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In-situ fluorescence monitoring of cyanobacteria: Laboratory-based quantification of species-specific measurement accuracy*

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ABSTRACT

In recent years, in-situ fluorometers have been extensively deployed to monitor cyanobacteria in near real-time. Acceptable accuracy can be achieved between measured pigments and cyanobacteria biovolume provided the cyanobacteria species are known. However, cellular photosynthetic pigment content and measurement interferences are site and species specific and can dramatically affect sensor reliability. We quantified the accuracy of an in-situ fluorometer compared with traditional methods using mono- and mixed cultures of four different cyanobacterial species. We found: (1) lower pigment content in cultures in stationary phase, (2) higher precision with the sensor compared to traditional pigment quantification methods of measuring phycocyanin and chlorophyll *a*, (3) species-specific relationships between sensor readings and measurements related to biovolume, (4) overestimation of pigments in mixed compared with mono cultures, (5) dissolved organic matter causing a loss in signal proportional to its degree of aromaticity, and (6) potential to quantify the degree of cell lysis with a fluorescent dissolved organic matter sensor. This study has provided important new information on the strengths and limitations of fluorescence sensors. The sensor readings can provide accurate biovolume quantification and species determination for a number of bloom-forming species when sensors are properly compensated and calibrated.

1. Introduction

Real-time monitoring of cyanobacteria in freshwater systems is of critical importance for many water utilities. Certain species of cyanobacteria can produce taste and odour compounds and toxins which pose health risks, such as skin rashes, liver failure, or neurodegenerative diseases, for water users/consumers (He et al., 2016; Sivonen and Jones, 1999). Due to changes in climate and shifts in human behaviour and water use preferences, it is expected that the frequency of harmful algal blooms and related illnesses will increase in the near future (Grattan et al., 2016; Huisman et al., 2018; Merel et al., 2013). The occurrence of blooms can also affect the removal efficiency of cyanobacteria in drinking water treatment plants, which usually rely on coagulation-flocculation-dissolved air flotation processes (Henderson et al., 2010). Thus, a rapid detection and response to blooms is critical to guarantee access to safe recreational and drinking water.

Real-time detection using in-situ fluorescence probes is an efficient method to monitor cyanobacteria concentrations and/or some of their by-products (Zamyadi et al., 2016b). However, a number of issues have emerged which pose serious limitations to the ability of in-situ fluorometers to achieve acceptable accuracy, unless appropriate and comprehensive compensation models are developed (Bertone et al., 2018; Zamyadi et al., 2016a).

Fluorometers do not directly measure cell counts or biovolume, but measure fluorescence intensity at particular wavelengths, which is then used diagnostically to calculate the pigment content, such as chlorophyll *a* (chl-a), *b* and *c* and carotenoids, as well as phycobilins (such as phycocyanin and phycoerythrin). The most commonly measured pigments are chl-a and phycocyanin (PC), with the latter only occurring in cyanobacteria. However, the PC and chl-a content per cell, as well as their ratios, vary among species, and with light regime, growth stage and other environmental conditions, as described in detail in previous review papers (Bertone et al., 2018; Zamyadi et al., 2016a). Thus, the conversion of PC and chl-a content to cyanobacterial biovolume or cell

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count is not reliable unless these factors are systematically and accurately accounted for. An additional source of error relates to a number of potential environmental interferences, which disrupt the ability of the sensors to accurately measure the pigment concentration. These interferences include the presence of eukaryotic algal species, turbidity, dissolved organic matter (DOM), and changes in water temperature. Very high cell counts or species mixtures can also introduce error (Bertone et al., 2018; Zamyadi et al., 2016a).

Previous studies have tried to quantify and model some of these interferences; for instance, by accounting for effects from chl-a of different phytoplankton, or from turbidity (Chang et al., 2012; Choo et al., 2018; Choo et al., 2019; Zamyadi et al., 2012a). There has been less research focused on the effects of DOM on chl-a (e.g. most recently ACTUS (2019a)), growth stage, mixed cultures, and in-situ or lab-based measurement errors, on the ability to accurately convert fluorescence measurements to cyanobacterial cell counts or biovolume. Therefore, the aim of this study was to test how such factors affect the accuracy of chl-a and PC fluorometer readings for different species of cyanobacteria with a range of morphologies, and including toxic species. We highlight the type and amount of experimental work required by those wishing to use sensors to achieve reliable measurements of cyanobacterial concentrations.

2. Methods

2.1. Cyanobacterial species and origin

The four non-axenic cyanobacterial species used in this experiment are common in reservoirs and rivers in Australia (Al-Tebrineh et al., 2012; Burford and O'Donohue, 2006). The species were isolated from different locations (Table 1) and maintained at the Australian Rivers Institute, Griffith University. The four species have a wide variation in cell morphology and size (Table 1). *Raphidiopsis raciborskii* produces cylindrospermopsins while the other species are non-toxic.

Stock cultures of all species were maintained in modified Jaworski's Medium (JM) (Andersen, 2005) under photosynthetic photon flux density of 20 µmol photons m^{-2} s^{-1} from a cool, white light source (LED) on a 12 h light/12 h dark cycle at a temperature of 28 °C. Cultures were gently manually shaken three times per week during incubations.

2.2. Growth experiments – monocultures

All cultures were subcultured to a desired optical density at 750 nm (OD_{750}) using a spectrophotometer (Novaspec II, Pharmacia Biotech) at the commencement of the experiment. Each species was grown in 4 × 500 mL tissue culture flasks (vented cap, Falcon) in sterile modified JM for ten days, reaching mid-exponential growth phase. All cultures were incubated under lab conditions described above. All cultures were scaled up to 2 L in modified JM media with three replicate cultures.

2.3. Mixed culture experiments

A mixed culture experiment was carried out on Day 22. One replicate of each species was diluted to 25% (v/v) of its original concentration, except *R. raciborskii*. This species was diluted to 12.5% of the original concentration due to extremely high cell densities. Cultures were then mixed in three ratios: 25/75, 50/50 and 75/25 using appropriate volume ratios to give a final volume of 400 mL. Eighteen treatments of mixed cultures were used in this experiment. After mixing the cultures, subsamples were collected for cell counting and biovolume estimation, chl-a and PC pigment extraction, and sensors readings. Table A1 provides further details on the ratios and cell densities for all the eighteen treatments.

2.4. Sample collection

Subsamples from each flask were collected on days 7, 11, 17, 22 and 36 to capture all growth phases. A 5 mL subsample was used for OD_{750} quantification, then preserved with Lugol's solution for cell counts and biovolume estimates. Two 40 mL subsamples were also collected for chl-a and PC extractions. Additionally, a 400 mL subsample was collected for measuring specified fluorescence parameters using a portable fluorometer.

2.5. Optical density

The optical density of the cultures was monitored by measuring OD_{750} using a spectrophotometer (Novaspec II, Pharmacia Biotech). The OD_{750} was measured immediately after subsampling flasks, after which Lugol's iodine solution was added (approximately 1% final concentration) to preserve the cells for enumeration. Growth duration was calculated as the number of days until culture density declined or stabilised. Exponential growth rates (y) during this phase were calculated for each species and treatment using the following equation:

$$y = \frac{\ln(N_2 - N_1)}{t_2 - t_1} \quad (1)$$

where N_2 and N_1 are the cell concentration at times 2 and 1 respectively; t_2 and t_1 represent respectively the end and the beginning of the exponential growth stage.

2.6. Cell enumeration and biovolume estimation

A 1 mL subsample from all Replicates #1 was loaded onto a Sedgewick-Rafter counting chamber and counted microscopically under an inverted microscope (Olympus CKX41) equipped with a DFK-41BF02 digital camera and an iMac computer. For each sample, 20 measurements were made for each individual species using PlanktoMetrix biovolume software (Zohary et al., 2016). PlanktoMetrix computed biovolume per cell for each individual measured, using the equations of Hillebrand et al. (1999). Counting was performed only once; cell count was derived by converting the number of cell units counted for a known area on a microscopic counting chamber to 1 mL. This derivation method has a counting error of ± 20%.

2.7. Chlorophyll a extraction

A known volume of each culture was filtered into glass fibre filters (Whatman GF/F, 25 mm) sonicating the glass fibre filters for 2 × 30 s with 30 s between each blast and removing any remaining particulate

Table 1
Characteristics of the four cyanobacterial species used in the experiments.

	Microcystis sp.	*Aphanocapsa* sp.	*Raphidiopsis raciborskii*	*Sphaerospermopsis* sp.
Taxonomical Order	*Chroococcales*	*Chroococcales*	*Nostocales*	*Nostocales*
Colony Morphology	Mostly single cells (in culture)	Mostly single cells	Trichomes	Trichomes
Cell Morphology	Coccoid	Coccoid	Cylinder shape	Cylinder shape
Cell size	Diameter 3 µm	Diameter ~1 µm	Length 7.5-10 µm, Diameter ~2.5 µm	Length 7.5-10 µm, Diameter ~3 µm
Origin	Baroon Pocket Dam, Queensland	Mt Isa, Queensland	Wivenhoe Dam, Queensland	Murray River, New South Wales

matter. Samples were then stored in 90% acetone (Chem Supply) in a −20 °C freezer overnight. The extracts were filtered through glass fibre filters and the absorbance of the extracts was measured at 750 and 665 nm, using a Shimadzu UV-VIS spectrophotometer (UV1800). Hydrochloric acid treatment was used prior to each spectrophotometer reading, to adjust chlorophyll values for phaeopigments. Chl-a concentration was calculated based on the absorbance reading (Jeffrey and Welshmeyer, 1997).

2.8. Phycocyanin extraction

Phycocyanin concentrations were quantified using the method described in Horváth et al. (2013). A known volume of culture was filtered through pre-combusted glass fibre filters (Whatman GF/F, 25 mm), with filters stored overnight in 15 mL of 0.05 mol L^{-1} of phosphate buffer (pH 6.8) in a −20 °C freezer. PC was extracted by sonicating the glass fibre filters for 2 × 30 s with 30 s between each blast, and removing any remaining particulate matter. The absorbance of these extracts was measured at 615 and 652 nm using a Shimadzu UV-VIS spectrophotometer (UV1800). PC concentrations were then calculated based on the absorbance readings (Siegelman and Kycia, 1978) as in Eq. (2):

$$PC = A_{615} - 0.474 \bullet \frac{A_{652}}{5.34} \quad (2)$$

where A_{615} is the absorbance at 615 nm and A_{652} is the absorbance at 652 nm.

2.9. Fluorometer measurements

An EXO2 sonde (YSI, Yellow Springs, OH, USA) equipped with KOR software (version 1.0.9) was used to collect data from the samples. The sonde had four sensors; two measure conductivity / temperature, and turbidity, and the other two measure fluorescence at appropriate wavelengths to estimate dissolved organic matter (fDOM) and total algae (i.e., chl-a and PC). Subsamples (400 mL) were used for measurements, and three replicate readings were taken at 1 min interval. The sensors were cleaned regularly with Milli-Q water (Sartorius) between samples. Modified JM and Milli-Q water were also measured for baseline correction.

2.10. DOM addition

In order to quantify the interference of the fluorescence signal from DOM, 100 mL of *Microcystis* sp., and of *R. raciborskii* (Day 36 of experiments) were each added to 300 mL of concentrated DOM from two different locations in southeast Queensland, Australia, i.e., North Pine reservoir and Lake Tingalpa. DOM was concentrated using modified reverse osmosis (Compact L300; ROWater Australia) based on the recommendations from Serkiz and Perdue (1990). DOM from Lake Tingalpa had a higher humic and aromatic content (SUVA = 4.21 L (mg m)$^{-1}$) compared to North Pine (SUVA = 2.44 L (mg m)$^{-1}$) (de Oliveira et al., 2018). DOM was measured using the aforementioned EXO2 probe, specifically using the EXO fDOM Smart Sensor (YSI, Yellow Springs, OH, USA), with output reported as relative fluorescence units (RFU). The sensor has an excitation/emission wavelength pairing of 365 ± 5 nm/480 ± 40 nm. A two-point calibration was performed, using as first standard ultrapure water (0 RFU) and as second standard a 300 µg L^{-1} quinine sulfate solution (Sigma-Aldrich, St. Louis, MO, USA), equivalent to 100 RFU. The concentrated DOM was then diluted to three x 300 mL volumes with different DOM concentrations, which were subsequently added to the 100 mL of *Microcystis sp*, and *R. raciborskii* cultures for each separate experiment. The concentrated DOM may be higher than what is generally measured in the selected water sources for most of the year, but our selected concentrations however, it allowed us to extrapolate beyond historical patterns and to understand the effects of potentially higher DOM levels in the future. Responses to lower DOM levels was also by successive dilutions of the concentrated DOM. In addition, by using higher concentrations, it is hypothesised that potential DOM interferences on the fluorescence signal would be much more evident and easier to model, as appears to be the case in previous studies (de Oliveira et al., 2018). The magnitude of the interference would be expected to be more clearly related to the character of the DOM only at higher concentrations (de Oliveira et al., 2018).

2.11. Data analysis

All data were collated and analysed in Microsoft Excel. Visual inspection, statistical tests (e.g. t-test) and regression analysis were deemed sufficient to extract information from the data. When the measurement/experiment involved all the replicates, the outputs of different replicates were averaged (and standard deviation calculated). When one replicate only was deployed for a particular experiment/measurement, either the measurement error or the standard deviation resulting from multiple measurements of the same sample was calculated and reported.

3. Results

3.1. Culture growth

Fig. 1 shows the cell concentration for the four different species (Replicate #1) over time. *Aphanocapsa* sp. is represented on a different axis due to proportionally higher cell counts. For all the following charts, the error bars either represent the actual measurement error (e.g. 20% for cell and biovolume) enumeration or, if the data points

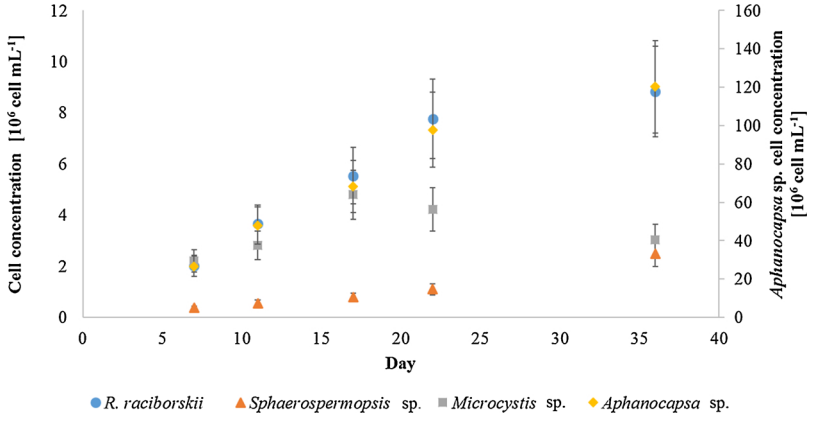

Fig. 1. Cell concentration [10^6 cell · mL^{-1}] over time for Replicate #1 of each of the four species. The span of the error bar is equal to 2 standard deviations.

were derived based on an average of multiple measurements (e.g. EXO sensor measurements).

R. raciborskii and *Aphanocapsa* sp. showed reasonably consistent growth over time, with maxima between days 17 and 22 and minima after day 22. *Sphaerospermopsis* sp., on the other hand, had a more regular increase until the end of the experiments. On the contrary, *Microcystis* increased until day 17, but decreased considerably after this time. Interestingly, the other two *Microcystis* sp. replicates "crashed" after day 11, but they recovered and continued to grow after day 17, until the end of the experiments. *Microcystis* sp. had the highest exponential growth rate (1.48 ln(cells) day $^{-1}$), followed by *R. raciborskii* (1.38 ln(cells) day $^{-1}$) and *Aphanocapsa* sp. (1.21 ln(cells) day $^{-1}$), with *Sphaerospermopsis* sp. (0.76 ln(cells) day $^{-1}$) exhibiting the slowest growth. Biovolumes were well correlated with cell densities for each species, although some species of varying morphology had better correlations than others (Fig. A1). The biovolume of *Aphanocapsa* sp. was not measured because individual cells are too small for standard geometric measurements by microscopy, hence a reference biovolume, previously measured and calculated (authors' obs.) was used. For other spherical species (*Microcystis* sp.), diameter was measured as part of the biovolume calculation. For Nostocales (*R. raciborskii* and *Sphaerospermopsis* sp.), two measurements (length and width) were required for biovolume estimation. In addition, for *R. raciborskii* the cell divisions are difficult to observe, and for *Sphaerospermopsis* sp. the vegetative cells' morphology can vary through time. These factors likely contributed to a higher cellular biovolume variation and thus a poorer correlation with cell counts for those species.

3.2. Correlation between laboratory and sensor measurements

Phycocyanin concentrations determined by spectrophotometer correlated well ($R^2 > 0.9$, both linear and non-linear regression) with PC determined by the EXO probe, with a similar slope of the regression line for each species except *Aphanocapsa* sp. ($R^2 = 0.55$) whose EXO readings were proportionally higher (Fig. 2). Chlorophyll *a* concentrations determined by spectrophotometer mostly correlated well ($R^2 > 0.9$ for all species) with chl-a determined by the EXO probe (Fig. 3). The correlation for *Sphaerospermopsis* sp. was quite different to the other two species. On average, the standard deviation ($n = 3$) related to the sensor readings was lower than for the spectrophotometer readings, especially for *R. raciborskii*. *Microcystis* sp. was not included in this analysis since only Replicate #1 had a stable growth rate and thus it was not possible to obtain multiple measurements to derive standard deviation between different replicates.

3.3. Correlation between biovolume and cell concentrations

Biovolume determined microscopically correlated well with PC readings from the EXO probe (R^2 = 0.71-0.81) although the slope of the best-fit line varied for each species (Fig. 4). By comparison, the correlation between biovolume and PC measured by the spectrophotometric method was poorer (R^2 = 0.5–0.8, Fig. A2). Based on the slope of the regression lines in Figs. A3–A4 it can be surmised that there is a similar ratio (i.e., 10–30 RFU mL 10^{-6} cell^{-1}) between PC, as measured by the sensor, and cell concentration for *R. raciborskii*, *Sphaerospermopsis* sp. and *Microcystis* sp., despite different cell sizes and shapes. Conversely, *Aphanocapsa* sp., with a smaller cell size than the other species, had a significantly different (i.e. lower) ratio (i.e. 0.3-0.7 RFU mL 10^{-6} cell^{-1}).

Figs. A5–A8 show the species-specific correlations between cell concentration and OD_{750}. The relationship is affected by the growth rate. In particular, when the culture reaches stationary phase (e.g., for *Aphanocapsa* sp. and *R. raciborskii*), OD_{750} increases proportionally more; in the case of *Microcystis* sp., whose cell concentration decreased after Day 17, OD_{750} still increased.

Fig. 5 illustrates the relationship between OD_{750} and PC readings from the EXO sensor. OD_{750} correlated well with PC measured by the EXO sensor up to OD_{750} of approximately 0.12. At higher concentrations, PC values "plateaued" while OD_{750} values continued to increase. The correlations varied between species, with *Sphaerospermopsis* sp. having a lower slope than the other species. For $OD_{750} > 0.06$, there is higher variability and broader confidence intervals compared to when lower OD_{750} were recorded.

3.4. Pigment content analysis

The ratios between chl-a and PC measured using the EXO sensor showed little variation over time for each species (Table A2). There was more fluctuation in the chl-a/PC ratio using the spectrophotometric method, although no consistent trends were noted. The ratio was often significantly different amongst species (Table A3). Although chl-a/PC is unitless, the ratios are substantially different between the sensors and the pigment extraction results from the lab.

The PC content per cell, using PC readings from the EXO probe, decreased as *Aphanocapsa* sp. and *Sphaerospermopsis* sp. cultures reached stationary phase, but increased for *Microcystis* sp. (Fig. 6). Similar patterns were noted for chl-a measured by the EXO probe compared with cell densities, which explains why the chl-a/PC ratio was rather constant throughout different growth stages. There was considerable variation in results for *R. raciborskii* and *Microcystis* sp.

There was an opposing behaviour between the rates of change of fDOM, as measured by the sensor, and cell concentration, except for *Aphanocapsa* sp. (Fig. 7). There was negligible fluctuation in the intra-replicate fDOM readings (i.e., low SD).

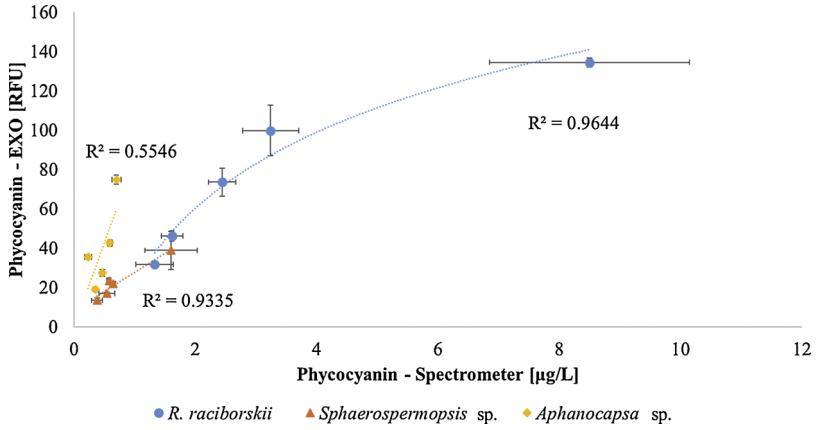

Fig. 2. PC determined by spectrophotometer vs PC from EXO sensor (mean and standard deviation of the three replicates for the same analysis day).

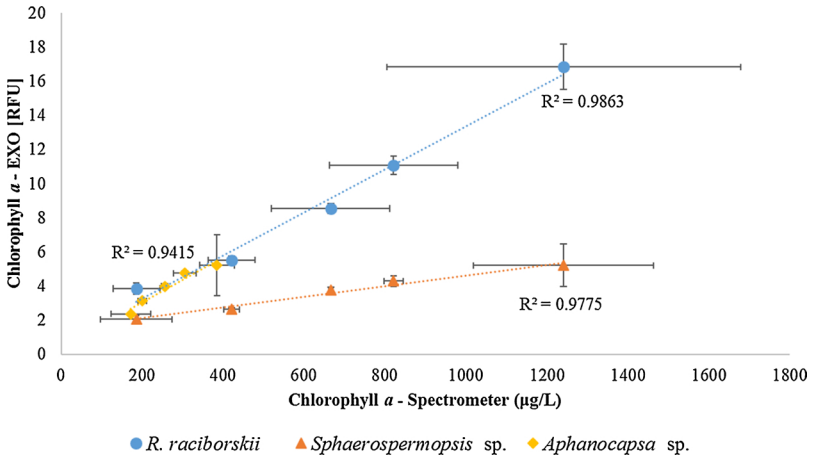

Fig. 3. Chl-a determined by spectrophotometer vs Chl-a from EXO sensor (mean and standard deviation of the three replicates for the same analysis day).

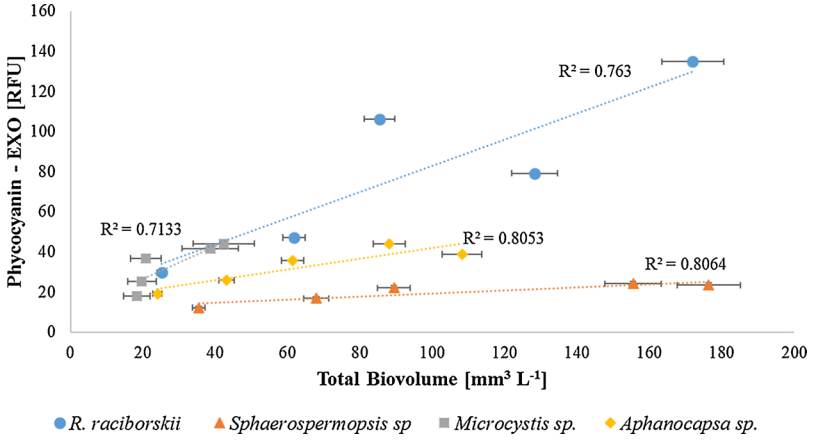

Fig. 4. Total biovolume vs. PC from EXO sensor (Replicate #1 for each species showing median and SD. SD for PC EXO was always < 1 RFU).

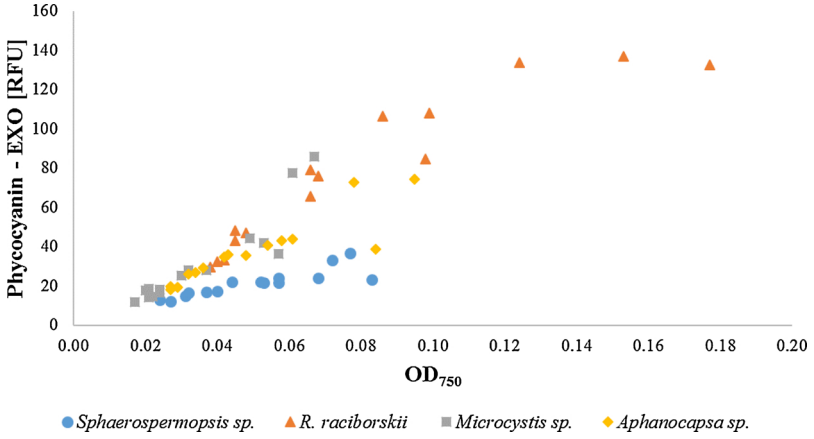

Fig. 5. OD_{750} vs. PC from EXO sensor (all 3 replicates of each species are shown as separate points).

3.5. Addition of dissolved organic matter

PC readings were lower than controls when concentrated DOM from Lake Tingalpa (TGL) and North Pine reservoir (NPR) was added to each sample (Fig. 8). In addition, DOM from TGL caused proportionally higher loss in signal than when an equivalent (as measured by the fDOM sensor) amount of DOM from NPR was added. Due to possible self-quenching effects because of the high cell density of the initial concentrated cultures, the calculated PC of the pure diluted culture (i.e., 20.7 RFU for *Microcystis* sp. and 32.5 RFU for *R. raciborskii*) was lower than expected that based on mathematical calculation (e.g., if 25% dilution, the expected reading would be 25% of the reading for the

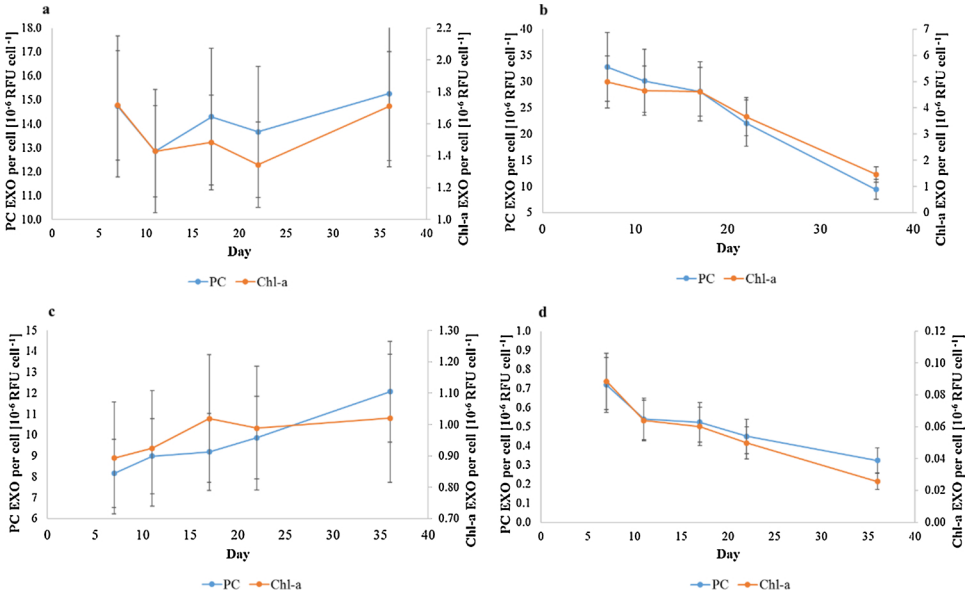

Fig. 6. Mean (± SD) PC and chl-a content per cell (EXO sensor measurement) for (a) *R. raciborskii*, (b) *Sphaerospermopsis* sp., (c) *Microcystis* sp., and (d) *Aphanocapsa* sp. for Replicates #1.

concentrated sample). Higher readings (i.e., > 25.1 RFU for *Microcystis* sp. and > 49.6 RFU for *R. raciborskii*) should have been recorded in the absence of DOM. Thus the charts below do not include these points.

3.6. Mixed cultures

The estimated PC readings from the EXO sensor, based on individual species readings and proportions in the mixed cultures, were always lower than the measured readings for the mixed cultures (Table 2). Additionally, the highest overestimations resulted from experiments where *Microcystis* sp. and *R. raciborskii* were mixed, although no statistically significant correlations could be derived. Chl-a measurements also showed similar results, with the estimated readings always lower than the actual readings. Other parameters (e.g. turbidity) had a more even distribution of over- and under-estimations.

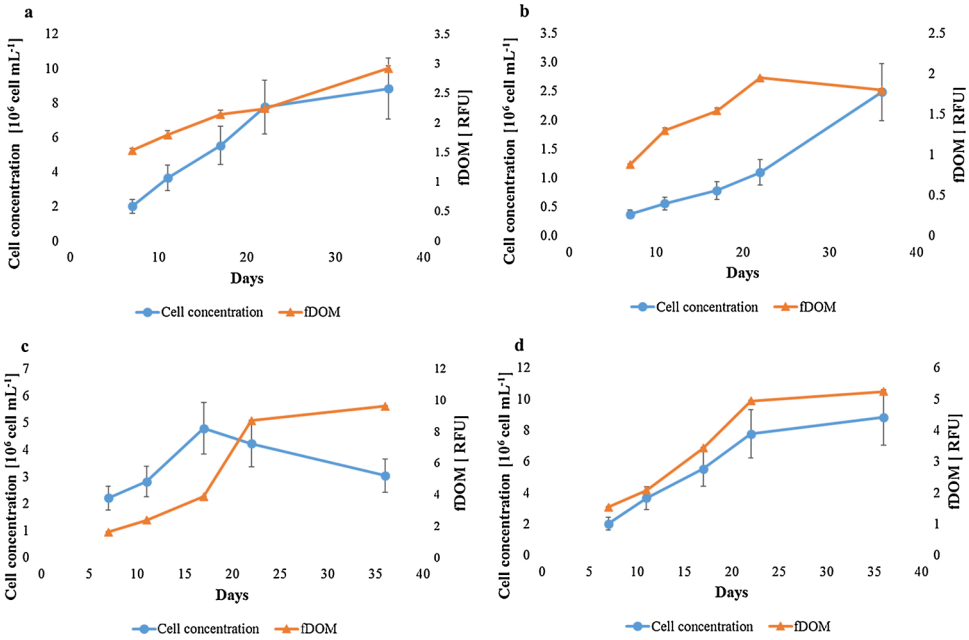

Fig. 7. Mean (± SD) cell concentration [10^6·cell· mL^{-1}] and fDOM [RFU] for (a) *R. raciborskii*, (b) *Sphaerospermopsis* sp., (c) *Microcystis* sp. and (d) *Aphanocapsa* sp. for Replicates #1.

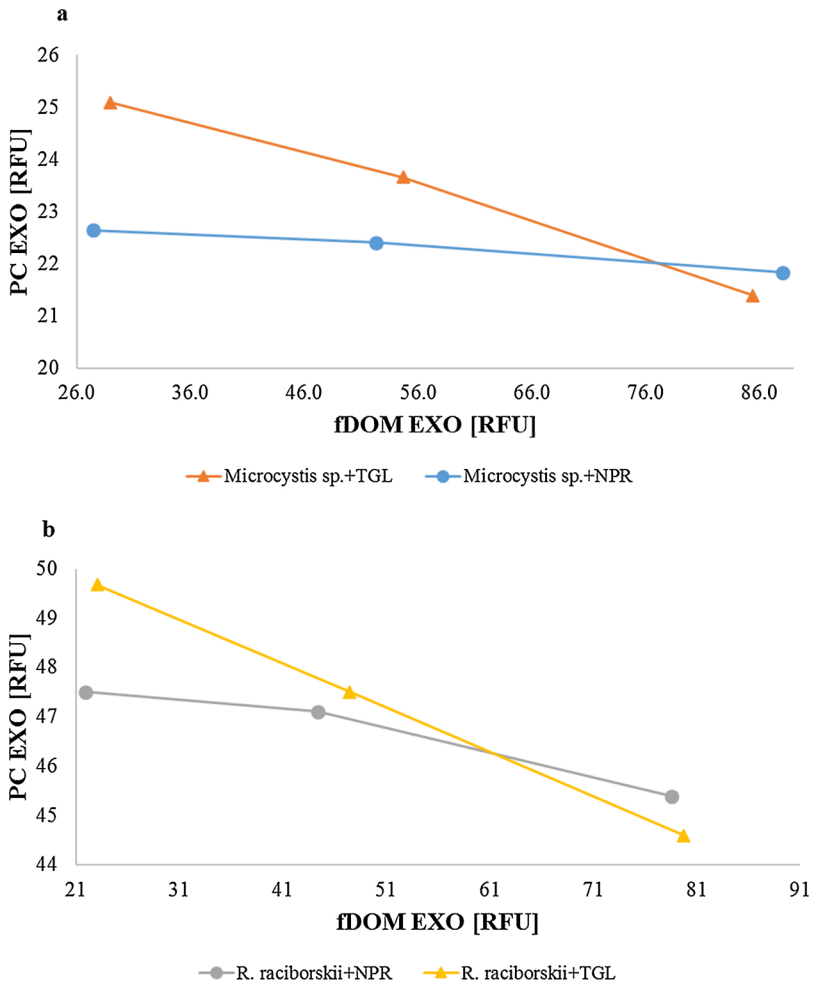

Fig. 8. Scatter plot PC readings (EXO sensor) vs. fDOM (EXO sensor), for (a) *Microcystis* sp. and (b) *R. raciborskii.* SD < 1% of the mean in all cases.

4. Discussion

Our results have a number of implications for the ability of fluorescence probes to provide reliable estimates of cyanobacterial biovolume or cell concentrations. Sensors do not measure the number of cells or the biovolume directly, but can be used to infer these measures based on fluorescence of diagnostic pigments (e.g. chl-a or PC). Previous studies have found that sensors provide a good estimate of the total biovolume, and they have therefore been recommended for real-time in-situ water monitoring (Bastien et al., 2011; Brient et al., 2008; Gregor and Maršálek, 2004). In most cases the recommendations were based on experiments for one species only. Our study with multiple species showed that the correlation between sensor readings and biovolume (in addition to, expectedly, cell concentration) was species-specific. Contrasting results are found in the literature for relationships between biovolume and fluorometer readings, with both strong (Kong et al., 2014; Zamyadi et al., 2012b) and weak (Jeffrey and Welshmeyer, 1997; Hodges et al., 2017) correlations estimated previously. Particular attention has to be dedicated to the method for concentration measurement, and the differences between microscopy and optical measurements.

Previous fluorescence research at a species level considered species such as *Microcystis* sp., which have higher PC content per cell (Chang et al., 2012) and lower chl-a content per cell (Ziegmann et al., 2010) during the stationary phase compared to the exponential growth phase. Hence one would expect a lower chl-a/PC ratio during stationary phases. The findings of this study are in contrast with previous research, since we noted that cultures in stationary phase generally produce less pigment (both PC and chl-a) per cell; unlike in Ziegmann et al. (2010) or Chang et al. (2012), we also have not found noticeable changes in the predominant pigment being produced with a change in the growth stage (e.g. from exponential to stationary). Interestingly, however, the ratio between chl-a and PC was often significantly different among species, in line with previous studies (Ziegmann et al., 2010; ACTUS, 2019b), thus potentially providing an important piece of information to discriminate between different species in situ using the EXO sensor (which measures both PC and chl-a). The ratios were substantially different between EXO readings and pigment extractions results. The cause of the variation might be due to the significant differences between the experimental procedures and the measurement outputs of the different approaches.

Growth stage seemed to also affect the relationship between cell count and optical density measurements, with OD_{750} increasing proportionally more when a culture reached stationary phase. A plausible

Table 2
Estimated and measured PC readings, and percentage measurement difference for PC, Chl-a and turbidity (EXO sensors) in mixed cultures (Replicates #1). Brighte red colours indicate higher variations between expected and measured values.

Volume ratios				Estimated PC EXO [RFU]	Measured PC EXO [RFU]	Δ PC EXO	Δ Chl-a EXO	Δ Turbidity EXO
Microcystis sp.	*R. raciborskii*	*Sphaerospermopsis* sp.	*Aphanocapsa* sp.					
25%	0%	0%	75%	10.8	10.8	0%	5%	-1%
0%	25%	0%	75%	11.5	11.6	1%	6%	-11%
0%	25%	75%	0%	7.9	8.0	2%	4%	10%
0%	75%	0%	25%	12.7	13.0	2%	7%	-9%
0%	50%	50%	0%	9.7	9.9	2%	7%	2%
0%	0%	50%	50%	8.5	8.7	2%	7%	9%
50%	0%	0%	50%	10.7	11.0	3%	6%	6%
75%	0%	0%	25%	10.6	10.9	3%	7%	0%
0%	50%	0%	50%	12.1	12.5	3%	8%	1%
0%	0%	25%	75%	9.7	10.1	3%	6%	0%
25%	0%	75%	0%	7.1	7.4	4%	7%	5%
0%	0%	75%	25%	7.3	7.6	4%	7%	4%
0%	75%	25%	0%	11.5	11.9	4%	9%	-3%
75%	0%	25%	0%	9.3	8.9	5%	5%	6%
50%	0%	50%	0%	8.2	8.7	5%	9%	7%
50%	50%	0%	0%	11.9	12.6	6%	10%	-11%
25%	75%	0%	0%	12.6	13.5	7%	11%	-27%
75%	25%	0%	0%	11.1	12.9	14%	18%	-16%
Mean				10.2	10.6	4.1%	7.7%	-1.6%
Standard Deviation				1.8	2.0	3%	3.1%	9.8%

explanation is that cell lysis may have occurred for those cultures, and optical measurements such as OD_{750} (but also PC and chl-a with the EXO sensor) would still be responsive to lysed cells, which are not accounted for when measuring cell count and biovolume by microscopy. The differences amongst measurements are important since it is possible to assume that the relationship between OD_{750} and PC is more reliable in detecting issues such as quenching, since both measurements are responsive to cell lysis and thus these other errors or interferences can be disregarded. The higher fDOM readings measured at stationary phases, supports the hypothesis of cell lysis and thus the detection of extracellular organic matter released by the lysed cells (Pivokonsky et al., 2016). The availability of other sensors (i.e., fDOM) that can potentially measure such algogenic organic matter released by lysed cells (Henderson et al., 2008), can help compensate the PC and chl-a readings and further improve the accuracy.

Our study suggests that background information on a system is needed prior to deploying fluorescence probes. Relevant information includes the dominant cyanobacteria species during a bloom (e.g. through routine traditional sampling, or a data-driven prediction model based on environmental and water quality input variables). This should allow site-specific, species-specific conversion models to be developed to estimate the total biovolume. Knowing when the bloom began, it would then be possible to estimate the growth phase, and thus adjust the model to account for the gradual loss in pigment content per cell over the course of the bloom.

It is clear from this study, that there are species-specific correlations

between the sensor results and laboratory results. At times the sensor readings achieved higher species-specific correlations with cell densities and biovolume than those in the laboratory. Traditional chl-a and PC estimations are also prone to errors and interferences, and therefore sensor measurements might not only be the most cost-effective and least time consuming option, but also the most accurate/precise in certain circumstances, with appropriate adjustment for interferences. Another important consideration is the behavioural difference between cyanobacteria grown in the lab and those observed in the field. For instance, most *Microcystis* cultures grown in the lab comprise solitary cells but *Microcystis* sp. in the field is usually colonial, thus creating further complexities and discrepancies when attempting to apply lab fluorescence findings to in-situ real-time monitoring. Future work should focus on numerically attempting to quantify how sensor measurements are affected by colony formation.

Regarding the addition of dissolved organic matter, PC readings decreased when concentrated DOM from Lake Tingalpa (TGL) and North Pine reservoir (NPR) was added. Quenching of phycobiliprotein and chl-a fluorescence by DOM has been noted in previous research (ACTUS, 2019a; Korak et al., 2015), however in this study DOM was measured using an EXO2 fDOM sensor, which can be installed in-situ in a vertical profiling system (VPS) next to a total algae (i.e. chl-a and PC) sensor. In line with previous studies, noting DOM interference on fluorescence intensity only at very high concentrations (de Oliveira et al., 2018), the magnitude of DOM interference on the total algae sensor became significant only for very high organic matter levels, which were only very rarely measured at the reservoirs of this study (Wang et al., 2019). Nevertheless, with DOM amount and character strongly correlated with storm events (Wang et al., 2019; Awad et al., 2017), it was important to understand and quantify such interference, since the frequency and magnitude of extreme wet weather events might increase in the future (Pachauri et al., 2014). The availability of both measurements in-situ and concurrently, can provide the opportunity to adjust PC and chl-a readings in real-time due to the presence of DOM. The nature of the DOM also affects the magnitude of the fluorescence loss, with more aromatic, humic DOM causing higher loss in measured PC. Provided that the fDOM sensor itself is properly compensated to account for interferences and inner filtering effects (de Oliveira et al., 2018), site-specific calibrations can be conducted to compensate for the presence of external sources of DOM on PC. However, due to the simultaneous presence of different sources of DOM in the natural environment, more sensors and further modelling are required to distinguish between the contribution of external sources and DOM released by lysed cyanobacteria cells.

In our experiments, the sensor was able to accurately measure species with very small diameter cells, such as *Aphanocapsa* sp. Previous studies have shown poor sensor detection of picoplankton (Pemberton et al., 2007; Seppälä et al., 2007). High cell concentrations (i.e. $OD_{750} > 0.06$) were a source of variability and reduced accuracy, consistent with previous research findings (Seppälä et al., 2007). Quenching was also noticed, with a loss in fluorescence signal at very high concentrations. The quenching effect can be modelled and accounted for, based on species-specific experimental results. For blooms where multiple species are present, there can be consistent overestimation of the pigment readings and in turn the actual cyanobacteria concentrations. Therefore, it is extremely important to be aware of the species present in situ during the bloom, by direct monitoring and also by modelling whenever possible.

In order to develop a comprehensive model, additional experiments need to be conducted to quantify, for specific species and reservoirs, other important effects, which have already been extensively studied in other locations. For example, interferences occur with turbidity (Zamyadi et al., 2012a; Beutler et al., 2002), green algae (Choo et al., 2019), or light exposure (Beutler et al., 2003; Gregor et al., 2007). Other commercially available fluorometers should be calibrated to better understand effects of different hardware. Without proper compensation for these effects and for the processes described above, such fluorometers would not be able to provide robust or reliable estimations of cyanobacteria, especially in environments where the dominant species varied. However, provided the species are known, there is potential, for a certain selected location, to run a number of experiments to model cyanobacteria behaviours and sensor interferences, and to dramatically increase the ability of the sensor to provide a reliable, real-time remote estimation of cyanobacterial cell concentrations at the designated site.

5. Conclusions

A number of controlled experiments were conducted to verify the ability of a commercial fluorometer, measuring chl-a and PC, to correctly estimate cyanobacterial concentrations. Results show that:

- The correlation between sensor readings and conventional chl-a and PC quantification methods was high, but species-specific.
- Strong correlations occurred between sensor readings and microscopy methods such as cell concentration or biovolume. However, the correlation between conventional chl-a and PC quantification methods and the cell concentration or biovolume was lower.
- Both cellular PC and chl-a decreased as cultures reached stationary phase. This change can be difficult to detect when cell lysis occurs, due to the measurement of pigments released by lysed cells, which however would quickly degrade.
- A simultaneous measurement of fDOM can help quantify the degree of cell lysis and thus compensate the PC and chl-a readings; however in the natural environment it would be important to distinguish between fDOM originating from lysed cells and fDOM from external sources.
- The presence of external DOM caused a loss in PC and chl-a fluorescence intensity; more humic DOM caused a higher signal loss.
- The sensor evaluated in our study was accurate at low cell concentrations, but showed higher variability and some quenching issues at $OD_{750} > 0.06$.
- When two species were mixed together, the sensor consistently overestimated PC and chl-a compared to the individual cultures.

Future work could usefully replicate such experiments with other available commercial sensors and additional cyanobacteria species. Results highlight how such sensors are generally unreliable and inaccurate without proper site-specific, species-specific calibration. However, if resources allow, it seems feasible to design a number of experiments whose results can be adapted into algorithms designed to reduce errors and provide a more useful, robust monitoring of PC and chl-a for real-time in-situ cyanobacteria assessment.

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Appendix A

Table A1
Species, ratios and cell densities, mixed cultures experiment.

Treatment	Species Name	Volume ratio	Cell Density [cells mL^{-1}]
Treatment 1	*Microcystis* sp.	0.25	215,638
	R. raciborskii	0.75	667,325
Treatment 2	*Microcystis* sp.	0.5	426,667
	R. raciborskii	0.5	403,292
Treatment 3	*Microcystis* sp.	0.75	235,391
	R. raciborskii	0.25	396,708
Treatment 4	*Aphanocapsa* sp.	0.25	13,382,010
	Microcystis sp.	0.75	158,354
Treatment 5	*Aphanocapsa* sp.	0.5	10,100,411
	Microcystis sp.	0.5	325,597
Treatment 6	*Aphanocapsa* sp.	0.75	9,386,666
	Microcystis sp.	0.25	504,033
Treatment 7	*Microcystis* sp.	0.25	212,675
	Sphaerospermopsis sp.	0.75	129,712
Treatment 8	*Microcystis* sp.	0.5	426,667
	Sphaerospermopsis sp.	0.5	160,329
Treatment 9	*Microcystis* sp.	0.75	645,926
	Sphaerospermopsis sp.	0.25	97,449
Treatment 10	*Sphaerospermopsis* sp.	0.25	134,650
	R. raciborskii	0.75	239,671
Treatment 11	*Sphaerospermopsis* sp.	0.5	106,008
	R. raciborskii	0.5	310,453
Treatment 12	*Sphaerospermopsis* sp.	0.75	66,470
	R. raciborskii	0.25	788,761
Treatment 13	*Aphanocapsa* sp.	0.25	4,243,771
	R. raciborskii	0.75	374,979
Treatment 14	*Aphanocapsa* sp.	0.5	15,846,296
	R. raciborskii	0.5	326,584
Treatment 15	*Aphanocapsa* sp.	0.75	9,928,395
	R. raciborskii	0.25	591,276
Treatment 16	*Aphanocapsa* sp.	0.25	19,911,111
	Sphaerospermopsis sp.	0.75	96,395
Treatment 17	*Aphanocapsa* sp.	0.5	8,133,333
	Sphaerospermopsis sp.	0.5	148,477
Treatment 18	*Aphanocapsa* sp.	0.75	12,082,962
	Sphaerospermopsis sp.	0.25	198,519

Table A2
Chl-a/PC ratio, measured with EXO sensor ("Sensor") and with spectrophotometer methods ("Lab"). Replicate #1 for *Microcystis* sp., average over the three replicates for *R. raciborskii*, *Sphaerospermopsis* sp. and *Aphanocapsa* sp.

Day	*R. raciborskii*		*Sphaerospermopsis* sp.		*Microcystis* sp.		*Aphanocapsa* sp.	
	Sensor	Lab	Sensor	Lab	Sensor	Lab	Sensor	Lab
7	0.12	140	0.15	570	0.11	303	0.13	488
11	0.12	261	0.16	502	0.10	285	0.11	429
17	0.12	273	0.17	613	0.11	238	0.11	1100
22	0.11	254	0.18	756	0.10	249	0.11	515
36	0.13	146	0.17	463	0.09	135	0.09	552
Mean	**0.12**	**215**	**0.17**	**581**	**0.10**	**242**	**0.11**	**617**
SD	**0.005**	**66**	**0.011**	**114**	**0.009**	**65**	**0.012**	**274**

Table A3
p-values resulting from t-tests for mean of chl-a/PC ratios. Above diagonal: based on "Sensor" data. Below diagonal: based on "Lab" data.

Species	*R. raciborskii*	*Sphaerospermopsis* sp.	*Microcystis* sp.	*Aphanocapsa* sp.
R. raciborskii	x	0.0001	0.0063	0.2070
Sphaerospermopsis sp.	0.0008	x	0.00002	0.0001
Microcystis sp.	0.5307	0.0012	x	0.3181
Aphanocapsa sp.	0.0332	0.7969	0.0406	x

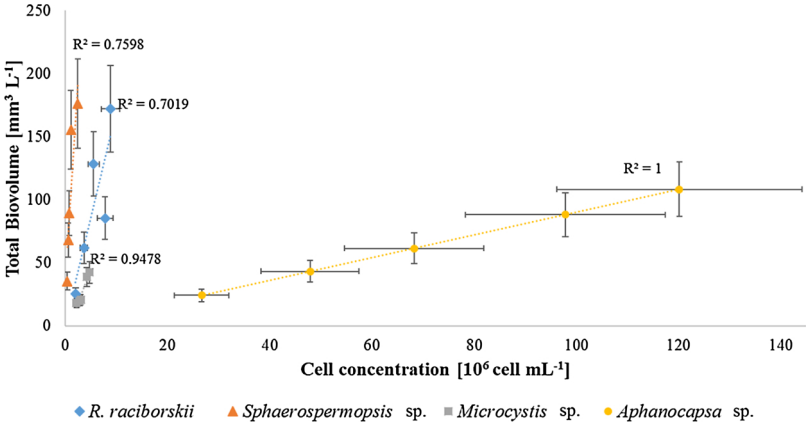

Fig. A1. Cell concentration vs biovolume for each species and respective correlations (Replicates #1, median and SD).

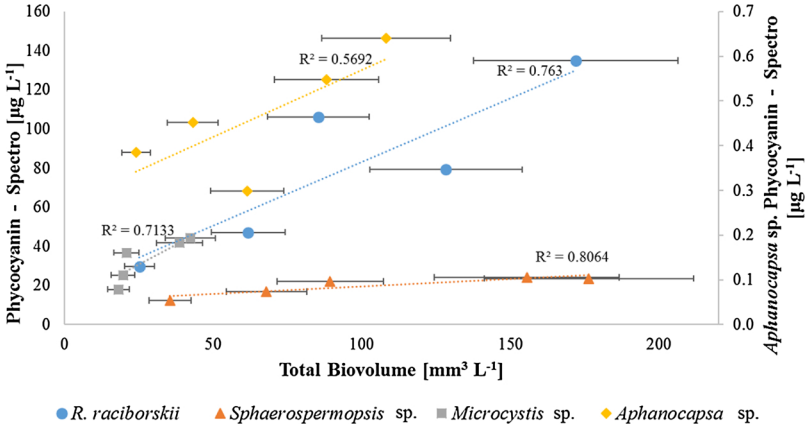

Fig. A2. Total biovolume vs PC as measured with extraction and spectrophotometer for all four species (Replicates #1).

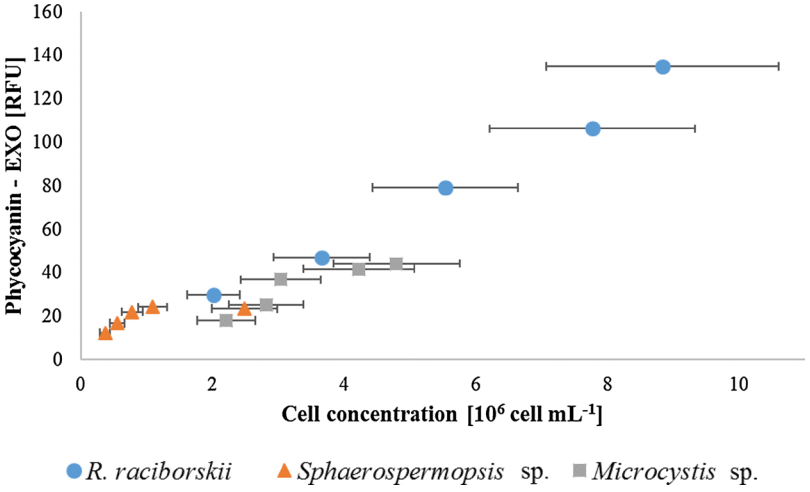

Fig. A3. Cell concentration vs PC as measured by EXO sensor for *R. raciborskii*, *Sphaerospermopsis* sp. and *Microcystis* sp. (Replicates #1; SD for PC EXO < 1% mean).

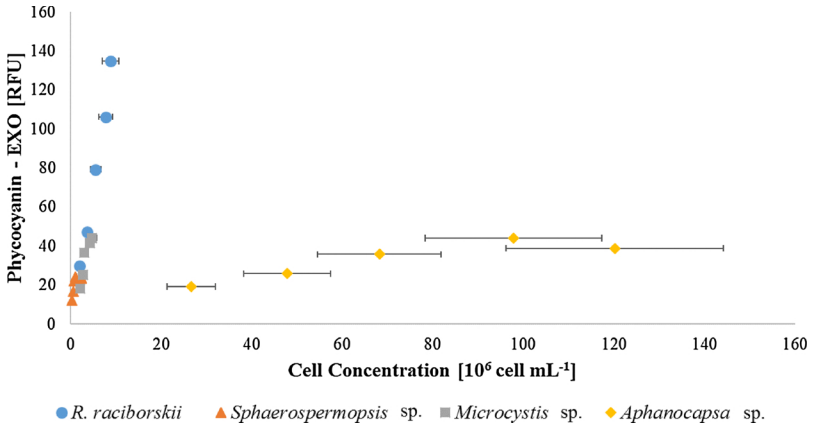

Fig. A4. Cell concentration vs PC as measured by EXO sensor for *R. raciborskii*, *Sphaerospermopsis* sp., *Microcystis* sp. and *Aphanocapsa* sp. (Replicates #1).

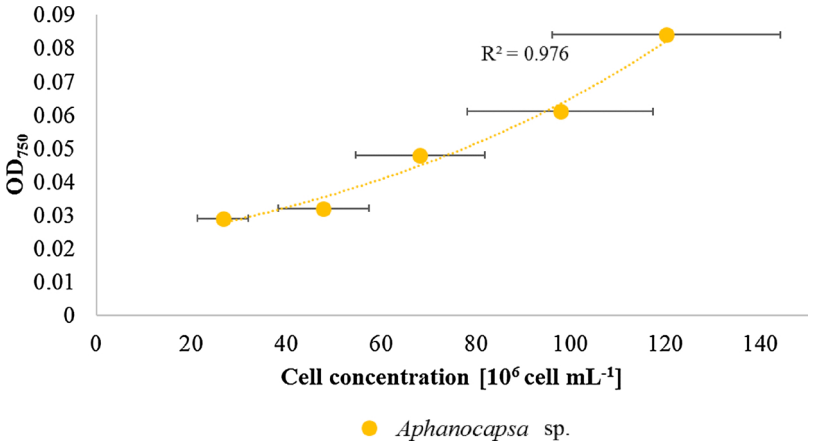

Fig. A5. Cell concentration vs OD_{750}, *Aphanocapsa* sp. (Replicate #1).

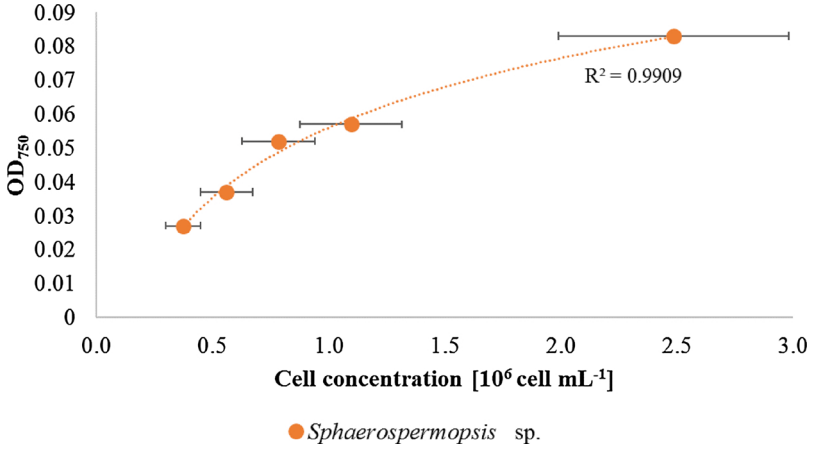

Fig. A6. Cell concentration vs OD_{750}, *Sphaerospermopsis* sp. (Replicate #1).

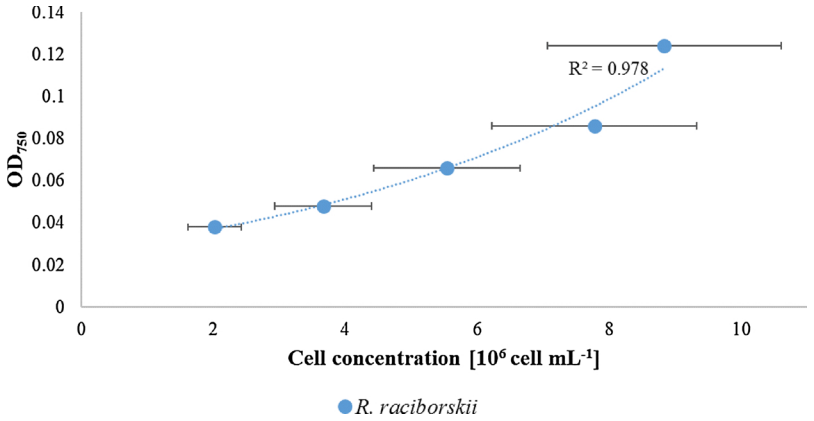

Fig. A7. Cell concentration vs OD_{750}, *R. raciborskii* (Replicate #1).

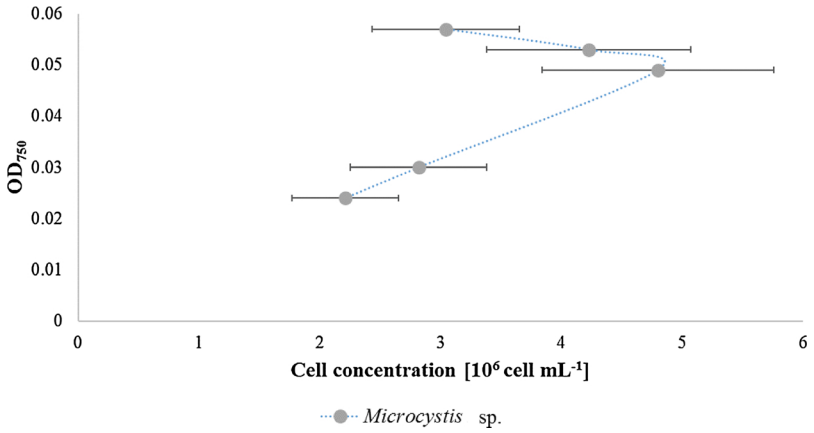

Fig. A8. Cell concentration vs OD_{750}, *Microcystis* sp. (Replicate #1); line connects successive measurements in time.

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