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REAL-TIME PHYTOPLANKTON QUANTIFICATION USING CHLOROPHYLL A AND PHYCOCYANIN FLUORESCENCE SENSORS

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SUMMARY: The management of fresh water bodies imposes to evaluate the presence of contaminants of both biological and anthropogenic origin and phytoplankton monitoring as the key element in the assessment of environmental water quality. Poor water quality and consequent cyanobacterial blooms represent a remarkable environmental risk with negative economic effects. Cyanobacteria are of an exceptionally high concern due to their ability of massive toxin production. Their early detection and prevention of bloom formation is of paramount importance. With the implementation of the Bathing water directive cyanobacterial monitoring becomes compulsory in the European Union. The project Innovative technology for cyanobacterial bloom control "LIFE Stop CyanoBloom" is a demonstration project co-financed by LIFE Environment financial mechanism of the European Commission. The aim of the project is a demonstration of remotely controlled vessel equipped with *on-line* sensors and embedded electrolytic cell for simple and effective determination of defined physical, chemical and biological parameters and prevention of cyanobacterial proliferation. The results obtained by using our fluorometric detection system showed high correlation with the data obtained by traditional methods with several advantages such as low costs of operation, high spatial and temporal resolution and real time information on the changes in plankton populations.

KEY WORDS: Harmful bloom, cyanobacterial bloom, fluorescence sensor, *in vivo* fluorescence, phytoplankton, bacterioplankton.

KVANTIFICIRANJE FITOPLANKTONA U REALNOM VREMENU UZ KORIŠTENJE FLUORESCENTNIH SENZORA ZA KLOROFIL A I FIKOCIJANIN

SAŽETAK: Upravljanje slatkovodnim tijelima nameće ocjenu prisutnosti kontaminirajućih tvari biološkog i antropogenog porijekla i monitoring fitoplanktona kao ključni element ocjene ekološkog stanja vode. Loša kakvoća vode i, kao posljedica toga, cvjetanje cijanobakterija predstavljaju velik rizik za okoliš s negativnim gospodarskim učincima. Cijanobakterije su iznimno veliki problem zbog njihove sposobnosti proizvodnje velikih količina toksina. Njihovo rano otkrivanje i sprječavanje pojave cvjetanje su od najveće važnosti.

Provođenjem Direktive o kakvoći vode za kupanje monitoring cijanobakterija postaje obvezan za EU. Projekt Inovativna tehnologija za kontrolu cvjetanja cijanobakterija "LIFE Stop CyanoBloom" je pilot projekt koji sufinancira LIFE, financijski instrument Europske komisije namijenjen aktivnostima zemalja EU u područjima zaštite okoliša. Cilj projekta je demonstriranje plovila na daljinsko upravljanje opremljenog *online* senzorima i s ugrađenom elektrolitskom jedinicom za jednostavno i učinkovito utvrđivanje definiranih fizičkih, kemijskih i bioloških pokazatelja i sprječavanje razmnožavanja cijanobakterija. Rezultati dobiveni primjenom našeg sustava za fluorometrijsku detekciju pokazali su veliku korelaciju s podacima dobivenim tradicionalnim metodama, uz nekolicinu prednosti poput niskih troškova rada, velike prostorne i vremenske rezolucije i informacija o promjenama planktonskih populacija u realnom vremenu.

KLJUČNE RIJEČI: štetno cvjetanje mora, cvjetanje cijanobakterija, fluorescentni senzor, *in vivo* fluorescencija, fitoplankton, bakterijski plankton.

1. INTRODUCTION

Phytoplankton monitoring is one of the key elements of water quality assessment and is essential in determining ecological status of lakes according to Water framework directive (Directive 2000/60/EC). Traditional methods of phytoplankton monitoring include the assessment of Secchi disk water transparency, phytoplankton sampling, species composition, taxonomic classification, biomass and chlorophyll *a* (Chl *a*) determination. Laboratory standardisation of Chl *a* determination and simplicity of Secchi disk use enabled the development of standards of general water trophic state (Caspers 1984). The microscopic counting and taxonomic classification give very accurate data on species composition and their biovolume in samples. However, there are several limitations of these approaches. Laboratory analyses are time consuming and highly trained personal is needed for species determination. Such monitoring therefore results in high costs, with the results that reflect only a current situation at the sampling site. Because of specific migration patterns and specific occurrence of cyanobacteria on a daily, seasonal and weather-induced basis, occasionally taken samples may give misleading results, underestimate or even overlook the presence of harmful cyanobacteria (Walsby *et al., 2004*).

Faster and simple monitoring methods are therefore needed, giving real-time results on a detailed spatial and temporal scale. Monitoring should reflect the continuous dynamics of plankton migration. One of such simple *in vivo* techniques is the use of *in vivo* fluorometry based on direct measurements of the fluorescence of photosynthetic and other accessory pigments of autotrophic planktonic organisms (Richardson *et al*., 2010). While Chl *a* is a primary photosynthetic pigment present in all photosynthetic organisms, specific accessory pigments are associated only to certain groups of algae and cyanobacteria. Cyanobacteria contain accessory pigments from the phycobiliprotein family, among which phycocyanin (PC) is predominant. Chl *a* and PC have a strong fluorescence response at different wavelengths of the visible part of the light spectrum, which enables differentiation between both groups of organisms with simultaneous estimation of their concentration (Gregor and Maršalek 2004; Gregor *et al*., 2007). The method enables processing of a large quantity of data on a real-time scale and therefore faster reaction

in the case of cyanobacterial exponential growth. Simultaneous implementation of Chl *a* and PC fluorescence sensors enables the distinction between phytoplankton and bacterioplankton.

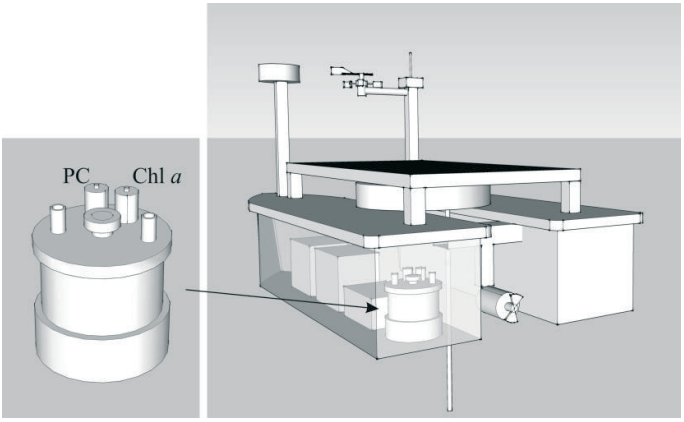

Figure 1. Flow-through chamber fitted with Chl a and PC sensors (left) and its position in autonomous vessel (right) (Arhel Ltd., Slovenia).

With the aim to improve phytoplankton monitoring techniques and demonstrate an efficient control of harmful cyanobacterial blooms, a demonstration project "LIFE Stop CyanoBloom" has been supported by the European Commission. The aim of the project is to present our autonomous remotely controlled vessel equipped with advanced on-line sensors and a device for suppressing the development of cyanobacterial blooms that is based on electrochemical principles. Two autonomous surface vessels (ASV) developed by Arhel Ltd. will be in operation on two Slovenian water bodies, Koseze pond in Ljubljana (Stop CyanoBloom 1) and Lake Bled (Stop CyanoBloom 2).
The goal of our first field trials was to validate the use of flow-through sensing chamber equipped with fluorescence sensors in the natural environment using the portable suitcase version of the sensing system. These activities enabled the upgrade of previous laboratory calibration experiments that were performed on single strain algal and cyanobacterial cultures, with data from the natural environment. These experiments were the first steps of the interpretation of obtained fluorescent signals for phytoplankton presence and structure determination. The transfer of the measuring chamber on board the autonomous vessel as a recessed version enables the implementation of multiple measurements that give us an excellent spatiotemporal resolution, which is not possible to achieve using traditional phytoplankton monitoring approaches.

2. MATERIALS AND METHODS

2.1. Site description

Sampling site 1: fishpond Koseze former clay pit (Latitude 46°4´2.51´´N, Longitude 14°28´7.92´´E, 305 m altitude, 37000 m^2, 55000 m^3, 3 m max depth).
Sampling site 2: Lake Bled (Latitude 46°21´50.15´´N, Longitude 14°5´40.09´´E, 475 m altitude, 1.43 km^2, 25.7 10^6 m^3, 30 m max depth).

2.2. Water sampling

Vertical profile of the water column has been sampled on the Lake Bled. The sampling station was located on the west side of the island. In fluorescence analysis experiment the water was pumped directly into the sensing chamber. Profiles were measured on 15th and 30th of September 2014 and on 29th of October 2014. The strategy of one sampling point in the middle of the water body has been applied on Koseze fishpond. Water samples were collected from the depth of 30 cm. The pond water was monitored on a weekly basis from May 2014 to end of September 2014. Sampling was conducted between 10 a.m. and 2 p.m..

2.3. Determination of chlorophyll a and phycocyanin

Chlorophyll a was extracted and the concentrations determined spectrophotometrically according to ISO 10260 (2001) (extraction with 90 % ethanol and determination at 665 and 750 nm) while PC was extracted according to the modified protocol from the intercalibration testing coordinated by Yéprémian (personal communication) with freezing-thawing-sonication procedure followed by spectrophotometric determination at 565, 620, 650 and 750 nm. Samples were prepared and measured in triplicate. The results were calculated according to SIST ISO 10260 (2001) and Lee at al. (1994) for Chl *a* and PC, respectively.

2.4. Taxonomic analysis

Samples were analysed with the use of an inverted microscope for phytoplankton and bacterioplankton species composition and abundance (John *et al*., 2002). All analyses were performed on the same water samples including fluorescence measurements.

2.5. Sensory device description

The portable KM 245 water quality system (Arhel Ltd., Slovenia) is equipped with Chl *a* and PC fluorescent sensors (Cyclops7, Turner, U.S.A.) in a flow-through dark chamber and with data transfer module. The system consists of a pump that delivers environmental water to the sensing chamber and a photovoltaic module with battery for undisturbed use also in field conditions. The sensors are equipped with a self-cleaning device. The PC probe excites the cyanobacterial PC at 595 nm and measures the fluorescence emission at 650 nm, whereas Chl *a* sensor excitation is at 460 nm and the emission measured at 685 nm. The KM 245 system is interconnected with RS232 interface to a personal computer using software for data transfer. The results are presented as relative units (r.u.).

2.6. Quantitative sensory testing

The samples from Koseze fishpond were kept in plastic containers and brought to the laboratory. The recordings of fluorescence were performed immediately after delivery. The samples were transferred into the sensing chamber and fluorescence recorded. For the recordings at the location of Lake Bled, the water was pumped directly into the chamber from different depths.

The sensory unit as it is in the portable water quality system KM 245 (Arhel, Slovenia) is also built-in the autonomous vessel for further demonstration purposes in the framework

of LIFE Stop CyanoBloom project. In situ fluorescence data measured during the field operations and data from laboratory measurements were compared with conventional sampling and taxonomic analysis.

3. RESULTS AND DISCUSSION

Toxic cyanobacterial blooms are a common phenomenon in the surface waters of Slovenia and Microcystis aeruginosa is the most frequent bloom forming cyanobacteria in eutrophic and hypertrophic water bodies. This is mainly due to intensive agricultural activities in our country (Sedmak and Kosi, 1997). The fishpond Koseze was the site of regular presence of cyanobacterial blooms until restoration measures in 2010. One of the reasons for choosing this fishpond for our field experiments is the likelihood of the possibility of repopulation by cyanobacteria. The radical clearing of foreshore and aquatic vegetation that plays an important role in self-purification of the water could favour the reappearance of cyanobacterial blooms. In 2014 the taxonomic analysis showed a predominance of phytoplankton with cyanobacteria barely reaching 1 % of the biomass (average 0.44 %, range 0.04-2.82 %). Traditional biomass determination that includes phytoplankton counting and the measurement of single representatives is time-consuming, laborious and therefore expensive. In spite of all, cell counting is not always accurate especially when the cell concentrations are below 105 cells/ml. Planktonic Chl *a* is recognized as a parameter of choice for describing water quality (Water framework directive, 2000/60/EC). It is also an important parameter in the OECD's (Organisation for Economic Cooperation and Development) eutrophication modelling approach. However, the amount of Chl *a* extracted from phytoplankton also depends on a variety of factors, including the variability of photobiont genera and species presence and most important of these, on extraction efficiencies. In our experiments the use of fluorescence sensors shows an excellent correlation (r^2=0.9678) between Chl *a* extraction and measurements with Chl *a* fluorescence *in vivo* (Figure 2). Because of the negligible presence of cyanobacteria the data from the PC sensor were very low and in the range of backscattering almost all the time of the field experiments.

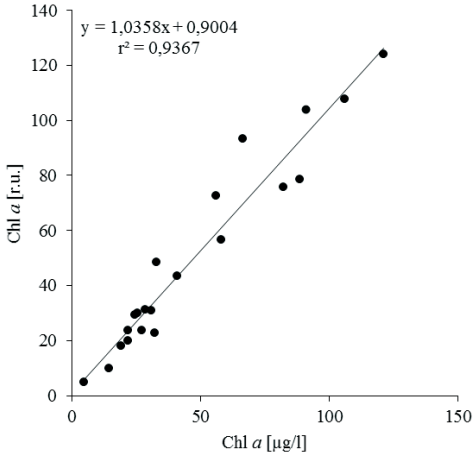

Figure 2. Correlation between the amounts of extracted chlorophyll and chlorophyll fluorescence determined in vivo in water samples from the fishpond Koseze.

Lake Bled in Slovenia is one of the few natural lakes in our country. It is a popular tourist destination what has become a heavy burden to the lake water environment. This relatively deep lake with characteristic thermal stratification is the site of regular cyanobacterial blooms (Vrhovšek *et al*., 1982; Sedmak and Kosi, 1997). Species that usually prevail because of their high proliferation are the filamentous Planktothrix rubescens (Oscillatoria rubescens has been redefined as Planktothrix rubescens), Anabaena flos-aquae, Aphanizomenon flos-aquae and occasionally representatives of the genus Microcystis spp. (Vrhovšek *et al*., 1982; Vrhovšek *et al*., 1985; Sedmak and Kosi, 1991; Sedmak and Kosi, 1998; Sedmak and Kosi, 2002).

Regular and persistent cyanobacterial blooms were the main reason for restoration measures applied to the lake in the past. The artificial inflow of Radovna River, the application of a modified Imboden model for hypolimnetic outflow as well as the reconstruction of the sewage system in the 1980s significantly improved the lake water quality (Imboden and Gächter 1978; Rismal, 1980; Vrhovšek *et al*., 1985). Also other sub-alpine lakes such as Lake Bourget (France), Lake Garda (Italy) and Lake Bodensee (Germany/Swizerland/Austria) that are major tourist attractions and constitute important drinking water resources suffer from the presence of cyanobacterial blooms (Salmasso *et al*., 2013; Jacquet, 2005). Programmes to restore the ecological health of the Lake Bourget to reduce nutrient loads and pollution in the lake have been implemented over the past two decades (Jacquet *et al*., 2005). Deep sub-alpine lakes have in common the regular presence of *Planktothrix rubescens* blooms as well as the production of [D-Asp^3] microcystin-RR as the major hepatotoxin (Grach-Pogrebinsky *et al*., 2003; Briand *et al*., 2005). Inspite of all restoration measures cyanobacteria blooms are still persistent on described locations.

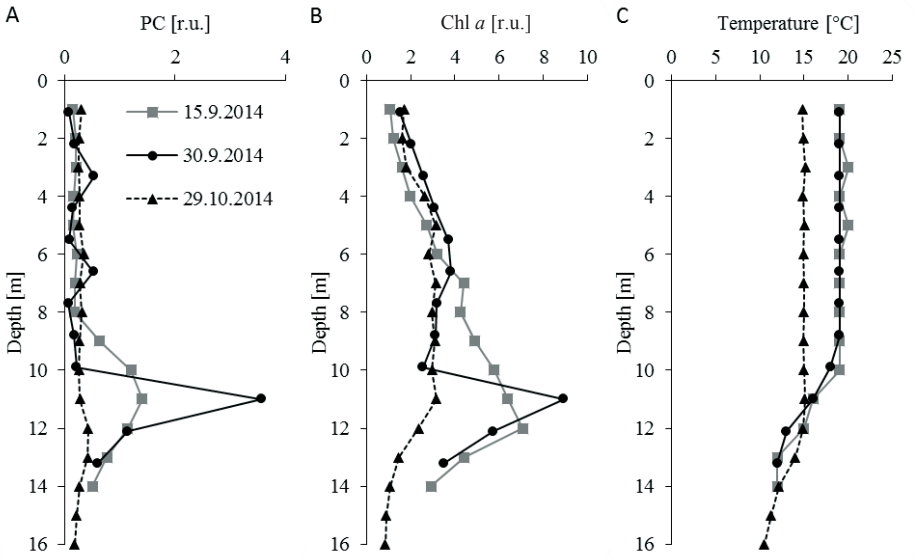

Figure 3. Phyto- and bacterioplankton distribution during the stratified period and during the transition to homogeneously mixed vertical water column. Panel A phycocyanin sensor readings, Panel B chlorophyll a sensor readings and Panel C temperature.

Typical dimictic subalpine lakes like Lake Bled mix from the surface to bottom twice a year in spring and autumn (overturn) and stratify in summer and in winter. These dynamic events strongly affect the distribution of the plankton community. To additionally complicate the situation the depth of the mixed layer (Zmix) additionally exhibits micro stratification that may change at scales of minutes rather than days or weeks (Coloso *et al*., 2011).

Autonomous mobile robotics has recently gained interest all over the world. As an example, scientists of the limnological station in Kilchberg Switzerland developed the autonomous surface vessel equipped with YSI 6600 multiparameter sensor for surveying toxic cyanobacterial blooms on the lake of Zurich (Hitz *et al*., 2012).

Real time detection of phyto- and bacterioplankton in Lake Bled was possible as the result of productive interdisciplinary cooperation. Cyanobacterial bloom is clearly detectable in the metalimnion during the stratification period and is distributed throughout the water column during the water mixing periods (Figure 3). At this point we are integrating high resolution data from fluorescence sensors with point measurements.

CONCLUSION

For modelling the Lake Bled ecosystem large amounts of data is needed due to system complexity and constantly changing patterns. The study of the planktonic community using conventional methods only, does not meet the needs of modern approaches. Observations of both, the microbial variables and the corresponding environmental parameters, are limited in resolution when obtained by conventional methods. Microorganisms like cyanobacteria that possess their own buoyancy mechanism, function at different spatiotemporal scales than other plankton organisms. Automated sensing technologies are therefore the only available technology able to collect large amounts of accurate and reliable data. So far, most systems for automated data acquisition are marine- and to a lesser extent freshwater stationary buoys that are not designed for gathering information of horizontal plankton distribution in lakes.

We present our new designed ASV that allows sampling at high spatiotemporal resolution and real time data transfer. It is equipped with two fluorescence sensors (Cyclops7, USA) in specially designed sensing chamber. Preliminary results from six months monitoring data on the fishpond Koseze show the seasonal dynamics and significant correlation in spite the phytoplankton seasonal succession. With the simultaneous application of Chl *a* and PC fluorescence sensors we were able to follow the succession of the phytoplankton community in subalpine Lake Bled during the autumn overturn).

Our main goal is to understand and control the cyanobacterial community for optimal performance in sanitation measures.

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