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Abstract

We present results that were obtained with a newly developed fluorometer, the 'PhytoSensor'. They are based on multi-wavelength excitation of chlorophyll fluorescence to detect the phytoplankton biomass and to identify main taxons (among cyanobacteria, green and brown microalgae). A method to evaluate the photosynthetic potential of the phytoplankton was established. Attention was focused on the measurement of the cyanobacterial biomass. A modelling to distinguish between the two spectral groups (blue and red) of cyanobacteria as a function of their pigments and physiological status is proposed. The main innovation of the device results in the recording of the fluorescence induction kinetics of the phytoplankton to confirm and refine the evaluation of the taxonomic composition. The PhytoSensor abilities were compared with pigment analysis, commercial fluorometers, particle and microscopic counting and identification. The PhytoSensor has been used with success to monitor the dynamics of phytoplankton in drinking-water supply reservoirs in Southeast Asia.

Key words: Bloom monitoring, Chlorophyll fluorescence, Fluorescence induction kinetics, Phytoplankton, Southeast Asia, Water supply.

Abbreviations: Chl, Chlorophyll; FIK, Chl *a* fluorescence induction kinetics; F_o and F_m, Minimal and maximal levels of chlorophyll fluorescence emission, respectively; HPLC, High-pressure liquid chromatography; LED, Light-emitting diode; PP, photosynthetic potential; PS II, Photosystem II.

1. Introduction

Chlorophyll *a* (Chl *a*) is an ubiquitous photosynthetic pigment present in all eukaryotic (algae) and prokaryotic (cyanobacteria) phytoplankton organisms. *In vivo* Chl *a* fluorescence has became an increasingly important tool for the assessment of both biomass and photosynthetic activity of phytoplankton *in situ* [1]. Very sensitive techniques have been developed [2] among which multi-wavelength fluorometers that allow *in vivo* and/or *in situ* taxonomic identification. The current systems are based on the discrimination of 'spectral groups' of phytoplankton (see [3]). These groups have specific accessory pigments which absorb light efficiently in different ranges of the visible light spectrum [4]. When they absorb a photon, these pigments become excited and they transfer their excitation energy from pigment to pigment to the Chl *a* molecules, the terminal acceptor of the excitation transfer channel, which emit fluorescence (reviewed in [5]). By use of light sources of different excitation wavelengths, an evaluation of the systematic composition of a mixed phytoplanktonic community can be obtained. With the progress in light-emitting diode optic, the feasibility of such an approach has been demonstrated [6]. Two devices, the Phyto-PAM (Heinz Walz, Germany) and the Fluoroprobe (BBE Moldaenke, Germany) [3], are currently available. They have from four to five excitation wavelengths and can detect from three to four spectral groups (green algae, cyanobacteria, 'brown microalgae' (mainly diatoms and dinoflagellates) and a 'mixed group' (cryptophytes)). Recent approaches have been developed to refine the discrimination between the two spectral groups of cyanobacteria (blue and red) and the cryptophytes [7,8].

One of the applications of these instruments is the monitoring of the algal and cyanobacterial proliferations (so-called blooms) in water resources used as drinking-water supplies and/or recreational areas [9]. Blooms are due to eutrophication and more than 40 % of worldwide lakes are eutrophic [10]. Because they can impair the water quality, blooms

have been of major concern for the water resource managers [11,12]. One of the sources of awareness is the synthesis of toxins by cyanobacteria which are responsible for human health hazards [13].

The present work is based on the measurement and interpretation of *in vivo* Chl *a* fluorescence emission by freshwater phytoplankton. The fluorometer, the 'PhytoSensor', uses the trichromatic excitation of Chl *a* fluorescence to differentiate three spectral groups: green and brown microalgae and cyanobacteria. A mathematical modelling based on pigment content and physiological status differences to discriminate between the blue and red groups of cyanobacteria is tentatively presented. A rough evaluation of the photosynthetic potential of the phytoplankton was also reached. The main novel aspect proposed by this device is the simultaneous combination of two independent measuring principles, the minimum level of fluorescence and the fluorescence induction kinetics, for a better identification of taxonomic groups. The PhytoSensor has been tested in natural and man-made (sub-) tropical reservoirs in Southeast Asia. Tropical water resources are more sensitive to eutrophication, they show a higher proportion of cyanobacteria and blooms occurring year round are often unpredictable [14,15]. The detection of the phytoplanktonic biomass, its proliferation, and the identification of the responsible taxa have been possible.

2. Materials and Methods

2.1. Culture of algae and cyanobacteria reference species

The strains of diatoms came from CCMP (Provasoli-Guillard Center for Culture of Marine Phytoplankton, USA), UTEX (University of Texas, USA) and Laboratoire Arago (France) collections, green algae from the University Technology of Malaysia (UTM) collection and cyanobacteria from the UTM and PCC (Pasteur Culture Collection, France) collections. All strains were grown photoautotrophically in continuously sterile air flushed

recipients. The following conditions were used. For marine isolates of diatoms: natural seawater F/2 medium, 18°C, light intensity of 40 $\mu E.m^{-2}.s^{-1}$ with a 16 h light / 8 h dark cycle. The freshwater isolate of *Phaeodactylum tricornutum* was grown in a Bristol medium. For green algae and the cyanobacteria *Anabaena* sp. and *Pseudoanabaena* sp.: Bold Basal medium, 27°C, 25 $\mu E.m^{-2}.s^{-1}$, 12 h light/12 h dark cycle. For the cyanobacteria *Synechococcus* PCC 7942 and *Synechocystis* PCC 6803, GN medium, 30°C, 60 $\mu E.m^{-2}.s^{-1}$ in continuous light. Cells were harvested during the exponential phase, or when indicated during the stationary phase of growth.

2.2. Cell enumeration, pigment analyses and spectroscopy

Algae and cyanobacteria were identified and counted as units with a Malassez hemocytometer counting chamber as described [16]. Particle counting was performed with a Particle Counter (PCX Hach) (resolution 1 µm, maximum size 500 µm) on 500 mL at 100 mL. min^{-1}. Chl *a* concentration was measured with a spectrophotometer (Varian DMS 90) after acetone 90 % extraction [17] and with a Datalink fluorometer (FL 100, Datalink, France). Pigment analyses were performed by HPLC [18], and the relative proportions of algae and cyanobacteria were determined as previously described [19]. Room temperature 680 nm fluorescence excitation spectra were measured with a F-4500 Hitachi spectrofluorometer [18].

2.3. Water sampling

Water was sampled in 1 L bottles at 0.5 m depth. In order to avoid the high light-dependent quenching of fluorescence [9,20], the water was not sampled around noon but instead in the morning (9 AM) and the evening (5 PM). Additionally, a preliminary dark incubation of 30 min before the measurement reduced most of the fluorescence quenching that could have developed [21] in spite of sampling precautions.

2.4. PhytoSensor device and data processing

1) PhytoSensor prototype: designed to be compact and portable in a suitcase (40 x 30 x 16 cm) for easy transportation. It consists of three parts (Fig. 1a). **i)** a data acquisition card (PCL 818, Advantech) inserted in a PC computer together with the 'Fluo' software (see § 2)); **ii)** the electronic devices; **iii)** a detector unit (Fig. 1b). It includes a sample compartment containing the water sample illuminated from three sides by blue, red and green lights. The sample compartment was isolated from ambient light by a cap. On the fourth side, chlorophyll fluorescence was detected by a miniature photomultiplier module (PM) (Hamamatsu H5701-50, together with an amplifier and a high-voltage supply) protected by an interference filter (Corion, S10 680 F, 680 ± 10 nm). Each light source consisted of an array of six light emitting diodes (LED) and a specific interference filter combination. Blue light, 15 $\mu E.m^{-2}.s^{-1}$ (maximum light intensity in the sample compartment): LED Everlight 383 UBC, band pass of 430 ± 30 nm obtained with filters Maier SPX R 500 + Andover 450 FL07 + Andover 600 FL07. Green light, 65 $\mu E.m^{-2}.s^{-1}$: LED Marl Ultrabright 'pure green' plus a 540 ± 15 nm filter Corion, XM 540. Red light, 250 $\mu E.m^{-2}.s^{-1}$: LED Hewlett Packard HLMP-DH08 plus a 625 ± 20 nm filter Corion, XM 625.

The light intensity was measured inside each light source by a photodiode (BPW 34, Siemens) and was kept constant during the illumination by a feed-back electronic loop (Fig. 2a). This was necessary since for a constant current a decrease of up to 20 % of the LED light intensity was recorded during the first second of illumination (Fig. 2b). This decrease is due to an increase in the temperature of the LED. The electric signal generated by the photodiode was injected at the entry of the circuit by the feed-back electronic loop which compensates the variations in light intensity by variations in the current.

The water samples were contained in cuvettes (Greiner, ref. 613101) placed as close as possible (1 cm) to the PM to improve the detection which can be disturbed by attenuation or diffusion of excitation and/or fluorescence lights. It was tested that the sensitivity of the measurement was not decreased up to a turbidity of 150 NTU artificially created with a bentonite powder (Sigma) (data not shown, the maximal turbidity during field assay period was 20 NTU). Since the bio-optical characteristics of phytoplanktonic organisms can modify the fluorescence emission signal [22], cyanobacteria and algae with different shapes (unicellular, colonies and filaments) and sizes (from 2 µm to hundreds of µm) were tested to not alter the sensitivity of the measurement (data not shown).

2) Software for the experimental procedure, data acquisition and data treatment: 'Fluo' software was developed in the Delphi 5 language (Borland, USA) and consists of three parts. **i)** the 'protocol' of the experiment; it was controlled through a set of specific macro-instructions. For each illumination sequence, the excitation wavelength, light intensity, time of illumination and PM voltage were pre-adjusted. **ii)** the 'evaluation table' of the Chl *a* concentration and of the relative proportions of algae and cyanobacteria; they were calculated by a mathematical modelling from the fluorescence signals obtained under blue, green and red low intensity excitations. The modelling was based on pre-determined proportionality factors between fluorescence and Chl *a* concentration measured on reference species. **iii)** the 'calculation table' of the photosynthetic potential (PP) index of the phytoplankton; it was calculated from the variable part of the fluorescence between the minimum fluorescence signal (F_o) and the maximum fluorescence signal (F_m).

3) Experimental protocol: measurements were done in three replicates for each sample. Samples were dark-adapted for 30 min before the start of the experiment; cuvettes were then

shaken to resuspend cells that had settled. A typical measurement lasted around 4-5 min. Three successive flash sequences of blue, green and red light of low intensity followed by a flash of high light were fired. Flash duration and irradiances were: **i)** blue: 0.2 s / 1 $\mu E.m^{-2}.s^{-1}$, **ii)** green: 0.5 s / 7 $\mu E.m^{-2}.s^{-1}$, **iii)** red: 0.2 s / 13 $\mu E.m^{-2}.s^{-1}$, and **iv)** red + blue: 0.2 s / 200 $\mu E.m^{-2}.s^{-1}$. The illumination sequence was first done on a blank of distilled water and thereafter on the water sample. At the end of the sequences, the signal from the blank was subtracted and the values were averaged. For low light intensity excitations, the best compromise was fixed between the light intensity/duration of illumination and signal-to-noise ratio in order to keep the flash as non-actinic as possible to obtain the closer and better approximation of the 'true' F_o value. The same remark is true for the F_m level; the high light intensity used here is not sufficient in some conditions to ensure a measurement of the 'true' F_m value but instead an approximation of it. Short illumination times were used to prevent variations in the fluorescence emission induced by the photochemistry processes [5]. The influence of one flash on the next was minimised by inserting between flashes dark periods from 5 s between low light flashes to 20 s after high light flash: they were tested to be long enough to allow the reoxidation of the plastoquinone pool between two successive flashes (data not shown).

4) Acquisition and software treatment of the fluorescence induction kinetics (FIK): a FIK represents the variation of Chl *a* fluorescence emission as a function of time during exposure to a continuous light intensity (see [5]). After 60 s dark adaptation following the last flash sequence, the FIK was recorded under a red light illumination of 130 s / 125 $\mu E.m^{-2}.s^{-1}$. Experimental FIK obtained from natural sample was compared with a software accessible library containing the kinetics of algae and cyanobacteria reference species (Fig. 3). The reference FIK were decomposed in elementary components susceptible to enter the

experimental curve. The sum of the reference inductions was then fitted to the experimental curve and the best combination chosen with a statistical test (the reduced quadratic error). By this approach, relative proportions of algae and cyanobacteria were obtained. Results were embedded in the 'evaluation table'.

3. Results and Discussion

The distinction between the spectral groups of phytoplankton [3] is based on one or two specific dominant accessory pigments. While green algae are rich in Chl *a* and *b* (which absorb in the blue and red ranges of the visible spectrum), brown microalgae (in this work diatoms were chosen as representative of the group) and cyanobacteria are rich in xanthophylls (which absorbs in the green range) and in phycobilins, respectively. There are two main types of phycobilins: phycocyanin present in all strains absorbs in the orange-red range, phycoerythrin absorbs in the green range. Two spectral groups of cyanobacteria can thus be defined: blue cyanobacteria (or BC) which have only phycocycanin and red cyanobacteria red (RC) which have both. Hence, the fluorescence excitation spectra of the four main spectral or taxonomic groups (Fig. 4) reflect the wavelength-dependence of fluorescence emission due to differences in pigment composition.

3.1. Modelling for the evaluation of the phytoplankton biomass and the taxonomic identification

Fluorescence emission intensity is a function of Chl *a* concentration. As it is complementary to the photochemical activity, the fluorescence emission is also depending upon the incident light intensity [5]. Under weak light and for dark-adapted cells, fluorescence emission is minimal (F_o); under saturating light (the photochemical activity being maximal), fluorescence increases to a maximum (F_m) when photosynthesis is saturated.

The F_o level is proportional to the number of photosystems (PS) II, which is not always the case for F_m because of fluorescence quenching mechanisms that develop under high light [20,23]. Therefore, the only, most reliable and simple base for the evaluation of Chl *a* concentration is the F_o level. F_o was measured under three low intensity wavelengths of blue (430 ± 30 nm), green (540 ± 15 nm) and red (625 ± 20 nm) (see Fig. 4) for the four reference organisms chosen (*Synechococcus* sp. and *Pseudoanabaena* sp. for cyanobacteria blue and red respectively, *Chlorella* sp. for green algae, *Phaeodactylum* sp. for diatoms, representative of the brown microalgae) and as a function of a range of Chl *a* concentration (0-50 µg Chl $a.L^{-1}$) likely to occur when a bloom starts. An example of results is shown in Figure 5. The sensitivity of the device was 2 µg Chl $a.L^{-1}$ in laboratory conditions (see Fig. 5). The slope of the linear relationship was the proportionality factor (Pf) between the F_o fluorescence emission and the Chl *a* concentration. It was determined for each organism at each excitation wavelength (Table 1).

The mathematical model used to evaluate the total Chl *a* concentration and the relative proportions of each group was a matrix of three equations:

$$Fb = (Pfb\ G \times G) + (Pfb\ D \times D) + (Pfb\ C \times C)$$

$$Fg = (Pfg\ G \times G) + (Pfg\ D \times D) + (Pfg\ C \times C)$$

$$Fr = (Pfr\ G \times G) + (Pfr\ D \times D) + (Pfr\ C \times C)$$

Pfb G, etc are the proportionality factors of Table 1 determined under blue (b), green (g) and red (r) lights. G, D and C are the concentrations (in µg Chl $a.L^{-1}$) of green algae, diatoms (representative of the brown microalgae) and cyanobacteria (blue, B, or red, R) deduced from the measurement of the fluorescence levels Fb, Fg and Fr with blue, green and red excitations, respectively. The sum G + D + C is the total phytoplankton biomass (in µg Chl $a.L^{-1}$).

The validity of the chosen reference organisms *Chlorella* sp. and *Phaeodactylum* sp. for the determination of the Pf was tested. The values obtained for *Chlorella* sp. were

compared to that of three other genera frequently encountered in freshwater reservoirs (Table 1). The Pf of all four genera were very similar and the reference species was determined as representative of the whole group. The same conclusion was reached for diatoms (Table 1). It confirms that under the same culture conditions, the fluorescence emission properties of different species of green algae and diatoms are very similar [4].

Unlike for algae, one set of Pf cannot be considered as representative for all cyanobacteria. Although the blue cyanobacteria (BC) group is the most frequent, the red group (RC) can be abundant in some conditions. As the fluorescence emission properties differ between the two groups, the identification procedure was then optimised as follows (Fig. 6):

Step 1- As the presence of cyanobacteria was always correlated with a high fluorescence under red light (Table 1), they were unequivocally detected even at low concentrations. If cyanobacteria were not the dominant taxon, the Pf of BC were used as the default set. It was verified that below a threshold of 20 % BC or RC in a mixed population, the Pf of BC and the Pf of RC sets gave equivalent results (data not shown). Beyond this threshold, the modelling was settled to discriminate between the two spectral groups.

Step 2- The distinction between the two types of cyanobacteria was based on the red light excited fluorescence over green light excited ratio (Fr/Fg) (Table 1). The evaluation was refined with the Pf of RC set when Fr/Fg was below 5 by referencing to *Anabaena* sp. (Fr/Fg = 5.6).

It has to be point out that a non negligible biomass of the brown microalgae (especially diatoms and cryptophytes) may disturb the discrimination between BC and RC because they absorb green light [7,8]. For this reason, this modelling would be reliable only if cyanobacteria are dominant which is often the case under tropical latitudes [14,15]. On the other hand, it has been observed that the presence of green light absorbing RC in non

negligible amounts disturbs the identification of the brown microalgae (data not shown), that is why the discrimination between BC and RC is essential over a certain threshold of cyanobacterial biomass.

When phytoplankton shifts from growth to stationary phase and cell death occurs (so-called 'ageing of the cells'), and to a larger extent when they are submitted to an environmental stress, the light absorption properties and Chl *a* fluorescence yield of their pigment antenna are modified [1]. Then, the proportionality factors between Chl *a* concentration and fluorescence determined for cells in the exponential phase of growth (Table 1) can be changed. The process is different among taxa. For green algae, fluorescence yields were not changed (see also [3]). In contrast, aged diatom cells showed a decreased Pf under green light (data not shown) possibly due to an increase in xanthophyll amount [18] and related fluorescence quenching [24], and/or to a decrease in antenna energetic coupling [25]. This uncertainty was not fully solved: in field experiments, diatoms were always in very low amounts as in most tropical resources [14]. The consequences of the cyanobacteria cell ageing on the variability in fluorescence emission are more dramatic because of the structure and location of their antenna, the phycobilisomes (PBS). Nutrient deficiency generates a degradation but also a partial disconnection of PBS from PS II producing fluorescence from the PBS itself [26]. As a consequence, in aged cyanobacteria, the fluorescence emitted under red and/or green lights was strongly increased (also observed by [7]) as well as the corresponding Pf (Table 1). In order to refine the identification and quantification of the cyanobacteria, the evaluation procedure was completed after Step 2 as follows (Fig. 6):

Step 3- The aged cells were distinguished with the Fr/Fg ratio (Table 1). For BC, Fr/Fg > 10 (by referencing to *Synechocystis* sp., Fr/Fg = 9.0) was indicative of their ageing and new Pf were used. For RC, the aged status was for Fr/Fg > 2. The choice for such thresholds was justified and illustrated by field experiments (see § 3.3.).

3.2. Laboratory and field tests

In order to test the sensitivity of the PhytoSensor and the modelling, the identification of the taxonomic groups was determined on laboratory culture mixtures of known composition and Chl *a* concentration (as measured spectrophotometrically) and compared to HPLC analyses. The Chl *a* concentration was set to 20 µg Chl $a.L^{-1}$ and three relative concentrations of each group were used: 12.5 %, 33 % and 75 % (Table 2). The Chl *a* concentration evaluated by the PhytoSensor was not significantly different from the spectrophotometry and HPLC measurements whatever the proportions of groups (Student test, $p < 0.05$). When groups were in equal proportions (33 % each) or one of them two times higher (50 %), the PhytoSensor evaluation as the HPLC analysis were very similar to the expected values (no more than 8 % difference); which made the PhytoSensor and HPLC evaluations as close as 3.9 ± 3.5 % in average (max 11 %). When a group was largely dominant (75 %), the difference between PhytoSensor evaluation and expected values was increased up to 10 % while HPLC analysis remained as close to the expected values as before. Under these conditions, the difference between the PhytoSensor and HPLC evaluations was nearly doubled (6.5 ± 3.5 % in average, max 12 %). Hence, evaluation of biomass and identification of the groups by the PhytoSensor was similar to that of HPLC measurements even in conditions where group proportion was low (12.5 %).

The reliability of the evaluation of Chl *a* concentration based on F_o measurement was further tested on several reservoirs used from drinking-water supply in China and Malaysia. PhytoSensor measurements were compared to particle counting (with a PCX, Hach) and Chl *a* evaluation by Turner (10-AU Digital) and Datalink (FL 100) fluorometers which are widely used to determine Chl *a* concentration as indicative of phytoplankton biomass in freshwaters [27]. An example of the results obtained is shown if Figure 7. It corresponds to the monitoring

of a Malaysian reservoir with variable Chl *a* concentrations (by a factor of up to 6) and over a three months period. The relationships between the Chl *a* concentration as measured with the PhytoSensor and both the Datalink Chl *a* evaluation and the number of particles showed a R factor of 0.98 and 0.93, respectively, with a slope close to 1 (0.96 and 0.88 respectively). The limit of sensitivity of 2 µg Chl *a*.L^{-1} (corresponding to 2-3 10^2 particles.mL^{-1}) was confirmed by field experiments (data not shown).

3.3. Bloom monitoring and the use of the chlorophyll fluorescence induction kinetics

A bloom with changes in the amounts of algae and cyanobacteria was monitored in the 'Cahaya Bahru' reservoir (Malaysia) over a one month period (Fig. 8a). The PhytoSensor Chl *a* concentration evaluation based on the F_o measurements was in agreement with the Datalink measurement (data not shown, slope = 1.01, R = 0.99). The ability of the PhytoSensor to identify the phytoplankton groups was compared to microscopic identification and counting (Fig. 8b). Despite a low sampling frequency before and after the bloom period, these data show that the procedure for the identification of phytoplankton groups, and especially cyanobacteria, was reliable. Cyanobacteria were determined by the PhytoSensor as aged red cyanobacteria. It is reliable with the observed continuously decreasing cyanobacterial population (Fig. 8a) and with the microscopic identification which revealed a predominance of the colony forming toxic genus *Microsystis* sp. for which a number of species have phycoerythrin [28].

In order to confirm and refine the discrimination of phytoplankton groups from the Phytosensor F_o measurement, the fluorescence induction kinetics (FIK) were recorded (Fig. 8c). A FIK (also called the 'Kautsky effect') represents the variation in the Chl *a* fluorescence emission as a function of time under exposure to continuous light (see [5]). The shape of the FIK is depending upon the redox state of the electron transport chain between the

PS II and PS I. The amplitude of the FIK is a function of the Chl *a* concentration and, as shown in Figure 3, its shape is characteristic for respective taxonomic group. FIK showed a maximum of emission already after 500 ms illumination in green algae and after 5 s for diatoms. For the two spectral groups of cyanobacteria, the shape of the induction was similar: flat with a maximum emission after approximately 20 s. Comparing the shape of the FIK from natural water samples (Fig. 8c) to the ones of model organisms (Fig. 3), it is clear that there is a majority of green algae and cyanobacteria. The results of the software FIK analysis are shown in Fig. 8b in grey. The relative proportions of the taxonomic groups evaluated with the two independent approaches (F_o and FIK) were similar. It was possible to monitor a change in biomass of the brown microalgae which represented less than 10 % of the phytoplankton. It is noteworthy that for the sampling of March 8, the two approaches greatly differed from the microscopic counting. It thus can be concluded that the counting might be false, which is not surprising since colony forming cyanobacteria can sometimes be over-estimated by the counting [29].

3.4. Evaluation of the photosynthetic potential of phytoplankton

During ageing of cells, F_m is usually lowered due to a decrease in photochemical activity. As a consequence, the variable part of the fluorescence emission ($F_v = F_m - F_o$) decreases (see [30]). The ratio F_v/F_m has been taken as an indicator of the photosynthetic capacity reflecting the global physiological status [1,2,5]. For algae, $F_v/F_m = 0.70 \pm 0.05$ and $F_v/F_m = 0.50 \pm 0.05$ are observed during the exponential and stationary phases of growth, respectively. Based on these observations, a rough measurement of F_v/F_m was done in order to evaluate the global photosynthetic potential (PP) index of the phytoplankton. For algae, two situations were defined: $F_v/F_m \geq 0.65$ (active growth, PP index: (+)), $F_v/F_m \leq 0.5$ (stationary growth, PP index: (-)); between these two values, the PP index was (+/-). For cyanobacteria in

active growth, F_v/F_m varied from 0.75 to 0.5 [26]. Then, when the amount of cyanobacteria was more than 20 %, different thresholds were used. In that case, it was defined as follows: (+) when $F_v/F_m \geq 0.5$ and (–) when $F_v/F_m < 0.5$.

The PP index was measured during the bloom event in the 'Cahaya Bahru' reservoir (Fig. 8b). It showed a change in the physiological status of the phytoplankton. At the beginning, the phytoplankton presented a PP index (+). Then, green algae produced a bloom. It is likely that the second sampling corresponds to the beginning of the decline of the green algae population as the PP index was (+/-). This decline is tentatively attributed to nutrient shortage because of the large consumption of nutrients during the bloom and/or to zooplankton grazing pressure [15]. The PP index further decreased to (-), indicating a breakdown of biomass as illustrated by the decrease in Chl *a* concentration (Fig. 8a). It was followed by an increase to a PP index (+) presumably because the end of the monitoring period likely corresponded to the starting of a new green algae bloom (as seen in the corresponding increase in the Chl *a* concentration, Fig. 8a).

4. Conclusions

We have described a new multi-wavelength fluorometer, the PhytoSensor, and a mathematical modelling to measure in few minutes the chlorophyll biomass, and to evaluate the taxonomic composition and the photosynthetic potential of water samples containing mixed phytoplankton organisms. The device detects biomass as low as 2□ $\mu g.L^{-1}$, discriminates between three groups (green and brown microalgae, and cyanobacteria) even in conditions where one is dominant, and monitors a change in biomass of one taxon which represents a small fraction (<10 %) of the total phytoplankton. A modelling, based on pigment and physiological status differences, allows the discrimination between blue and red cyanobacteria when cyanobacteria are dominant. Its main innovation results from the

simultaneous combination of two independent measuring principles, the minimum level of fluorescence and the fluorescence induction kinetics, for a better identification of the taxonomic groups. Combining all parameters it was possible to monitor a bloom in a reservoir used as a drinking-water supply in Southeast Asia.

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Figures legends

Fig. 1. (a) Schematic representation of the PhytoSensor device top viewed. PM: photomultiplier, WS: water sample. (b) Picture of the detector unit with the cuvette containing the water sample illuminated with red light.

Fig. 2. (a) Schematic representation of the feed-back electronic loop that allows the regulation of the light intensity emitted by the LED. (b) Light emission signal (in arbitrary units) of blue LED during a 100 s illumination measured with (dark symbols) and without (grey symbols) the feed-back electronic loop system. The decrease in light intensity was due to an increase in the temperature of the LED (see the text for details).

Fig. 3. Chlorophyll fluorescence (in arbitrary units) induction kinetics during a red light excitation (50 µg Chl $a.L^{-1}$) of (a) blue cyanobacteria (BC) (*Synechococcus* sp.), diatoms (D) (*Phaeodactylum* sp.) representative of the brown microalgae and green algae (G) (*Chlorella* sp.). Curves are normalised to their maximum of emission.

Fig. 4. Room temperature 680 nm fluorescence excitation spectra ('photosynthetic action spectra') of green algae (G) (*Chlorella* sp.), diatoms (D) (*Phaeodactylum* sp.) representative of the brown microalgae, blue cyanobacteria (BC) (*Synechococcus* sp.) and red cyanobacteria (RC) (*Pseudoanabaena* sp.). The wavelength ranges of the blue, green and red excitation lights used by the PhytoSensor are shown below the spectra.

Fig. 5. Chlorophyll fluorescence emission under low light excitation (F_o in arbitrary units) as a function of the Chl *a* concentration (in $\mu g.L^{-1}$) for blue cyanobacteria (*Synechococcus* sp.). The slopes of the linear fits correspond to the proportionality factors (Pf) under blue (●),

green (▲) and red (■) lights listed in Table 1. Standard variation was not more than 5 % for blue and red lights and 10 % for green light.

Fig. 6. The three steps of the evaluation procedure. The modelling evaluates the proportions of green algae (G), diatoms (D) representative of the brown microalgae, and cyanobacteria (C) blue (BC) or red (RC) combining the pre-ajusted proportionality factors (Pf) and the fluorescence (F) measurements under blue (b), green (g) and red (r) light excitations. STEP 1: if BC < 20 %, cyanobacteria were negligible and the modelling was settled to use the Pf of blue cyanobacteria (BC) by default. If BC > 20 %, an optimisation of the evaluation of the cyanobacteria was necessary through steps 2 and 3. STEP 2: if Fr/Fg > 5, BC was the dominant group: the modelling kept using the Pf BC. In contrast, if Fr/Fg < 5, red cyanobacteria (RC) were dominant: the modelling moved to the Pf RC set and calculated the new values of G, D, C and Chl *a* concentration. STEP 3: when BC were dominant and if Fr/Fg > 10, they were evaluated as aged and then the set of Pf BC aged was preferred to Pf BC in order to refine the evaluation procedure. Similarly, when RC were dominant and if Fr/Fg > 2, the set of Pf RC aged was used by the modelling instead of Pf RC (see the text for details).

Fig. 7. Particle number (per mL) (∆), and Chl *a* concentration (in $\mu g.L^{-1}$) as measured by the PhytoSensor (●) and the Datalink fluorometer (□) from 31/01/2001 to 02/04/2001 in the 'Administrative Pond' reservoir (Masai, Malaysia).

Fig. 8. (a) Chl *a* concentration (in $\mu g.L^{-1}$) of green algae (grey), cyanobacteria (dark) and brown microalgae (white) as measured by the PhytoSensor from 27/02/2001 to 03/04/2001 in the 'Cahaya Bahru' reservoir (Masai, Malaysia). (b) % of green algae (○), cyanobacteria (∆)

and brown microalgae (◊) as evaluated by the PhytoSensor from the F_o measurement (black symbols), by the PhytoSensor from the fluorescence induction kinetics (FIK) (grey symbols), and the unit microscopic counting and identification (white symbols). PP: photosynthetic potential index of the phytoplankton (see the text for details). (c) FIK of the phytoplankton normalised to their maximum of emission. Data are mean value (± SD) of three measurements.

Table 1

Proportionality factors between F_o chlorophyll fluorescence emission and Chl *a* concentration of different species of green algae, diatoms and cyanobacteria. [a]

	Pfb	Pfg	Pfr	***Fr/Fg*** [b]
Green algae (G), *Chlorella* sp.	**1.7**	**0.6**	**1.9**	/
Scenedesmus sp.	1.6	0.6	1.5	/
Ankistrodesmus sp.	1.7	0.5	1.8	/
Monoraphidium sp.	1.9	0.5	1.9	/
Diatoms (D), *Phaeodactylum* sp. freshwater strain	**5.9**	**7.8**	**4.9**	/
Phaeodactylum sp. seawater strain	6.2	7.4	4.7	/
Cylindrotheca sp.	5.6	7	4.8	/
Thalassiosira sp.	5.6	7.4	4.9	/
Blue Cyanobacteria (BC), *Synechococcus* sp.	**0.7**	**2.3**	**16.4**	***7.1***
Anabaena sp.	1.4	4.2	23.7	***5.6***
Synechocystis sp.	0.6	1.8	16.2	***9.0***
BC **aged** [c], *Synechococcus* sp.	0.6	2.0	26.0	***13.0***
Red Cyanobacteria (RC), *Pseudoanabaena* sp.	**1.3**	**16.3**	**20.5**	***1.3***
RC **aged**, *Pseudoanabaena* sp.	2.0	27.5	60.4	***2.2***

[a] The parameters are labelled as Pf for proportionality factor between Chl *a* concentration and fluorescence; b, g and r for blue, green and red lights respectively; and G, D, C for green algae (reference species: *Chlorella* sp.), diatoms (reference species: *Phaeodactylum* sp.) representative of the brown microalgae, and cyanobacteria, respectively. For cyanobacteria, two spectral groups were distinguished: the blue cyanobateria, BC (reference species: *Synechococcus* sp.) which absorb preferentially in the orange-red range (absence of

phycoerythrin) and the red cyanobacteria, RC (reference species: *Pseudoanabaena* sp.) which additionally absorb in the green range (presence of phycoerythrin).

[b] The values of Fr/Fg were used to refine the cyanobacteria evaluation procedure: when Fr/Fg < 5, the set of Pf RC was substituted to the set of Pf BC (see the text for details).

[c] Aged cyanobacteria designate cells which are in the stationary phase of growth for days.

Table 2

Chl *a* concentration and % of green algae (G) (*Chlorella* sp.), diatoms (D) (*Phaeodactylum* sp.) representative of the brown microalgae, and blue cyanobacteria (BC) (*Synechococcus* sp.) as measured by the PhytoSensor and the HPLC analysis from mixtures with different proportions.

	Chl *a* (µg. L^{-1})	% G	% D	% BC
Expected values	**20**	**33**	**33**	**33**
PhytoSensor [a]	20,4 ± 1,0	34 ± 2	34 ± 1	32 ± 2
HPLC analysis	18,5	31	41	28
Expected values	**19.0**	**50**	**25**	**25**
PhytoSensor [a]	17.4 ± 0.3	57 ± 2	27 ± 2	26 ± 3
HPLC analysis	18.0	57	21	22
Expected values	**19.0**	**75**	**12.5**	**12.5**
PhytoSensor [a]	15.7 ± 0.2	65 ± 1	19 ± 1	16 ± 1
HPLC analysis	17.2	75	11	14
Expected values	**21.0**	**25**	**50**	**25**
PhytoSensor	19.0 ± 1.0	32 ± 5	45 ± 2	23 ± 3
HPLC analysis	18.0	32	45	23
Expected values	**20.3**	**12.5**	**75**	**12.5**
PhytoSensor	17.6 ± 0.9	18 ± 1	70 ± 2	12 ± 1
HPLC analysis	19.8	13	77	10
Expected values	**20.0**	**25**	**25**	**50**
PhytoSensor	18.9 ± 0.9	23 ± 2	24 ± 2	53 ± 4
HPLC analysis	18.7	31	27	42
Expected values	**18.1**	**12.5**	**12.5**	**75**
PhytoSensor	19.3 ± 0.3	9 ± 2	10 ± 2	81 ± 4
HPLC analysis	20.2	17	14	69

[a] PhytoSensor data are mean value (± SD) of three measurements.

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Figure 1

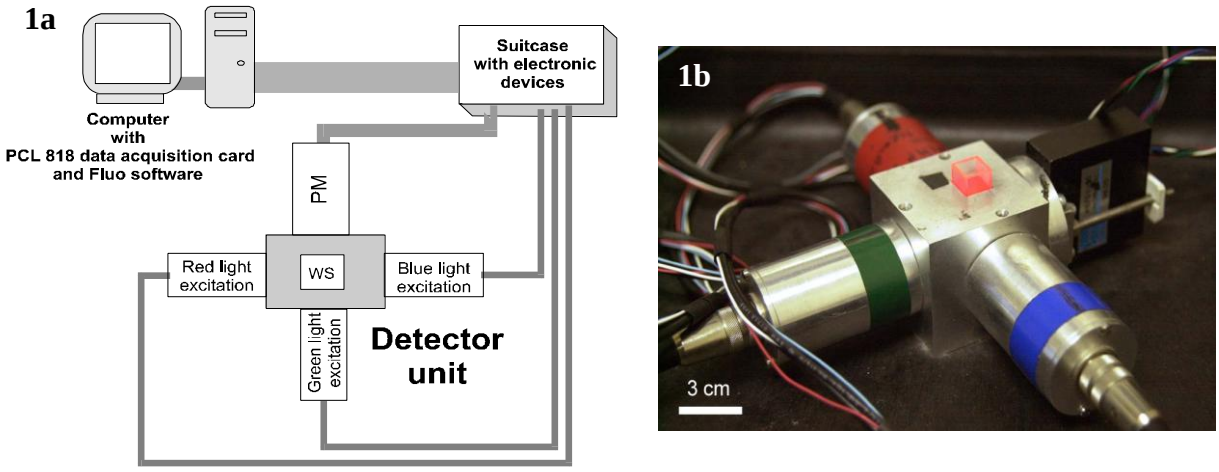

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Figure 2

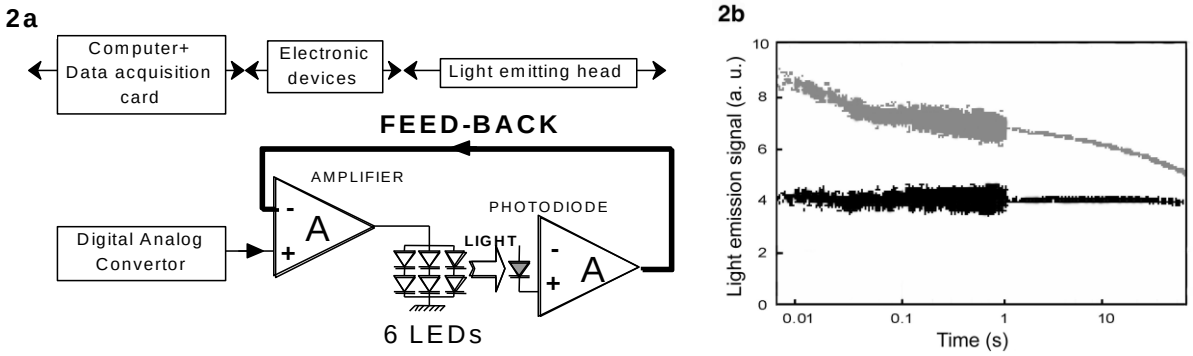

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Figure 3

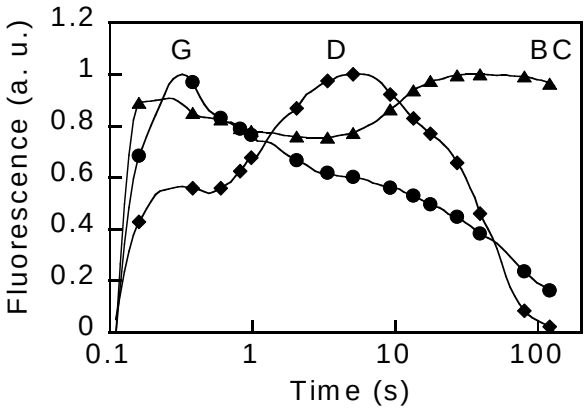

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Figure 4

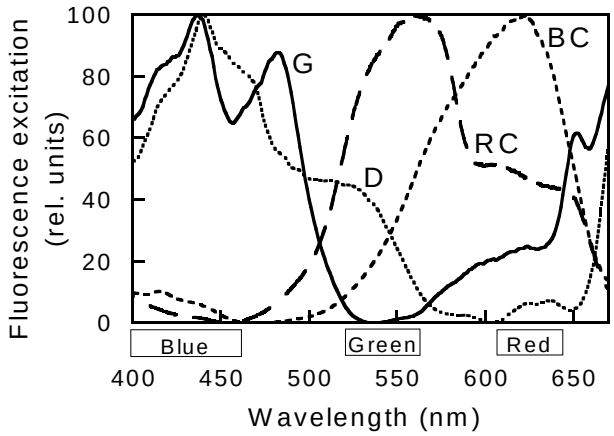

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Figure 5

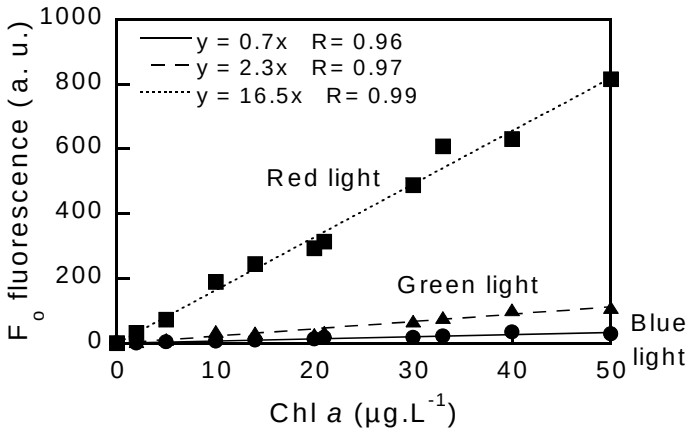

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Figure 6

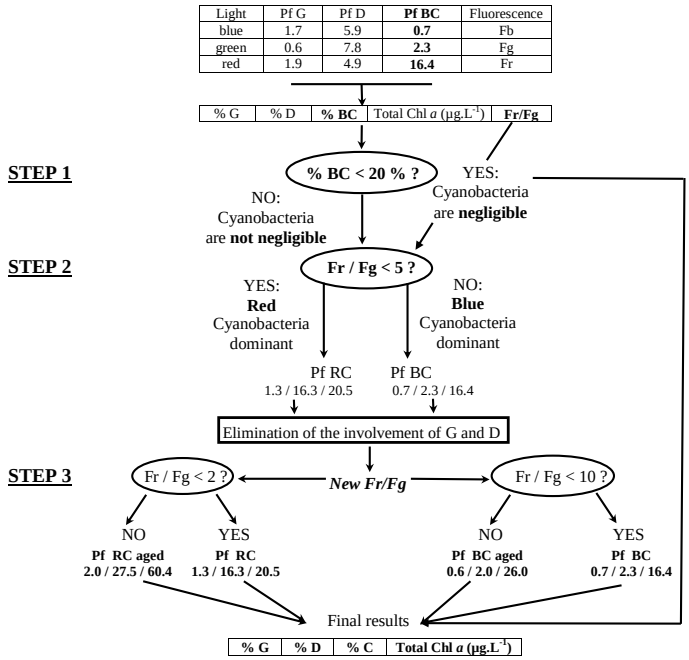

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Figure 7

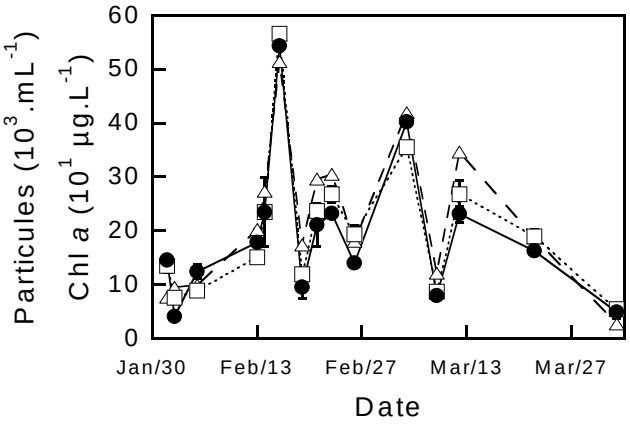

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Figure 8