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Early warning method for cyanobacteria toxin, taste and odor problems by the evaluation of fluorescence signals

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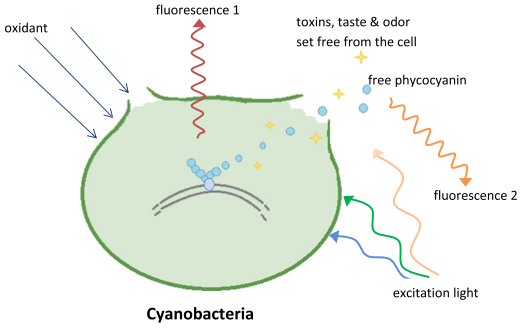
HIGHLIGHTS

- Stressed cyanobacteria can release toxins. An early warning system is discussed
- Stressed cyanobacteria in drinking water treatment plants affect water, air and health
- An apparatus analyzing optical spectra from cyanobacteria and released, free phycocyanin was used
- The appearance of free phycocyanin can be an indicator for released toxins etc.
- A model explains the energy distribution between the pigments in cyanobacteria and disconnected phycocyanin

GRAPHICAL ABSTRACT



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ABSTRACT

Permanganate and ozone are often used in drinking water treatment plants for the oxidation of taste and odor compounds, toxins, and algae as well as the reduction of mussel activity. The disadvantage of an overuse of such oxidants is the potential lysis of cyanobacterial cells. Cell lysis causes taste and odor components as well as toxins to be released into the water, which results in the need for even more treatment to remove these compounds completely. Our research in the CLIENT-SIGN project investigated an innovative method to monitor the lysis of cyanobacteria cells: increases in a specific fluorescence emission spectrum of the cyanobacteria pigment phycocyanin were used as a proxy for cell lysis and other compounds (taste/odor, toxins) leaving the cells. We call this form of phycocyanin "free phycocyanin" or "unbound phycocyanin". By monitoring free phycocyanin via a relatively fast and inexpensive measurement, water utilities will be better able to optimize the dosage of pre-oxidation compounds to remove extracellular compounds while preventing the lysing of cells. Laboratory studies and a case study at Yangcheng Lake (adjacent to Lake Taihu, Yangcheng Lake Water Treatment Plant, Suzhou Industrial Park, China) are presented herein. An online surveillance system that monitors incoming raw water and the water after pre-oxidation is proposed to better cope with changing water conditions.

1. Introduction

It is well known that the damage to the cell wall during cell lysis is a major player in the appearance of cyanotoxins as well as taste and odor (T&O) compounds and by-products during the water treatment process (Fang et al., 2010; Zamyadi et al., 2013; Ma et al., 2013). Further, the

Abbreviations: BAC, Biological Activated Carbon; Chl-a, chlorophyll; F_s, steady-state fluorescence; PSI, PSII, photosystem I and II; PC, phycocyanin; T&O, taste and odor compounds; IOM, intracellular organic matter.

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United States Environmental Protection Agency (EPA), (2014) note (810F11001) states that care has to be taken to use a correct concentration for pre-oxidation treatment in order to avoid lysing the cyanobacteria cells. It is also known that more distinctive treatment considerations are needed to find the optimal treatment process, as proposed in Ho et al., 2009. The optimal process will not cause cells to lyse, and thus avoids the increase or even start of problems during the drinking water treatment process. The use of excess oxidant(s) not only increases the costs of water treatment, but it may also be the cause for the formation of toxic by-products during the process, and should therefore be avoided whenever possible. Water treatment operators currently do not have a device that will provide a quick check of sufficient treatment levels regarding cyanotoxins and T&O compounds.

One possible device is an online fluorometer in the treatment train. Li et al., 2018 reported there is good correlation between the changes in the intensity of cyanobacterial pigments that fluoresce with cell inactivation due to oxidative treatment and the release of 2-methylisoborneol (2-MIB), thus making fluorescence an applicable monitoring method. Simis et al. (2012) previously described the role of cyanobacteria pigments and how the differences in their pigmentation are used to differentiate between taxonomic phytoplankton groups in applications that range from microscopy to the remote sensing of water color. The highest level of pigment differentiation between phytoplankton groups is found between prokaryotic cyanobacteria and the vast majority of algal taxa. Chlorophylls and carotenoids are the dominant pigments in algae while phycobilipigments (phycoerythrin, phycoerythrocyanin, phycocyanin and allophycocyanin) are the main light harvesting pigments in cyanobacteria, cryptophytes (prochlorophytes excepted) and red algae. Absorption and fluorescence techniques can be used to detect and determine the amount of biomass at both the community and subcommunity level based on differences in pigmentation between the major phytoplankton groups (Yentsch and Yentsch, 1979; Kolbowski and Schreiber, 1995; Beutler et al., 2002; Beutler et al., 2003; Seppälä and Olli, 2008). The distribution of chlorophyll-a (Chl-a) between photosystems I and II (PSI, PSII) is fundamentally different in these phytoplankton groups (Johnsen and Sakshaug, 1996, 2007), and would require special consideration in all aspects of fluorescence measurements of phytoplankton communities; for the sake of simplicity this is not done in this paper. Variable fluorescence methods relate the fluorescence that occurs with the "closure" of PSII centers under saturating light conditions or blockages to the energy flow in PSII (Kautsky and Hirsch, 1931; Genty et al., 1989; Kalaji et al., 2017). This can cause deviations in the estimation of the chlorophyll content. Algae class differentiating fluorometry is already widely being used for water quality risk assessment (Kalaji et al., 2016; Szymański et al., 2017). There are also approaches that use fluorescence in combination with other parameters, such as TP, DOC, turbidity etc., to create models that predict the appearance of T&O compounds (Bertone et al., 2018). The combination of these models with the technique proposed herein could be helpful to improve the prediction of critical extracellular compounds.

Advances in light-emitting diode (LED) manufacturing have resulted in readily available, high-power excitation light sources of high efficiency and stability. A method that uses discrete excitation-emission-fluorescence-matrices was used for the detection of pure phycocyanin (PC) (PhycoLA, bbe Moldaenke, Germany).

The present work applies a simplification to the work of Simis et al. (2012) as previously published by Moldaenke (2008) and Schmidt et al. (2009). An additional fingerprint for non-variable phycocyanin fluorescence was introduced and used to estimate its contribution to the amount of "PSII-fluorescence", even in mixed communities. For this purpose, extracted phycocyanin was used to extend the set of fingerprints (according to green algae, cyanobacteria, group of diatoms/dinoflagellates etc., cryptophytes and yellow substances, Beutler et al., 2002, Szymański et al., 2017) that are used to analyse a water sample and assign chlorophyll concentrations accordingly. Yellow substances herein represent degraded organic matter that results in fluorescence signals according to the applied excitation and emission wavelengths. They are measured in relative units [r.u.] and are mainly used to deduct its signals from the algae fluorescence. At the same time, the intensity of the concentration of the non-variable fluorescing phycocyanin in relation to the concentration of cyanobacteria was used as an indicator for the appearance of unbound, "free" PC. We will therefore herein discuss the potential role of free PC as an early warning parameter for lysed cells.

The cyanobacteria intracellular organic matter (IOM) was analysed by Wert et al., 2014, Li et al., 2012 and Fang et al., 2010. Cyanobacteria are known to produce a wide range of toxins (Lyra et al., 2001) and can produce T&O compounds like geosmin, 2-MIB and β-cyclocitral (as stated in the reviews Jüttner and Watson, 2007 and Lee et al., 2017, Zhang et al., 2013). These substances can pose a threat to human health and have a negative influence on drinking water quality. Water processing of IOM can also lead to the generation of oxidation by-products that will also impact water quality (Zamyadi et al., 2013).

The practical application of this work is the potential use of a fluorometer for the detection of fluorescence from chlorophyll disconnected phycocyanin. These molecules can serve as an early warning system for the presence of cyanobacterial T&O compounds and cyanotoxins in finished drinking water. This fluorescence cannot be used to determine a quantity of toxins and T&O products; the goal of this screening method is to use the parameter "free PC" to monitor and anticipate, and thus minimize, potential T&O and cyanotoxin problems during drinking water treatment and production. The monitoring targets are the complementary cyanobacterial T&O compounds (e.g. 2-MIB, geosmin) and cyanotoxins, based on the assumption that the release of phycocyanin from the cell is accompanied by the release of other compounds, such as cyanotoxins and T&O compounds. For demonstration purposes, we used the lab instrument bbe PhycoLA, but real-life conditions would require an online monitor that would allow water treatment operators to timely react to changing conditions. This instrument would be used to monitor the raw water, and ideally, in parallel, the water as it exits the pre-oxidation treatment and, if possible, after further treatment steps. Such an instrument is the bbe Phycosens, which has recently become available.

To show that free PC can serve as such an indicator, we analysed the fluorescence behaviour of cyanobacteria containing water samples as described above. We will show that this analysis discriminates between:

- PC that has probably left the cell (free PC) or is at least disconnected from chlorophyll but still in the cell (unbound PC);
- PC that is still bound to the photosystem.

This free PC signal can therefore provide a measure of potential risk in incoming raw water. This monitoring would allow for an early warning system, giving water treatment operators sufficient time to adjust the various treatment steps as needed. Additional monitoring along the treatment train would allow for optimization of the treatment process, saving money but also alleviating problems from arising later in the process.

2. Materials and methods

2.1. Cyanobacteria cultures and growth conditions

The cyanobacteria cultures of *Microcystis aeruginosa* SAG 46.80 were grown in 250 mL of HUB-Voll medium in culture flasks at 22 °C. Daylight fluorescent tubes (18 W BIO Vital 1125Lm, Narva, Germany) in a 12 h:12 h light-dark cycle were used, which resulted in light conditions of 10–40 μmol photons m^{-2} s^{-1} (LI-190/R sensor; LI-250A Light Meter; LI-COR Inc. Lincoln, Nebraska, USA). The algal cells used in these experiments were in a stationary growth phase.

2.2. Cyanobacteria treatments

Different methods were used to cause the cyanobacteria to undergo physiological stress that led to cellular disruption. Mechanical stress was caused by sonication of the cells. Chemical stress was mimicked by exposing the cells to potassium permanganate.

2.2.1. Sonication

For the ultrasound experiments, a 0.5 L sample that contains approximately 40 μg Chl-a/L of *M. aeruginosa* in HUB medium was prepared and divided in 15 mL samples. Each sample was placed in a 10 °C water bath and subjected to sonication (Bandelin Sonoplus HD2070, Sonotrode UW2070) for various lengths of time and amplitudes. The cycle was set to 50% for all samples. The amplitude and treatment time were, respectively: 10%, 35%, 70%, 100% and 0 s, 150 s, 300 s, 600 s. For analysis, the sample was diluted 1:10 with medium to a final volume of 150 mL and divided in half. One half of the sample was directly measured for pigment concentrations with bbe PhycoLA and for organic matter with bbe FluoSens. The second half of the sample was filtered through a 0.45 μm cellulose nitrate filter (Sartorius AG, Goettingen, Germany) from which a 0.5 mL sample was analysed for microcystins, the rest was measured again with PhycoLA and FluoSens. Only the control sample and the 10% 150 s, 70% 150 s, 100% 300 s and 100% 600 s samples were analysed for microcystins.

2.2.2. Permanganate

An experiment was performed to mimic the chemical stress that is caused by the addition of potassium permanganate to raw water. In this experiment, a cyanobacteria culture in medium was subjected to treatment with potassium permanganate as a surrogate for chemicals that are added during the pre-treatment process in water treatment plants.

The treatment was performed on a sample spiked with *M. aeruginosa*. The sample was analysed before treatment (0 min) and directly after 30, 60, 120, 210, 360, and 1440 min. Each time a sample was taken, and any remaining $KMnO_4$ residual was quenched with excess $Na_2S_2O_3$. The samples were analysed with bbe PhycoLA before and after filtration, using a 0.45 μm cellulose nitrate filter (Sartorius AG, Goettingen, Germany). A sample of 0.5 mL of the filtrate was taken for extracellular microcystins analysis.

2.2.3. Chlorination

500 mL of *M. aeruginosa* solution was added to chlorinated drinking water (estimated chlorine content was 0.4 mg/L) at a 1:1 ratio. The samples were measured in 25 mL cuvette in PhycoLA after 0, 5, 15, 25, 35, 45 min and 345 min.

2.3. Water treatment plant monitoring

Samples were collected after intermediate process steps at the Yangcheng Lake Water treatment Plant. A schematic of the water treatment process is shown in the Fig. 7. The sampling points were located before the pre-ozonation step (raw water), after pre-ozonation, at the outlet of the sedimentation tank, after sand-filtration, after post-ozonation, after Biological Activated Carbon (BAC), and after the clean water tank. The fluorescence with bbe PhycoLA was measured within 1 h of taking the sample. The samples were stored at 4 °C until they were analysed for 2-MIB and geosmin content. Part of each sample was filtered with 0.45 μm membrane filter. Filtered and unfiltered samples were analysed within a day of collection.

2.4. Chemical analysis

2.4.1. Determination of 2-MIB and geosmin

The analysis for 2-MIB and geosmin was performed following the standard GB/T 32470–2016. Extraction was performed using headspace solid-phase micro-extraction (SPME) with divinylbenzene/carboxen/polydimethylsiloxane (DVB/CAR/PDMS) fibers (aging temperature 230–270 °C for 1 h for the fiber's first use). Sample extracts were injected into the gas chromatography/mass spectrometry (GC (7890B) - MS (7000C), Agilent, USA) in splitless mode with an inlet temperature of 250 °C. The carrier gas was ultrapure helium at 56.6 kPa, and the temperature for the column (Agilent HP-5, 30 m × 0.25 mm × 0.25 μm) was programmed for an initial temperature of 60 °C for 2.5 min and then a temperature increase to 250 °C at a rate of 8 °C/min, and then maintained for 5 min. The analysis was performed with 2-isobutyl-3-methoxypyrazine (IBMP) as the internal standard.

2.4.2. Determination of microcystins

Extracellular toxins in the samples were tested with the use of immunochromatographic test strips for the detection of microcystins and nodularin in finished drinking water samples (Microcystins Strip Test Abraxis, Warminster, PA, U.S.). The samples were diluted 1:4 for analysis.

2.5. Extraction of phycobiliproteins (PBP)

50 mL of a *M. aeruginosa* culture was centrifuged with a Varifuge K (Heraeus Christ GmbH, Osterode, Germany) at 3500 rpm for 20 min. The cyanobacteria pellet was re-suspended in 10 mL of 0.05 M phosphate buffer set to pH 6.8. The suspension was subjected to a freeze-thaw cycle, where it was frozen at −18 °C and thawed at 13 °C. To clarify the sample, the sample was filtered with a 0.45 μm syringe filter (cellulose acetate membrane, Rotilabo®). Phycocyanin content and purity in the PBP extract was determined with the use of a Lambda 25 spectrophotometer (Perkin Elmer Inc., Waltham, Massachusetts, U.S.).

2.6. bbe PhycoLA

A bbe fluorometer as described in the work of Beutler et al. (2002) was used with a second detector to measure fluorescence at 650 nm (Beutler et al., 2003). The resulting instrument, bbe PhycoLA, is a cuvette fluorometer that also consists of 7 excitation LEDs and 2 detection photomultipliers. The instrument is capable of measuring photosynthetic yield, as described by Genty et al. (1989), and transmission light at all excitation wavelengths and scattered light at 700 nm.

The PhycoLA calibration was done by using five different algae-representing spectral groups (Beutler et al., 2002). The calibration was extended with the use of previously extracted phycocyanin.

2.7. bbe FluoSens

bbe FluoSens expands an LED fluorometer principle (Beutler et al., 2002) used in the bbe PhycoLA to the UV-VIS range to offer an instrument for dissolved organic matter analysis. The principle is a simplification described in the work of Wagner et al. (2016). FluoSens records on-line discrete EEM matrix at 6 excitation wavelengths (245, 255, 285, 315, 430, 525, and 610 nm) and 4 detection wavelengths (328, 429, 511, 700 nm). The measurement of transmission at excitation wavelengths allows for the correction due to inner filtering effects (Lakowicz, 2008) and to determine traditional parameters like Spectral Absorption Coefficient at 254 nm. The collected data allows for the detection and differentiation of fluorescence from protein-like substances (biopolymers) and high and low molecular humic substances as well as from chlorophyll.

3. Results and discussion

Several types of instruments have been designed for the purposes of monitoring and evaluating algae and free phycocyanin fluorescence. The types of systems that use this technology are a lab system, a profiling system, and an online system. The system used in our study is the

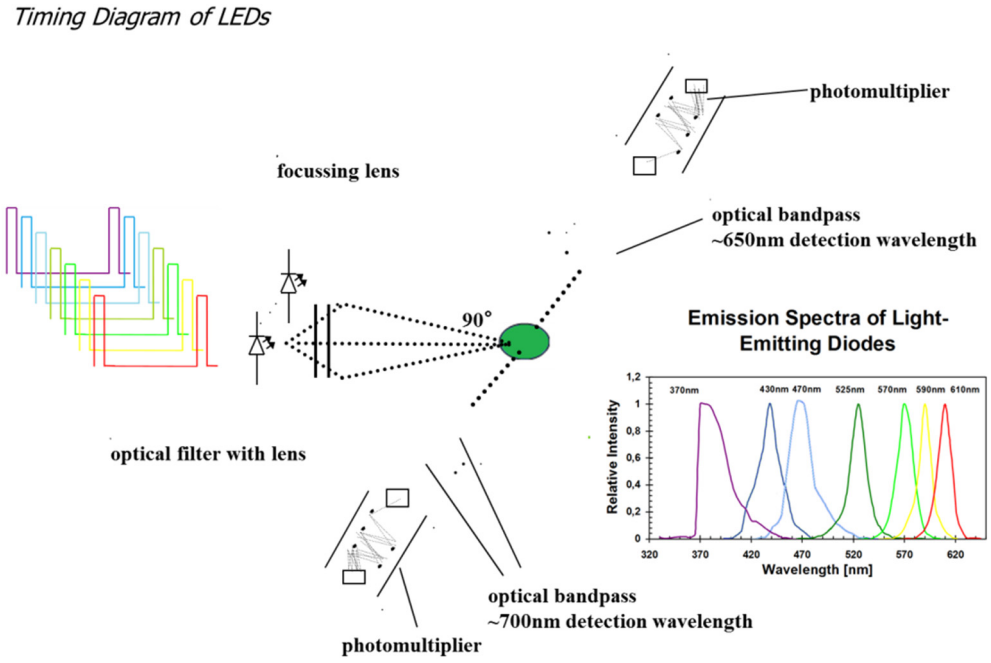
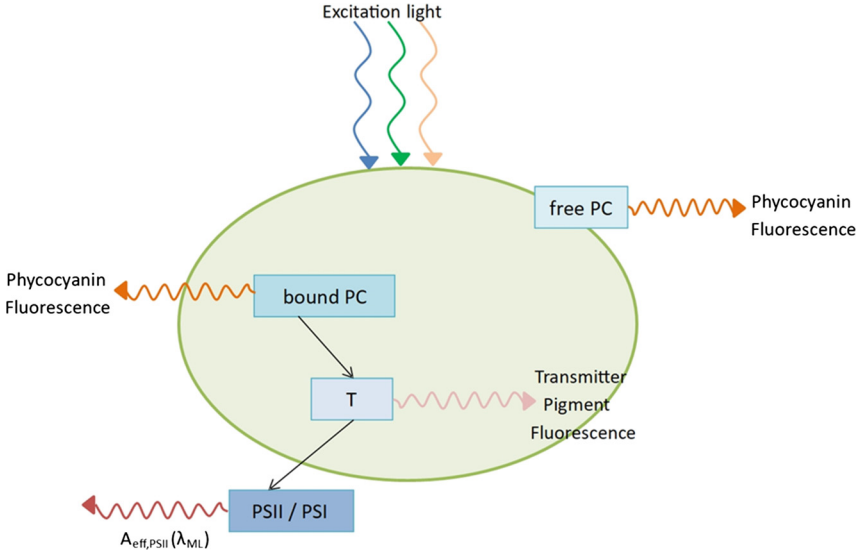


Fig. 1. Schematic of the new discrete band fluorescence-spectrometer.

lab system (PhycoLA). All of these systems have a system of light emitting diodes and sensors (Fig. 1) in common. This is essentially the core of the evaluation system for chlorophyll and phycocyanin fluorescence. Seven LEDs with peak wavelengths at 370, 430, 470, 525, 570, 590 and 610 nm are used to induce specific excitations of algae pigments and humic substances. This is an extension of work previously done by Beutler et al. (Beutler et al., 2003).

In addition to a detector at about 700 nm that mainly traps the fluorescence from the PSII system, a (mainly) phycocyanin fluorescence sensor with the central wavelength of 650 nm was used. We propose a new simplified model (Fig. 2) of the energy pathways that are possible for phycocyanin containing algae. It shows the different possibilities that are available to distribute light-induced energy once absorbed by phycocyanin. The fluorescence, F, is measured in steady state and refers to F_S as defined by van Kooten and Snel, 1990 and Kalaji et al., 2017, here at about 10 μmol/m^2/s photons.

The following consideration applies to the fluorescence emitted by PC and chlorophyll: the change of the energy from both of these emitters can have mechanical, protective and damage-related reasons and will cause reduced or increased PC and chlorophyll fluorescence.



$$F_{tot}(\lambda_{ML},\lambda_{em}) = F_{PSII}(\lambda_{ML},\lambda_{em}) + F_{PSI}(\lambda_{ML},\lambda_{em}) + F_{PC,bound}(\lambda_{ML},\lambda_{em}) + F_{PC,free}(\lambda_{ML},\lambda_{em}) + F_{Transmitter}(\lambda_{ML},\lambda_{em})$$

Fig. 2. Sketch of the energy pathways that exist within phycocyanin (PC) and chlorophyll, respectively, photosystems (PSII, PSI). The excitation light at the wavelength relevant to PC is absorbed by the pigment and then transferred via a transmitter system to the photosystems, which leads to fluorescence, F. Alternatively, decoupled PC (unbound or free) adds its own fluorescence. The formula expresses the intensity of fluorescence responses based on the measuring light (ML, excitation and intensity) and emission wavelength (λ_{em} = 650 nm and 700 nm).

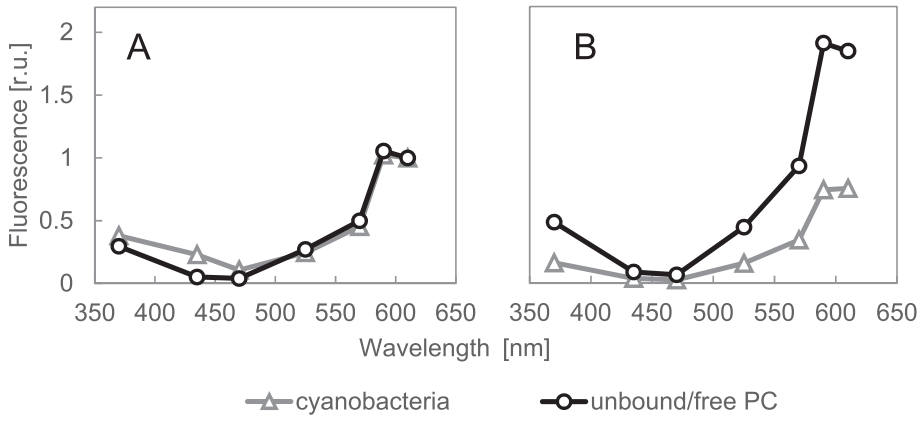


Fig. 3. Fingerprints of cyanobacteria (Δ) and unbound and free phycocyanin (PC) (o) at (A) 700 nm and (B) 650 nm detection. The data is normalized to the 610 nm excitation and 700 nm emission point, but are not corrected for LED brightness and different detector sensitivities.

Examples of patterns of PC and chlorophyll fluorescence at varying conditions can be observed when looking at time-dependent spectroscopy data (Wlodarczyk et al., 2012). The magnitude of the chlorophyll decay function changes based on the degree of coupling between PC and chlorophyll.

As observed by Six et al. (2007), the dismantling of PC can be detected per fluorescence at 650 nm.

The system used here analyses the excitation spectra at 650 nm and 700 nm emission wavelength. A set of fingerprints is used for the analysis via the solution of the Gaussian equation (Beutler et al., 2002). For the estimation of the contribution of free PC to the total fluorescence spectrum, PC was isolated from *M. aeruginosa*, and its spectrum was added to the set of fingerprints (Fig. 3). This approach is a rough approximation; it doesn't consider changes in the fluorescence from other pigments in the phycobiliprotein complex. The additional fingerprint for free PC was proven to be linearly independent, which is an important prerequisite for the applicability of the used model. Another investigation (Simis et al., 2012) considered the role of each component of the complex, and provided methods with the unique focus on uncoupled PC. The model described below is a simplification of the actual process where e.g. the influence of PSI fluorescence is neglected. However, it will be shown that this simplification is helpful for the evaluation of algae conditions in a water treatment plant. The model which allows to differentiate characteristic norm spectra and to determine densities of algae classes, free phycocyanin and yellow substances is described in the appendix. An evaluation using the proposed model can describe the observed fluorescence spectra with a low error.

3.1. Lab tests of the parameter "free" or "unbound" phycocyanin

The lysing process of cyanobacteria and its potential to release IOM such as cyanotoxins and T&O components is shown below. This knowledge can be integrated into water treatment processes and can potentially lead to optimized procedures for pre-oxidation.

A very simple trial shows how cell decay is tied to the resulting signal change (Fig. 4). Chlorinated drinking water (estimated chlorine content was 0.4 mg/L) was mixed 1:1 with a *M. aeruginosa* suspension. The bbe PhycoLA was used to monitor the mixture and its filtrate. Intact cells were removed via filtration, and only extracellular phycocyanin was detected. PC is decoupled from photosystems and can be detected according to its fluorescence properties. It can also be detected in the filtered solution, which indicates that the model is useful. The chlorophyll fluorescence expressed by the coefficient $a_{cyanobacteria}$, which refers to the cyanobacteria fingerprint in Eq. (5), also increases in the first minutes. This is probably caused by effects on metabolic processes and the function of the electron transport chain, what can temporarily lead to an increase of the steady state fluorescence. They investigated the change of the chlorophyll fluorescence and the parallel release of 2-MIB and other metabolites. Depending on the oxidant (ozone, chlorine, permanganate) and its concentration, they also found significant differences in the reaction time.

Mechanical stress can also cause such changes in energy distribution. The application of ultrasound can disrupt the cell integrity (Bandelin Sonoplus HD2070), of PC and other phycobiliproteins (unbound phycoerythrin can also act as an indicator, data not shown).

Fig. 5 shows the result of cell lysis caused by sonication. This treatment mimics the mechanical stress (senescence and cell aging) experienced by cells in the raw water during the water treatment process. The test shows that before treatment the sample contains 100% cyanobacteria and no free PC (A). This slowly changes as the sample is treated for 2 min (100% of 100 W). Cells lyse and free PC appears in both the treated and the 0.45 μm filtered sample, which indicates that free PC has partially left the cell. This trend continues and becomes more obvious after 12 min of treatment, which demonstrates that cell lysis can be monitored with the use of fluorescence detection methods.

The characterisation of algal IOM and its release due to oxidative stress is widely described (Wert et al., 2014, Li et al., 2012, Fang et al., 2010). Fig. 5(A) demonstrates that when cells lyse and release molecules (biopolymers such as proteins, peptides and amino acids), the

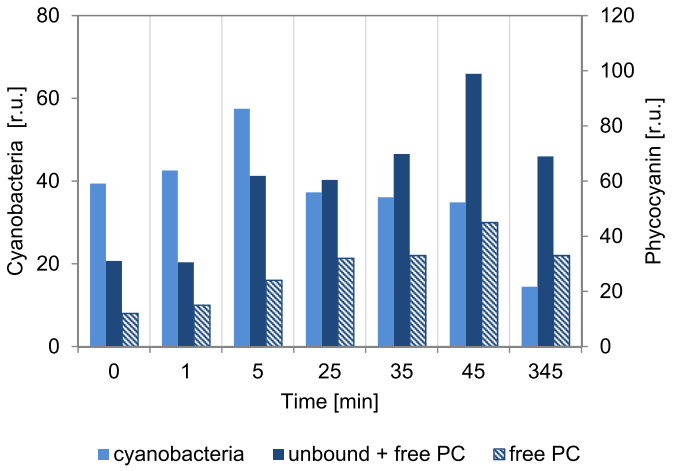


Fig. 4. Chlorinated tap water is spiked with a cyanobacteria culture (*M. aeruginosa*) at time 0. Data show fluorescence (light blue) and unbound plus free phycocyanin (dark blue blocks). Free phycocyanin (striped blocks) measurements were collected after filtration. The cyanobacteria chlorophyll fluorescence is expressed by the coefficient $a_{cyanobacteria}$ which refers to the cyanobacteria fingerprint in Eq. (5). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

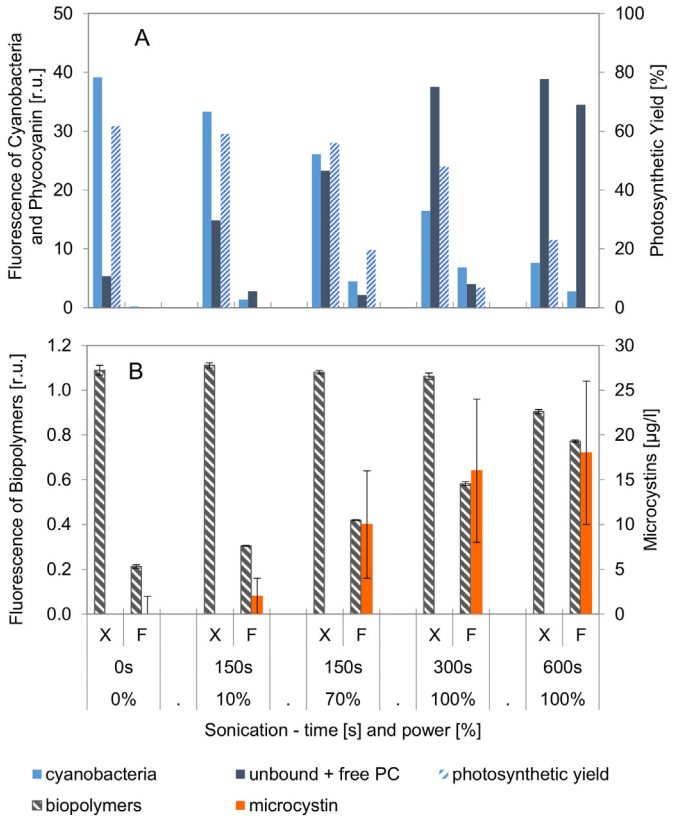


Fig. 5. The lysis of the cyanobacteria cells treated with ultrasound. (A) Recorded with PhycoLA, fluorescence of cyanobacterial Chl-a (light blue), sum of free and unbound PC (dark blue) and photosynthetic yield (striped). Second graph (B) presents FluoSens measurements of biopolymers (striped gray) and results of microcystins test (orange). In both cases, the results are shown for non-filtered ("X") and 0.45 µm filtered samples ("F"). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

molecules can pass through the 0.45 µm filter. Further ultrasound energy disrupts more cell membranes, which causes more components to leave the cells. At the same time, however, the concentration of the biopolymers in the samples that were treated with varying energies remains the same, as was expected.

We also investigated the behaviour of the microcystins present in the algae in this study. Fig. 5(B) shows the appearance of these interesting cellular components. In this test, microcystins are at a high enough level that they can be measured in the filtered phase after increased ultrasound treatment time, which shows that the appearance of free PC can serve as a warning signal for the presence of cyanotoxins and T&O compounds. If free PC appears in the matrix, the current water treatment should be under scrutiny:

Within these detection cycles, there is a delay between the appearance of free PC in the unfiltered samples and the appearance of free PC and microcystins in the filtered samples. The reasons for this are not yet well understood. One possibility is that microcystins exits the cells easier and thus earlier than the disrupted PC. This could be due to the difference in their sizes. The molecular weight of microcystins is between 800 and 1100 Da (van der Merwe, 2015), PC is about 100 times heavier (Dasgupta, 2015) and therefore substantially bigger.

Zamyadi et al., 2012 observed that inefficiencies in the water treatment processes can lead to breakthrough of cyanobacterial toxins into the treated drinking water. One such treatment that has the potential of the dose being too low or too high is the pre-oxidation. The pre-oxidation step often occurs at the beginning of the water treatment train, and if it leads to cell disruption and IOM being released from the cell, it can have severe effects on the water quality. As a consequence, a toxin and T&O breakthrough can occur as well as the formation of by-products (Fang et al., 2010; Zamyadi et al., 2013; Ma et al., 2013).

Next, an example of oxidative stress was analysed with the use of potassium permanganate, $KMnO_4$, on samples with *M. aeruginosa*. The results are displayed in Fig. 6.

In this trial the cell lysis allows for the detection of free plus unbound PC in the original sample as well as extracellular microcystins and free PC in the filtered sample. Parallel to the increase of unbound and free PC in the original solution, free PC and microcystins in the filtered solution increased. When lysis started after 120 min, the permanganate is mostly consumed and PC is no longer severely affected. Residual oxidant and the natural decay of PC leads to its decrease after 6 h. The results are in good agreement to those presented in previous publications that analysed the release of toxin and the kinetics of cell inactivation as a result of exposure of *M. aeruginosa* to permanganate (L. Li et al., 2014a, 2018). This method demonstrates its advantage when using chemicals like ozone and permanganate because it only takes that concentration of the oxidation chemical in account that is available to affect the cell membranes. Yellow substances and other extracellular compounds are competitive reaction partners for the oxidant permanganate. If this concentration is low, permanganate can oxidize cells at concentrations down to 0.2 mg/L; at high concentrations of humic substances, the effect can be delayed by hours and only happens at permanganate concentrations of >8 mg/L (data not shown). Other important factors that must be taken into consideration for an effective oxidation are the physiological conditions, concurrent other algae and the formation of colonies. All these parameters can affect the release of toxins, T&O compounds and PC.

The exact relationship of the appearance of free or unbound PC and toxins, and T&O components in the water is hardly predictable. First of all the concentration of all components (total algae/total biomass, cyanobacteria, and other physical parameters) vary naturally over time. These variations can affect the oxidation capacity strongly, e.g. if the biomass rises but the added oxidants remain constant, or only the toxin content per cell rises. The expression of cyanotoxins and T&O compounds can vary very widely depending on the species (Jüttner and Watson, 2007) and natural conditions (Oh et al., 2017). Further, even if the retention time in the coagulation/sedimentation basin is, as given here, 2 h, portions of algae will remain in the basin for a longer time. Cyanobacteria will also be retained in the sedimentation basin and can release IOM (X. Li et al., 2014b). Lastly, the contact time plays an important role. At low concentrations of the applied oxidant in relationship to the total organic matter, the oxidant can be consumed within minutes (L. Li et al., 2014a, 2018). This is very often the case.

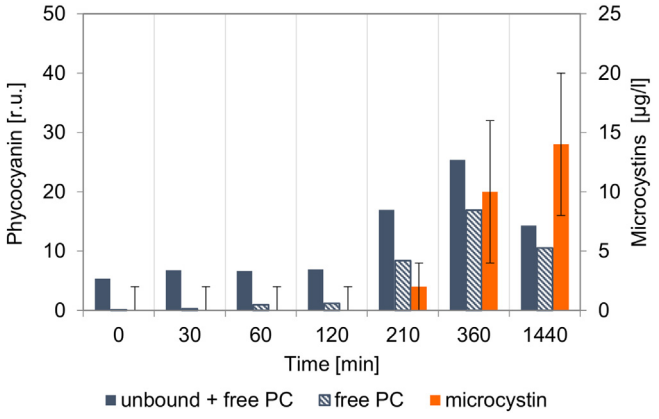


Fig. 6. Application of 4 mg/L $KMnO_4$ on a microcystins containing *M. aeruginosa* suspension with estimated 50 µg/L chlorophyll-a. The solid blue bars represent unbound plus free phycocyanin. Free phycocyanin (striped) and microcystins (orange) were measured after filtration. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

The cell wall can nevertheless be affected and it can start lysing after a few minutes to hours; the free PC does not react with substantial amounts of the oxidant and thus can show life times of several hours. Fig. 4 provides such an example. Chlorine is consumed before cell lysis becomes obvious. PC, which can easily be oxidized by chlorine (Schmidt et al., 2009), is still detected for hours. Only an overdose of the oxidant(s) will oxidize the PC as well, but in this case it can cause by-products that have to be avoided.

3.2. Field test of the parameter "free" or "unbound" phycocyanin

In a practical application, we investigated the behaviour of free PC in the Yangcheng Lake Water Treatment Plant in Suzhou Industrial Park in China. The process of the plant is shown in Fig. 7. In previous years *M. aeruginosa* blooms were identified as a source of T&O in nearby Lake Taihu (Ma et al., 2013 and Zhang et al., 2013). The presence of cyanobacteria in this plant was generally not of any concern from 2015 to 2018, except for a few minor events with values below World Health Organisation thresholds (WHO, 1998). One event is shown in Fig. 8. On June 15, 2015, free phycocyanin appeared in a greater magnitude after ozonation. For some of these days, free phycocyanin was already a substantial part of the raw water. Based on this, we assume that the water treatment plant was dealing with a small dying cyanobacterial bloom during these days, which caused increased values of free PC.

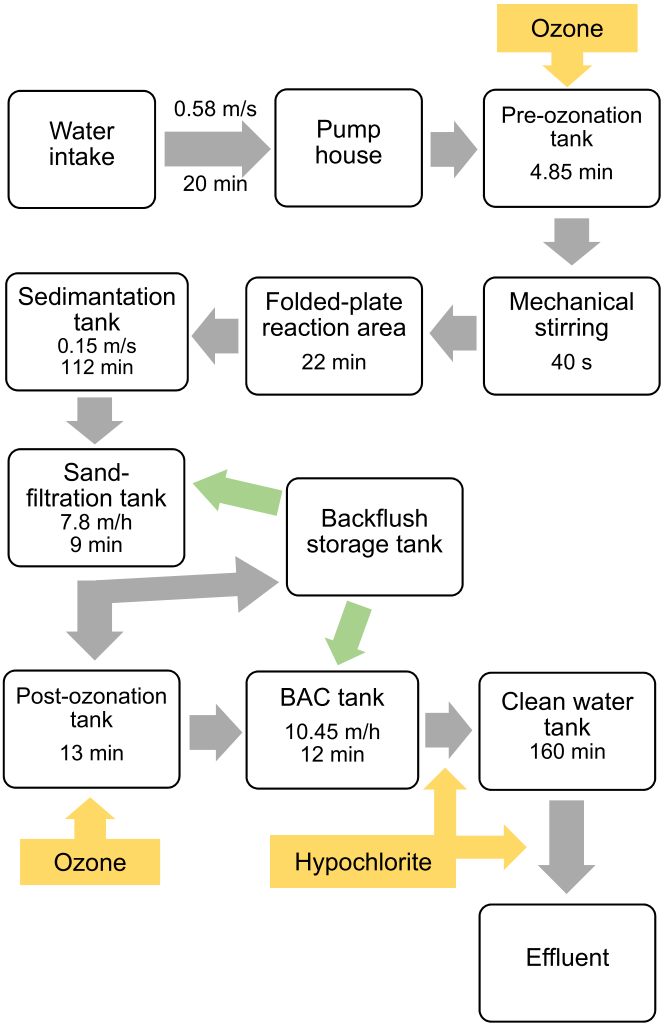


Fig. 7. The water treatment train in Yangcheng Lake Water Treatment Plant in Suzhou Industrial Park in China. In the pre-ozonation step 1.1 mg/L ozone was added.

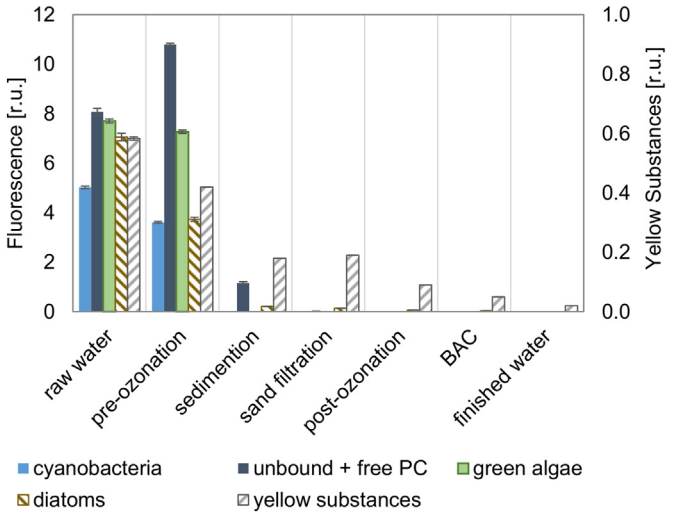


Fig. 8. Analysis of samples taken at several steps of the Yangcheng Lake Water Treatment Plant in Suzhou Industrial Park in China.

Raw water was treated with ozone in the pre-ozonation tank. The treatment process resulted in an increased amount of free and unbound phycocyanin in the water after pre-ozonation. In the next step PC passed the sedimentation basin but was adsorbed in the filtration step. We assume natural decay is the dominant reason of its reduction. None of these components were detected in the finished water possibly because of low cell density or a lack of toxins and T&O products. Yellow substances were also reduced within the treatment train. This might serve as an indication that the oxidation procedures were sufficient.

The lysis effect of cyanobacteria at Yang Cheng Lake could be observed more concisely in Fig. 9. The pre-oxidation triggers the lysis of cyanobacteria so that free phycocyanin is released after 30 min. At the same time the chlorophyll fluorescence of the algae cells is decreasing. After another 120 min retention time in the sedimentation basin, the chlorophyll and phycocyanin concentration is reduced to almost zero. Fig. 9(A) illustrates the lysis of cyanobacteria, whose chlorophyll fluorescence is already disappearing during ozonation, and instead a peak of free PC was observed. In the present case, other algae species appear to be more resistant to pre-oxidation.

The investigation of 2-MIB and geosmin in parallel shows that odorous substances are already present intracellularly and extracellularly in the raw water (Fig. 9B). With pre-oxidation, almost all cyanobacteria appear to lyse, as an increase in extracellular concentrations close to the total concentrations are observed. At the same time as the increase in the extracellular concentrations occurs, the content of free PC also reaches its maximum. In the course of further water treatment, both the free PC and, albeit only with post-oxidation, the odorous substances are completely oxidized.

4. Conclusions

Given that about 220 T&O components and about 80 cyanobacterial toxins have already been identified, water treatment operators cannot and do not know whether cyanotoxins and/or T&O components are present in the raw water unless they have access to a time consuming, detailed and comprehensive chemical analysis. In addition it is well known that it is more difficult to remove toxins and T&O compounds from the water once they are no longer contained in the cells, as it moves through the treatment train and exits the water treatment plant. A quick and sensitive indicative test is needed to monitor the water for proper treatment levels. Lab studies showed that fluorescence detection could be used for an absence/presence indication

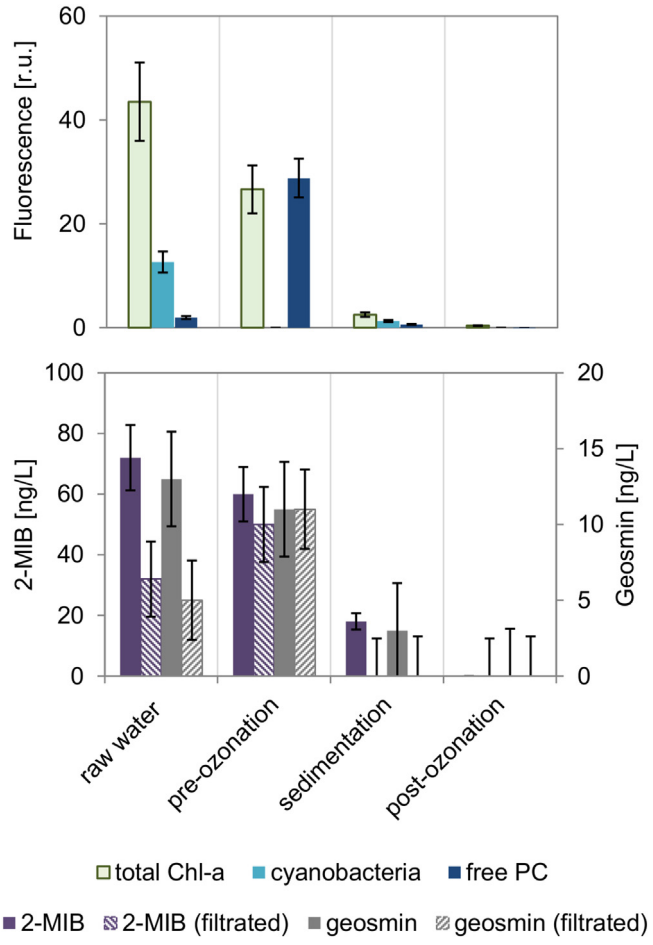


Fig. 9. Monitoring of several treatment steps of the water treatment plant. (A) The degradation process of total chlorophyll-a (green), chlorophyll-a containing cyanobacteria (light blue) and free phycocyanin (dark blue) during the water treatment process. (B) Presentation of the odorous substances geosmin (gray) and 2-MIB (violet) during treatment steps. The total concentrations (solid blocks) and the concentrations in solution measured after sample filtration (extracellular; striped blocks) are compared. The transition of the odorous substances into solution is accompanied by the occurrence of free phycocyanin. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

of microcystins. Successful detection of free PC in the filtered phase after inappropriate treatment demonstrates that the appearance of free PC can be used as a warning signal. This method was used in a field study in an actual water treatment plant, where operators were able to detect free PC as a warning sign for release of T&O compounds into the water column. The monitoring described herein provides operators an early warning system that will give them sufficient time to adjust various treatment steps to ensure optimal treatment of the water and avoid costly problems later.

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Appendix A. Proposed model and its evaluation

A model was developed that uses normalized spectra of algae classes and phycocyanin to estimate their density in a water sample. The sum of the multiples of the spectra has to be close to the measurement $\underline{M}$ with

$$\underline{M} = (F_{i,j}) \tag{1}$$

for which $F_{i,j}$ are the fluorescence contributions at the excitation wavelengths $i = 370, 430, 470, 525, 570, 590$ and 610 nm and the detection wavelengths are $j = 650$ and 700 nm.

The normalized curve of an algae class can be represented by:

$$n_k = \left(f_{k,i,j}\right) \tag{2}$$

for which $f_{k,i,j}$ is described by the chlorophyll fluorescence of the algae class k for the excitation wavelength i and the detection wavelength j. The individual values are normalized against the fluorescence values for 1 μg/L chlorophyll and 1 μg/L phycocyanin.

The coefficient a_k represents the multiple of the normalized concentration of the chlorophyll of each algae class in order to estimate the value for M, ideally, with the use of a linear equation:

$$M = \Sigma a_k\, n_k \tag{3}$$

Inaccuracies during the measurement that are dependent on the wavelengths and the algae species allow us to only approximate the measurement $\underline{M}$, and the difference χ^2 between the measured and estimated fluorescence values should be minimized.

$$\chi^2 = \left(\underline{M} - M\right)^2 \tag{4}$$

After the introduction of weighting factors $\sigma_{k,i,j}$ that represent different reliabilities of the norm curve $n_k = f_{k,I,j}$ at specific wavelengths i and j, χ^2 can be calculated as

$$\chi^2(a_1, a_2, \ldots) = \sum_{i=1}^{I}\sum_{j=1}^{J}\left(\frac{F_{i,j} - \sum_{k=1}^{K} a_k f_{k,i,j}}{\sum_{k=1}^{K} a_k \sigma_{k,i,j}}\right)^2 \tag{5}$$

χ^2 is the value for the deviation between the estimated and measured values and must be minimized with the use of a suitable algorithm (Beutler et al., 2002). The standard deviations $\sigma_{k,i,j}$ are not easy to determine. In principle they represent the variation of the various components in the total of all forms of a class of algae fingerprints. The sigma values can also be determined for the various isolated fingerprints such as free PC and yellow substances. The PSI fluorescence and the transmitter pigment fluorescence are not taken into consideration. The fit was carried out by placing newly determined a_k's back into the numerator, and recalculating a_k. This procedure is described by Beutler et al. (2002).

To control the quality of each fit, the relative lack of fit (Wagner et al., 2016) is used to relate the sum of the deviations between measured and estimated data to the sum of measured data

$$LoF_{rel} = \sqrt{\frac{\left(M - \underline{M}\right)^2}{\underline{M}^2}} \tag{6}$$

This parameter indicates how much of the measured data cannot be explained by the model. Only measurements where the lack of fit doesn't exceed 10% are taken into consideration in the tests. This choice was justified using the sonication experiment shown in Fig. 5. Fig. A.1 compares measured with modelled data under different treatment conditions. Fig. A.1(A) and (B) show good conformity of both curves using an untreated cyanobacteria sample (LoF_{rel} = 2,55%) as well as after the application of high power ultrasound sonication (LoF_{rel} = 4,74%) where free phycocyanin was released.

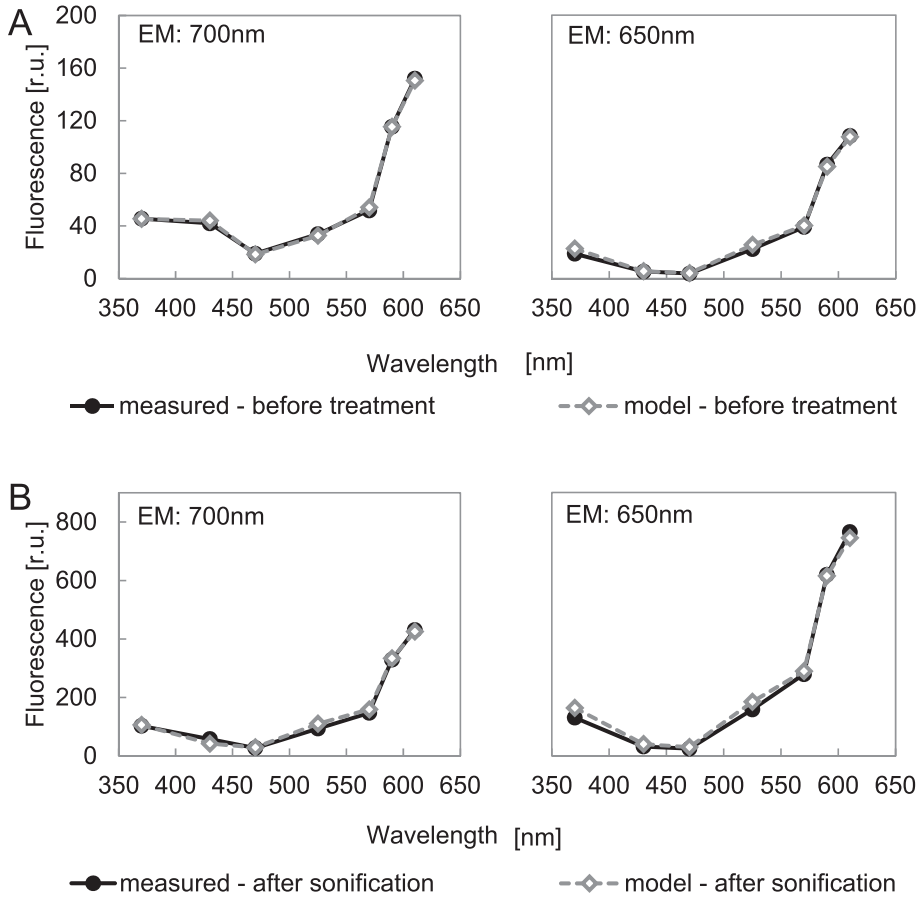

Fig. A.1. Measured excitation spectra (black) at 700 nm (left) and 650 nm (right) emission overlaid by the modelled data (gray) for (A) untreated suspension of *M. aeruginosa* and (B) after 600 s sonication treatment at 100% amplitude. Data were collected with bbe PhycoLA and not normalized on the same brightness of each excitation wavelength.

The LoF_{rel} for all sonication experiments is presented in Table A.1. The data show that the measured fluorescence can be explained well by the proposed model. Deviations are possible if the applied fingerprints do not fit to the algae species whose behaviour is investigated. The lab studies show a much smaller error because the exact fingerprints from the cultures are well known. In treatment plants, the calibration would need to be repeated, mainly for cyanobacteria, once the error exceeds the limit, either in the raw or treated water. It was found that all data with a $LoF_{rel} < 10\%$ gave results that coincided with other observations such as the appearance of free PC. Also, the data are still useful if the LoF_{rel} is low in the raw water, but higher in treated water as might be the case when the PC is mostly stripped from the chlorophyll. In such a case, the error increases because the remaining cyanobacteria fingerprint changes and it can no longer be properly modelled by the currently applied set of fingerprints. Nevertheless, the appearance of free PC was well detected.

Table A.1
Lack of fit as defined in Eq. (6) calculated for the individual data sets collected during sonication study.

Sample	Sonication parameter		Lack of fit [%]
	Power	Treatment time [s]	
Before sonication	–	–	2.55
Sonication	10%	150	4.54
Sonication	70%	150	5.12
Sonication	100%	300	4.83
Sonication	100%	600	4.74

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