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Combined use of chlorophyll a and phycocyanin fluorescence sensors for quantification and differentiation of phytoplankton: a useful approach for small surface water bodies

Kombinirana uporaba klorofilnega in fikocianinskega senzorja fluorescence za kvantifikacijo in kvalifikacijo fitoplanktona: uporaba v majhnih vodnih telesih

Tinkara Rozina[a], Tina Eleršek[b], Maja Zupančič Justin[c], Andrej Meglič[d*]

[a] Envit d.o.o., Vojkova cesta 63, 1000 Ljubljana, Slovenia
[b] Department of Genetic Toxicology and Cancer Biology, National Institute of Biology, Večna pot 111, 1000 Ljubljana, Slovenia
[c] Arhel d.o.o., Pustovrhova ulica 15, 1000 Ljubljana, Slovenia
[d] Biotechnical Faculty, University of Ljubljana, Večna pot 111, 1000 Ljubljana,

*correspondence: andrej.meglic@bf.uni-lj.si

Abstract: Sensors based on *in vivo* measurements of photosynthetic pigments fluorescence enable real-time phytoplankton monitoring with high spatial and temporal resolution. A combination of chlorophyll a (CHL) and phycocyanin (PC) fluorescence sensors was used for phytoplankton quantification and differentiation in two small water bodies, Koseze Pond and pond in Hotinja vas. The high correlation of CHL and PC fluorescence signals with biovolume was confirmed during the two-year monitoring in a natural pond environment in spite of a seasonal succession of the phytoplankton. Additionally, disturbances of the sensors were investigated. Water bodies containing predominantly algae yielded false positive signals of the PC sensor, which reached up to 1% of the intensity of the CHL signal. Similarly, underestimated counts of cyanobacteria measured with CHL fluorescence sensor can be adjusted using PC fluorescence sensor.

Keywords: small water bodies, algae, cyanobacteria, fluorescence sensors, biovolume

Izvleček: Senzorji za merjenje *in vivo* fluorescence fotosintetskih pigmentov omogočajo meritve fitoplanktona z visoko časovno in prostorsko ločljivostjo. Kombinacijo klorofilnega (CHL) in fikocianinskega (PC) senzorja smo uporabili za kvantitativno in kvalitativno spremljanje fitoplanktona v dveh majhnih vodnih telesih, Koseškem bajerju in ribniku v Hotinji vasi. Kljub spreminjanju vrstne sestave je ostala korelacija med signalom CHL senzorja in biovolumnom visoka. Opredelili smo tudi napake senzorjev. V primeru vodnih teles z visoko koncentracijo zelenih alg je PC senzor dajal lažen pozitiven signal v velikosti 1 % signal CHL senzorja. S kombinacijo obeh senzorjev je mogoče odpraviti tudi podcenitev koncentracije cianobakterij, do katere pride ob uporabi le CHL senzorja.

Ključne besede: mala vodna telesa, alge, cianobakterije, senzorji fluorescence, biovolumen

Introduction

Phytoplankton determination is according to EU Water Framework Directive (Directive 2000/60/EC) compulsory for the evaluation of ecological water status. Spectrophotometric analysis following extraction with organic solvents provides good estimates of the total chlorophyll *a* (CHL). However, CHL as an indicator of the total phytoplankton biovolume does not enable distinguishing between different groups of phytoplankton, for example between algae and cyanobacteria. The later are problematic due to formation of biologically active and often toxic substances and therefore deserve special attention and faster identification. Because of specific migration patterns on a daily, seasonal and weather-induced basis, occasionally taken samples in traditional monitoring approaches may give misleading results of phytoplankton and cyanobacteria population (Walsby et al. 2004).

Monitoring of the presence of toxic cyanobacteria became compulsory with the Bathing Water Directive 2006/7/EC. However, the Directive only requires a control of the presence of cyanotoxins. Monitoring sites with other recreational activities than bathing are not included in the Directive (Brient 2008). Among them are small lakes, ponds and urban water bodies covering only a few hectares or less. Despite their prevalence and rich biological diversity, they have received little attention in the EU Water Framework Directive (Directive 2000/60/EU, Biggs et al. 1999, Oertli et al. 2002, Williams et al. 2003). They are subjected to increasing negative environmental impacts, such as stormwater nutrient and contaminant loading. One of the greatest concerns in such water bodies are cyanobacterial blooms, leading to unpleasant odours and occasional animal poisoning (Sedmak et al. 1994, Lürling and Faassen 2013). In such sites, faster and simple monitoring methods are needed, giving real-time results on a detailed spatial and temporal scale.

One of the characteristics of cyanobacteria are accessory photosynthetic pigment. With their help, the available light can be used more efficiently than other phytoplankton organisms (Raps et al. 1983). In fresh waters, cyanobacteria are the only organisms producing significant amounts of PC (Wetzel 2001). In the marine environment, PC is also present in Cryptophyceae and Rodophyceae (Wetzel 2001).

In recent years, advances in technology allow fluorometric measurements *in situ*. Submersible PC fluorescence sensors enable quantitative evaluation of cyanobacteria abundance in freshwater bodies (Kong et al. 2013, Kasinak et al. 2015, Zamyadi et al. 2014). Field probes measuring the *in-vivo* fluorescence of CHL and PC may present an interesting approach for fast detection of changes in the planktonic population (Bastien et al. 2011, Seppälä et al. 2007).

For proper use of fluorescence field sensors in water monitoring, their limitations should be considered. The signal can be affected by different factors such as water turbidity, uneven distribution of cyanobacteria due to the formation of colonies, the presence of biological and mineral particles in the water and the growth status of cyanobacteria and algae (Chang et al. 2012, McQuaid et al. 2011, Zamyadi et al. 2012). Errors can also occur if sensors are used without proper calibration (Bowling et al. 2012, Song et al. 2013).

In this study, we weekly measured CHL and PC fluorescence in Koseze Pond and compared the results with phytoplankton biovolume – a combination that gave the highest uniformity in the previous study (Rozina et al. 2017). Due to the negligible occurrence of cyanobacteria we took additional samples of water from the pond in Hotinja vas with a predominant population of cyanobacteria and also measured some laboratory algae and cyanobacteria cultures. We consider the necessity of simultaneous measurements with PC and CHL sensors to avoid the underestimation or overestimation of cyanobacterial biomass.

Materials and methods

Sampling sites

Koseze Pond is a small, shallow artificial water body used also as a fishpond, situated in Ljubljana, Slovenia (46°04` 02.33``N; 14°28` 07.78``E) and is a part of the recreational area. The pond was selected due to the occasional presence of cyanobacteria in the past. It has an approximately 37,000 m^2 surface area and 55,000 m^3 volume. Maximum depth is 3 m. Water samples were collected weekly

from beginning of May to the end of September in 2014 and 2015 from the middle of the pond. As the preliminary tests showed even distribution of phytoplankton on different locations and depths of the pond, grab water samples were collected from a depth of 30 cm. Samples were kept in plastic containers and measured immediately after arrival in the laboratory.

The pond in Hotinja vas (46° 28` 03.85``N; 15° 40` 39.67``E) is a small, shallow water body situated in the centre of the village in the eastern part of Slovenia, with a 9,300 m^2 surface area, not deeper than one meter and used also as a fishpond. Untreated surrounding sewage is occasionally discharged into the water body leading to regular large-scale occurrences of cyanobacteria. Sampling was performed four times in summer 2015. Both water bodies are not included in the national monitoring.

In addition to environmental samples, we also measured laboratory cultures of green algae and cyanobacteria. We used *Desmodesmus communis* 276-4b, *Chlorella vulgaris* 211-11b ans *Arthrospira platensis* 85.79 from the SAG collection (Goettingen, Germany) and *Microcystis aeruginosa* PCC 7806 from the Institute Pasteur (Paris, France). All laboratory cultures were grown at room temperature and maintained under axenic conditions in 100 mL flasks in 50 mL Jaworski's medium exposed to natural daylight.

Fluorescence measurements

For fluorescence measurement a portable KM 245 water quality flow-through chamber (Arhel, Slovenia) fitted with CHL and PC fluorescent sensors (Cyclops 7, Turner, U.S.A.) were used. The sensors were installed in a black, non-reflective measuring chamber, equipped with a brush that automatically cleaned the lenses of the sensors (Fig. 1). The CHL sensor excites CHL at 465 ± 85 nm and measures emission at 696 ± 22 nm while the PC sensor excites the cyanobacterial PC below 595 nm and measures fluorescence emission above 630 nm. To prevent interferences optimal distance from the walls of the chamber and between both sensors was determined in a series of tests.

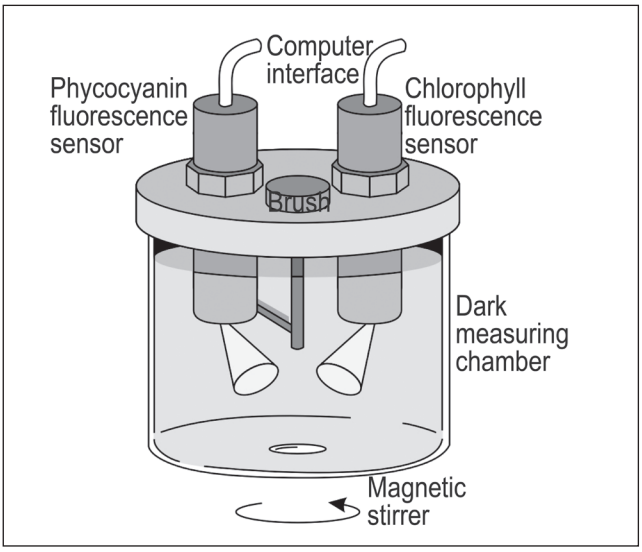

Figure 1: Portable KM 245 water quality system (Arhel, Slovenia) with dark measuring chamber fitted with CHL and PC fluorescent sensors, magnetic stirrer, brush and interface to personal computer, which serves as data logger and data display.

Slika 1: Prenosni sistem KM 245 za nadzor kvalitete vode (Arhel, Slovenija). V črni merilni komori sta CHL in PC senzor fluorescence, magnetno mešalo, metlica in povezava z osebnim računalnikom, ki je namenjen shranjevanju in prikazovanju podatkov.

For experimental purposes, 800 mL of water sample was poured into the measuring chamber. Settling was prevented using a magnetic stirrer. Data sampling frequency was set to 4.5 Hz. Each sample was measured for 5 minutes, and the average signal was calculated. The results were presented in relative units (r.u.) corresponding the voltage output of the sensor. The same water sample was used for biovolume and taxonomic determination.

Phytoplankton quantification and taxonomic determination

Environmental samples were concentrated in 100 mL glass cylinders (Hydro Bios, Germany) under darkness for 24 hours. Plankton was taxonomically determined under an Eclipse TE300 inverted microscope (Nikon, Japan). Material fixed in 4% (v/v) formalin was used for counting and biovolume calculations. Depending on the phytoplankton composition and concentration, a Nageotte (Assistent, Germany) or Sedgewick-Rafter (PhycoTech, U.S.A.) counting chamber was used. The biovolume of phytoplankton culture was calculated from the average biovolume of individual cells, taking into account the geometry of the cells (Hillebrand et al., 1999). General procedures for the use of sedimentation chambers, preservation and storage of samples and evaluation of cell density were followed according to the European standard (CEN EN 15204, 2006). Algal species were identified following the keys and articles of John et al. (2002), Komárek and Anagnostidis (2000, 2005) and Hindak (1976).

Results

Correlation between CHL fluorescence and biovolume

Measurements of CHL and PC fluorescence, expressed in relative units (r.u.), were compared to total biovolume. The seasonal dynamic of phytoplankton in the Koseze pond was followed from the beginning of May to the end of September in 2014 and 2015 (Fig. 2). The peak of the phytoplankton density was in August 2014 and 2015. In later the phytoplankton development was slightly slower and reached lower pick values. The CHL fluorescence followed the biovolume (Fig. 2).

Throughout the entire 2014 and 2015 monitoring period, the measurements of CHL fluorescence showed a high correlation (r=0.95, n=21 for 2014 and r=0.92, n=18) with phytoplankton biovolume (Fig. 3). Moreover, most of the measurements from 2015 fall into the prediction band made for 2014.

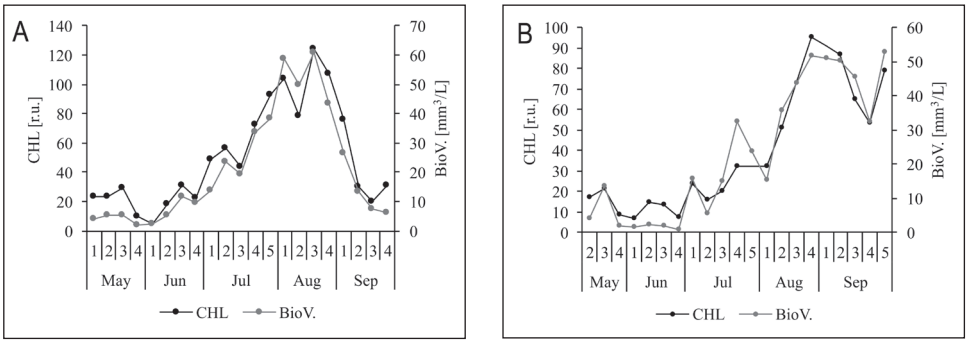

Figure 2: Seasonal changes of chlorophyll (CHL) fluorescence and total phytoplankton biovolume (BioV) in Koseze Pond from the beginning of May to the end of September 2014 (A) and 2015 (B). Numbers on x-axis indicate week of the month.

Slika 2: Sezonsko spreminjanje fluorescence CHL in celokupnega biovolumna fitoplanktona (BioV.) v Koseškem bajerju med začetkom maja in koncem septembra 2014 (A) in 2015 (B). Številke na x osi označujejo tedne v mesecu.

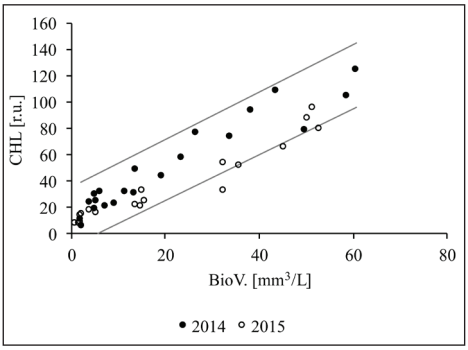

Figure 3: Chlorophyll (CHL) fluorescence as a function of total phytoplankton biovolume (BioV). Black circles represent samples taken from Koseze Pond in season 2014 and hollow circles samples from the 2015 season. Grey lines indicate prediction band of 2014 measurements.

Slika 3: Fluorescenca klorofila (CHL) kot funkcija celokupnega biovolumna fitoplanktona (BioV.). Črni polni krogi predstavljajo vzorce Koseškega bajerja iz sezone 2014, prazni krogi pa iz sezone 2015. Sivi črti označujeta predikcijski interval meritev iz 2014.

The correlation between CHL fluorescence and total biovolume in the pond remained high during both observation periods (Fig. 3), in spite of the phytoplankton seasonal succession (Fig. 4). In the 2014 season, a significant change in species composition occurred in the second week of July, with a growing abundance of filamentous green algae *Planctonema lauterbornii* (*Trebouxiophyceae*) (Fig. 4A). This species dominated in the 2015 summer season as well (Fig. 4B).

The taxonomic analyses from 2014 and 2015 showed that the cyanobacteria population barely reached 2% of the total biovolume (average 0.45%, range 0.04-2.82% for 2014 and average 0.41%, range 0.01-2.0% for 2015). In addition, values from the phycocyanin sensor were also low, most of the time below 1% of the CHL fluorescence indicating almost complete absence of cyanobacteria in the pond. Monthly taxonomic analysis have shown the presence of turbulent and the absence of colonial species of cyanobacteria.

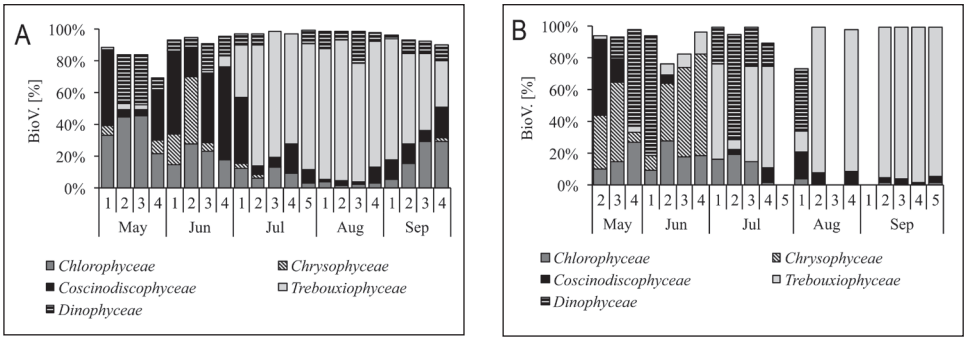

Figure 4: Seasonal succession of the biovolume (BioV) in Koseze pond in 2014 (A) and 2015 (B). Data are represented as a relative share of five main phytoplankton classes. The class *Trebouxiophyceae* was represented by the species *Planctonema lauterbornii.*

Slika 4: Sezonsko spreminjanje biovolumna (BioV.) v Koseškem bajerju leta 2014 (A) in 2015 (B). Podatki so predstavljeni kot relativen delež petih najpogostejših razredov fitoplanktona. Razred *Trebouxiophyceae* je zastopala vrsta *Planctonema lauterbornii.*

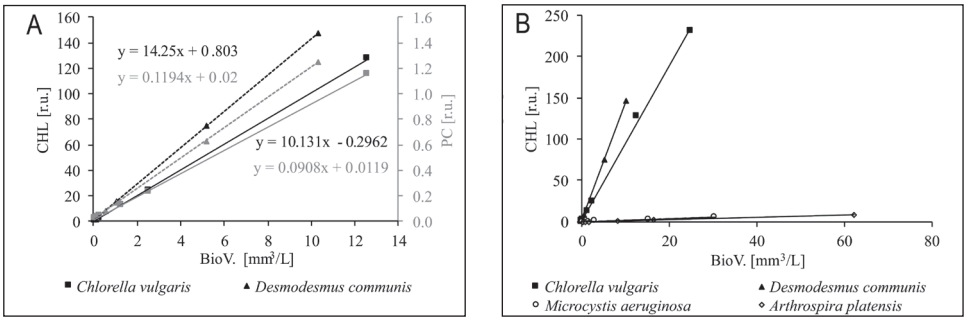

Figure 5: Laboratory cultures. (A) Phycocyanian (PC) - grey lines and chlorophyll (CHL) fluorescence - black lines - of green algae *Desmodesmus communis* and *Chlorella vulgaris* as a function of cell biovolume. The ratio between PC and CHL fluorescence signals is approximately 1:100. (B) Relationship between CHL fluorescence and biovolume (BioV.) of four axenic laboratory cultures – green algae *Desmodesmus communis* and *Chlorella vulgaris* and cyanobacteria *Microcystis aeruginosa* and *Arthrospira platensis*. The suspension of each culture was gradually diluted to 1000 cells/ml.

Slika 5: Laboratorijske kulture. (A) Fluorescenca fikocianina (PC) – sive črte in klorofila (CHL) – črne črte pri algah vrste *Desmodesmus communis* in *Chlorella vulgaris* v odvisnosti od biovolumna. Razmerje med fluorescenco PC in CHL je približno 1:100. (B) Odvisnost fluorescence CHL od biovolumna (BioV.) štirih akseničnih laboratorijskih kultur – zelenih alg vrste *Desmodesmus communis* in *Chlorella vulgaris* ter cianobakterij vrste *Microcystis aeruginosa* in *Arthrospira platensis*. Suspenzija vsake kulture je bila postopoma redčena na 1000 celic/ml.

CHL and PC fluorescence in cyanobacterial reach pond

Four water samples from the pond in Hotinja vas with cyanobacteria biovolume ranging from 53 and 81 percent were analysed. Although the correlation between CHL fluorescence and biovolume remained high (r=0.96) the slope of the linear fit was much lower than in the case of Koseze Pond (0.6 compared to 1.6). The correlation between the PC fluorescence signal and cyanobacteria biovolume was high (r=0.95) although higher number of samples should be collected to confirm the significance (Fig. 5).

Discussion

Linear relationship and high correlation between fluorescence and biovolume have been confirmed in many studies. However, most of the studies with PC and CHL fluorescence sensors were made using laboratory phytoplankton cultures and only a few were made using environmental samples (Loisa 2015, Kong 2013, Izydorczyk 2009).

In the case of Koseze Pond, the measurements of CHL fluorescence showed a high correlation with phytoplankton biovolume throughout the entire 2014 and 2015 monitoring period. Calibration curve from 2014 could be used for 2015 samples, although, for a more accurate calculation of the biovolume, calibrations in the environmental water sample should be repeated every season. The fluorescence system therefore constitutes the upgrade to the current monitoring system, providing information of phytoplankton dynamics in real time. The correlation stayed high despite the changes in the phytoplankton composition. Different species composition should also result in different slope of a linear fit because the concentration of CHL depends on phytoplankton species. This can be explained by the adaptation of the pigment concentration of the phytoplankton organisms according to environmental light conditions and was also confirmed by Gregor et al. (2007) with measurements performed at different lake depths. In our case, all the samples were taken from the same pond location and depth. Organisms in a small turbid shallow water body, as in our case, were exposed to the same environmental condi-

tions. It can be concluded that due to comparable ecological niches, the *in-vivo* CHL fluorescence of various species was similar and the correlation with biovolume remained high. This additionally argues in favour of fluorescent measurements and their application for biovolume quantification. Results of fluorescence measurement on green algae (laboratory cultures) have showed low PC signal although green algae do not contain PC (Fig. 5a). Gregor et al. (2007) also reported in their study that falsely positive signals of the PC sensor at a high eukaryotic algae presence in water might occur. There was about 10-11 fold higher PC signal response from cyanobacteria than algae and vice versa, a much higher CHL signal response received from eukaryotic algae than cyanobacteria in their study. In the measurements with our equipment and settings, the PC signal of the green algae represented only 1% of their CHL signal. Therefore, the ratio between the CHL and PC signals should be taken as an important criterion in the evaluation of the cyanobacteria presence. By using both PC and the CHL sensors during on-site monitoring, we can avoid overestimation of the presence of cyanobacteria.

Long-term observations in Koseze Pond, confirmed that in the case of a parallel increase of PC and CHL fluorescence signals, with a 100 times lower PC signal than the CHL one, the real presence of cyanobacteria is probably negligible and the value of the PC signal shows only interferences.

An opposite problem is found in water bodies with the prevailing cyanobacteria population (Hotinja vas fishpond). Despite their high biomass, cyanobacteria have a low CHL fluorescence response (Figure 5b) that can be explained by a weak absorption of blue light (Campbell et al. 1998, Gregor et al. 2007). In contrast to green algae where the antenna pigments that harvest light for photosystem II are CHL, accessory chlorophylls and carotenoids (Green and Durnford 1996), cyanobacterial photosystem II contains only 10 to 20 % of the total CHL (Bryant 1986). The major part of cyanobacterial CHL is located in photosystem I, which does not dissipate energy through fluorescence. The measurement of the PC fluorescence is, therefore, a better approach to cyanobacterial biovolume determination compared to CHL fluorescence measurements as this prevents cyanobacteria underestimation.

Performed measurements demonstrated the need for simultaneous measurements with PC and CHL fluorescence sensors to accurately quantify cyanobacteria and green algae in mixed populations.

Conclusion

The study demonstrates that seasonal phytoplankton development and the presence of cyanobacteria can be monitored with combined use of PC and CHL fluorescence sensors. Correlation between CHL fluorescence and biovolume in remained stable even after significant changes in the phytoplankton species composition and allowed relatively accurate quantification of cell biovolume. This reduces the need for standard analyses and allows qualitative differentiation of phytoplankton with a reliable prediction of cyanobacteria even in small ponds and lakes with quickly changing biovolume and species composition. False positive signals of the PC sensor in the case of the predominant eukaryotic algal population in the water body, reaching up to 1% of the intensity of the CHL signal, as it was the case with our instruments and settings, could be recognised and disregarded. Similarly, underestimated counts of cyanobacteria measured with CHL fluorescence sensor can also be adjusted using PC fluorescence sensor.

Povzetek

Cvetenje cianobakterij predstavlja izjemno tveganje za okolje. Njihovo zgodnje odkrivanje in nadzorovanje je zato ključnega pomena za upravljanje z vodnimi telesi. Standardne metode spremljanja fitoplanktona imajo številne omejitve, zato je potrebna nadgradnja z metodami, ki dajejo rezultate v realnem času. V našem delu smo pokazali, da lahko z uporabo CHL in PC senzorja fluorescence določimo koncentracijo alg in cianobakterij v vodnem telesu. Meritve fluorescence CHL na Koseškem bajerju so pokazale visoke korelacije z biovolumnom fitoplanktona skozi celotno dvoletno obdobje izvajanja meritev kljub spremembam v vrstni sestavi. Opredelili smo tudi napake senzorjev. V primeru vodnih teles z visoko koncentracijo zelenih alg je PC

senzor dajal lažen pozitiven signal v velikosti 1 % signala CHL senzorja. Po drugi strani pa pride do podcenitve koncentracij cianobakterij ob uporabi le CHL senzorja. Ker so cianobakterije in zelene alge prisotne v istih okoljih je upoštevanje te napake pri meritvah bistvenega pomena. S sočasno uporabo CHL in PC S sočasno uporabo CHL in PC senzorja se lahko izognemo tako podcenitvi kot precenitvi številčnosti cianobakterijske populacije.

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