

Freshwater phytoplankton quantification by chlorophyll *a*: a comparative study of in vitro, in vivo and in situ methods

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Abstract

Standard ISO method for chlorophyll *a* quantification (extraction into ethanol, spectrophotometrical quantification at 665 and 750 nm), spectrofluorometry (reader for 96 wells, excitation 410 nm, emission 670 nm), and a submersible fluorescence probe for in situ phytoplankton quantification (excitation 410, 525, 570, 590, and 610 nm, emission 685 nm) were compared in different freshwater environments—reservoirs and rivers.

The ISO method is accepted as a standard method but requires sample handling and transport to the laboratory. Spectrofluorometry is a sensitive method, even for natural phytoplankton populations. Nevertheless, it cannot be recommended for the quantification of cyanobacterial water blooms because colonial and filamentous species such as *Microcystis*, *Anabaena*, or *Aphanizomenon* display unacceptable variability (18–33%).

The submersible probe featured high correlation with a standard ISO method ($r = 0.97$, $P < 0.05$). This probe can provide the selective measurement of technologically important phytoplankton groups like cyanobacteria, diatoms, green algae, and cryptophytes in lake vertical profiles of up to 100 m. The limitation of this instrument is the possible reabsorption of the light signal, e.g. in the presence of humic substances, or dense algal blooms. The use of submersible probes for in situ phytoplankton quantification can be recommended as a sensitive tool for water management, especially in the case of drinking water resources.

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1. Introduction

Chlorophyll *a* is a photosynthetic pigment present in all species of phytoplankton, including eukaryotic (algae) and prokaryotic organisms (cyanobacteria) and thus it is a reliable and commonly used proxy for total phytoplankton biomass. Most methods of chlorophyll quantification are based on its extraction from disintegrated cells in an organic solvent such as methanol,

ethanol, or acetone and on its subsequent determination by spectrophotometry [1–3] and fluorometry [4], or chromatography [5,6]. These methods have been routinely used for decades but they are time-consuming and require a standard sampling procedure and transport to a laboratory, as well as an experienced analyst. Furthermore, all parts of the process from water sampling to the final determination of the chlorophyll *a* content can be subject to variability. Other disadvantages include a large volume of samples often needed (thus a limitation in their number) and possible quantitative changes during sample storage.

One of the key characteristics of chlorophyll *a* is its fluorescence. Photosystem II (PS II), which is mainly responsible for chlorophyll fluorescence, consists of a peripheral and core antenna. The former contains a

Abbreviations: LED, Light-emitting diode; HPLC, High-pressure liquid chromatography; PSII, Photosystem II; FP, FluoroProbe

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species-dependent light-absorbing pigment, the latter an evolutionary conserved molecule of chlorophyll *a* [7]. Most of the energy transferred from the peripheral antenna to the core is used for photochemistry and thermal decay and a variable percentage is re-emitted as red light (approx. 685 nm). The magnitude of this light signal serves as a tool for the *in vivo* determination of chlorophyll *a*.

This method is often used in laboratory experiments when estimating algal or cyanobacterial biomass, e.g. in tests of toxicity [8,9]. These are studies in laboratory research; however, the use of chlorophyll *a* fluorescence is possible in field studies, directly in the water column. Connecting the fluorometer in a continuous or stop flow mode to a pumping system that brings water to the measuring cell of the fluorometer is one possible way of the on-line monitoring of chlorophyll *a* [10,11]. Asai et al. [12] used a sensor with two fluorescence channels, the first for detecting chlorophyll *a* of eukaryotic algae (excitation 440 nm, emission 680 nm), and the other for detecting the phycocyanin of cyanobacteria (excitation 620 nm, emission 645 nm). The *in situ* fluorometer with three excitation bands and detection of emission from 546 to 733 nm was also designed [13]. The possibility of the use of absorbance and fluorescence spectra for discrimination among algal phylogenetic groups was investigated, e.g. by Millie et al. [14].

Submersible probes for detecting one or more phytoplankton classes have been also commercially available [7,15]. They contain diodes emitting light for the excitation of pigments of phytoplanktonic organisms in the water column and their response in fluorescence of chlorophyll *a* is measured. These instruments are usually connected to a portable computer from which they are operated and where measurements are sent and stored. This design allows for the submersion of a probe to a selected depth, which is limited only by the length of the probe cable. Continuous measurement presents a big advantage over discrete sampling, since some species of phytoplankton (e.g. cyanobacteria) can be concentrated in a very thin layer.

We tested a submersible probe—the FluoroProbe (bbe-Moldaenke, Kiel, Germany)—in the determination of total chlorophyll *a* in three reservoirs and rivers with

varying levels of eutrophication. This probe contains five diodes emitting light for the excitation of accessory pigments present in a variety of phytoplankton groups, thereby allowing for a complex estimation of chlorophyll *a*. These results are compared to (1) the standard extraction ISO method with a spectrophotometrical endpoint and (2) measurements of phytoplankton chlorophyll fluorescence intensity taken on a fluorescence reader.

2. Materials and methods

2.1. Sampling

Three reservoirs (Brno, Hubenov, and Mostišť) and three rivers (the Svatka, the Morava, and the Dyje) in Southern Moravia (Czech Republic) were sampled between July and September 2002. The data from the reservoirs represent surface water (the Brno reservoir, 6 samples) as well as various depths (the Hubenov reservoir—8 measurements from within 0 to 14 m, the Mostišť reservoir—4 measurements from within 0 to 8 m). Rivers were sampled as follows: the Dyje—25 km, 4 sites; the Morava—96 km, 7 sites; and the Svatka—52 km, 10 sites. All reservoirs and the Svatka River (on which the Brno reservoir is situated) suffer from extensive cyanobacterial blooms in the summer. The features of the study reservoirs and rivers are given in Table 1.

2.2. FluoroProbe

In situ measurements were carried out with a submersible fluorescence probe for determination of chlorophyll *a* (FluoroProbe, bbe-Moldaenke, Kiel, Germany). This probe contains five light emitting diodes (450, 525, 570, 590, and 610 nm) for the excitation of pigments present in the phytoplankton and a 370 nm diode for the excitation and subsequent subtraction of the fluorescence of dissolved organic matter (“yellow substances”). Chlorophyll *a* fluorescence is measured at 685 nm. The excitation spectrum obtained is compared to so called normal curves stored in the probe and the amount of chlorophyll *a* of four different phytoplankton

Table 1
Features of study reservoirs/rivers and some physical and chemical parameters on the sampling day

	Brno (30.7)	Hubenov (12.9)	Mostišť (12.9)	Dyje (22.7)	Morava (22.7)	Svatka (4.9)
Volume (mm ³)/length (m)	21.6	3.39	11.94	306	353	174
Area/catchment area (km ²)	2.59	0.55	0.93	13 419	26 580	7 119
Max. depth (m)	18	16	26	2.6	3.1	1.5
Flow velocity (m ³ s ⁻¹)	8.27	0.11	1.43	43.89	120	27.24
Temperature (°C)	25.8	18.8	19.2	23.1–24	19.8–22.2	19.9–20.8
Dissolv. O ₂ (mg ml ⁻¹)	8.1	9.2	9.5	6.9–7.5	7.2–8.7	7.4–10
pH	10.7	9.4	9.7	7.6–8.1	7.2–7.8	8.1–8.9

spectral groups—“blue” (Cyanobacteria), “green” (Chlorophyta), “brown” (Chromophyta and Dinophyta) and “mixed” (Cryptophyta), as well as the total chlorophyll *a* concentration—is calculated. The data are transferred every 2–3 s to a PC connected to the probe where the results are shown immediately as tables and graphs. See [7] for details.

In reservoirs, the phytoplankton was measured by the probe directly in the water column and 11 samples for a laboratory analysis were taken with a Friedinger sampler. River water samples were taken with a 20 l barrel into which the probe was submersed for obtaining measurements and from which 11 samples for the laboratory analysis were taken.

2.3. Determination of chlorophyll *a* concentration

Samples of 100–150 ml were filtered through a Whatman GF/C filter, which was cut into small pieces and chlorophyll was extracted in 90% ethanol at 75°C for 5 min. The extract was filtered and 10 ml was taken for acidification by 10 µl of 3 mol l⁻¹ HCl. The absorbance of both extracts was measured at 665 and 750 nm and the concentration of chlorophyll *a* was determined according to ISO 10 260 [16].

2.4. Fluorescence reader

Measurements were carried out using a microplate fluorescence reader GENios (Tecan, Maennedorf, Switzerland). Samples (96 well white microplate for fluorescence measurements, 250 µl in each well) were measured with the following parameters: excitation wavelength, 410 nm (40 nm bandpass); emission wavelength, 670 nm (25 nm bandpass); gain, 140; number of flashes, 3; lag time, 0; integration time, 40 µs; top read mode; shake duration, 10 s (inside).

3. Results and discussion

3.1. Reservoirs

In reservoirs, phytoplankton was dominated by cyanobacteria and a smaller number of green algae.

Diatoms were present rarely, probably because of their suppression by cyanobacteria that produce toxic substances (Table 2). The concentration of chlorophyll *a* was between 3.49 and 47.34 µg l⁻¹ (taken from spectrophotometric values). Spectrophotometry and the FluoroProbe method were highly correlated, with similar trends of the determination of total chlorophyll *a* ($r = 0.97$, $p < 0.05$, $n = 18$), shown in Fig. 1. However, the values provided by the FluoroProbe were significantly lower than those obtained from the standard method.

The FluoroProbe determines the actual amount of chlorophyll *a* according to calibration parameters that are stored in the device and are based on HPLC analyses [7]. HPLC generally provides lower results than spectrophotometry due to allomers and other chlorophyll derivatives which are detected at 665 nm but are separated chromatographically by HPLC [17]. This could be one reason for the lower content determined by the FluoroProbe compared to the standard spectrophotometry method that we used. In the study of Le Boulanger et al. [15], using acetone as an extractant and spectrophotometrical endpoint according to Strickland and Parsons [18], the ratio of chlorophyll *a* determined by

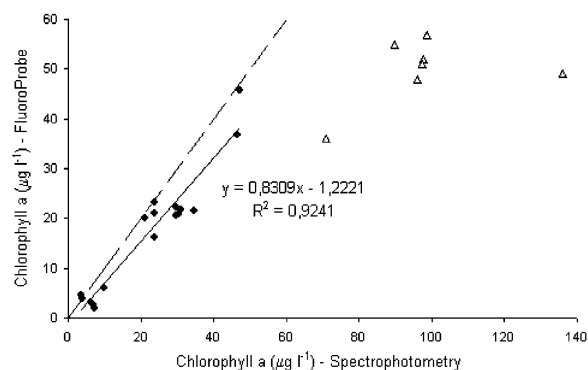


Fig. 1. Correlation of chlorophyll *a* values determined by the FluoroProbe with a correction for “yellow substances” and spectrophotometry in reservoirs. The dashed line presents an “ideal” curve for the estimation of chlorophyll *a* by two methods ($y = x$). Regression is applied only to values of up to 50 µg l⁻¹ (full circles). A non-linear response by the FluoroProbe at higher levels of chlorophyll *a* is shown (open triangles).

Table 2

Taxonomic composition of sampled reservoirs and rivers expressed as % of total cell counts

	Brno (30.7)	Hubenov (12.9)	Mostiště (12.9)	Dyje (22.7)	Morava (22.7)	Svratka (4.9)
<i>Cyanobacteria</i>	98.46	72.82	99.67	50.32	64.18	92.14
<i>Dinophyta</i>	0.00	0.03	0.00	0.00	0.72	0.00
<i>Cryptophyta</i>	0.00	0.12	0.01	0.06	0.14	0.00
<i>Chromophyta</i>	0.80	1.67	0.00	8.37	7.39	4.01
<i>Euglenophyta</i>	0.00	0.03	0.03	0.13	0.00	0.00
<i>Chlorophyta</i>	0.74	26.95	0.28	41.12	27.56	3.85

the FluoroProbe and spectrophotometry method was approx. 1.03. In our measurements, this ratio was approx. 0.83. However, standard methods based on pigment extraction and spectrophotometry or fluorometry endpoint show high within sample variance depending on the organic solvent and equation used [19]. Consequently, we used the standardized ISO method, in this respect our results comparing the FluoroProbe and this standard method appear consistent.

Our results obtained from the whole year measurements in several reservoirs with varying levels of chlorophyll *a* and phytoplankton floras indicate that the FluoroProbe is most suitable for use in water bodies with lower levels of chlorophyll *a* and differentiated phytoplankton flora. In the case of high phytoplankton biomass, the underestimation of the chlorophyll *a* level can appear. When cyanobacteria are dominant and form water blooms, the upper limit, based on our whole-season data, is approximately $50 \mu\text{g l}^{-1}$, especially when colonial species are present (see Fig. 1 and a relation of FP to spectrophotometry at higher concentrations). On the other hand, the FluoroProbe was able to measure up to $300 \mu\text{g l}^{-1}$ chlorophyll *a* in a dense, natural sample of natural phytoplankton containing unicellular species of algae and diatoms (unpublished results), which corresponds to the results by Beutler et al. [7] who found a linear response ranging from approximately $1\text{--}400 \mu\text{g l}^{-1}$. It should be noted that the instrument used in the study of Desiderio et al. [13] recorded no linearity in the *in vivo* fluorescence response of the *Dunaliella tertiolecta* culture already at concentrations of around $10 \mu\text{g l}^{-1}$. Optical density (caused by the suspended particles or high amounts of phytoplankton) is the limiting factor of such methods. Light emitted by the instrument or phytoplankton can be shaded, scattered, or re-absorbed, which can cause non-linear response to the phytoplankton quantity.

Values for chlorophyll *a* given by the FluoroProbe can be affected by the fluorescence of various chemical compounds present in water, illustrated in Figs. 1 and 2, the former with and the latter without correction for “yellow substances”, i.e. dissolved organic matter. At lower concentrations of chlorophyll *a* particularly, the main fluorescence output belongs to these substances and can be falsely interpreted as fluorescence of phytoplanktonic origin.

3.2. Rivers

Chlorophyll *a* concentrations in rivers fluctuated between 14.0 and $86.16 \mu\text{g l}^{-1}$; the respective taxonomic composition is also shown in the Table 2. The data obtained from the *in situ* fluorescence and spectrophotometry correlated closely ($r = 0.91$, $p < 0.05$, $n = 21$). However, in contrast to the reservoirs, we

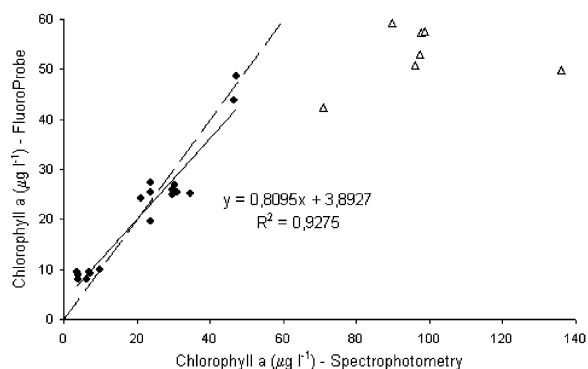


Fig. 2. Correlation of chlorophyll *a* values determined by the FluoroProbe without a correction for “yellow substances” and spectrophotometry in reservoirs. The dashed line presents an “ideal” curve for the estimation of chlorophyll *a* by two methods ($y = x$). Regression is applied only to values of up to $50 \mu\text{g l}^{-1}$ (full circles). A non-linear response by the FluoroProbe at higher levels of chlorophyll *a* is shown (open triangles).

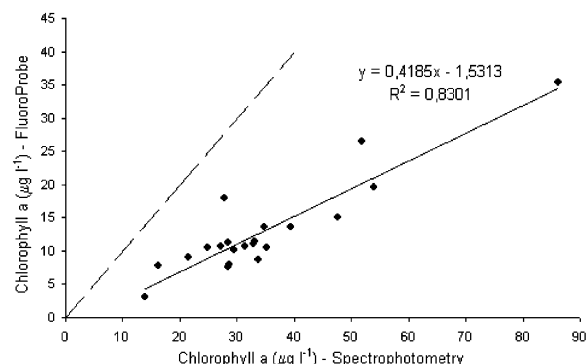


Fig. 3. Correlation of chlorophyll *a* values determined by the FluoroProbe and spectrophotometry in rivers. The dashed line presents an “ideal” curve for the estimation of chlorophyll *a* by two methods ($y = x$).

detected approximately 60% underestimation by the FluoroProbe (Fig. 3). The average coefficient of underestimation (Chl *a* by FP/Chl *a* by spectrophotometry) was 0.39. This effect was probably caused by fluorescence quenching that appears in shallow waters and subsurface layers due to high irradiance since the depth of sampled rivers is very low (up to 1 m). Another problem could be lower transmission in river water, which reached an average of 59.08%, while in reservoirs this figure was 70.85%. Suspended particles in water probably scatter excitation light from the diodes as well as fluorescence response from the cells. This problem does not prevent the use of the FluoroProbe in rivers and other turbid waters if a method of calibration is used for correction. Manufacturers of other submersible

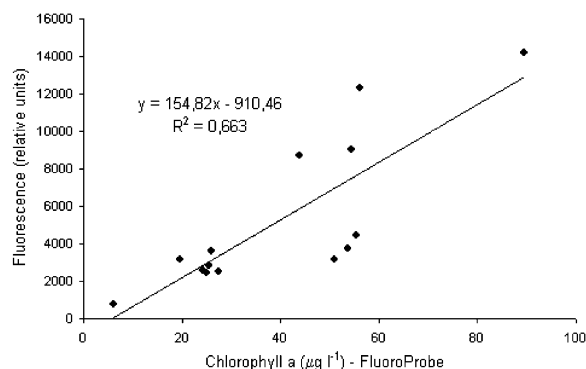


Fig. 4. Correlation of the FluoroProbe and a fluorescence reader.

probes for the detection of chlorophyll *a* generally recommend this approach, with the recalibration of the probe for different localities and phytoplankton assemblages.

3.3. Comparison of the *in situ* probe and fluorescence reader

Results obtained from the fluorescence reader showed relatively high variability (approximately 25% in average) probably caused by the presence of colonial cyanobacteria (their abundance in the sample was not fully homogenous). The correlation was significant ($r = 0.81$, $p < 0.05$, $n = 14$) but not as obvious as the comparison of the probe and the standard method (Fig. 4). This result was surprising—these two methods (the probe and the fluorescence reader) work on the same principle. One reason for the difference could be that the reader has only one excitation wavelength and thus its ability to respond to changes in the phytoplankton composition is limited. The same conclusion was made by Pinto et al. [10] when explaining a weak correlation between fluorometer *in vivo* measurement and chlorophyll *a* content in waters with cyanobacterial dominance. Fluorometers, of course, allow for the change of excitation filters, which, however, makes measurement much less comfortable and still does not solve problems with samples where the co-dominance of more phytoplanktonic groups (e.g. green algae and cyanobacteria) appears.

Conclusions

The determination of chlorophyll *a* based on *in vivo* fluorescence using a submersible probe seems a promising method for the routine monitoring of phytoplankton biomass in reservoirs and (after device re-calibration or the subsequent re-counting of results from the probe)

ivers. It provides results comparable to standard spectrophotometry methods. One of the biggest advantages of using a probe is the greater volume of data obtained quickly and on-line. Taxonomic determination is not necessary for all samples, only for localities or depths where changes in phytoplankton composition and biomass were detected by the probe. The probe is suitable for monitoring natural phytoplankton communities with variable composition because it contains five diodes with wavelengths covering excitation spectra for the main pigments present in important groups of phytoplankton (cyanobacteria, diatoms, green algae, and cryptophytes). We found the most reliable results to be obtained in waters with high light transmission and a chlorophyll *a* content of up to $50 \mu\text{g l}^{-1}$, especially in the case of water blooms.

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