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Nikodem SZYMAŃSKI[1], Piotr DĄBROWSKI[2], Magdalena ZABOCHNICKA-ŚWIĄTEK[3], Balaji PANCHAL[4], Detlev LOHSE[5], Hazem M. KALAJI[6]

[1]Falenty Institute of Technology and Life Sciences (ITP)
[2]Department of Environment Improvement, Warsaw University of Life Sciences – SGGW
[3] Institute of Environmental Engineering, Czestochowa University of Technology
[4]Nurture Earth R and D Pvt. Ltd.
[5]bbe Moldaenke GmbH
[6]Department of Plant Physiology, Warsaw University of Life Sciences – SGGW; Falenty Institute of Technology and Life Sciences (ITP)

Taxonomic classification of algae by the use of chlorophyll *a* fluorescence

Key words: AlgaTorch, algal bloom, FluoroProbe, water quality

Introduction

Lakes and reservoirs are abundant natural systems that provide fundamental ecosystem services to human populations, such as water production, fish farming, recreational activities and patrimonial value (Downing et al., 2006). In the last decades, these water bodies have suffered from rapid eutrophication (Søndergaard & Jeppesen, 2007). In response to the degradation of water resources, the European Community has expressed its will to ensure the sustainable use of both marine and freshwater ecosystems through the UE Water Framework Directive – WFD (Directive 2000/60/EC). Degraded quality of water, especially eutrophication, promote the development and persistence of algal blooms (Lenzi et al., 2015). Eutrophication is mainly caused by high concentrations of biogens (nitrogen, phosphorus, potassium) that could be found in contaminated water bodies. Knowledge of algal species composition and their population dynamics is necessary for understanding the development of algal blooms (Catherine et al., 2012). Algae can assimilate biogens in the processes of photosynthesis and biosorption (Zabochnicka-Świątek, 2017). A number of algal species can be grown in darkness on organic substrates (heterotrophy) and growth in light on

carbon dioxide (autotrophy) (Kim, Park, Cho & Hwang, 2013; Krzemińska, Pawlik-Skowrońska, Trzcińska & Tys, 2014; Zabochnicka-Świątek, 2015). The iron--rich water promotes the growth of algae (Concas, Steriti, Pisu & Cao, 2014).

There are a few traditional group--specific assessments of microalgae, such as microscopy or high performance liquid chromatography – HPLC (Wong & Wong, 2003; Larson & Passy, 2005). These methods are often labor-intense, time-consuming and the samples must be collected before obtaining the results. Most importantly, these traditional methods cannot provide real-time and *in situ* measurements due to the time lag between sample collection and sample analysis. It is difficult to obtain fine spatial and temporal dynamics of microalgae because samples are often integrative by nature and enumeration of samples are time-consuming (Edgar & Laird, 1993).

Chlorophyll fluorescence analysis offers an alternative approach and potentially allows *in situ* estimate of algal concentration. In this work the fluorometric differentiation of algal population was presented.

The concept of fluorometric differentiation of algal population

The concept is based on insights on energy transfer and fluorescence emission of photosynthetic organisms (Beutler, Wiltshire, Meyer, Moldaenke & Dau, 1998). For algae and cyanobacteria, the FCh is mainly emitted by chlorophyll *a* of photosystem II (PSII), which consists of an evolutionary conserved chlorophyll *a*-containing core and species-dependent peripheral antenna. In Figure 1, A_{peri} (λ_{ML}) and A_{core} (λ_{ML}) represent the absorption of the peripheral and core antennae, respectively. The species-dependent A_{peri} (λ_{ML}) affects both the chlorophyll fluorescence excitation spectrum.

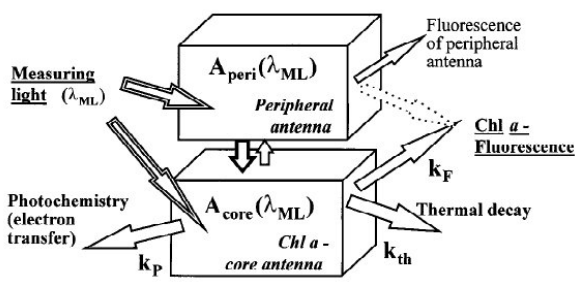

FIGURE 1. General model for PSII antenna systems. A_{peri} (λ_{ML}) and A_{core} (λ_{ML}) denote the absorption cross-section of the peripheral and core antennae, respectively (Dau, 1994)

Algal color is a useful taxonomic criterion and algal taxonomic classes differ significantly in their fluorescence excitation spectrum. Each of the five spectral algal classes is characterized by similar fluorescence excitation spectra resulting from the composition of their peripheral antenna (the Table).

In the green class, the peripheral antenna contains chlorophyll *a*, chlorophyll *b*

TABLE. Spectral class of microalgae (van den Hoek, Mann & Jahns, 1995)

Spectral class	Pheripheral antenna	Division
Green	chlorophyll *a*/*b* (carotenoids)	Chlorophyta
Blue	phycobilisomes (phycocyanin)	Cyanobacteria
Brown	chlorophyll *a*/*c*	Glaucophyta Heterokontophyta Haptophyta Haptophyta Dinophyta
Red	phycobilisomes (phycoerythrin)	Rhodophyta (some Cyanobacteria)
Mixed	chlorophyll *a*/*c* (phycobiliprotein)	Cryptophyta

and xanthophyll. In the blue class, phycobilisomes function as peripheral antennae and contain mainly phycocyanin. The members of the brown class contain chlorophyll *a*, chlorophyll *c* and xanthophylls (fucoxanthin, fucoxanthin derivates or peridinin). The peripheral antennae of the red class are composed of phycobilisomes, as in the blue class. But the phycobiliprotein phycoerythrin dominates in the red class instead of the phycocyanin. The mixed class has a combination of chlorophyll *a* and chlorophyll *c* with one phycobiliprotein that can be either phycoerythrin or phycocyanin. Here, just the phycoerythrin-containing members of the mixed class are considered.

A laboratory-based instrument – the AlgaeLabAnalyser – and a submersible fluorometer – the FluoroProbe – were developed by bbe Moldaenke GmbH, Germany, for a rapid measurement of the chlorophyll *a* fluorescence intensities excited at five distinct wavelengths. The evaluation of the chlorophyll concentration associated with individual algal class is based on a fit of the measured spectra by so-called norm curves.

In order to measure the norm spectra and to carry out laboratory and *in vivo* experiments a bench-top fluorometer was set up as shown in Figure 2, which illustrates the function of the fluorometer. The following wavelengths of light-emitting diodes (LEDs) were employed for excitation of pigment complexes: 370, 450, 525, 570, 590 and 610 nm, at light intensities at 3–7 $\mu mol \cdot m^{-2} \cdot s^{-1}$, respectively. For excitation, the LEDs are switched on sequentially at a frequency of 5 kHz. The measuring pulse duration is 0.1 ms. Chlorophyll fluorescence (ChF) emitted by the algal suspension is detected with a Hamamatsu photomultiplier in combination with a band pass filter. The signal was digitized by an AD converter and processed by an internal microcontroller. A glass cuvette filled with 25 ml of algal suspension was placed in the measuring chamber (Beutler et al., 2002b).

Depth profiles obtained from *in situ* experiments were recorded with the submersible probe sketched in Figure 3 and briefly described below. The instrument set-up of Figure 3 was mounted inside a carbon enforced tube. A light shield in the form of an outer tube prevents the incidence of direct sunlight. Measurement of water pressure enables calculation of the submersion depth. Sample water flows across the measuring cavity

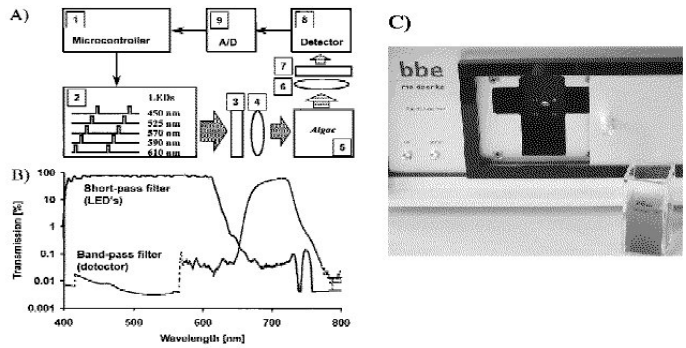

FIGURE 2. The AlgaeLabAnalyser fluorometer: (A) components, (B) transmission spectra of optical filter, (C) housing. In A: (1) microcontroller; (2) light-emitting diodes; (3) short-pass filter to block red and IR emission (see B); (4) focusing lens (f = 25 mm); (5) sample volume containing algal suspension; (6) focusing lens; (7) band-pass filter (see B); (8) integrated photomultiplier; (9) 12-bit AD converter (conversion rate 100 kHz). (B) Transmission spectra of the short-pass filter (3) in A and the band-pass filter (7) in A

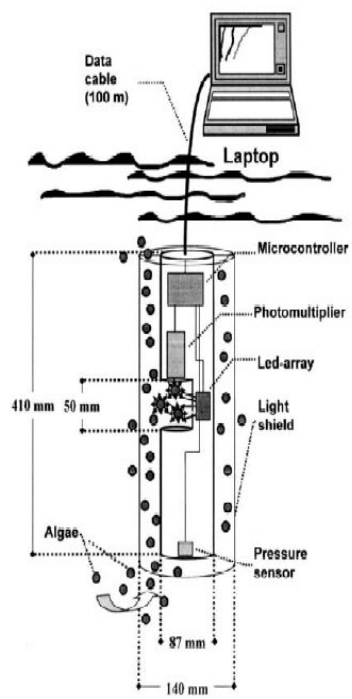

FIGURE 3. The submersible FluoroProbe. All electronic and optical components depicted in Figure 2 are enclosed in a carbon enforced housing with an open measuring cavity (50 mm height) as indicated. The stainless-steel housing is encased in a black polyethylene cylinder (diameter of 140 mm), open at the bottom and top, serving as a light shield. Data are transferred on-line via a RS 485 interface and cable to a personal computer, on board a research vessel. The measured pressure (piezo sensor) is used to calculate the actual water depth; deconvolution of the data is done with the connected PC

when the instrument is lowered into the water. The recommended vertical velocity of the probe during depth-profiling is 30 $cm \cdot s^{-1}$.

A selection of cultured microalgae provided the information about the spectral features of the four distinguishable algal divisions (Beutler et al., 2002b): Green spectral group (*Chlorella vulgaris*), blue spectral group (*Synechococcus leopoliensis*), brown spectral group (*Cyclotella* sp.), and mixed spectral group. A fifths class of fluorescence excitation was implemented due to the interference of "Yellow Substances" (YS) which is used to compensate the influence of YS on chlorophyll analysis.

The *in vivo* method is based on the analysis of chlorophyll fluorescence from PSII measured around 685 nm at physiological temperatures. For chlorophyll *a*-containing photoautotrophs, the mathematical approach is based on the assumption of a constant photosystem II / photosystem I (PSII : PSI) ratio; a constant fluorescence excitation spectra (independent, of the physiological status of the cells and the individual strain within the class) and a linear independence of the normalized algal spectra. Beutler et al. (2002a) could give good evidence for reliable norm spectra and the linear relationship in a series of dilution experiments. Figure 4 displays the norm spectra of the individual algae classes.

The fluorometer determines the emission intensity of a natural sample on LED excitation with the internal photomultiplier. All data are converted and analysed on the basis of algal class norm spectra. The five-point mixed excitation spectra of the sample are deconvoluted by internal CPU calculating applying a least square fitting model by use of the norm spectra. By means of the deconvolution approach, for each spectral algal

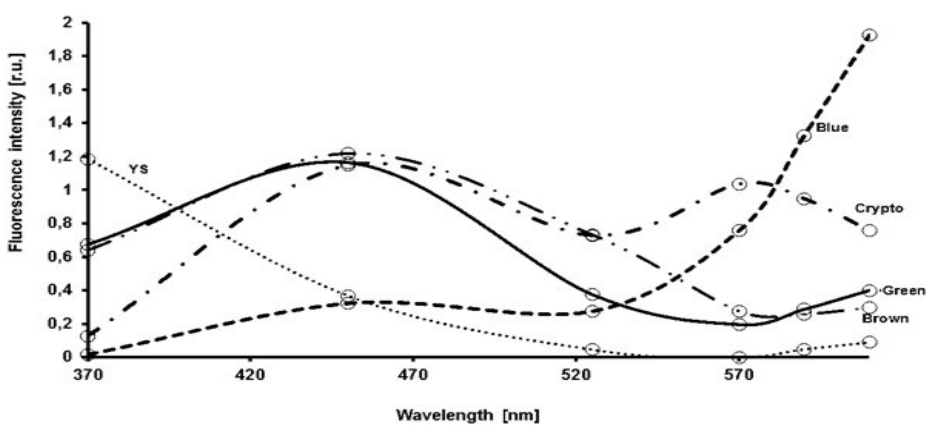

FIGURE 4. Normalized excitation spectra for four spectral algal class (norm spectra) and "Yellow Substances" (YS). Excitation spectra are determined for several species per spectral algal class from different divisions. Data points shown are mean values. The measured fluorescence intensities are normalized to the chlorophyll content and the light intensity of the respective LED. The spectra from members of each spectral algal class are averaged. Units on the y-axis: digitized photomultiplier voltage (digits) per measuring light intensity [$\mu mol \cdot m^{-2} \cdot s^{-1}$] and chlorophyll concentration of the algal sample [$\mu g \cdot l^{-1}$] (Beutler et al., 2002a)

class an estimate of the corresponding chlorophyll *a* concentration is obtained (μg of chlorophyll *a* per l l per spectral algal class in the measuring volume).

For the calibration of the fluorometers following algal cultures were employed: green class, *Chlorella vulgaris*; blue class, *Microcystis aeruginosa*; brown spectral class, *Cyclotella meneghiniana*; mixed spectral class, *Cryptomonas* sp.

Some examples of fluorometric application

A submersible fluorescent probe (FluoroProbe) was configured and used to survey the vertical distribution of the deep-living toxic and filamentous cyanobacterium *Planktothrix rubescens* among the autochthonous algal communities in Lake Bourget, France (Leboulanger et al., 2002). The *in situ* measuring spectrofluorometer provided a realistic estimation of the abundance and dynamics of the cyanobacterial population that is known to produce the hepatotoxic heptapeptides microcystin RR and LR. Data provided from in-line measurements using the probe and from *P. rubescens* cell counts obtained by discrete sampling were closely correlated ($r = 0.899$, $P < 0.01$). A survey conducted from December 1999 to May 2001 revealed that *P. rubescens* exhibits a deep maximum level (typically 10–15 m) in spring and summer (reaching concentrations up to 20 μg equivalent chlorophyll *a* per 1 l, i.e. 27,000 cells per 1 ml), whereas it spreads from the surface either to the top of the thermocline or to the bottom of the lake, in autumn and winter respectively. The probe could be used as a powerful tool for assaying the occurrence and dynamics of microalgal blooms, typically toxic cyanobacteria that call for accurate and rapid monitoring to assess the health of the ecosystem and to alert the authorities about potential risks regarding pumping and use of the lake water for drinking water.

In order to improve the water quality monitoring program developed for the Danube Delta, during 2008 a new system was tested for assessing the development of phytoplankton biomass (Török, 2009). Data were obtained by the use of a submersible spectrofluorometer at sampling sites along the main branches of the Danube River. Assessment of water quality using bbe FluoroProbe for algae differentiation allowed a fast and reliable detection of phytoplankton in the Danube Delta Biosphere Reserve. This enables to fulfil the requirements of the EU Water Framework Directive for at least four sampling dates per year. Application is suitable for an efficient and conclusive classification of phytoplankton in reophillic ecosystems of the Danube Delta.

The suitability of using the FluoroProbe for monitoring the dynamics of the haptophyte *Phaeocystis globosa* in the coastal waters of the eastern English Channel was examined (Houliez, Lizon, Thyssen, Artigas & Schmitt, 2012). The FluoroProbe was recalibrated by recording a new fingerprint for *P. globosa*. The new fingerprint was tested through a series of laboratory and *in situ* experiments. The annual dynamics of *P. globose* estimated using the FluoroProbe and by flow cytometry were similar. A strong relationship was found between the FluoroProbe estimates of *P. globosa*

biomass expressed in terms of chlorophyll *a* and flow cytometric cell counts ($r = 0.889$, $P < 0.001$). It is important to note that the detection of *P. globosa* at the species level was possible in the eastern English Channel because it was the only haptophyte species present with a biomass sufficient to be detected by the FluoroProbe. In areas where several haptophyte species are simultaneously present, their discrimination will be impossible. In such situations, the FluoroProbe can be used to monitor the dynamics of the combined haptophyte group.

As microscope analysis of phytoplankton does not allow for high frequency (spatial and/or temporal) data acquisition, fluorescence-based approaches that use selective excitation of pigment antennae have spread rapidly. The ability of spectral fluorescence to provide accurate estimates of phytoplankton biomass and composition is still under debate. An extensive data collection from samples of the Ile-de-France region, North Central France, was used to assess the ability of the bbe Moldaenke FluoroProbe to estimate phytoplankton community composition in lakes and reservoirs (Catherine et al., 2012). FluoroProbe data yields better estimates of total phytoplankton biovolume than do spectrophotometric chlorophyll *a* measures and that FluoroProbe data can be further corrected using the average chlorophyll *a* to biovolume ratio among phytoplankton groups. Overall, group-specific relationships between FluoroProbe and biovolume data were consistent. For euglenophytes the variation in photosynthetic apparatuses in response to changing environmental conditions affects the biovolume determination. The misattribution toward the "red" group of phycoerythrin-containing cyanobacteria requires correcting procedures to improve the data quality. Strong scattering in the relationship between the FluoroProbe vs. biovolume of the "blue" group that can be partly attributed to the occurrence of large colony-forming cyanobacteria (e.g. *Microcystis* spp., *Aphanizomenon flos-aquae*).

An example of distribution profiles recorded with the submersible probe in Lake Plußsee, Northern Germany, shows the vertical migration of dinoflagellates (Beutler et al., 1998) – Figure 5. Both measurements were made at the same location at 9:30 and 14:00. The phytoplankton consisted of dinoflagellates (*Ceratium* spp.), chlorophyta (*Phacotus* sp.), blue-green algae/cyanobacteria (*Microcystis* spp., *Anabaena* spp.) and cryptophyta (*Cryptomonas* spp.). Dinoflagellates were dominating. At 9:30 most of the dinoflagellates are situated at the surface (0–2 m) of the lake. A maximum for cryptophyta was found at approximately. At 14:00 the dinoflagellates were moving downwards in water layers with higher nutrient concentrations. Their maximum concentration could be found at 3 m depth while the other algal classes did not migrate (Beutler et al., 1998).

The filamentous red cyanobacteria *Planktothrix rubescens* is well-known for the potential production of cyanotoxins. The occurrence of the hepatotoxic microcystin poses a challenge to water works when reservoir water contains *P. rubescens*. In spring 2013 the growth

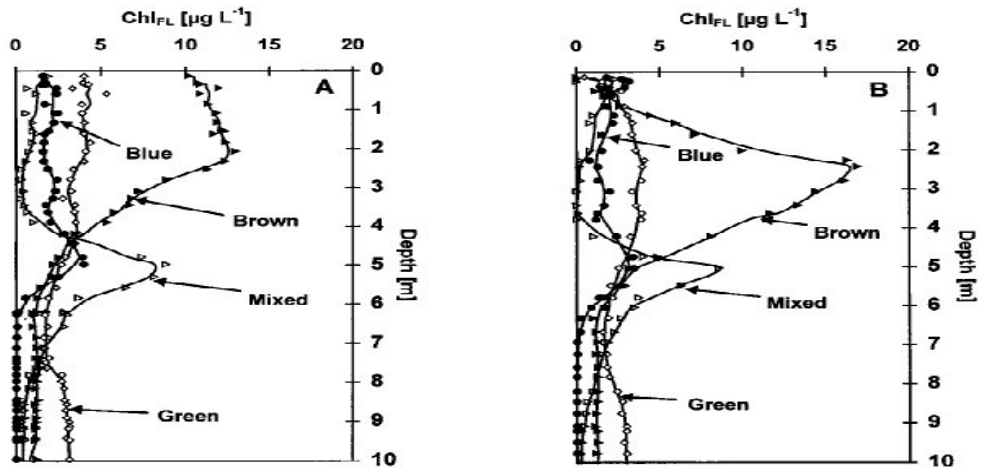

FIGURE 5. Example of distribution profiles recorded with the submersible probe in Lake Plußsee (Northern Germany) at 4 August 1998 showing vertical migration of dinoflagellates (Beutler et al. 1998)

of cyanobacteria was observed in the Sengbach Reservoir of the Glüder Waterworks, Germany (Mokros & Kobarg, 2013). The management at the waterworks became particularly alert and started cooperation with the health authority in Solingen. Since May 2013, daily laboratory tests (cell counting) have been supplemented by continuous monitoring for cyanobacteria using a flow through AlgaeOnlineAnalyser (Fig. 6) adjusted for the detection of *Planktothrix rubescens*. The measurement is based on multi-wavelength excitation and chlorophyll *a* fluorescence emission. Sampling was done at the water intake of the waterworks.

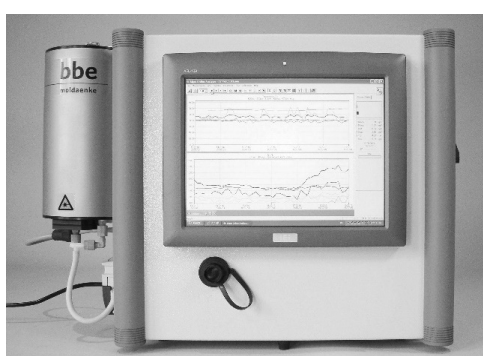

FIGURE 6. AlgaeOnlineAnalyser (AOA) for continuous monitoring of algae and cyanobacteria in water (bbe Moldaenke, Germany)

The recorded chlorophyll concentration in the observed period were far below the alarm level set issued by the WHO guidelines (Fig. 7). The recommended alarm thresholds for cyanobacteria equivalent to chlorophyll *a* are concentrations of 20 and 50 µg·l^{-1}, respectively. At peak times, just above 20 µg·l^{-1} were measured. Since cyanobacteria algae can reproduce very fast, the installed monitoring system is highly important for security and the drinking water supply. The AlgaeOnlineAnalyser permanently monitors the cyanobacteria and algae concentrations at the intake of

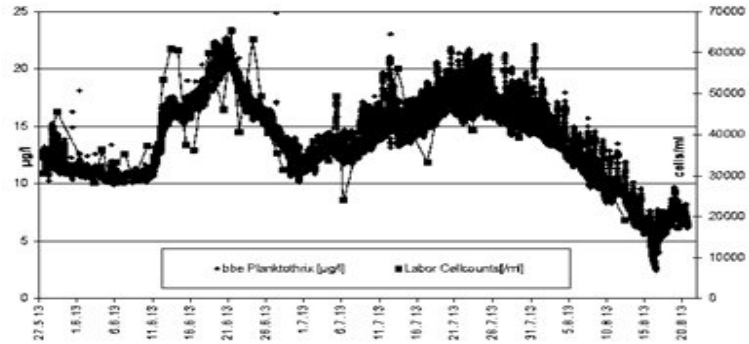

FIGURE 7. Comparison of laboratory measurement values and AOA chlorophyll measurements, May – August 2013, Güder Water Works, Germany (Mokros & Kobarg, 2013)

the waterworks and transmits the records to a control center. In contrast to microscopic analysis of daily spot sampling with cell counting the AlgaeOnlineAnalyser provides a 24/7 continuous survey.

Conclusions

Algal blooms are problematic in polluted water. The knowledge of algal species composition is necessary for understanding this process. Chlorophyll fluorescence analysis offers an alternative approach and potentially allows *in situ* estimation of algal concentration. This technique can be used to monitoring natural water reservoirs. In this work the fluorometric methods to estimate algae content in water and to differentiate algal population was presented. Based on examples which were presented in this work, chlorophyll fluorescence analysis can be proposed for wide use in monitoring water ecosystems.

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Summary

Taxonomic classification of algae by the use of chlorophyll *a* fluorescence. Natural water reservoirs are very important ecosystems thus they should be under continuous monitoring and protection. In water of low quality, the algal blooms develop develops vastly. The knowledge of algal species composition is necessary for understanding this process. There are a few traditional group-specific methods of microalgae classification, but they are often labour-intense

and time-consuming. Moreover, the samples must be prepared and/or collected before getting any results. Non-invasive chlorophyll fluorescence analysis offers an alternative approach and potentially allows *in situ* estimation of algal concentration. In this work the fluorometric methods to estimate algae content in water and to differentiate algal populations is presented.

Authors' address:
Nikodem Szymański
Instytut Technologiczno-Przyrodniczy
w Falentach
al. Hrabska 3, 05-090 Raszyn
Poland
e-mail: nikodem.szymanski@wp.pl