Journal of the Serbian Chemical Society
JSCS-info@shd.org.rs • www.shd.org.rs/JSCS

J. Serb. Chem. Soc. *79* (2) 185–198 (2014)
JSCS–4575

UDC 579.8:544.537:543.426.1
Original scientific paper

Investigation and detection of cyanobacterial Cr-phycoerythrin by laser-based techniques

BRATISLAV P. MARINKOVIĆ[1], AMBRA DELNERI[2], MAJA S. RABASOVIĆ[1], MIRA TERZIĆ[3], MLADEN FRANKO[2] and DRAGUTIN ŠEVIĆ[1]*

[1]*Institute of Physics, Belgrade, P. O. Box 68, University of Belgrade, Serbia,* [2]*Laboratory for Environmental Research, University of Nova Gorica, Slovenia and* [3]*Faculty of Science, University of Novi Sad, Serbia*

(Received 17 April 2013)

Abstract: The application of the high-sensitivity method of time resolved laser induced fluorescence (TR-LIF) and a flow-injection system by thermal lens spectrometry (FIA-TLS) for the analysis of Cr-phycoerythrin (Cr-PE) isolated from a proprietary cyanobacterium is presented. In the excitation wavelength range (340–470 nm), the fluorescence spectra exhibited a pronounced maximum at 575 nm. Another maximum at about 600 nm could also be observed. The obtained results were used to verify the technical parameters of the employed thermal lens technique, which is complementary to spectrofluorimetry and subject to lower sensitivity in the case of high fluorescence quantum yields and photolability of the measured compounds.

Keywords: time resolved laser induced fluorescence spectroscopy; thermal lens spectroscopy; phycoerythrin; cyanobacteria.

INTRODUCTION

Cyanobacteria are photosynthetic microorganisms that are worldwide distributed in marine and fresh waters. During their ageing and decay, they release secondary metabolites (cyanotoxins) that are toxic to the environment and humans. Cyanotoxins have dermatotoxic, hepatotoxic and neurotoxic effects[1] and can also lead to death.[2] Moreover they are tumor promoters and recently they have been related to neurodegenerative diseases, such as amyotrophic lateral sclerosis and Alzheimer's disease.[3] Under particular conditions (for example eutrophication and rising temperature), cyanobacteria can grow abnormally, leading to so called harmful algal blooms (HABs) with a consequent release of large amounts of cyanotoxins during their senescence and death.[4] During such events, the mass of cyanobacteria in water can reach over 100 g m^{-3}.[5] Concern

* Corresponding author. E-mail: sevic@ipb.ac.rs
doi: 10.2298/JSC130417088M

about HABs has grown in the last decades due to their increase in occurrence and severity and because cyanotoxins are not efficiently removed by conventional water treatment technologies; hence, they can be found in recreational and drinking waters. In 2004, the World Health Organization (WHO) set a provisional limit of 1 μg L^{-1} for the presence of microcystin-LR (the most harmful among the cyanotoxins) in drinking water.[6] Cyanobacteria represent a serious threat to the environment and human health, and therefore, the development of sensitive and rapid analytical tools that could be used in early warning systems for the detection of cyanobacteria is of high interest.

Phycoerythrin (PE), isolated from cyanobacteria, is a highly fluorescent pigment with a broad and high absorption of light over a wide range of the visible spectrum. Its content in cyanobacteria is about one percent dry weight, but in some species, up to 8 % PE can be found.[7] The spectral properties of PE depend on the native structure of the polypeptide and interaction with different chromophores. PE was among the first molecules to be observed at the single-molecule level using laser-induced fluorescence.[8] PE fluoresces in a spectral region that is distinct from the region of emission of the simple organic dyes commonly used as fluorescent indicators. Therefore, PE is commonly used for fluorescent immuno-labeling, particularly in applications involving fluorescent-activated cell sorting.[9,10] Sometimes, the relatively high-molecular weight of PE may be problematic in a spectrofluorimetric detection system, due to steric hindrance caused by conjugation to other proteins.[11]

Among the PEs, Cr-phycoerythrins (Cr-PE) provide additional spectral characteristics that can complement the common phycoerythrins (B-PE and R-PE). Besides this, the Cr-PEs have a lower molecular weight (around 40 kDa). Due to these features, Cr-PEs can provide additional functionality to the phycobiliprotein pigments when multiplexing assays or the introduction of the dye into cells is concerned. In addition Cr-PE could find many applications in cellular analysis, flow and laser scanning cytometry.[11,12] Similarly, PEs and other cyanobacterial pigments could serve as early indicators of the presence of cyanobacteria and of the associated risk of cyanotoxins in water, as was shown for phytoplankton cell lysis based on release of carotenoids and the application of thermal lens spectrometry.[13,14]

According to literature data, three different Cr-PE types (Fig. 1) are recognized. They are designated based on their characteristic absorption maxima, λ_{max}.[15–17]

In the present study, a commercially available Cr-PE, a low molecular weight phycobiliprotein (40 kDa) with an absorption maximum at 550 nm, was examined. The characteristics of this Cr-PE, *i.e.*, high extinction coefficient and high fluorescence quantum yield, make it an ideal fluorophore for use in immunoassays.[10,18] To date, not much data for this phycoerythrin type has been pub-

lished. However, information on the fluorescence quantum yield would be valuable for the development of an alternative method for detection of PEs in water, such as, for example, thermal lens spectrometry (TLS). In such a case, however, higher fluorescence quantum yields result in lower sensitivity of the method, which is based on the indirect measurement of absorbance through radiationless de-excitation processes. It is therefore important to estimate eventual differences in the fluorescence quantum yields for excitation in the main PE absorption peak at 550 nm, which is accessible by Ar-ion laser lines (458.9–514.5 nm) and the PE absorption peak at 375 nm, reachable by Kr laser lines (406.7 and 413.1 nm) in the visible spectral range, which are the most frequently used in TLS spectrometry.[19]

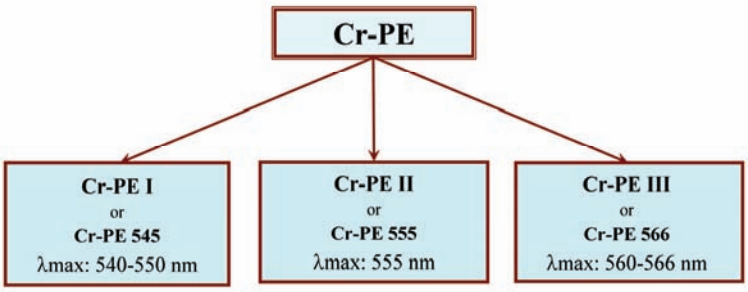

Fig. 1. Schematic illustration of Cr-PE types, modified from the literature.[15-17]

Two laser-based techniques were used for the characterization and detection of Cr-PE: laser-induced fluorescence (LIF)[20] and flow-injection analysis with thermal lens spectrometric detection (FIA-TLS).[21] The fluorescence technique is based on measurement of photons emitted by the excited fluorophore. Detection of absorbed energy by thermal lens techniques is achieved by measurement of heat released by non-radiative relaxation of the excited fluorophore.

The obtained results were used to verify the technical parameters of the employed thermal lens technique, which is complementary to spectrofluorimetry and subject to lower sensitivity in the case of high fluorescence quantum yields and photolability of measured compounds under irradiation by intensive light sources such as lasers.

EXPERIMENTAL

The study was realized using phycobiliprotein Cr-PE I type with a molecular weight of 40 kDa, isolated from a proprietary cyanobacterium. The pigment Flogen® Cr-PE was supplied by FEBICO Taipei (Taiwan) in: 100 mM potassium phosphate buffer, pH 7.0, with 60 %, saturated $(NH_4)_2SO_4$, 1 mM EDTA and 1 mM sodium azide. The absorption spectrum of Cr-PE was characterized by an absorbance maximum at 550 nm. The purity ratios $A_{550}/A_{280} \geq$ ≥ 5.5 correspond to pure Cr-PE and $A_{620}/A_{550} \leq 0.005$ demonstrates the absence of phycocyanin contamination.

Time resolved laser-induced fluorescence

The TR-LIF experimental setup used in this work, shown on Fig. 2, was described previously.[22] Solutions of Cr-PE in quartz cuvette were illuminated using a tunable Nd:YAG laser system (Vibrant model 266-I, Opotek, Inc.). This system includes an optical parametric oscillator (OPO) that is pumped by the fourth harmonic of the Nd:YAG Brilliant laser at 266 nm and control electronics. The output of the OPO can be continuously tuned over a spectral range from 320 nm to 475 nm, providing pulses of 5.4 ns and an energy per pulse of between 5.5 and 4 mJ at a 10 Hz repetition rate.

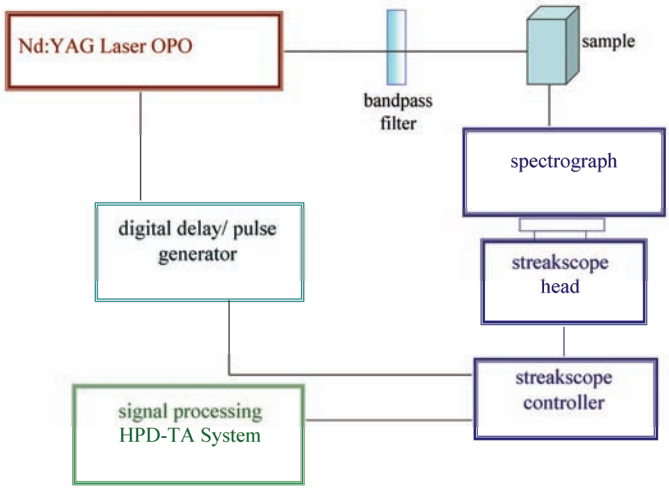

Fig. 2. Experimental set for TR-LIF spectrometry.

The fluorescence signals from Cr-PE solution were measured using a streakscope (Hamamatsu model C4334-01) with an integrated video streak camera. For all measurements of the Cr-PE spectra, the emission was collected in the right angle arrangement and dispersed by a 0.3 m focal length triple grating imaging spectrograph (SpectraPro-2300i). The fluorescence data was acquired in the photon-counting mode using Hamamatsu HPD-TA software. All the measurements were performed at room temperature.

Flow-injection system with thermal lens spectrometric detection

The FIA-TLS experimental set-up used in this work is shown in Fig. 3. The sample is injected into the stream of carrier buffer pumped at 1 mL min^{-1} by an HPLC pump (Knauer Smartline pump 1000). A Knauer injector (Knauer A1358) equipped with a 200 µL injection loop was used for this purpose. The detection was realized in an 8 µL flow-through sample cell (Hellma 176.050-QS) by a dual-beam (pump/probe) thermal lens spectrometer.[19] The pump beam (514.5 nm) was derived from an Ar-ion laser (Inova 90, Coherent) with 100 mW power measured at the laser head. A helium–neon laser (632.8 nm, 2 mW) (Meles Griot, 1103P) was used as a source of the probe beam. The pump and probe beams were properly focused with respect to the sample cell by a set of lenses and their collinear propagation through the sample cell was assured by a dichroic mirror. The pump beam, modulated by a mechanical chopper (Scitec Instruments) at 40 Hz, induces the photo-thermal effect, consisting of periodic changes in the refractive index gradient in the sample, which is related to the

concentration of the sample. The refractive index gradient causes defocusing of the probe beam, resulting in changes of the probe beam intensity at its axis, which can be sensed by a PIN photodiode connected to a lock-in amplifier (Stanford Research, SR830). The signal from the lock-in amplifier is then recorded, stored and later elaborated by a computer.

For batch mode measurements, a 1 mL cuvette (1 cm path length) (Hellma 101-QS) was used, and was placed at the position of the flow-through cell in the FIA-TLS system.

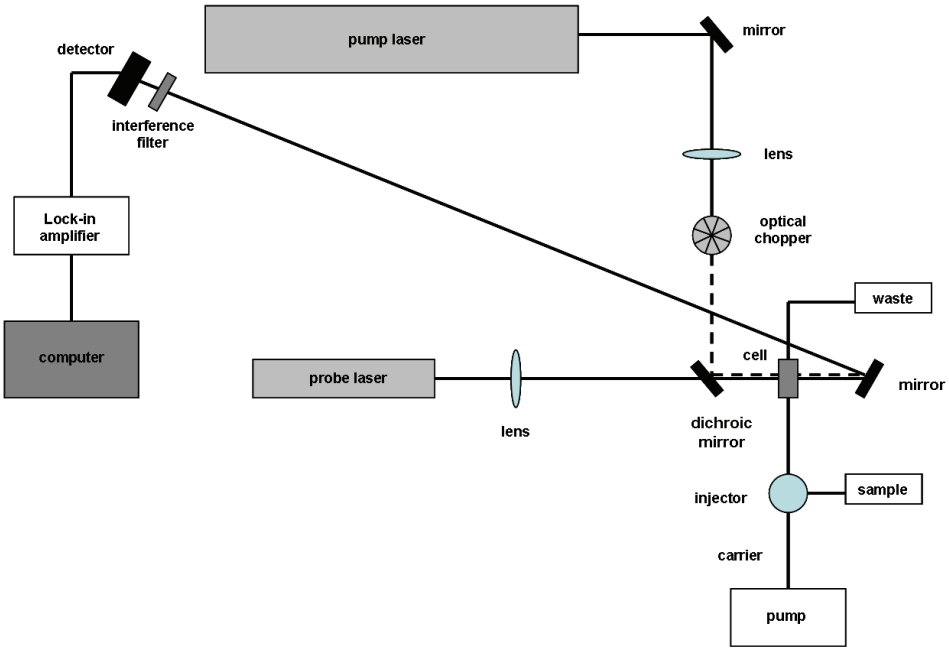

Fig. 3. Experimental set-up for FIA-TLS.

RESULTS AND DISCUSSION

TR-LIF

The fluorescence spectra of Cr-PE (50 μg mL^{-1}) excited at 400 nm are presented in Fig. 4. The spectra exhibit a strong emission maximum around 575 nm, which is expected for this phycoerythrin. Two additional maxima located below and above 575 nm could also be observed. Similar behavior was found for other phycoerythrins.[7,23,24] In order to locate the position of the maximum and their relative contribution in a fluorescence spectrum, the spectra were analyzed by the Gauss peak spectrum (GPS) method[25] for pigment quantification. The method is based on the description of each pigment spectrum by a series of Gaussian peaks. The experimental data were deconvoluted into three emission bands (Fig. 4). A high correlation (R^2 = 0.93) was observed. The three overlapping Gaussians were resolved at 530.2, 575.9 and 592 nm. The two most dominating peak were at 575.9(3) nm (full width at half maximum (FWHM) about 27 nm)

and a shoulder at 592.5(18) nm (FWHM about 115 nm). These components contributed 34.5 and 63.8 % to the total area of the fluorescence emission spectrum, respectively. The third, minor peak at 530.2(18) nm (FWHM about 13 nm) is actually the second harmonic of the laser, not attenuated enough in this measurement.

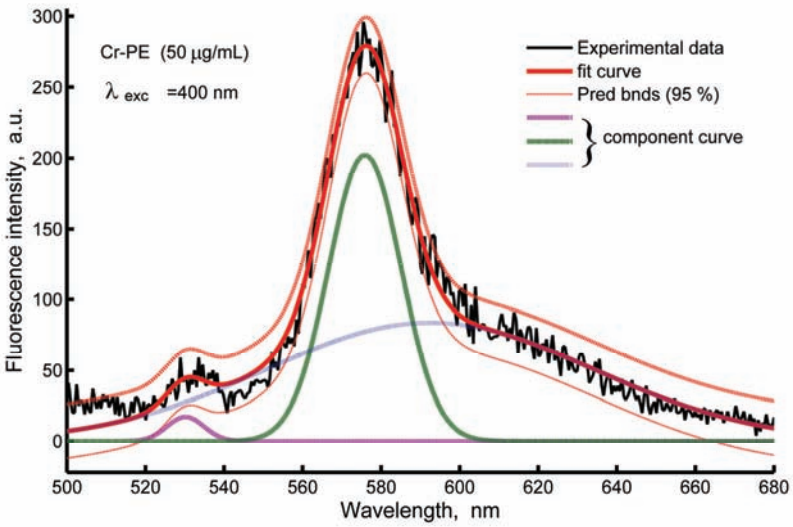

Fig. 4. Emission spectra of Cr-PE (50 µg mL^{-1}) at 400 nm OPO excitation and deconvolution of the emission spectra.

The emission spectrum of the R-PE subunit also showed a maximum at 575 nm and a shoulder at 593 nm.[26] Noticeably, the difference between these two peaks for Cr-PE and R-PE is nearly the same (about 17 nm). Chekalyk and Hafe[27] found a peak at 589 nm for phycoerythrin. An additional fluorescence peak (at 511 nm) before the main peak (555 nm) for PE2b was also detected.[28] The authors indicated that a PE isoform might have been present. The difference between these two fluorescence peaks for PE2b was about 44 nm. A somewhat larger difference between the two peaks was measured for Cr-PE (585 nm and 675 nm for absorption maximum at 550 nm).[15]

In solutions at 25 µg mL^{-1} (diluted in potassium phosphate (KPi), 0.1 M, pH 7), fluorescence spectra were induced by varying the excitation wavelength in 10 nm steps between 340 and 470 nm to obtain the corresponding excitation–emission matrixes (EEMs). The absorption of Cr-PE in this excitation wavelength range is weak. For lower excitation wavelengths (λ_{exc} < 44 nm), an emission maximum appears at about 576 nm and shoulder at about 599 nm. Hence, the fluorescence spectra for 340 ≤ λ_{exc} ≤ 430 nm were deconvoluted into two overlapping Gaussian peaks (Fig. 5), which were resolved at 575.9 nm (FWHM about

34 nm) and 600 nm (FWHM about 109 nm). These components contributed 41 and 59 % to the total area of the fluorescence emission spectrum, respectively. A third peak in the fluorescence spectra appeared at longer excitation wavelengths ($\lambda_{exc} \geq 440$ nm. The position of this peak is at about 520 nm.

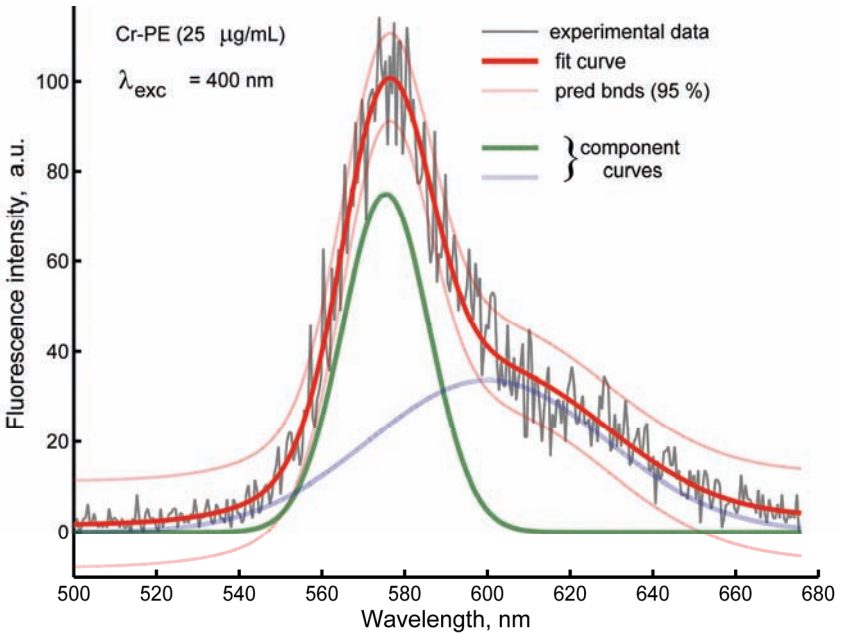

Fig. 5. Emission spectra of Cr-PE (25 µg mL^{-1}) at 400 nm OPO excitation and deconvolution of the emission spectra.

The main advantage of EEMs is that more information about the fluorescent species can be extracted, because the bands arising over a wider area are considered. The three-dimensional EEM of Cr-PE (25 µg mL^{-1}) recorded by measuring the samples within the spectral excitation and emission ranges 340–470 nm and 500–660 nm, respectively, are shown in Fig. 6.

HPD-TA software was applied to determine the fluorescence lifetime of Cr-PE (λ_{exc} = 400 nm, 50 µg mL^{-1}). The best exponential fit deconvoluted with OPO gave a fluorescence lifetime of 2.44 ns. This is in good accordance with literature data for PE, where values in the range of 1.7–4.0 ns were reported.[26]

To the best of our knowledge, the value of the fluorescence quantum yield of Cr-PE has not been published. A simple indirect method was used to determine the fluorescence quantum yield of Cr-PE. Rhodamine B has similar wavelengths for absorption and emission maxima as Cr-PE. The second harmonic of a Nd:YAG laser (532 nm, close enough to absorption maxima of both pigments) was used as an excitation source. An ethanolic solution of rhodamine B and a buffered solu-

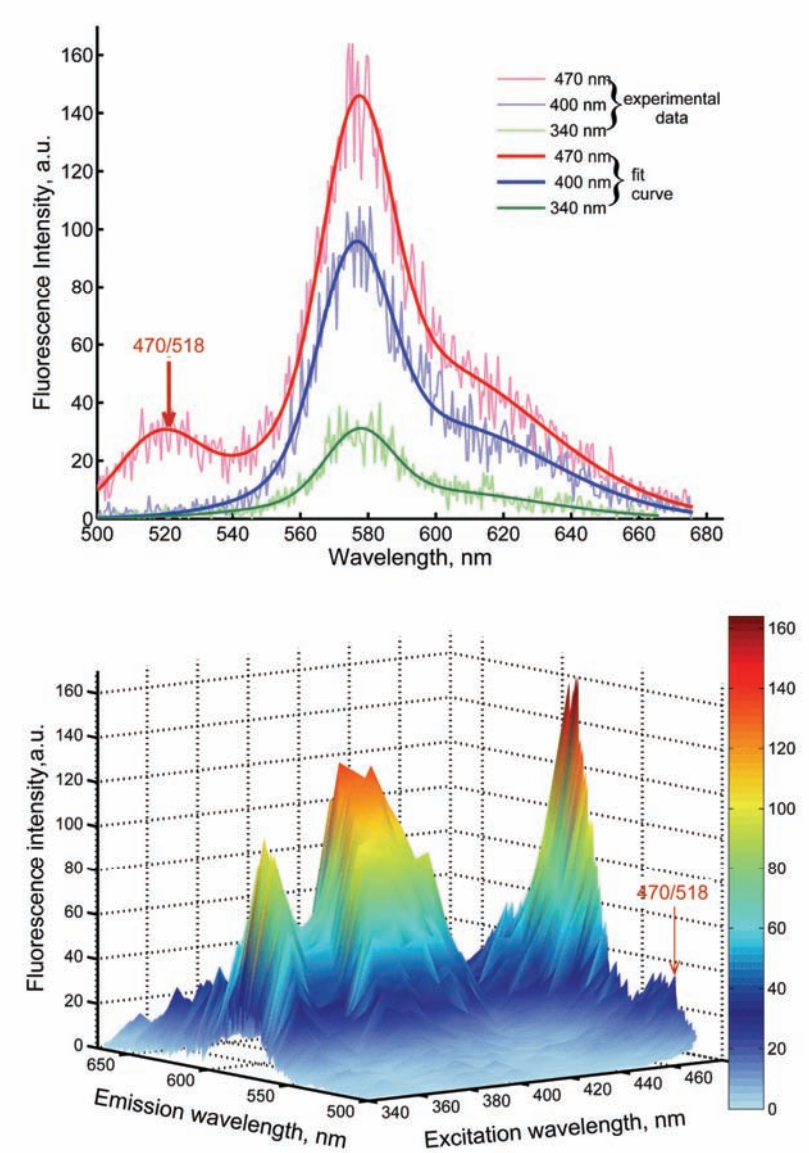

Fig. 6. Emission–excitation spectra of Cr-PE (25 μg mL^{-1}), bottom part. The top part shows the recorded fluorescence spectra, curve fitted spectra at 470, 400 and 340 nm OPO excitation. As an example, the third emission/excitation peak for excitation at 470 nm is marked.

tion of PE were diluted in 10 mm cuvettes until the absorption values measured with an Ocean Optics spectrometer at 532 nm were about 0.2. After that, streak images of both solutions were obtained using 532 nm excitation. The obtained

images are shown in Fig. 7. As can be seen, the streak images of both pigments look very similar, so it seems that an appropriate reference standard for the indirect measurement of the quantum yield of Cr-PE was chosen. Taking into account the value of the quantum yield of an ethanolic rhodamine B solution of 0.7,[29] and comparing the integrated areas of fluorescence on both images, the fluorescence quantum yield of Cr-PE was estimated to be 0.68.

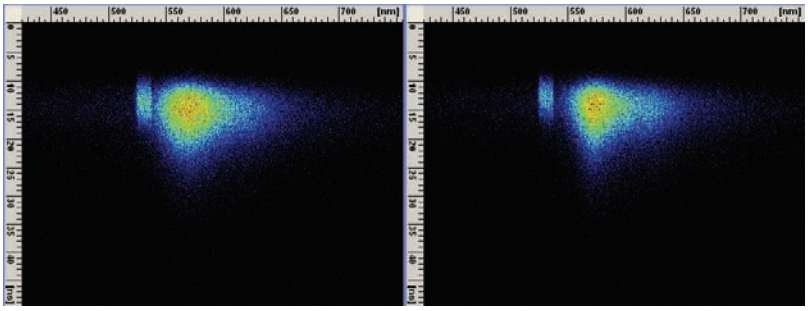

Fig. 7. Streak images of an ethanolic rhodamine B solution (left) and a buffered phycoerithrin solution (right), excitation at 532 nm (second harmonic of the Nd:YAG laser).

Most importantly, by comparison of fluorescence resulting from excitation at selected wavelengths in the two absorption maxima (375 and 550 nm), the ratio of the fluorescence quantum yields for various excitation wavelengths was calculated. For the wavelengths of interest for TLS reachable by the used lasers (458.9, 476.0 nm and 514.5, 406.7 and 413.1 nm) the ratio of the fluorescence quantum yields for excitation at 470 and 410 nm was found to be F_{470}/F_{410} = = 1.6±0.2. Assuming a similar fluorescence quantum yield for Cr-PE when excited at 550 nm as when excited at 532 nm (0.68), it was calculated that the conversion of absorbed energy into heat could be by a factor of 1.8 higher (58 compared to 32 %) when exciting in the 375 nm absorption band (Kr laser emission lines) instead of in the 550 nm absorption band (Ar lasers emission lines). However, the sensitivity of TLS measurement can not be improved due to lower absorbances at the Kr laser lines as compared to the 476 nm ($A_{476.0}/A_{406.7}$ = = 1.86) and particularly the 514.5 nm Ar laser line ($A_{514.5}/A_{406.7}$ = 12.57). It was therefore decided that the Ar laser would be used for excitation in the FIA-TLS system for the detection of phycoerythrin.

FIA-TLS

Batch mode measurements of phycoerythrin showed a considerable degree of photodegradation of the pigment under the intensive irradiation by the tightly focused pump laser beam. As can be observed in Fig. 8, the TLS signal decreases by 0.48 mV over a period of 400 s. Taking into account the 0.22 mV background signal, this represents a 72.4 % decrease of the signal from phycoerythrin. This

can affect the sensitivity as well as the reproducibility of the method, therefore all further measurements were performed in a flow injection mode to reduce the exposure of the pigment to the intense laser light (Fig. 9). The residence time of the sample (8 μL) within the flow through the cell under the given experimental conditions was only 480 ms. Under such conditions, a limit of detection (*LOD*) of 81 μg mL^{-1} was obtained for phycoerythrin.

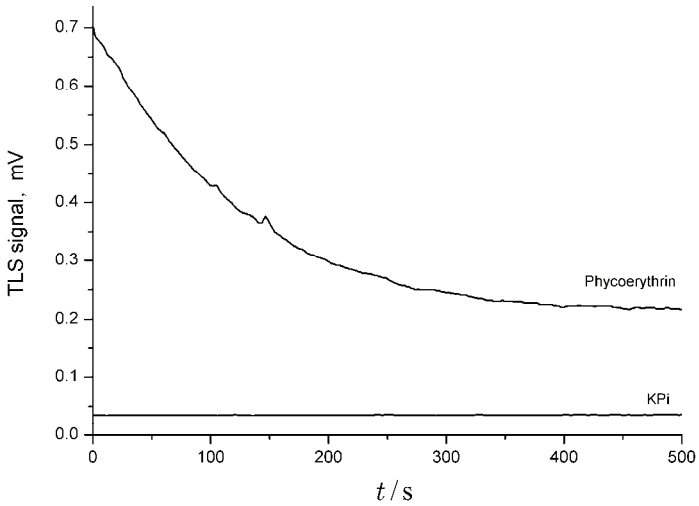

Fig. 8. Batch mode experiments showing photodegradation of phycoerythrin (20 μg mL^{-1}) which resulted in a decrease of the thermal lens signal as opposed to the stable signal of the blank (phosphate buffer-KPi).

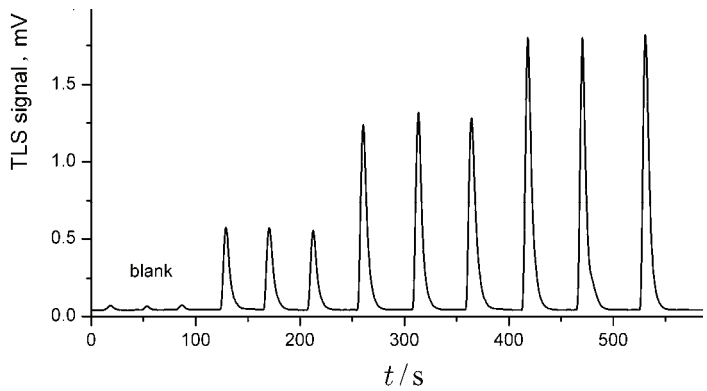

Fig. 9. Thermal lens signal in a flow injection system. Signals corresponding to injection of the blank, and of 5, 10 and 15 μg mL^{-1} of phycoerythrin are shown.

To reduce the loss of absorbed energy and therefore to increase the degree of radiationless de-excitation processes, which are directly related to the magnitude

of thermal lens effect, the effect of KI as a fluorescence quencher was tested. An obvious decrease in fluorescence efficiency was observed (Fig. 10) and a 1.7-fold decrease in the fluorescence intensity at the emission maximum (580 nm, excitation at 540 nm) was calculated. Assuming again that the fluorescence quantum yield was 0.68, it was calculated that 59.5 % of the absorbed energy was converted to heat in the presence of KI. Based on this, an increase in sensitivity of 1.9 times is expected in comparison to phycoerythrin in buffer solution (32 % of the absorbed energy was released as heat). As calculated from the ratio of slopes of calibration curves for phycoerythrin in 0.5 M potassium iodide solution and in buffer (Fig. 11), the sensitivity of phycoerythrin determination by TLS increased by 2.25 times, lowering the *LOD* to 36 μg mL^{-1} which exceeds the predicted increase of 1.9 times by 18 %. This additional increase could be attributed to an increase in the thermal lens enhancement factor (increase in the temperature coefficient of the refractive index and a decrease in thermal conductivity) due to the high concentration of KI, as could be extrapolated from literature data where a 2.6 times enhancement of the thermal lens signal, as compared to water, was reported for 2.5 M KI.[21,30,31] It was also interesting to observe, that the photodegradation of the pigment in batch mode measurements was reduced in the presence of KI (as demonstrated by a constant value of the TLS signal over 75 s, Fig. 8).

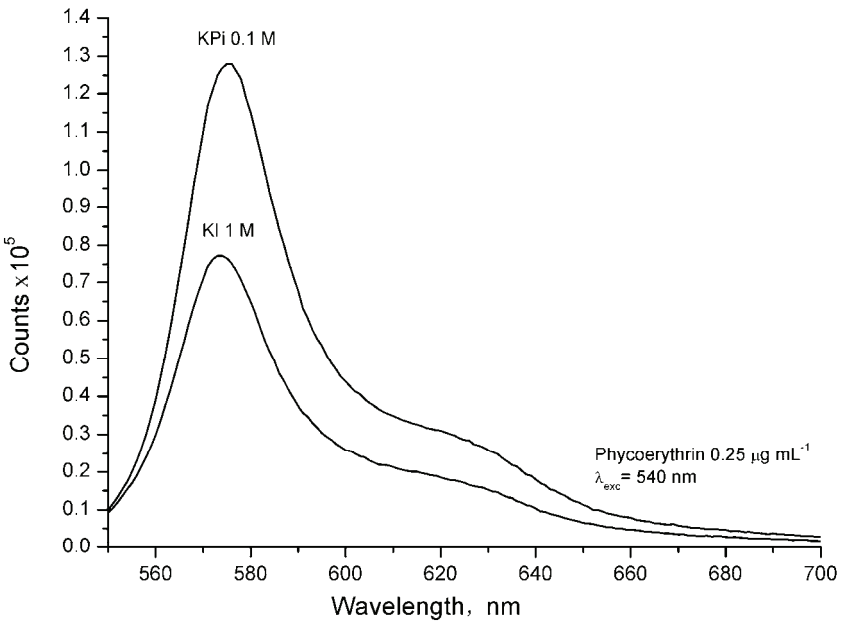

Fig. 10. Fluorescence spectra of Cr-PE in phosphate buffer and in 1 M KI.

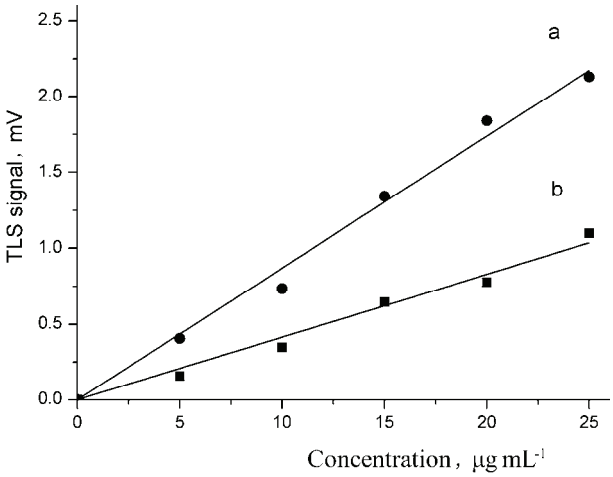

Fig. 11. Calibration curves for phycoerythrin in a) 0.5 M potassium iodide (k = 0.09 VL g^{-1}, R^2 = 0.9846) and b) in phosphate buffer – KPi, 0.1M (k = 0.04 VL g^{-1}, R^2 = 0.9734).

Currently, *LOD*s achieved by FIA-TLS compare favorably with recently reported techniques,[32] which could be used as early warning systems for microcystins and are based on detection of cyanopigments. Taking into account that on average one cyanobacterial cell (60 μm^3) produces about 0.2 pg of microcystin,[33] then 5000 cells mL^{-1} are required to reach the limit of 1 μg L^{-1} for microcystin in water. Using a conservative estimation of 1 % PE in the dry weight of cyanobacterial cells, which can actually reach up to 8 %,[7] and accepting that on average cyanobacteria contain 264 g dry weight μm^{-3},[34] then a comparable concentration of 0.8 μg L^{-1} PE for the maximum contaminant level (*MCL*) of microcystin could be calculated.

As demonstrated in this work, such low concentrations of PE are within the reach of FIA-TLS, which can easily detect early onsets of massive HABs in natural waters, where concentrations of cyanobacteria can peak at up to 1000 times higher levels as required to reach the *MCL* for microcystin.

It is however expected that further improvements in the sensitivity of phycoerythrin detection by TLS could be achieved by using higher concentrations of KI or other compounds for fluorescence quenching and to improve the thermo-optical properties of the analyzed samples, which will be the focus of future research. The achieved *LOD*s already indicate that TLS could represent a powerful tool for the detection of cyanobacteria in water.

CONCLUSIONS

The performances of two laser-based systems: TR-LIF and FIA-TLS for the detection of Cr-PE are presented. The fluorescence and thermal lens signals are complementary. The first one is generated *via* radiation relaxation and the second

via nonradiative relaxation. Hence, the combination of these two laser-based techniques could be suitable for the measurement of various physical quantities of phycobiliproteins with the aim of detecting the presence of cyanobacteria in water.

Acknowledgment. This work was realized within the projects MES RS OI 171020 and COST Action FA0906 "UV-B radiation: A specific regulator of plant growth and food quality in a changing climate (UV4growth)".

ИЗВОД

ИСПИТИВАЊЕ И АНАЛИЗА Cr-ФИКОЕРИТРИНА ИЗ ЦИЈАНОБАКТЕРИЈЕ ЛАСЕРСКИМ ТЕХНИКАМА

БРАТИСЛАВ П. МАРИНКОВИЋ[1], AMBRA DELNERI[2], МАЈА С. РАБАСОВИЋ[1], МИРА ТЕРЗИЋ[3], MLADEN FRANKO[2] и ДРАГУТИН ШЕВИЋ[1]

[1]*Институт за физику, Београд, п. пр. 68, Универзитет у Београду,* [2]*Laboratory for Environmental Research, University of Nova Gorica, Slovenia и* [3]*Природно–математички факултет, Универзитет у Новом Саду*

Представљена је примена метода велике осетљивости – временски разложена ласерски индукована флуоресценција (TR-LIF) и систем за убризгавање у проток са спектрометријом термичког сочива (FIA-TLS) – на анализу Cr-фикоеритрина (Cr-PE) изолованог из посебне врсте цијанобактерије. У опсегу побуде таласних дужина (340–470 nm) флуоресцентни спектри показују изразит максимум на 575 nm. Запажен је такође и други максимум, на око 600 nm. Добијени резултати се користе за проверу техничких параметара за употребљену технику термичког сочива. Она је комплементарна спектрофлуорометрији, са мањом осетљивошћу у случају високих флуоресцентних квантних приноса и фотолабилних једињења.

(Примљено 17. априла 2013)

REFERENCES

1. M. E. van Apeldoorn, H. P. van Egmond, G. J. A. Speijers, G. J. I. Bakker, *Mol. Nutr. Food Res.* **51** (2007) 7
2. W. W. Carmichael, S. M. Azevedo, J. S. An, R. J. Molica, E. M. Jochimsen, S. Lau, K. L. Rinehart, G. R. Shaw, G. K. Eaglesham, *Environ. Health Perspect.* **109** (2001) 663
3. J. Pablo, S. A. Banack, P. A. Cox, T. E. Johnson, S. Papapetropoulos, W. G. Bradley, A. Buck, D. C. Mash, *Acta Neurol. Scand.* **120** (2009) 216
4. K. G. Sellner, G. J. Doucette, G. J. Kirkpatrick, *J. Ind. Microbiol. Biotechnol.* **30** (2003) 383
5. K. D. Hambright, T. Zohary, *Limnol. Oceanogr.* **45** (2000) 110
6. *Guidelines for drinking-water quality*, 4th ed., World Health Organization, Geneva, 2011
7. H. Rodriguez, J. Rivas, M. G. Guerrero, M. Losada, *Appl. Environ. Microbiol.* **55** (1989) 758
8. D. C. Nguyen, R. A. Keller, J. H. Jett, J. C. Martin, *Anal. Chem.* **59** (1987) 2158
9. S. Sekar, M. Chandramohan, *J. Appl. Phycol*, **20** (2008) 113
10. M. N. Kronick, *J. Immunol. Methods* **92** (1986) 1
11. W. G. Telford, M. W. Moss, J. P. Morseman, F. C. T. Allnutt, *Cytometry* **44** (2001) 16

12. D. A. McCarthy, in *Flow Cytometry: Principles and applications*, M. G. Macey Ed., Humana Press, Totowa, 2007, p. 78
13. M. B. Zrimec, J. Kožar Logar, A. Zrimec, L. Drinovec, M. Franko, A. Malej, *Cent. Eur. J. Biol.* **4** (2009) 313
14. J. K. Logar, A. Malej, M. Franko, *Instrum. Sci. Technol.* **34** (2006) 23
15. D. R. A. Hill, K. S. Rowan, *Phycologia* **28** (1989) 455
16. G. Novarino, *Hydrobiologia* **502** (2003) 225
17. P. Sanchez-Baracaldo, N. A. El Semary, P. K. Hayes, in *Unravelling the algae: the past, present, and future of algal systematics*, J. Brodie, J. Lewis, Eds., CTC Press, New York, 2007, Ch. 5, p. 93
18. L. Sun, S. Wang, L. Chen, X. Gong, *IEEE J. Selec*t. *Top. Quant. Electron*. **9** (2003) 177
19. M. Franko, C. D. Tran, *Rev. Sci. Instrum.* **67** (1996) 1
20. J. L. Lakowicz, *Principles of fluorescence spectroscopy*, 3rd ed., Springer, Berlin, 2006
21. M. Franko, *Appl. Spectrosc. Rev*. **43** (2008) 358
22. M. S. Rabasović, D. Sević, M. Terzić, B. P. Marinković, *Nucl. Instrum. Meth., B* **279** (2012) 16
23. C. H. Mazel, *Mar. Ecol. Prog. Ser.* **120** (1995) 185
24. H. M. Shapiro, *Practical flow cytometry*, 4th ed., Wiley-Liss, Hoboken, New York, 2003
25. H. Küpper, S. Seibert, P. Aravind, *Anal. Chem*. **79** (2007) 7611
26. J. Sepúlveda-Ugarte, J. E. Brunet, A. R. Matamala, J. Martínez-Oyanedel, M. Bunster, *J. Photochem. Photobiol., A* **219** (2011) 211
27. A. Chekalyuk, M. Hafe, *Limnol. Oceanogr-Meth.* **6** (2008) 591
28. H. Küpper, E. Andresen, S. Wiegert, M. Šimek, B. Leitenmaier, I. Šetlík, *Biochim. Biophys. Acta, Bioenerg.* **1787** (2009) 155
29. F. López Arbeloa, I. Urrecha Aguirresacona, I. López Arbelo, *Chem. Phys*. **130** (1989) 371
30. S. E. Bialkowski, *Photothermal Spectroscopy Methods for Chemical Analysis*, Wiley, New York, 1996
31. C. D. Tran, M. Franko, in *Encyclopedia of Analytical Chemistry*, R. A. Meyers, Ed., Wiley, Chichester, 2010
32. W. Schmidt, H. Petzoldt, K. Bornmann, L. Imhof, C. Moldaenke, *Water Sci. Technol*. **59** (2009) 1531
33. W. Hoogenboezem, A. J. Wagenvoort, K. Blaauboer, *The occurrence of toxic cyanobacteria in some Dutch surface waters used for the production of drinking water*, Vereniging van Rivierwaterbedrijven (RIWA), Nieuwegein, 2004
34. D. M. Mahlmann, J. Jahnke, P. Loosen, *Eur. J. Phycol*. **43** (2008) 355.