. raciborskii*. (A: freezing-thawing method; B: grinding; C: sonication; D: homogenization with Polytron). Error bars are standard deviations with n=3.

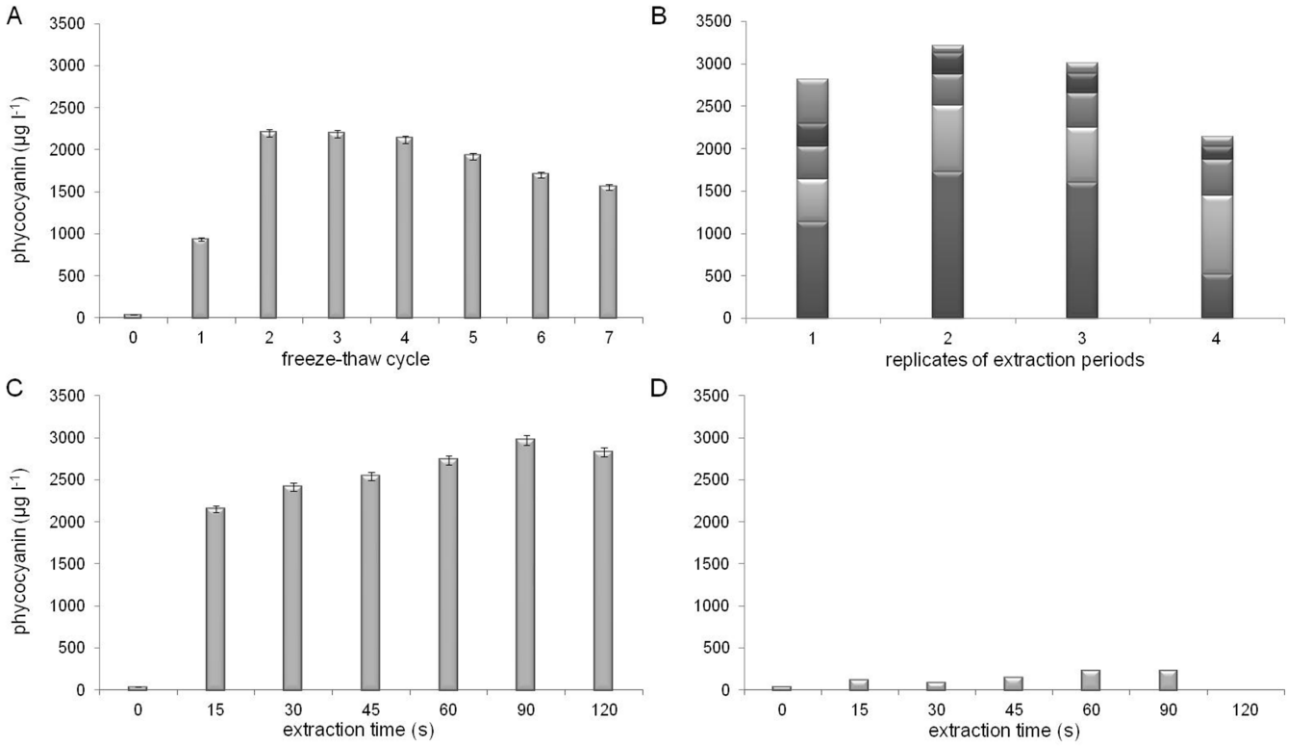

Fig. 5: Changes in the extracted phycocyanin concentration during sonication (A: *C. raciborskii*, B: *A. spiroides*, C: *Aph. flos-aquae*, D: *Aph. issatschenkoi*). Error bars are standard deviations with n=3.

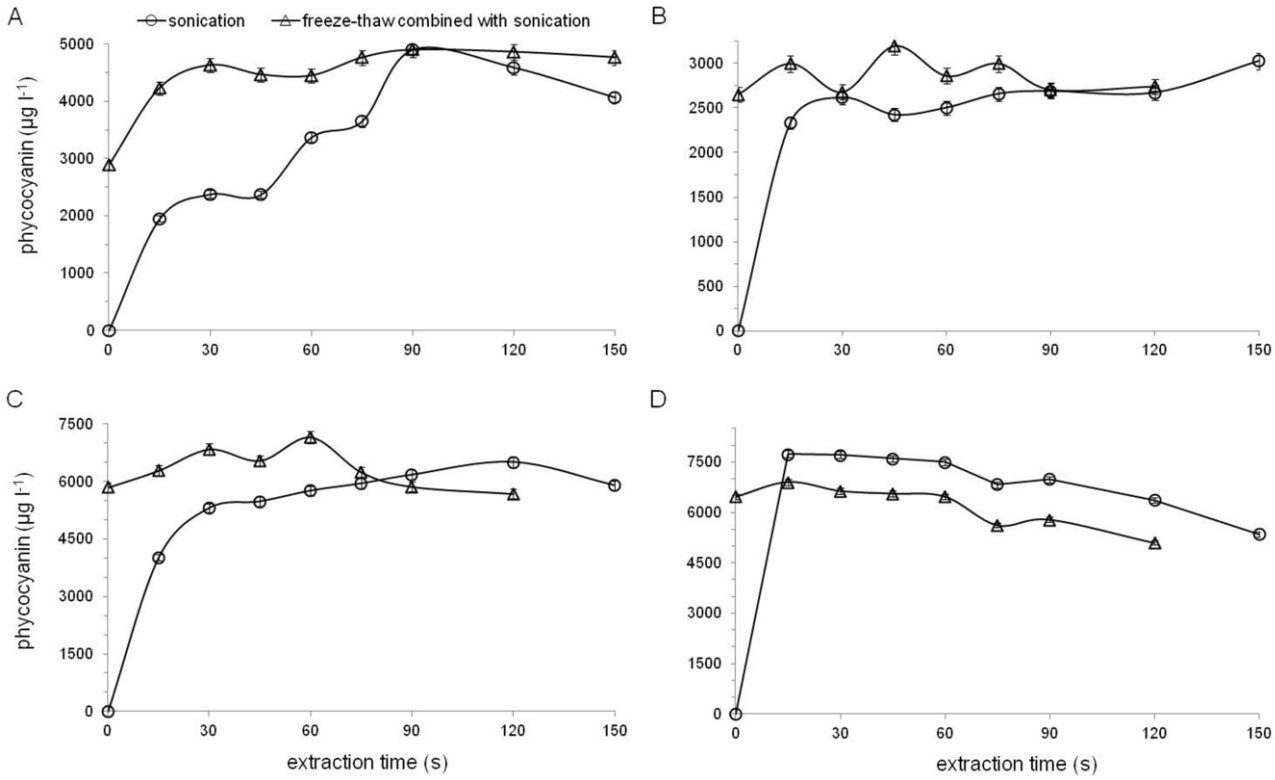

Fig. 6: Cyanobacterial biomass versus phycocyanin concentration (SD = 1.7%) in Lake Balaton.