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Evaluation of cyanobacteria biomass derived from upgrade of phycocyanin fluorescence estimation

Vrednotenje biomase cianobakterij na osnovi nadgradnje ocene fluorescence fikocianina

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Abstract: The number of harmful cyanobacterial blooms has increased significantly at the global level in recent years. One of the characteristics of cyanobacteria that gives them advantage over other phytoplankton organisms are auxiliary photosynthetic pigments, such as phycocyanin. This fluorescent pigment emits light at a different wavelength as chlorophyll and can therefore be used for detection of cyanobacteria *in situ*. In this study we used submersible phycocyanin fluorescence sensors and compare their voltage output to concentration of extracted phycocyanin, cell counts and biovolume. The relation was linear in all three cases; however, the variability of regression line slopes between different cyanobacteria strains was high in the case of PC extract concentration and cell count. The highest uniformity in the linear fits was between fluorescence signal and biovolume therefore making it the best candidate for fluorescence sensor voltage output conversion. In the context of this work we also compared different methods for PC extraction. Modifying the equations by subtracting the absorption at 750 nm almost entirely reduces the false PC concentration estimation due to sample turbidity.

Keywords: fluorescence measurements, phycocyanin, cyanobacteria

Izvleček: V zadnjih letih se število škodljivih cvetenj cianobakterij na globalni ravni močno povečuje. Ena od značilnosti cianobakterij, ki jim prinaša prednost pred ostalimi fitoplanktonskimi organizmi, so pomožna fotosintezna barvila, med katerimi prevladuje fikocianin. Fikocianin fluorescira pri drugi valovni dolžini kot klorofil, zato lahko s pomočjo meritve fluorescence ugotavljamo prisotnost cianobakterij v vodnem okolju *in situ*. S potopnim senzorjem fluorescence fikocianina smo opravili meritve dveh sojev cianobakterije *Microcystis aeruginosa* in nitaste cianobakterije vrste *Arthrospira platensis*. Odnos med koncentracijo ekstrahiranega fikocianina, številom celic in njihovim biovolumnom ter fluorescenco fikocianina je bil v vseh treh primerih linearen, vendar pa je bila variabilnost naklonov regresijske premice v

primeru koncentracije fikocianinskega ekstrakta in števila celic med različnimi vrstami cianobakterij visoka. Najvišje ujemanje naklonov linearnih regresij je bilo med signalom senzorja in biovolumnom, zaradi česar je najboljši kandidat za pretvorbo izhodne napetosti fluorescenčnega senzorja v limnološko pomembno količino. V okviru tega dela smo primerjali tudi različne protokole za ekstrakcijo fikocianina. Obstoječe enačbe za pretvarjanje absorpcije v koncentracijo fikocianina smo dopolnili z odštevanjem absorpcije vzorca pri 750 nm in s tem zmanjšali zavajujočo oceno koncentracije.

Ključne besede: meritve fluorescence, fikocianin, cianobakterije

Introduction

Cyanobacteria are a part of the phytoplankton community in each water body. Problems occur if their concentration increases. Most cyanobacterial genera produce cyanotoxins – a very diverse group of toxic substances, which pose a threat to the environment, animals and people. The number of blooms at the global level has significantly increased (Paerl et al. 2011) also due to nutrient load from anthropogenic sources.

Reliable and accurate information on cyanobacteria concentration in water bodies enables us to respond appropriately and prevent risks to animal and human health. Traditional methods of phytoplankton monitoring have been available for many years, as part of national legislations in many countries, and from 2000 a part of official monitoring procedures within the EU Water Framework Directive (Directive 2000/60/EC). Precise determination of phytoplankton species composition can be achieved with microscopic examination. The method is time-consuming and requires specialized knowledge. Moreover, the overall apprehension on spatial and temporal distribution of phytoplankton in water body is very limited.

One of the characteristics of cyanobacteria that gives them advantage over other phytoplankton organisms are auxiliary photosynthetic pigments such as phycocyanin (PC), alophycocyanin, and phycoerythrin. They allow cyanobacteria to use the available light more efficiently (Raps et al. 1983). PC is a fluorescent pigment, which emits light at a different wavelength as chlorophyll so the PC fluorescence can be used to detect the presence of cyanobacteria in the aquatic environment *in situ* and discriminate them from other phytoplankton.

Field sensors measuring *in vivo* fluorescence have been successfully applied in various oceanographical and limnological studies, giving real-time results on a detailed spatial and temporal scale. Detection limits and correlation between PC signal and biovolume have also been determined (Kong et al. 2013, Kasinak et al. 2015). Despite the advantages, limitations in the estimation of cyanobacterial abundance with PC fluorescence sensors have been reported (Gregor et al. 2007, Chang et al. 2012).

In this study we used submersible phycocyanin fluorescence sensors to measure concentration of three different cyanobacterial strains. We compared the results with cell counts, biovolume and concentration of extracted PC in order to find the most suitable parameter for converting the voltage output of the sensors into limnological values. In the context of this work we also compared different methods for PC extraction. In contrast to chlorophyll the extraction of phycocyanin is not standardized.

Materials and Methods

Laboratory cultures

Three different axenic cell lines of cyanobacteria and one of green algae were used: two different strains of unicellular *Microcystis aeruginosa* (microcystin-producing strain PCC 7806 and non-producing strain PCC 7005) from the Institute Pasteur (Paris, France), *Arthrospira platensis* SAG 85.79, a filamentous representative of cyanobacteria, and green algae *Desmodesmus communis* 276-4b from the SAG collection (Goettingen, Germany). The cyanobacteria and green algae were grown and maintained under

sterile conditions in 100 mL flasks with 50 mL Jaworski medium at room temperature exposed to natural daylight.

Fluorescence measurements

For fluorescence measurements a portable KM 245 (Arhel, Slovenia) system equipped with submersible PC fluorometer Cyclops 7 (Turner, USA) was used. A magnetic stirrer (C-MAG MS4, IKA, Germany) was used to prevent settling. The PC sensor excites the cyanobacterial PC at 590 nm (FWHM 13 nm) and measures fluorescence emission above 630 nm. The volume of measured sample was 800 mL. Each sample was measured for 5 minutes with 4.5 Hz sampling frequency and then the average signal was calculated. The results are presented in relative units [r.u.] that correspond to the voltage output of the sensor.

Cell counts and biovolume

The cell counts were determined with a Bürker-Türk haemocytometer (Brand, Germany) under an inverted Eclipse TE300 microscope (Nikon, Japan). The biovolume was calculated from the average biovolume of individual cells estimated by shape assimilation to known geometric forms and measurement of the main dimensions in more than100 randomly selected cells of each species (Hillebrand et al. 1999).

Determination of PC concentrations

We tested three different methods, using two different saline buffers and two methods for cell lysis. All tests were done on *M. aeruginosa* PCC 7806. Cell concentration was 5 x 10^6 cells/mL.

Method A - modified protocol by Horváth et al. (2013)

We centrifuged *M. aeruginosa* PCC 7806 culture with LC-321, (Tehtnica Železniki, Slovenia) at 4000 rpm for 15 min, removed the supernatant and substituted it with phosphate buffer (concentration of KH_2PO_4 was 0.1 mol/L, pH 6.8). We sonicated the sample with CV 18 ultrasonic homogenizer (tip diameter 6 mm, power 22 W, frequency 20 kHz, sonication volume 10 mL; Sonics and Materials, USA) for 10 minutes. After centrifugation (4000 rpm, 5 min), absorbance was measured with Nanocolor VIS (Macherey-Nagel, Germany) spectrophotometer.

Method B - protocol by Meriluonto et al. (2017)

Samples with different concentration of cells were filtered through a GF/C glass-fibre filter (Sartorius Stedim Biotech, Germany). The maximum vacuum during filtration did not exceed 31 kPa in order to avoid cell lysis. The filter with the cells was freeze-thawed to induce lysis. A saline buffer was added to glass tubes with filters and shaken until the filters broke down. The buffer was prepared by dissolving 8.77 g (0.15 mol/L) NaCl, 2.01 g (27 mmol/L) KCl, 11.36 g (80 mmol/L) Na_2HPO_4, 2.72 g (20 mmol/L) KH_2PO_4, 3.73 g (10 mmol/L) Na_2EDTA in distilled water to a final volume of 1 L. pH was adjusted using 5 M NaOH to 7,45 - 7,50. After dissolving the sample in the buffer, the tube was kept at 4 °C for 16 hours, after which the sample was shaken and sonicated for 20 min in an ultrasonic bath DC200H (mrc, Israel) with tap water and ice. After centrifugation (4000 rpm, 20 min), absorbance of supernatant was measured.

Method C

Method C was the same as method A, except that we used the buffer described in the method B.

Extractions were done under dim light to avoid photochemical degradation of PC. The concentrations of PC (in mg per L of water sample) were calculated according to de Marsac and Houmard (1988), Bennet and Bogorad (1973) and Fujita (1979), respectively:

$$PC\ [mg/L] = \frac{(A_{620} - 0.7 \times A_{650}) \times Ve}{7.38 \times Vs \times l} \quad \ldots 1$$

$$PC\ [mg/L] = \frac{(A_{615} - 0.474 \times A_{652}) \times Ve}{5.34 \times Vs \times l} \quad \ldots 2$$

$$PC\ [mg/L] = \frac{(198 \times A_{620} - 133 \times A_{650} - 0.190 \times A_{565}) \times Ve}{Vs \times l} \quad \ldots 3$$

Ve is the volume of buffer extract (mL), *Vs* volume of water sample (L), *l* optical path length (cm) and *A* absorbance at different wavelengths. Molar extinction coefficients and molecular weight of the phycocyanin are incorporated in the equations.

Results

PC extraction method

The difference between the minimum and maximum PC concentration obtained from the three methods using the same equation was between 17 and 18 % (depending on the equation) and was not statistically significant ($p > 0.1$). On the other hand, the choice of equation had larger impact on the estimation of PC concentration. The difference between concentration estimations from equation 1 and equations 2 and 3 was approximately 60% and was statistically significant ($p < 0.05$), whereas the estimations from equations 2 and 3 were not significantly different ($p > 0.05$). PC extraction from similar biovolume of green algae *Desmodesmus communis*, which does not contain PC, showed values ranging from 17 to 33 % of PC concentration in *M. aeruginosa*. To reduce the overestimation of PC concentration due to the nonspecific turbidity of the sample we have modified the equations similarly as for the chlorophyll extraction (ISO 10260, 1992) and subtracted absorbance at 750 nm which is caused by suspended solids. These are present in spite of centrifugation and are composed mainly of residues of the glass-fibre filter. Absorption spectra showed that the photosynthetic pigments absorb very little light at this wavelength.

$$PC\ [mg/L] = \frac{((A_{620}-A_{750})-0.7\times(A_{650}-A_{750}))\times Ve}{7.38\times Vs\times l} \quad \ldots 1m$$

$$PC\ [mg/L] = \frac{((A_{615}-A_{750})-0.474\times(A_{652}-A_{750}))\times Ve}{5.34\times Vs\times l} \quad \ldots 2m$$

$$PC\ [mg/L] = \frac{(198\times(A_{620}-A_{750})-133\times(A_{650}-A_{750})-0.190\times(A_{565}-A_{750}))\times Ve}{Vs\times l} \quad \ldots 3m$$

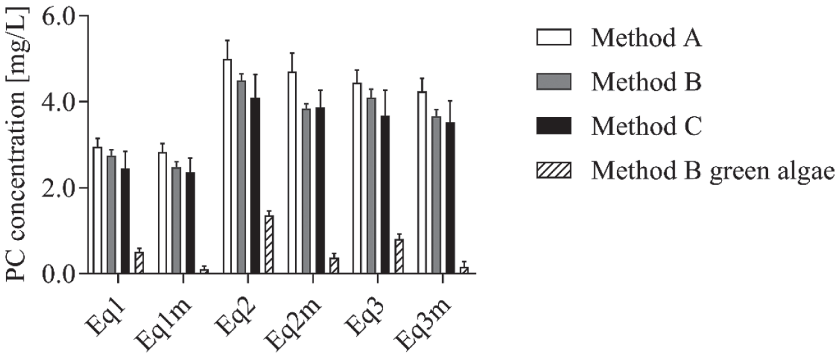

Figure 1: Comparison of PC extraction using three different methods and three different equations. Standard equations (Eq1, Eq2 and Eq3) were compared with modified equations (marked with m), where we subtracted the absorbance at 750 nm, which is indicative for nonspecific turbidity. Cyanobacteria *Microcystis aeruginosa* PCC 7806 and green algae *Desmodesmus communis* were used.

Slika 1: Primerjava treh različnih metod ekstrakcije PC in treh različnih enačb. Obstoječe enačbe (Eq1, Eq2 and Eq3) smo primerjali z dopolnjenimi, kjer smo odšteli absorpcijo vzorca pri 750 nm. Uporabili smo cianobakterije *Microcystis aeruginosa* PCC 7806 in zelene alge *Desmodesmus communis.*

Modification of equations resulted in 3.6 to 5-fold decrease of virtual PC concentration estimation in green algae sample. The decrease was statistically significant ($p < 0.01$). The decrease in PC concentration estimation in *M. aeruginosa* was around 10 %. The decrease in PC concentration using the modified equations was not statistically significant except in the case of method B, using equations 2 and 3.

Laboratory cultures

Three axenic lines of cyanobacteria, differing in shape, size and structure were used. Both strains of *M. aeruginosa* are unicellular and spherical. Average cell size of PCC 7806 was 28.81 ± 12.39 µm^3 and of PCC 7005 22.49 ± 7.66 µm^3. Cyanobacteria *A. platensis* is filamentous, the cell size was 163 ± 63 µm^3, filament length was 212 ± 96 µm. Concentration of extracted PC per cell was the lowest in PCC 7806 (0.55 ± 0.03 pg/cell). Concentration of extracted PC in PCC 7005 was 0.05 ± 0.01 pg/cell and 3.3 ± 0.3 pg/cell in *A. platensis*. PC was extracted according to method B and equation 1.

PC fluorescence of different laboratory strains

The same sample measured with fluorescence sensor was used for PC extraction, cell count and biovolume determination. The PC fluorescence intensity was in positive linear correlation with the concentration of extracted PC, cell count or biovolume (Fig. 2 A, B, C). The slopes of the linear fit were the most diverse when comparing PC fluorescence to concentration of extracted PC (Fig. 2 A). The increase in PC fluorescence was greater in *M. aeruginosa* PCC 7005 (k was 171 ± 16) than in PCC 7806 (k was 10.4 ± 0.4), despite the smaller size and 10-fold lower average PC

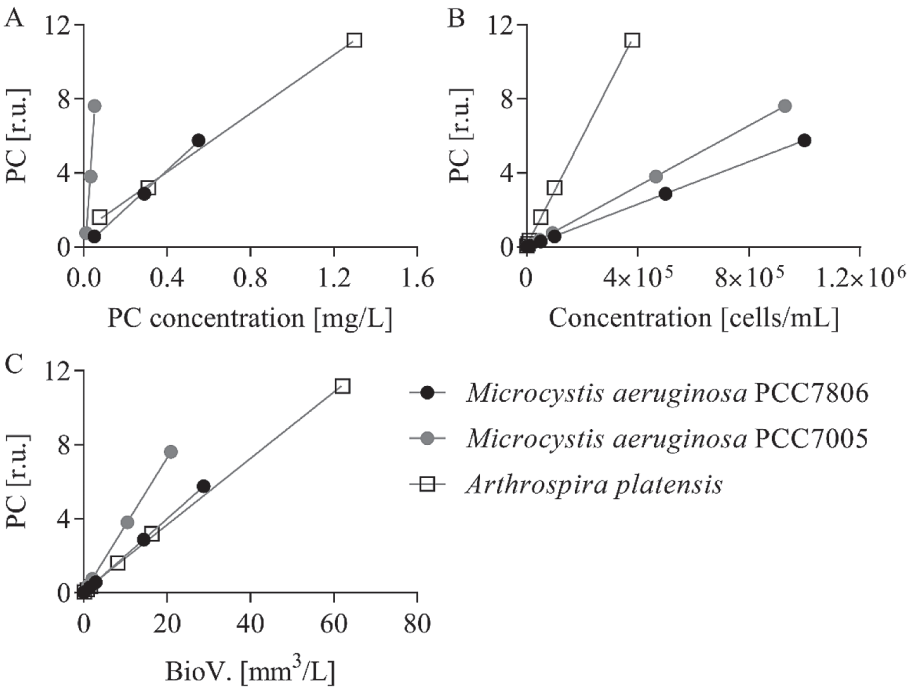

Figure 2: Relationship between phycocyanin (PC) fluorescence of three laboratory cultures and (A) extracted phycocyanin, (B) cell count and (C) total biovolume.

Slika 2: Odnos med fluorescenco fikocianina treh laboratorijskih kultur ter (A) koncentracijo ekstrahiranega fikocianina, (B) številom celic ter (C) biovolumnom.

content per cell. The slope of the linear fit was similar in filamentous *A. platensis* (k was 7.9 ± 0.2) and spherical *M. aeruginosa* PCC 7806. The average content of PC per cell in *A. platensis* was 6 fold higher than in PCC 7806.

The order of the linear slopes was different when we compared PC fluorescence to cell count. The increase in cell count resulted in the highest increase of PC fluorescence in *A. platensis* (k was $2.93 \times 10^{-5} \pm 3 \times 10^{-7}$). Slopes for the *M. aeruginosa* PCC 7806 and PCC 7005 were similar ($5.75 \times 10^{-6} \pm 8 \times 10^{-9}$ and $8.18 \times 10^{-6} \pm 1 \times 10^{-8}$ respectively).

Differences in the slopes of the linear fit were most similar when we compared PC fluorescence to biovolume. The highest slope (k was 0.3641 ± 0.0005) was calculated for *M. aeruginosa* PCC 7005, *M. aeruginosa* PCC 7806 was in the middle (k was 0.1996 ± 0.0003) and *A. platensis* had the lowest slope (k was 0.179 ± 0.002).

Discussion

We compared three different PC extraction methods and three different equations for assessing PC concentration. The difference between the minimum and maximum PC concentration obtained from the three methods was around 17 % and was not statistically significant. The largest difference in concentration was between method A and C that only differ in the buffer composition. Although there is no significant difference in the estimation of the concentration, method B has some advantages over method A. A combination of sonication with one freeze–thaw cycle enables shorter extraction time than sonication alone, and freezing the samples allows them to be stored.

The largest differences in estimation of PC concentration were due to different equations - 60%. By subtracting the absorbance at 750 nm from the absorbance at other wavelengths we almost entirely reduced the false PC concentration estimation. This was the most effective when we used equation after de Marsac and Houmard (1983). The modification did not statistically significantly influence the estimation of PC concentration in samples with cyanobacteria but reduced the virtual PC concentration estimation in green algae sample. This enables us to evaluate and minimize the disturbances that arise from the extraction of mixed samples from water bodies without any additional procedures. Nevertheless, additional studies should be made to confirm the modification and show influence of substances present in natural water samples.

PC can also be detected through the measurement of its fluorescence *in situ* as has been shown in the field and the laboratory. To translate the signal into limnological language we have to calibrate the sensor. Methods for calibration are different and may compromise the utility of these tools. In some studies, manufacturer settings are used without additional calibrations (Bowling et al. 2012) or the output of the sensor is calibrated in respect to the PC concentration (Song et al. 2013). Purchased solutions with defined PC concentration are usually used. Impurity of the solutions can reduce the precision of calibration. We have tested the relation between sensor output and cell count, biovolume and concentration of PC extracted from the same samples. The slopes of the linear fit were the most diverse when comparing PC fluorescence to the concentration of extracted PC. There was no obvious order in the slopes from different cyanobacteria species: *M. aeruginosa* PCC 7005 had the steepest slope when we compared PC fluorescence to extracted PC despite 10 fold lower PC cell content. Difference between slopes was almost 22 times. The differences were smaller (5 fold) when we compared PC fluorescence to cell count.

The highest uniformity in the linear fits was achieved when we compared PC signal with the cell biovolume. Similar results have been demonstrated in other studies (Kasinak et al. 2015). Our results show that the PC fluorescent signal is species dependent. Calibration of the PC fluorescent sensors with only one species, typically *M. aeruginosa* (Bastien et al. 2011), is inadequate for reliable conversion between PC fluorescence and biovolume of cyanobacteria.

Conclusions

- A combination of sonication with one freeze–thaw cycle is the most effective PC extraction method

- The largest differences in estimation of PC concentration are due to different equations used for calculating PC concentration from absorbance
- Modifying the equations by subtracting the absorption at 750 nm almost entirely reduces the false PC concentration estimation
- Relation between PC fluorescence signal and cell count, concentration of extracted PC and biovolume is linear
- The highest uniformity in the linear fits is between PC fluorescence signal and biovolume

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