

## Performance evaluation of *in situ* fluorometers for real-time cyanobacterial monitoring

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### Abstract

Detecting the presence of cyanobacteria is an integral part of maintaining high water quality standards. *In situ* fluorometers are tools which may allow for the detection of cyanobacteria in real-time but there are few studies that review fluorometer performance. A systematic study that evaluated the performance of a range of fluorometers using key cyanobacterial species of interest and two known sources of interference (green algae and added turbidity) was undertaken. Specifically, six fluorometers and four cyanobacterial species were investigated. A good correlation ( $R^2 \geq 0.92$  and  $p$ -value of  $<0.001$ ) was obtained for mono cell culture suspensions, with robust performance exhibited for all fluorometers. Limits of detection for the fluorometers and multiplier factors which enable direct comparison of fluorometers were developed. The addition of green algae caused fluorometer performance to decrease by either overestimating or underestimating the concentration of cyanobacteria in a cellular suspension. Some fluorometers were more susceptible to these interference sources; the magnitude of the fluorometer measurement inaccuracy was dependent on cyanobacteria concentration and interference source. This study indicates that while there are inherent problems with fluorometers, the extent of the impact from interference sources can be characterised and potentially corrected to enable successful cyanobacteria detection in the field.

**Key words:** *in situ* probe, phycocyanin, source management

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### INTRODUCTION

Cyanobacterial bloom management remains a challenge for drinking and recycled water treatment plants. Recent research has demonstrated a need for a real-time cyanobacteria monitoring system that will facilitate real-time adjustment of the treatment process to ensure removal of these

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microorganisms and their harmful metabolites and guarantee consumers' health (Loisa *et al.* 2015; Zamyadi *et al.* 2016a). *In situ* fluorometers have been proposed as a viable option for this purpose (Zamyadi *et al.* 2016a). Although previous studies have investigated *in situ* fluorometers as a real-time monitoring tool under laboratory and field conditions (Loisa *et al.* 2015; Zamyadi *et al.* 2016b), they have been limited in the number of cyanobacterial species and fluorometers assessed. The aim of this study was to systematically compare the performance of six *in situ* fluorometers for the detection of seven different species and strains of cyanobacteria in real time and in the presence of known interference sources, including two species of green algae. This was achieved by: (1) testing the accuracy and precision of the *in situ* fluorometers using mono phytoplankton suspensions; (2) determining limits of detection for the species examined; and (3) investigating the impact of green algae and turbidity on the fluorescence measurement accuracy.

### The need for real time cyanobacterial monitoring

Climate change and a growing human population have exacerbated the frequency of cyanobacterial blooms. This poses a concern for drinking water treatment because certain cyanobacterial species can produce taste and odour compounds which degrade water quality aesthetics and can also generate cyanotoxins which can cause irreversible health effects (Paerl & Paul 2012; Merel *et al.* 2013). Cyanobacterial monitoring in water sources can range from sample analysis in laboratories (NHMRC 2013) to full field remote sensing (Simis *et al.* 2005; Randolph *et al.* 2008). Laboratory monitoring strategies include direct analytical detection such as microscopic enumeration (NHMRC 2013), taxonomic identification, GC-MS (taste and odour), HPLC-UV (toxin), HPLC-MS/MS (toxin), FlowCAM or indirect detection through pigment quantification such as chlorophyll-*a* (Chl-*a*) extraction (Kaushik & Balasubramanian 2013; Bullerjahn *et al.* 2016). The time required for sample preparation analysis and delivery make current laboratory methods unfavourable for real-time monitoring (Izydorczyk *et al.* 2009; Zamyadi *et al.* 2016a). While Chl-*a* measurements are used as an indicator for cyanobacteria (Kasinak *et al.* 2015), Chl-*a* is found in other phytoplankton groups such as diatoms and green algae. On the other hand, phycocyanin is a blue pigment predominantly found in fresh water cyanobacteria (Zamyadi *et al.* 2016a) and can be a potential pigment identifier for certain types of cyanobacteria. Although extraction methods have been developed to determine phycocyanin concentrations in samples, there are still high levels of uncertainty pertaining to the data obtained and they are time-consuming processes (Zimba 2012; Horváth *et al.* 2013).

### *In situ* fluorometers as a real-time monitoring system

Real-time monitoring can be achieved through submersible *in situ* fluorometers (Zamyadi *et al.* 2016a). Light is emitted over a specific wavelength range which excites electrons within the pigment molecules (such as Chl-*a* and phycocyanin) of target phytoplankton cells and measures the relative fluorescence that results from the electrons returning to a lower state (Pires 2010; Zamyadi *et al.* 2016a).

Nevertheless, there are limitations associated with using *in situ* fluorometry (Chang *et al.* 2012; Kring *et al.* 2014; Zamyadi *et al.* 2016a). The excitation and emission peak maxima wavelengths for Chl-*a* are 470 and 660 nm for the two excitation peaks and 680–685 nm for the emission wavelength, respectively, while those for phycocyanin are 590–610 nm and 660–685 nm, respectively. Chl-*a* has a double peak (at an excitation wavelength range of approximately 250–525 nm for peak 1 and approximately 550–680 nm for peak 2) where peak 2 can overlap with the phycocyanin peak. Consequently, fluorometers (especially those with wide excitation and emission wavelength bandpasses) may potentially measure overlapping wavelengths released by the excited Chl-*a* and phycocyanin pigments (Waters 2009). Additionally, previous studies have indicated that pigment

production rate in cells (Ziegmann *et al.* 2010; Zamyadi *et al.* 2016a), light intensity, temperature, turbidity, cell morphology and aggregation (Catherine *et al.* 2012; Chang *et al.* 2012; Kring *et al.* 2014; Zamyadi *et al.* 2016a) and biomass composition (Richardson *et al.* 2010; Ziegmann *et al.* 2010) can contribute to fluorometer measurement inaccuracy (Izydorczyk *et al.* 2009; Chang *et al.* 2012). However, the impact of these factors has not been investigated through a systematic laboratory study.

Cyanobacterial species, population density, shape, fluorometer model, light saturation and environmental conditions need to be investigated further since these parameters have been shown to influence correction factors (Chang *et al.* 2012; Zamyadi *et al.* 2016a). Additionally, previous studies used only a limited number of fluorometers (maximum three different models) and cyanobacterial species (maximum three genera) (Bastien *et al.* 2011; Chang *et al.* 2012; Zamyadi *et al.* 2012). The limited number of fluorometers and species tested in previous studies makes direct comparison of the fluorometer performance difficult. Consequently, a comprehensive and systematic study is needed to properly evaluate the fluorometers' performance for real-time monitoring.

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## MATERIALS AND METHODS

### Materials

MLA medium (Bolch & Blackburn 1996), ASM-1 medium (Gorham *et al.* 1964) and Jaworski's media (CCAP 2014) were prepared for cyanobacteria and green algae. All chemicals used for the preparation of culture media were analytical grade and supplied by Sigma-Aldrich (Missouri, USA), Thermo-Fischer Scientific (Massachusetts, USA) and Orica (Victoria, Australia). The media were prepared under sterile conditions. Kaolin and bentonite were supplied by Sigma-Aldrich (Missouri, USA). Rhodamine WT and C- phycocyanin were supplied by Sigma-Aldrich (NSW, Australia) where secondary standard solutions and serial dilutions using phosphate buffer were undertaken for chemical calibrations. The C-phycocyanin was stored at 4 °C in a darkened room prior to experimentation. A stock solution of C-phycocyanin was made using phosphate buffer in a darkened room, after which it was covered in aluminium foil and placed in a darkened room to slow down the degradation. The stock solution was diluted to ten approximate standards between 0 µg/L to 500 µg/L. Each standard was prepared fresh in a darkened room for each round of fluorometer measurements to minimise the degradation of the C-phycocyanin. Measurements were conducted in a calibration cup that came with the fluorometer which was wrapped in a dark cloth.

### *In situ* fluorometers and experimental setup

The six *in situ* fluorometers (Supplementary information Table S1) used throughout this study were the: EXO2 (YSI, USA), V6600 (the predecessor to the EXO2, YSI, USA), Algaetorch (AT) (bbe Moldanke, Germany), Microflu (MF) (TriOS, Germany), Fluoroprobe (bbe Moldanke, Germany) (FP) and DS5 (Hach, USA). For this study, phycocyanin and Chl-*a* sensors were attached to each fluorometer, turbidity sensors were added to the EXO2 and V6600 while the AT fluorometer had an inbuilt turbidity sensor. The FP was the only fluorometer which had a system correcting the fluorescence signal based on turbidity measurements. The raw fluorescence readings were recorded and used directly in the data analysis. The fluorometers were rinsed with distilled water before and after each experiment.

The cyanobacteria used in this study included (1) three strains of *Dolichospermum circinale* (reference strain ANA188B and ANA131CR, Australian Water Quality Centre (AWQC), Adelaide SA, Australia and reference strain CS-553, Australian National Algae Culture Collection (ANACC), sourced from a water body in Western Australia); (2) two strains of *Cylindrospermopsis raciborskii*

(reference strain CYP011 K, AWQC, Adelaide SA, Australia and reference strain CS-508, ANACC, sourced from a water body in Queensland, Australia); (3) two strains of *Microcystis aeruginosa* (reference strain MIC338, AWQC, Adelaide SA, Australia and reference strain CS-564, ANACC, sourced from a water body in Tasmania, Australia); and (4) *Microcystis flos-aquae* (reference strain MIC052F, AWQC, Adelaide SA, Australia). The green algae used were *Chlorella vulgaris* (reference strain CS-42, ANACC) and *Ankistrodesmus* sp. (reference strain ANK001A, AWQC, Adelaide SA, Australia). CS-555, CS-508 and CS-564 were grown in MLA medium, ANA188B, ANA131CR, CYP011 K and ANA001A were grown in ASM-1 medium, and CS-42 was grown in Jaworski's medium. Growth curves were monitored and cell cultures in the mid-stationary phase were used for this study. Cultures were maintained at 21 °C under a 16/8-hour white fluorescence lamp-based light cycle; the lamp emitted a light intensity of approximately 600 μmole/s (as photosynthetic photon flux).

The suspensions were measured using the fluorometers in a covered 10 L container containing de-chlorinated tap water and varying concentrations of cyanobacteria and green algae. The fluorometers were hung from a scaffold to ensure that (a) the fluorometers emitted and received light unblocked, (b) the fluorometers remained submerged during the experiment, and (c) depth was eliminated as a variable in the measurements. The water was allowed to reach room temperature (22 ± 2 °C) prior to experimentation.

### Laboratory fluorometer calibration

Two methods of basic calibration were applied to all the fluorometers used in this study:

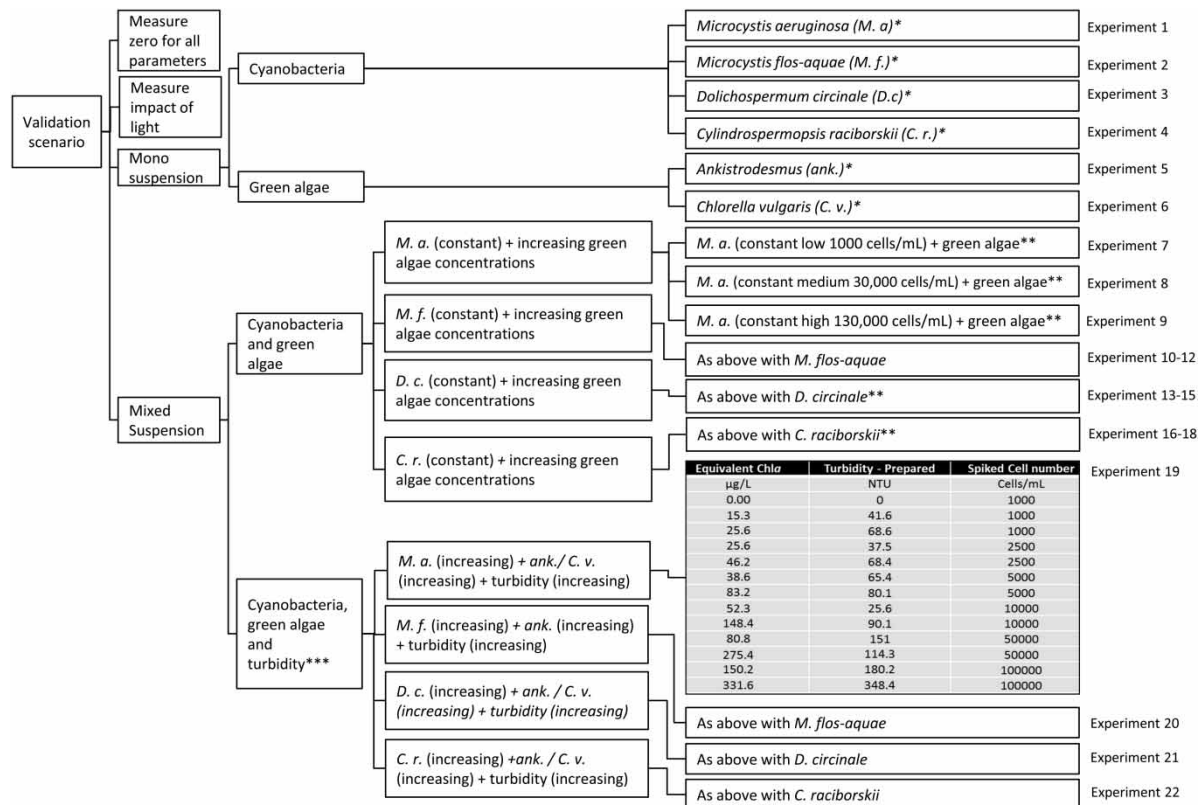
- **Manufacturer's calibration:** A one-point calibration with deionised water was conducted with all the fluorometers. This calibration was also used to establish the baseline and limits of detection for two of the methods reported in the section 'Limits of detection calibration'.
- **Two-point calibration using rhodamine and phycocyanin:** For this calibration, two EXO2 total algae sensors were used placed on the same EXO2 sonde. One EXO2 total algae sensor was calibrated using rhodamine while the other sensor was calibrated using phycocyanin to determine the impact of different chemical standards on fluorometer measurements. One fluorometer only was chosen for this experiment due to logistical and experimental restraints. The EXO2 was selected as multiple total algae sensors hold their respective chemical calibration even when attached to a single EXO2 sonde for direct comparison. A direct comparison between the two sensors was achieved by testing the sensors in serial dilutions of rhodamine and phycocyanin (section 'Materials'). A calibration curve for the EXO2, AT, MF and V6600 using serial dilutions of phycocyanin was also generated in this experiment which will be used to determine limits of detection (see section 'Limits of detection calibration').

### Experimental procedure

#### Mono species suspension analyses

Six species specific calibration curves were prepared for suspensions containing a single species (mono suspensions) using serial dilutions of *M. aeruginosa* (MIC338, CS-564), *M. flos-aquae* (MIC052F), *D. circinale* (ANA188B, CS-533), *C. raciborskii* (CYP011 K, CS-507), *C. vulgaris* and *Ankistrodesmus* sp., (see [Figure 1](#), Experiments 1–6).

For each suspension, a known aliquot of cyanobacterial cells was diluted with de-chlorinated tap water in a container that was covered with a darkened cloth. A magnetic stirrer was used to ensure the cell suspension was well mixed. For each calibration curve, seven cell concentrations (from 0 cells/mL, 2,000 cells/mL, 10,000 cells/mL, 20,000 cells/mL, 65,000 cells/mL, 100,000 cells/mL and 170,000 cells/mL) were prepared for fluorometer measurements. The volume of culture stock



**Figure 1** | Experimental design. For the mono suspensions, nine experiments were prepared using serial dilutions of cyanobacteria. \*For each experiment, seven cell suspension concentrations (2,000–170,000 cells/mL) were prepared for the phycocyanin (PC) measurements; for the mixed suspensions, seven experiments were prepared. \*\*For these experiments, an increasing concentration of green algae (2,000–170,000 cells/mL) was added to three concentrations of cyanobacteria (low – 1,000; medium – 30,000; high – 130,000, respectively). \*\*\*Laboratory validation chart for mixed suspension; increasing cyanobacteria, turbidity and green algae concentration. The concentrations of cyanobacteria, green algae and turbidity were based on previous work by Chang *et al.* (2012).

required for each cell suspension concentration was predetermined through microscopic enumeration and through a dilution equation.

The EXO2, V6600, AT, MF and FP fluorometers were used for single species calibrations (Experiments 1–6). Measurements were recorded two minutes after fluorometer submersion. After the fluorometer measurements, samples were collected for microscopic enumeration and turbidity determination. Biovolume was used to represent cell density in this study as a more relevant unit of measurement when comparing data from different cyanobacterial species (Zamyadi *et al.* 2016a). These mono species suspension calibration curves were also used to determine the limits of detection (LoD) for the fluorometers (section ‘Limits of detection calibration’) and to calculate a multiplier factor to convert fluorometer output to a cell biovolume equivalent measurement for fluorometer comparison (section ‘Multiplier factor and biovolume equivalent calculation’).

### Impact of interference sources on fluorometer performance

*Impact of green algae in mixed cell suspensions:* The impact of green algae through the addition of *Ankistrodesmus* sp. and *C. vulgaris* on fluorometer measurements was investigated. This experiment used the same experimental setup as the section ‘Mono species suspension analyses’ but with increasing concentrations of green algae (1,000–170,000 cells/mL) added to a predetermined concentration *M. aeruginosa* (MIC338, low concentration – 1,000 cells/mL) (Figure 1, Experiment 7). Fluorometer measurements were recorded and samples taken for microscopic enumeration and turbidity

measurement. This experiment was repeated using 30,000 cells/mL (medium concentration) and 130,000 cells/mL (high concentration) (Figure 1, Experiments 8 and 9). The EXO2, V6600, AT and MF were used in these experiments and samples were collected for cell enumeration and turbidity after all the fluorometer measurements were recorded for each cell suspension (and for all subsequent experiments). This experiment was repeated using the remaining cyanobacterial species (*M. flosaquae* MIC052F, *D. circinale* ANA188B and ANA131CR and *C. raciborskii* CYP011 K) at the three concentrations of cyanobacteria (low, medium and high) (Figure 1, Experiments 10–18).

*Influence of turbidity increases on mixed suspensions:* The impact of both green algae and turbidity was investigated. This experiment used the same setup as the section ‘Mono species suspension analyses’ but Chl-a (as the green algae *Ankistrodesmus* sp.) and turbidity (as 4 g/L bentonite or kaolin solution in milli-Q water) was added to suspensions spiked with a predetermined concentration of *M. aeruginosa* (MIC338) (Figure 1, Experiment 19). In this experiment, the EXO2, V6600, AT and MF fluorometers were used and the experiment was repeated using *M. flos-aquae* (MIC052F), *D. circinale* (ANA188 and ANA131CR) and *C. raciborskii* (CYP011 K) (Figure 1, Experiments 20–22). All fluorometer measurements were conducted in triplicate and in the absence of light.

## Analytical methods

### Cell enumeration

Phytoplankton suspensions and culture samples were preserved in Lugol’s iodine supplied by Sigma-Aldrich (Missouri, USA) and counted using a LEICA light microscope (The Netherlands) or Nikon Eclipse 50i (Nikon, Japan) and a Sedgewick Rafter Counting Cell (Pyser-SGI, UK). The samples were allowed to settle in the cell chambers for 20 minutes before enumeration. The cells were counted using the procedure for randomly selected fields outlined by Chorus & Bartram (1999).

### FEEM (fluorescence excitation emission matrix) analysis

The FEEMs were obtained by analysing 10 mL of an unfiltered sample of culture using an Aqualog spectrofluorometer (Horiba, Japan). The cultures used for the FEEMs were (a) *M. aeruginosa* and (b) *C. vulgaris*.

### Turbidity analysis

For the experiments investigating the impact of green algae and turbidity, 30 mL of the cell suspension sample and turbidity (bentonite/kaolinite) were analysed with a TN-100 turbidity meter (Eutech, USA).

### Biovolume determination

Cell density was converted into biovolume using calculations supplied by DEPI (2014). Measurement inaccuracy and relative measurement error (based on fluorometer output) in this study was defined in Equations (1) and (2):

$$\begin{aligned} \text{Measurement accuracy} &= (\text{Biovolume equivalent derived from the fluorometer output}) \\ &\quad - (\text{Biovolume derived from cell enumeration}) \end{aligned} \quad (1)$$

$$\text{Relative measurement error} = \frac{\text{Biovolume equivalent}_{\text{mixed}} - \text{Biovolume equivalent}_{\text{mono}}}{\text{Biovolume equivalent}_{\text{mono}}} \quad (2)$$

where *Biovolume equivalent<sub>mixed</sub>* refers to the biovolume equivalent for a specific cyanobacteria concentration found in the mix suspension experiments and *Biovolume equivalent<sub>mono</sub>* refers to the biovolume equivalent for a specific cyanobacteria concentration found in the mono suspension experiments.

### Limits of detection calculation

For this study, three different methods for calculating the limit of detection were compared: the method outlined by Armbruster & Pry (2008), the EPA (2016) and Shrivastava & Gupta (2011).

The limit of blank (LoB) is defined as the highest apparent analyte concentration found when testing blank samples (Armbruster & Pry 2008). The limit of detection (LoD) is defined as the lowest feasible analyte concentration that is distinct from the limit of blank (Armbruster & Pry 2008; EPA 2016). The EPA (2016) defines the method of detection limit (MDL) as the minimum concentration (with 99% confidence) of an analyte that is distinct from the blank results. The methods for calculating the detection limit are shown below:

Method one (Armbruster & Pry 2008):

$$LoB_1 = \bar{X}_{blank} + 1.645 \times STD_{blank} \quad (3)$$

$$LoD_1 = LoB_1 + 1.645 \times STD_{low\ concentration} \quad (4)$$

where 20 replicates were used to determine the LoB and LoD.

Method two (EPA 2016):

$$LoD_{2,s} = MDL_s = t_{n-1,0.99} \times STD_{spiked\ sample} \quad (5)$$

$$oD_{2,b} = MDL_b = \bar{X}_{blank} + t_{n-1,0.99} \times STD_{blank} \quad (6)$$

The highest LoD value is chosen as the limit of detection.

Method three (Shrivastava & Gupta 2011):

$$LoD_3 = 3 \times \frac{\text{Standard error of regression}}{\text{slope of regression}} \quad (7)$$

where  $\bar{X}$  is the mean of the replicates, STD is the standard deviation of replicate and  $t_{n-1,0.99}$  is the Student t-test value for the degrees of freedom ( $n$  is the number of replicates).

The first two methods rely on the use of blanks and spiked samples to determine the estimated LoD. The third method determines the LoD through linear regression. LoD<sub>1</sub> and LoD<sub>2</sub> were determined with at least seven replicate blank samples of deionised water and four replicate samples of spiked phycocyanin concentration or cyanobacterial cells for all the fluorometers tested. The linear regression of the calibration curves found from using phycocyanin and cyanobacteria (*Microcystis*, *Cylindrospermopsis* and *Dolichospermum*) were used to determine LoD<sub>3</sub>. The LoB<sub>1</sub> and LoD<sub>1</sub> were determined for all six fluorometers while the LoD<sub>2</sub> and LoD<sub>3</sub> were determined for the MF, EXO2, AT and V6600. The highest value for each method and fluorometer was chosen as the reporting LoD.

### Multiplier factor and biovolume equivalent calculation

In order to convert fluorometer measurements into a common unit (biovolume equivalent), a multiplier factor was established. For this study, the multiplier factor ( $m$ ) is the slope found between the

fluorometer output and true biovolume in the mono cyanobacteria experiments through linear regression. Biovolume equivalent can be found using the equation below.

$$\text{Biovolume equivalent} = \frac{\text{raw fluorometer output}}{m} \quad (8)$$

This approach to directly compare fluorometer performance by converting them to biovolume equivalent is adapted from Zamyadi *et al.* (2012).

## RESULTS AND DISCUSSION

### Evaluation of fluorometer limitations using standard calibration and limit of detections

The results from the manufacturer's calibration check and the calculated LoD are summarised in Table 1. A correlation between the estimated phycocyanin concentration and the readings from the rhodamine calibrated EXO2 sensor (excitation wavelength at 530 and emission wavelength at 555 nm) was obtained with a  $R^2 > 0.99$  and  $p$ -value  $< 0.0001$  (through weighted regression); similarly, the phycocyanin calibrated EXO2 sensor obtained a correlation of  $R^2 > 0.99$  ( $p$ -value  $< 0.0001$ ) (Figure 2).

Rhodamine has an excitation wavelength at 550 nm and an emission wavelength at 573 nm. It was therefore concluded that rhodamine can be used as a substitute for sensor calibration. Additionally, the two EXO2 calibrated sensors showed a similar trend between the chemical calibrant and raw fluorometer readings. However, when comparing the fluorometer algorithm corrected output, there was a noticeable disparity between the trends of the phycocyanin and rhodamine calibrated sensor measurements (Figure 2). All the fluorometers adjust the raw phycocyanin readings to a corrected output by embedded software. The FP has an in-depth explanation of the algorithm embedded in the software involved in adjusting the output (Bbe Moldaenke 2011). The AT has an offset correction factor by adjusting the correction factor in the system (default set to 1) (Bbe Moldaenke 2014). The V6600 has an option of correcting phycocyanin readings offset by temperature (YSI Incorporated 2006). Hence, the raw readings were considered more reliable than the conversion supplied by the manufacturer and thus used in this study.

The fluorometer which had the lowest LoD based on the phycocyanin calibration curves and was distinguishable from the blank was the MF (1.10  $\mu\text{g/L}$ ); the fluorometer with the highest relative LoD was the AT as the LoD was indistinguishable from the blank. The lowest LoD for this experiment was

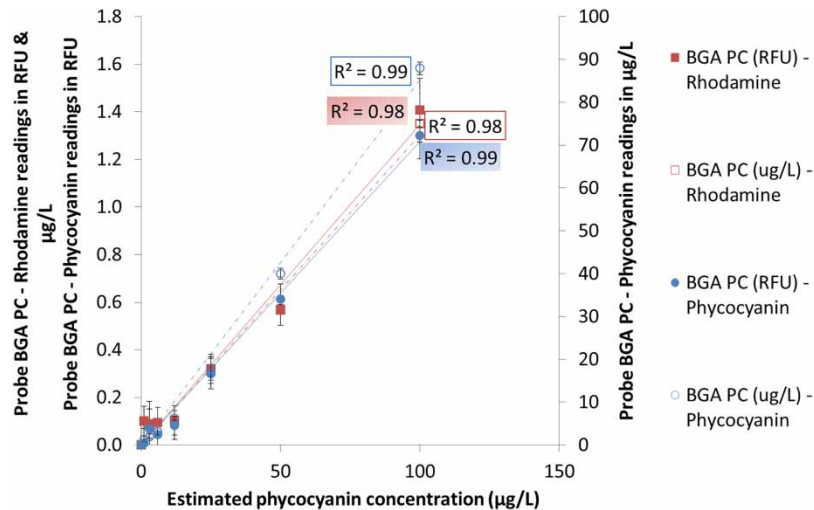
**Table 1** | Manufacturer calibration check using deionised water and subsequent calculations for the detection limit of the fluorometers using the methods developed by Armbruster & Pry (2008), EPA (2016) and Shrivastava & Gupta (2011)

| Calibrant   | Method           | Reference                              | Fluorometer            |                    |                        |             |                                 | DS5 (V)   |
|-------------|------------------|--|------------------------|--------------------|------------------------|-------------|---------------------------------|---|
|             |                  |  | MF ( $\mu\text{g/L}$ ) | EXO2 (RFU)         | AT ( $\mu\text{g/L}$ ) | V6600 (RFU) | Fluoroprobe ( $\mu\text{g/L}$ ) |   |
| Blank       | Blank check      | EPA (2016) and Armbruster & Pry (2008) | $0.5 \pm 0.4$          | $-0.11^a \pm 0.05$ | $2.7 \pm 0.3$          | $0 \pm 0.1$ | $0 \pm 0.0$                     | $2.726 \times 10^{-3} \pm 1.671 \times 10^{-3}$ |
|             | MDL_b            | EPA (2016)                             | 1.52                   | 0.12               | 3.5                    | 0.2         | 0                               | $7.737 \times 10^{-3}$                          |
|             | LoB              | Armbruster & Pry (2008)                | 1.14                   | $-0.03^b$          | 3.2                    | 0.1         | 0                               | $5.476 \times 10^{-3}$                          |
| Phycocyanin | LoD <sub>1</sub> | Armbruster & Pry (2008)                | 1.77                   | 0.05               | 3.7                    | 0.2         | 0                               | $8.225 \times 10^{-3}$                          |
|             | LoD <sub>2</sub> | EPA (2016)                             | 1.10                   | 0.27               | 2.1                    | 0.6         |                                 |   |
|             | LoD <sub>3</sub> | Shrivastava & Gupta (2011)             | 2.64                   | 0.09               | 7.2                    | 0.2         |                                 |   |

<sup>a</sup>The values refer to the unchanged baseline signal of the fluorometers in deionised water where some of the values are negative.

<sup>b</sup>Refer to footnote a.





**Figure 2** | Phycocyanin and rhodamine calibration curves for fluorometer EXO2. BGA PC is the phycocyanin measurement output (BGA PC) used by the EXO2.

determined by calculating the relative difference between the LoD and the fluorometer blank measurement. Overall, the fluorometers exhibited a good linear regression curve with the phycocyanin dilution solutions regardless of the calibrant used. However, the raw fluorometer readings were more reliable than the fluorometer adjusted values. Based on the phycocyanin dilutions, the MF had the lowest LoD.

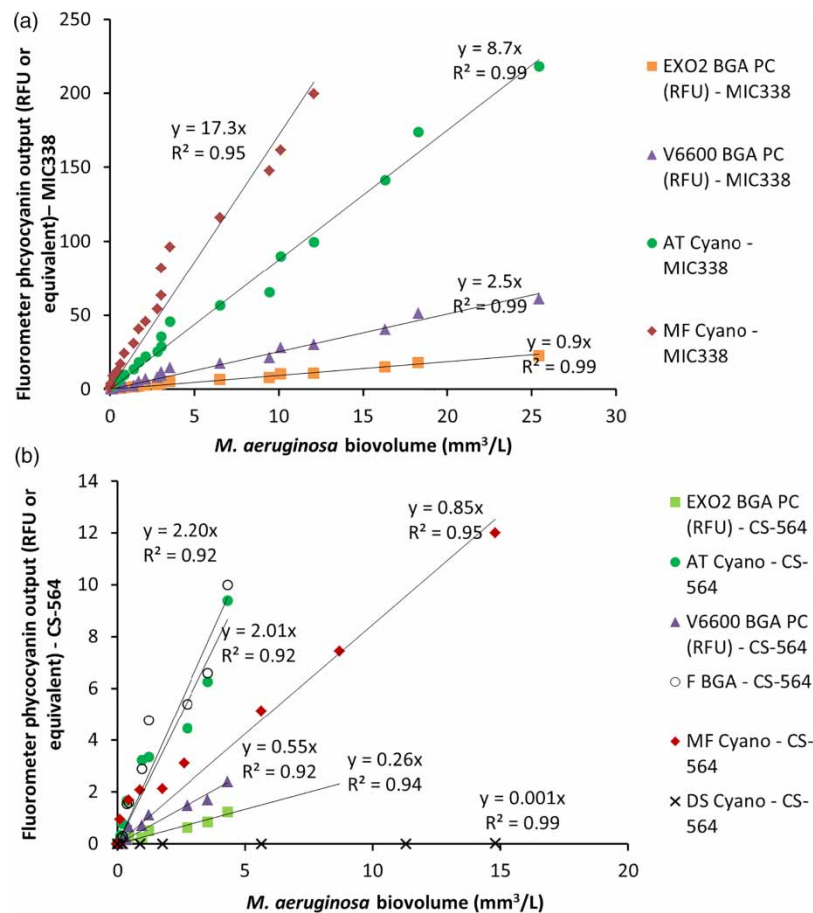
### *In situ* fluorometer laboratory calibration using mono suspensions

#### Linear regressions

All the fluorometer raw readings measured using the phycocyanin fluorometer increased proportionally with *M. aeruginosa* cyanobacteria biovolume (Figure 3(a) and 3(b)) with a correlation of  $R^2 \geq 0.92$  and  $p$ -value  $< 0.001$ ; similar results were seen for all the species tested (Supplementary information, Figures S1, S2 and S3).

The data generated in this study are consistent with other studies that looked at the relationship between raw fluorescence readings and cyanobacteria biovolume, despite the fact that previous studies did not cover the same range of fluorometers and species (Bastien *et al.* 2011; Chang *et al.* 2012; Zamyadi *et al.* 2012). For example, the correlations from the V6600 and Microflu using *M. aeruginosa* in the research of Bastien *et al.* (2011) ( $R^2 = 0.996$ ,  $R^2 = 0.998$ , respectively, and SE  $< 1\%$  for both fluorometers) and correlations from the Model 10 Series (Turner Design, USA) and 6600 V2 sonde (V6600) using *M. aeruginosa*, *Anabaena circinalis* (*D. circinale*) and *Planktothricoides raciborskii* in the research of Chang *et al.* (2012) ( $R^2 = 0.976$  to  $0.999$  and the SE was reported to be  $< 10\%$ ) were similar to the correlations found in this study.

Furthermore, different detection limit thresholds were found for all fluorometers depending on the cyanobacterial species tested. The fluorometer with the lowest LoD based on the three cyanobacterial species tested was the V6600 where the LoD was 2.81 RFU (approximately 18,000 cells/mL based on fluorometer output), then the MF with 15.63  $\mu\text{g/L}$  (at 20,000 cells/mL), EXO2 with 1.08 RFU (23,000 cells/mL) and AT with 18.6 (47,000 cells/mL, see Table 2 for all the calculated LoDs). Methods one and two gave relatively similar LoDs (compared to Method three) as both methods calculate detection limit using fluorometer blanks. The relatively high LoDs from Method three may be due to the use of cyanobacterial cells for validation instead of a calibrant or blank. Although Method



**Figure 3** | Relationship between the raw fluorescence output of various fluorimeters against biovolume of seven mono suspensions using *Microcystis aeruginosa* (CS-564 (a) and MIC338 (b)).

three resulted in the highest LoDs, the LoDs calculated through linear regression offer a more realistic idea of the fluorometer performance and limitations.

*D. circinale* gave the lowest LoD for the MF, AT and V6600 while *C. raciborskii* gave the lowest LoD for the EXO2. From the data, it also appears that some fluorometers have more difficulty in detecting certain cyanobacterial species than other fluorometers. For instance, the MF, EXO2 and V6600 had the highest detection limit for *M. aeruginosa* while the AT had the highest detection limit for *D. circinale*. Overall, the best performing fluorometer was the MF as it had one of the lowest LoDs but had the smallest standard deviation. From this study, it is apparent that some fluorometers may be more suited for monitoring certain types of cyanobacterial species. For instance, some fluorometers are more accurate for filamentous cyanobacterial species such as *D. circinale*. Overall, the fluorometers exhibited good linear regression for all the species tested; however, the LoD of the fluorometers can change depending on the fluorometer, the species and the method to determine LoD applied.

#### Evaluation of the multiplier factor from the regression curves

Different multiplier factors (slopes) were observed for each fluorometer when analysing the relationship between cyanobacteria biovolume and fluorometer reading (Figure 3). The slope ranged from 0.1 to 1.1 for the EXO2, 0.04 to 3.4 for the V6600, 0.5 to 8.1 for the AT and 0.9 to 16.1 for the MF depending on the species examined (*M. aeruginosa*, *M. flos-aquae*, *D. circinale* and *C. raciborskii*) (Table 3). For example, the slope for all the fluorometers varied from 0.001 to 13.2 for *M. aeruginosa*, 0.6 to 7.3

**Table 2** | Calculations for the detection limit of the fluorometers based on cyanobacterial species using the methods developed by Armbuster & Pry (2008), EPA (2016) and Shrivastava & Gupta (2011)

| Cyanobacterial species                                 | Method                        | Reference                  | Parameter  | Fluorometer                            |   |  |  |
|--|-------------------------------|----------------------------|--|--|---|--|--|
|  |                               |                            |  | MF<br>µg/L (for<br>unshaded<br>values) | EXO2<br>RFU (for<br>unshaded<br>values) | AT<br>µg/L (for<br>unshaded<br>values) | V6600<br>RFU (for<br>unshaded<br>values) |
| <i>M. aeruginosa</i>                                   | LoD <sub>2</sub> <sup>a</sup> | EPA (2016)                 | As fluorometer output<br>As cells/mL of<br><i>M. aeruginosa</i>  | 1.38<br>$1.2 \times 10^5$              | 0.41<br>$5.3 \times 10^3$               | 0.8<br>$1.1 \times 10^3$               | 0.4<br>$1.7 \times 10^3$                 |
|  | LoD <sub>3</sub> <sup>b</sup> | Shrivastava & Gupta (2011) | As fluorometer output<br>As cells/mL of<br><i>M. aeruginosa</i>  | 22.57<br>$2.0 \times 10^4$             | 1.31<br>$1.7 \times 10^4$               | 11.6<br>$1.6 \times 10^4$              | 4.0<br>$1.8 \times 10^4$                 |
| <i>D. circinale</i>                                    | LoD <sub>2</sub>              | EPA (2016)                 | As fluorometer output<br>As cells/mL of<br><i>D. circinale</i>   | 2.94<br>$4.0 \times 10^3$              | 0.05<br>$1.2 \times 10^3$               | 1.2<br>$3.2 \times 10^3$               | 0.2<br>$2.1 \times 10^3$                 |
|  | LoD <sub>3</sub>              | Shrivastava & Gupta (2011) | As fluorometer output<br>As cells/mL of<br><i>D. circinale</i>   | 10.80<br>$1.5 \times 10^4$             | 1.11<br>$2.8 \times 10^4$               | 37.6<br>$1.0 \times 10^5$              | 1.3<br>$1.2 \times 10^4$                 |
| <i>C. raciborskii</i>                                  | LoD <sub>2</sub>              | EPA (2016)                 | As fluorometer output<br>As cells/mL of<br><i>C. raciborskii</i> | 1.57<br>$2.8 \times 10^3$              | 0.15<br>$4.6 \times 10^3$               | 0.2<br>$5.0 \times 10^2$               | 0.3<br>$2.0 \times 10^3$                 |
|  | LoD <sub>3</sub>              | Shrivastava & Gupta (2011) | As fluorometer output<br>As cells/mL of<br><i>C. raciborskii</i> | 13.53<br>$2.4 \times 10^4$             | 0.81<br>$2.5 \times 10^4$               | 6.6<br>$2.2 \times 10^4$               | 3.1<br>$2.4 \times 10^4$                 |
| LoD (selected as highest value for fluorometer output) |                               |                            | As fluorometer output  | 22.57                                  | 1.31                                    | 37.6                                   | 4.0                                      |
| LoD (selected as highest value for cell counts)        |                               |                            | As cells/mL  | $2.4 \times 10^4$                      | $2.8 \times 10^4$                       | $1.0 \times 10^5$                      | $2.4 \times 10^4$                        |

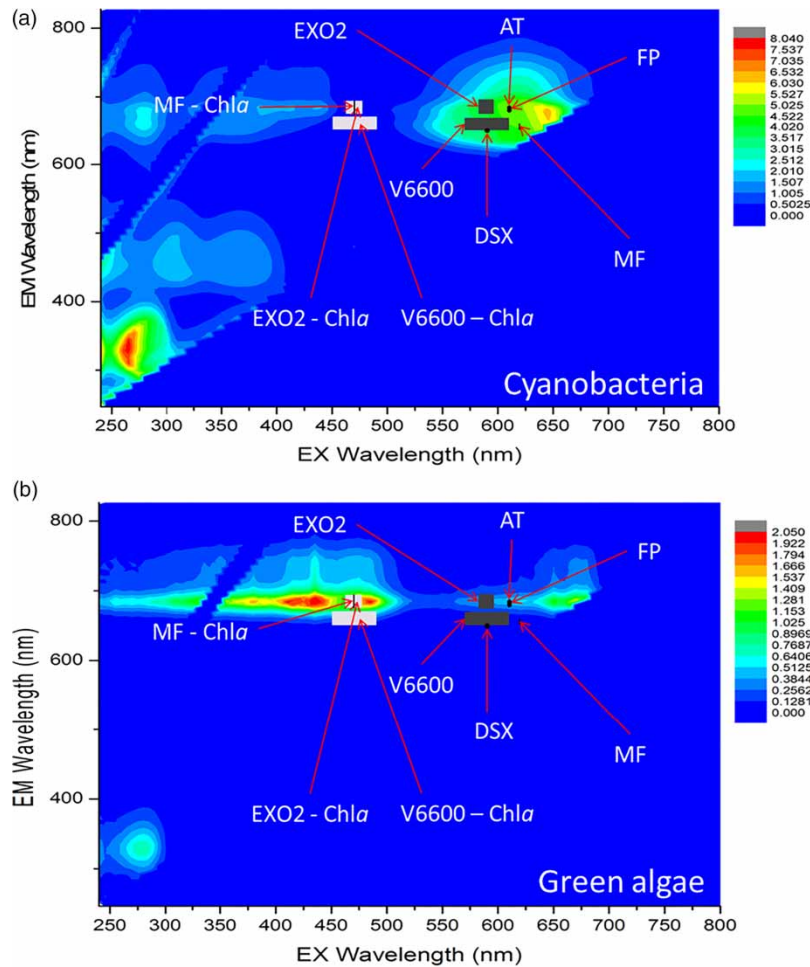
<sup>a</sup>The limit of detection developed by the EPA (2016), see Equations (6)–(8).<sup>b</sup>The limit of detection developed through the linear regression of mono cyanobacteria species, see Equation (7).

for *M. flos-aquae*, 0.001 to 3.3 for *D. circinale* and 0.005 to 16.1 for *C. raciborskii* (Table 2). Hence, for the same cyanobacterial species, the fluorometers gave slopes that varied by more than two orders of magnitude. Furthermore, two *M. aeruginosa* strains, MIC338 and CS-564, gave slopes of 1.0 and 0.3, respectively, when measured by the EXO2, indicating that fluorometer output depends on the cyanobacterial species detected even if measuring at the same cell concentration. This trend is also consistent with the other cyanobacteria strains and can be seen with the other fluorometers investigated (Table 3).

One source of variation in the slopes may be the optics of the fluorometers as the excitation wavelengths of the light applied and the bandpass of the wavelengths can affect the fluorometer measurements (see Figure 4).

**Table 3** | Species and strains used in this study; strains were sourced from two locations. The multiplier factor was derived from the mono suspension calibration curves by finding the gradient of the linear regression and setting the intercept to zero

| Cyanobacteria         | Species | Strain               | Location | Phycocyanin <i>in situ</i> fluorometer |       |     |     |      |       |
|-----------------------|---------|----------------------|----------|--|-------|-----|-----|------|-------|
|                       |         |                      |          | EXO2                                   | V6600 | AT  | FP  | MF   | DS5   |
| <i>M. aeruginosa</i>  |         | MIC338               | AWQC     | 1.0                                    | 2.6   | 8.1 | –   | 13.2 | –     |
|                       |         | CS-564               | ANACC    | 0.3                                    | 0.5   | 2.0 | 2.2 | 0.9  | 0.001 |
| <i>M. flos-aquae</i>  |         | MIC052F              | AWQC     | 1.0                                    | 0.6   | 7.3 | –   | –    | –     |
| <i>D. circinale</i>   |         | Ana188B and ANA131CR | AWQC     | 0.2                                    | 0.5   | 1.7 | –   | 3.3  | –     |
|                       |         | CS-533               | ANACC    | 0.1                                    | 0.7   | 1.5 | –   | 1.2  | 0.001 |
| <i>C. raciborskii</i> |         | CYP011 K             | AWQC     | 1.1                                    | 3.4   | 7.9 | –   | 16.1 | –     |
|                       |         | CS-508               | ANACC    | 0.1                                    | 0.04  | 0.5 | –   | 0.9  | 0.005 |



**Figure 4** | The excitation and emission wavelength of the available fluorometers in relation to the fluorescence of phycocyanin and Chl-*a* plotted against the FEEMs (fluorescence excitation-emission matrix) of (a) *M. aeruginosa* (cyanobacteria) and (b) *C. vulgaris* (green algae).

For example, the EXO2 and V6600 (Table 3) had slopes of 1.0 and 2.6 for the same *M. aeruginosa* strain (strain MIC338), respectively. Both have an excitation wavelength of 590 nm (with bandpass of 15 nm) but different emission wavelengths of 685 nm and 620 nm, respectively, which could account for the discrepancy. When examining Figure 4, MF, FP and AT detect fluorescence emission closer to the phycocyanin peak than the EXO2, DS5 and V6600; this is consistent with the measurement slopes found in the experiment, where the fluorometers that measured closer to the phycocyanin peak had larger measurement slopes. For example, the measurement slope for the MF, AT, EXO2 and V6600 was 13.2, 8.1, 1 and 2.6 for *M. aeruginosa* MIC338. Similarly, the AT and FP fluorometers had similar slopes of 2.0 and 2.2, respectively, for the same strain (CS-564), which may be attributed to the fact that the excitation/emission wavelength pairs (including bandpass width) were similar (AT: 610/685 nm and FP: 610/680 nm). These observations emphasise the importance of understanding the optics of the system.

Overall, the intensity of the measured fluorescence will be dependent on where on the fluorescence spectrum the fluorometer is focused (Figure 4), as well as other factors (light source and detector type) (Pires 2010). This is best observed in Figure 3(b) when comparing the fluorescence intensity of the MF and EXO2. The average of the ratio between the raw fluorescence MF readings and EXO2 readings (fluorescence intensity ratio) is approximately 15.3. Since the fluorometer output changes based on the cyanobacterial species tests, there is a need to convert the raw fluorometer output to a biovolume equivalent measurement to determine the concentration of cyanobacteria in prospective experiments.

As noted by Kasinak *et al.* (2015) and Zamyadi *et al.* (2012), converting to biovolume can reduce the measurement inaccuracy but this study showed it cannot fully eliminate the inaccuracy. Previous research showed that normalising the fluorometer output for biovolume was able to reduce the inaccuracy better than normalising the measurements to cell density (Zamyadi *et al.* 2012; Kasinak *et al.* 2015). Additionally, Chl-*a* and cell enumeration are not indicative of the presence of toxic cyanobacterial blooms (Macário *et al.* 2017). This is due to two factors: (1) each species has a specific phycocyanin production rate (Mulders *et al.* 2014) and (2) cell size between species can differ by several orders of magnitude (Kasinak *et al.* 2015). In this study, the slopes found in this experiment will be used as a multiplier factor to determine the biovolume equivalent (section 'Fluorometer performance in the presence of sources of interference').

### Phycological factors impacting fluorometer measurements

As noted in the section 'Evaluation of the multiplier factor from the regression curves', the measurement inaccuracy exhibited by the fluorometers is mainly influenced by the different optic specifications between the models' other factors, such as variation in phycocyanin production rates can impact the efficacy of fluorometers. Single species calibration of the cyanobacteria found that, for different strains of cyanobacteria, the regressions can differ by an order of magnitude (Figure 3). The concentration of phycocyanin detected between the two strains of *M. aeruginosa* and the fluorometers differed by five to twenty times (Figure 3). This is most likely explained by the differences in phycocyanin production rates between different species and strains (Eriksen 2008; Whitton & Brian 2012; Mulders *et al.* 2014; Macário *et al.* 2017). Previous studies have shown that production rates varied between five species of *Spirulina* and four species of *Athrospira* (Eriksen 2008). Variation in pigment production rates can explain the differences in slopes for each fluorometer and cyanobacterial strain tested (Figure 4, Supplementary information Figures S1–S3). This shows the limitation of the fluorometers: fluorometers detect the total phycocyanin concentration which makes fluorometer outputs susceptible to fluctuations or changes in the cyanobacterial bloom diversity. That is, distinction between different species and strains is difficult and can influence the interpretation of the fluorometers.

Light intensity (Takano *et al.* 1995; Eriksen 2008; Whitton & Brian 2012; Mulders *et al.* 2014), presence of organics (Eriksen 2008; Whitton & Brian 2012) and nitrogen (Whitton & Brian 2012) can impact the phycocyanin content rates in cyanobacteria species. Light intensity is the main factor that can influence phycocyanin production rates in cyanobacteria cells where low light intensity may stimulate phycocyanin production (Chorus & Bartram 1999; Eriksen 2008; Mulders *et al.* 2014). Certain species (such as *Microcystis*) have been known to degrade phycocyanin as a protection mechanism from exposure to high intensity light such as UV-B (Mulders *et al.* 2014). Cyanobacteria can potentially alter phycocyanin production rates through chromatic acclimation adaption (CCA) (Montgomery 2017). That is, certain species of cyanobacteria with the phycocyanin and phycoerythrin pigments may undergo a process to change the ratio of phycocyanin and phycoerythrin to absorb light efficiently in their current environment. However, in this study, none of the species tested are known to undergo CCA; *Microcystis* was found to have phycoerythrin but CCA (Otsuka *et al.* 1998) is not confirmed for this genera. Species such as *Phormidium* sp. and *Psuedanabaena* sp. (Tandeau de Marsac 1977) have been known to undergo CCA. The impacts of light intensity should be considered for field fluorometer application and that should still be noted for future fluorometer use.

Presence of organics (such as organic matter) can also impact phycocyanin production. For instance, Eriksen (2008) and Whitton & Brian (2012) noted that *Dolichospermum* experienced stimulated phycocyanin production rates in the presence of organics but not in *Athrospira* which shows potential interspecies phycocyanin production rates. Similarly, nitrogen can also impact phycocyanin

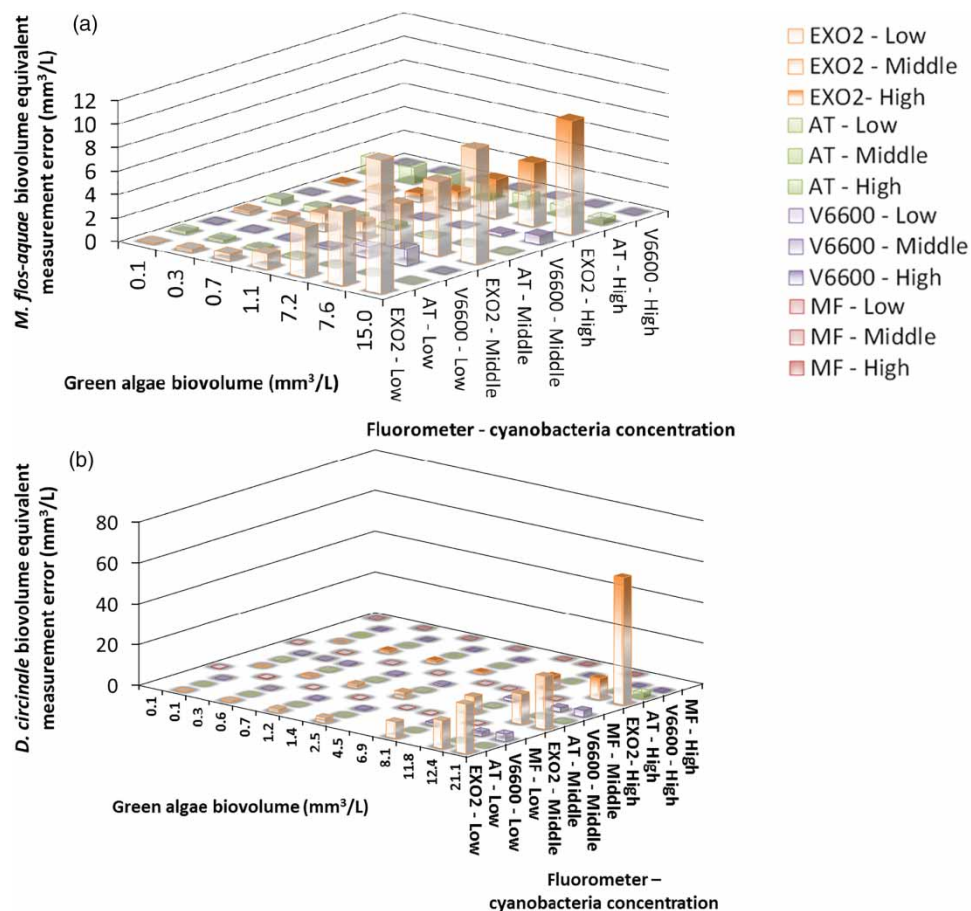
production rates (Whitton & Brian 2012; Mulders *et al.* 2014). Cyanobacteria can use phycocyanin as a nitrogen storage reserve where phycocyanin production rates increase in a nitrogen-rich environment and phycocyanin is degraded in a nitrogen-starved environment (Mulders *et al.* 2014). The presence of environmental factors can indirectly impact fluorometer output and should be considered for field applications.

## Fluorometer performance in the presence of sources of interference

### Investigating fluorometer performance in the presence of green algae

An increase in green algae concentration resulted in poorer performance for all the fluorometers. This was more apparent at high green algae concentrations where the measurement inaccuracy was at its highest for all the fluorometers (Figure 5(a) and 5(b), Supplementary information Figures S4 and S5).

Gregor *et al.* (2007) also noted that high concentrations of eukaryotic algae can affect phycocyanin results even if the excitation wavelength used is exclusive for cyanobacteria. Measurement inaccuracies (based on biovolume values) varied between a lower limit of 0 mm<sup>3</sup>/L and an upper limit of 15–100 mm<sup>3</sup>/L depending on the initial cyanobacterial species examined. However, as the phytoplankton suspensions contain different cyanobacteria concentrations, the relative measurement error based on the initial concentration of cyanobacteria present can vary between a lower limit of 0% and an upper limit of 200–130,000% (Supplementary information Tables 2(a)–2(d)). The lowest interference was observed for *M. flos-aquae* while the largest was observed for *D. circinale* (Figure 5(a)



**Figure 5** | Difference in the biovolume readings for the fluorometers in mono suspensions of cyanobacteria ((a) *M. flos-aquae* and (b) *D. circinale*) and mixed suspensions of increasing cyanobacteria and green algae. Underestimation was not shown in the figure but underestimated measurement inaccuracy did not exceed 5 mm<sup>3</sup>/L for all the species and fluorometers.

and 5(b)). The concentration of cyanobacteria or green algae present affected the fluorometer performance. For the same concentration of green algae tested, the measurement inaccuracy increased as the cyanobacteria concentration increased (for *M. aeruginosa*, *M. flos-aquae*, *D. circinale* and *C. raciborskii* (Supplementary information Figures S4 and S5)).

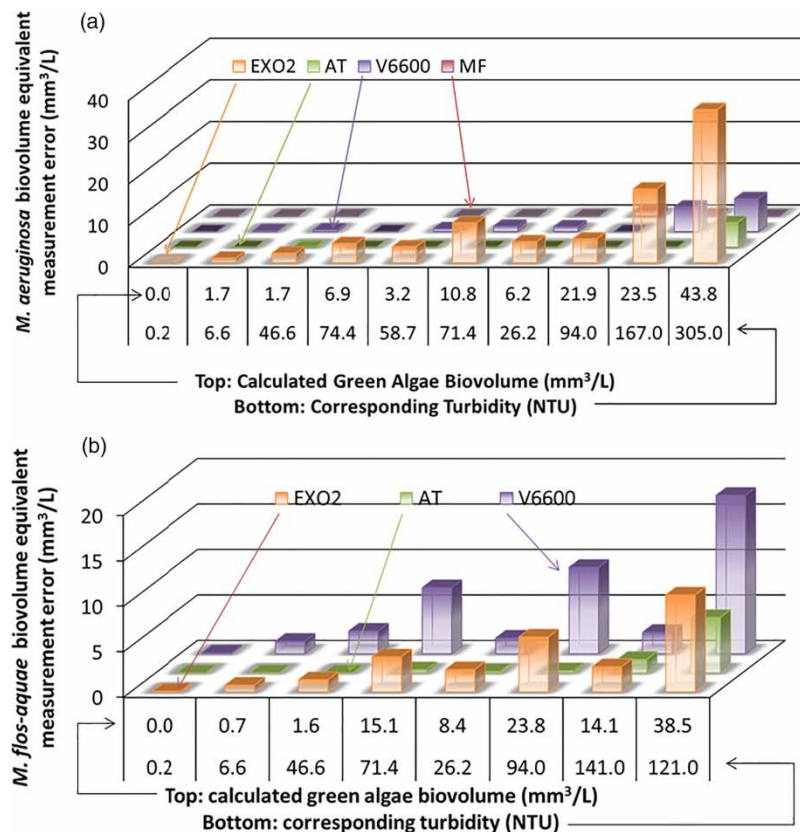
The measurement inaccuracy range varied from an underestimation of 3.5 mm<sup>3</sup>/L to an overestimation of 80 mm<sup>3</sup>/L for all the species and fluorometers examined. Over-estimations were lowest for the MF for *M. aeruginosa*, *D. circinale* and *C. raciborskii* (overestimations of up to 3.5 mm<sup>3</sup>/L equivalent and 18% relative measurement error). By contrast, the AT and V6600 overestimated the biovolume by up to 8 mm<sup>3</sup>/L equivalent, while the largest measurement inaccuracy was observed for the EXO2, where the fluorometer overestimated the cyanobacteria concentration by up to 80 mm<sup>3</sup>/L equivalent with a corresponding relative measurement error of 405% (see Figure 5(b) and Supplementary information, Table S2c). The V6600, MF and AT tended to underestimate the cyanobacteria present in the sample; the greatest and most frequent underestimation was exhibited by the MF (up to 3.5 mm<sup>3</sup>/L equivalent, 200% relative measurement error). Refer to Supplementary information, Tables S2a, S2b, S2c and S2d for the relative measurement errors of other species. For the low cyanobacteria and green algae experiments, the AT was the best performer. For the medium and high cyanobacteria and green algae experiments, the MF was the best performer. The highest measurement inaccuracy in most of the experiments was given by the EXO2. The average rate of change in measurement inaccuracy at low, medium and high concentrations of *M. aeruginosa* and green algae concentrations was 0.3, 0.6 and 0.4 (cyanobacteria equivalent biovolume/green algae biovolume), respectively (Supplementary information, Table S3). For all the species, the differences in the rate of change for the measurement inaccuracy in all the fluorometers were statically significant ( $p < 0.05$ ). Fluorometers estimated the filamentous cyanobacteria concentration most inaccurately, with measurement inaccuracy reaching an additional 60 mm<sup>3</sup>/L equivalent with respect to the cyanobacteria concentration tested; almost 400% overestimate of true cyanobacteria concentration.

A likely reason for the measurement inaccuracy is the misidentification of green algae as cyanobacteria (Beutler *et al.* 2002; Catherine *et al.* 2012). Again, this may be a result of the different fluorometer designs (emission and excitation wavelength and bandpass filters). The EXO2 and V6600 have a wider emission and excitation bandpass filter while the AT and the MF have a shorter excitation wavelength filter. On the other hand, cases of underestimation may arise from the self-shading of cells (such as in colonies of *Microcystis*) which may result in a partial excitation of all the target cells (Catherine *et al.* 2012). Another possible cause for these inaccuracies is that some fluorometers have the Chl-*a* and phycocyanin sensors integrated or have both sensors active simultaneously during measurements. As a result, operation of the fluorometers could contribute to false positive phycocyanin fluorescence due to Chl-*a* fluorescence. It is likely that methods to compensate for Chl-*a* interference to the phycocyanin measurements can be developed in which simultaneous measurement of Chl-*a* can be used to develop correction factors to reduce the occurrence of false positives.

### Investigating fluorometer performance in the presence of both green algae and turbidity

The addition of turbidity and green algae together further increased the magnitude of cyanobacteria overestimation and also the frequency of cyanobacteria underestimation (Figure 6(a) and 6(b), Supplementary information Figures S6 and S7).

Overall, the measurement inaccuracy ranged from an underestimation of 3.5 mm<sup>3</sup>/L to an overestimation of 200 mm<sup>3</sup>/L equivalent while the relative error with respect to true cyanobacteria concentration ranged from an underestimation of 1,400% to an overestimation of 7,000% (Supplementary information, Tables S4a-S4d) for all the fluorometers and species tested (following the



**Figure 6** | Difference in the biovolume readings for the fluorometers in mono suspensions of cyanobacteria ((a) *M. aeruginosa* and (b) *M. flos-aquae*) and mixed suspensions of increasing cyanobacteria, green algae and turbidity. Underestimation was not shown in the figure but underestimated relative measurement inaccuracy did not exceed 5 mm<sup>3</sup>/L equivalent for all the species and fluorometers.

baseline for the fluorometer measurements, Table S5). Measurement inaccuracy for individual fluorometers varied from an underestimation of 0.6 mm<sup>3</sup>/L to an overestimation of 200 mm<sup>3</sup>/L depending on the initial cyanobacterial species examined. When comparing fluorometer performance across all the different cyanobacterial species tested, the lowest interference range was observed for the MF and the largest was observed for the EXO2 (Figure 6(a) and 6(b)). Interestingly, the AT showed decreases in measurement inaccuracy when the green algae concentration increased and turbidity was present in the sample. The V6600 saw a reduction in measurement inaccuracy as the turbidity concentration increased. This does not imply that the addition of turbidity will reduce measurement inaccuracy; the more likely reason is that turbidity could cause the fluorometers to underestimate the cyanobacteria concentration in the sample.

For instance, an increase in turbidity does not necessitate an increase in relative measurement error, as seen in Figure 6(b). In the case of *M. flos-aquae* (Figure 6(b)), at similar green algae concentrations (15.1 mm<sup>3</sup>/L, 71.4 NTU) but different turbidity concentrations (14.1 mm<sup>3</sup>/L, 141.0 NTU), a 97% increase in turbidity saw a 28% and 67% decrease in relative measurement error for the EXO2 and V6600, respectively. On the other hand, the same turbidity increase resulted in a 400% increase in measurement error for the AT. Hence, the combination of green algae and turbidity could potentially interfere unpredictably with the measurements of different fluorometers.

In summary, the addition of turbidity as an interference source compounded the extent of the measurement inaccuracy exhibited by the fluorometers. The addition of turbidity also contributed to an increase in cases of underestimation. Additionally, the presence of turbidity had different impacts on different fluorometers as an increase in turbidity did not necessarily correspond to an increase in measurement inaccuracy.



## Implications for field fluorometer application

Previous studies have used various *in situ* fluorometers as a monitoring and detection tool for cyanobacteria (Beutler *et al.* 2002; Waters 2009; Richardson *et al.* 2010; Bastien *et al.* 2011; Catherine *et al.* 2012; Chang *et al.* 2012; Zamyadi *et al.* 2012, 2016b; Kring *et al.* 2014; Nguyen *et al.* 2015). Ideally, the fluorometers should be able to detect cyanobacteria at the lowest alert limit. The cyanobacteria alert levels from various frameworks are listed in Table 4. The fluorometers give the LoD to be approximately 500–5,000 cells/mL for LoD<sub>2</sub> and at least 20,000 cells/mL for LoD<sub>3</sub>. The LoD<sub>2</sub> (EPA 2016) method gave the lowest LoDs for all the fluorometers and species while, on average, the calculated LoD<sub>3</sub>s were roughly five times larger than LoD<sub>2</sub>s. The LoD<sub>2</sub>s calculated were low enough for most of the selected cyanobacteria alert limits while the LoD<sub>3</sub>s were only low enough for medium to high alerts (see Table 4).

The fluorometers will not be able to detect cyanobacteria at the detection or lowest alert levels (Table 4) if using LoDs calculated from the linear regression of mono cyanobacterial calibrations. However, if the fluorometers were applied for long-term monitoring at a particular site, this would allow the detection limits to be re-evaluated using the LoD<sub>2</sub> method, thus achieving detection limits low enough for the lowest alert limits in the alert level frameworks. This is because the LoD<sub>2</sub> method (EPA 2016) determines the LoD based on the fluorometer blank and a spiked sample. It is also appropriate for use for ongoing data collection, requiring yearly validation. However, the LoD<sub>3</sub> detection limits and grab sampling can still have an important role in field monitoring; they could be applied for grab sampling for when cyanobacteria concentrations are closer to medium and high alerts.

A future direction that this research should explore is the development and validation of correction factors to mitigate measurement inaccuracy that has been found in previous studies (McQuaid *et al.* 2011; Catherine *et al.* 2012; Chang *et al.* 2012; Zamyadi *et al.* 2012; Loisa *et al.* 2015) and confirmed here in this study. As the measurement inaccuracy is inherently systematic, correction methods can be applied to reduce the measurement error. There have been minor differences noted between chlorophyll and phycocyanin concentration ratio (Myers & Kratz 1955). A tandem measurement between chlorophyll and phycocyanin from the fluorometers can be potentially used to further correct the measurement exhibited by the fluorometers as part of Chl-*a* correction. Previous studies have started applying and developing their correction methods; however, validation of previous correction factors have not been covered extensively and will need to be conducted.

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## CONCLUSION

This study aimed to lay the framework for an ongoing investigation of using *in situ* fluorometers for real-time monitoring. The key message from this study is that the fluorometers cannot be the only method applied for detecting cyanobacteria as they cannot distinguish between different species which makes it difficult to estimate the true cell density. Rather, they are more suited to: (a) provide early warnings that cells are reaching low alert levels if applied *in situ* and (b) give a rapid indication of approximate cell numbers for medium to high alerts through grab sampling. Additionally, this study found that:

- The highest LoD for the fluorometers were calculated using linear regression, while the lowest LoD was determined using a combination of the fluorometer blank and spiked measurements.
- Good linear regression ( $R^2 > 0.9$ ,  $p$ -value  $< 0.001$ ) was found between fluorometer output and bio-volume for all the fluorometers (MF, EXO2, V6600, AT) and species (*M. aeruginosa*, *M. flos-aquae*, *D. circinale*, *C. raciborskii*) tested.

**Table 4** | Summary table of the suitability of fluorometers for selected cyanobacterial species of interest and recommended alert level frameworks

| Parameter  | Alert level Framework (ALF)   | Range/Threshold  | Cyanobacterial species of interest | Is the fluorometer sensitive enough to detect the cyanobacteria cells for the following alert limits: Yes or No, if Yes – for each LoD method what is the lowest alert level the fluorometers can detect? |                                  |                                  |                                  |
|--|---|--|------------------------------------|---|----------------------------------|----------------------------------|----------------------------------|
|  |   |  |                                    | AT  | EXO2                             | MF                               | V6600                            |
| Precision: Limits of detection (LoD)   | Australian Drinking Water Guidelines (NHMRC 2013)   | Detection level: <b>1,000 cells/mL</b>   | <i>M. aeruginosa</i>               | No  |                                  |                                  |                                  |
|  |   |  | <i>D. circinale</i>                | No  |                                  |                                  |                                  |
|  | Management Strategies for Cyanobacteria (Blue-Green Algae) and their Toxins: A Guide for Water Utilities (Newcombe <i>et al.</i> 2009)  | Alert level 1: <b>2,000–6,500 cells/mL</b> ; Alert level 2: <b>6,500–65,000 cells/mL</b> ; Alert level 3: <b>≥ 65,000 cells/mL</b> | <i>C. raciborskii</i>              | Yes – LoD <sub>2</sub>  | No                               |                                  |                                  |
|  |   |  | <i>M. aeruginosa</i>               | Yes   |                                  |                                  |                                  |
|  |   |  | <i>M. aeruginosa</i>               | LoD <sub>2</sub> – Alert level 1  | LoD <sub>2</sub> – Alert level 2 | LoD <sub>2</sub> – Alert level 1 | LoD <sub>2</sub> – Alert level 1 |
|  |   |  | <i>D. circinale</i>                | LoD <sub>3</sub> – Alert level 3  |                                  |                                  |                                  |
|  |   |  | <i>D. circinale</i>                | Yes   |                                  |                                  |                                  |
|  |   |  | <i>D. circinale</i>                | LoD <sub>2</sub> – Alert level 1  | LoD <sub>3</sub> – Alert level 2 | LoD <sub>3</sub> – Alert level 3 | LoD <sub>3</sub> – Alert level 3 |
|  | Cyanobacterial Incident Management Framework (CIMF) and Alert Levels Framework for cyanobacteria Chorus & Bartram (1999), Du Preez & Van Baalen (2006)                          | Alert level 1: <b>≥ 2,000 cells of cyanobacteria</b> ; Alert level 2: <b>≥ 100,000 cells/mL</b>                                    | <i>M. aeruginosa</i>               | Yes   |                                  |                                  |                                  |
|  |   |  | <i>D. circinale</i>                | LoD <sub>2</sub> and LoD <sub>3</sub> – Alert level 1   |                                  |                                  |                                  |
|  |   |  | <i>C. raciborskii</i>              | Yes   |                                  |                                  |                                  |
|  | The development of an alert levels and response framework for the Management of blue-green algal blooms Burch (1993)  | Alert level 1: <b>500–2,000 cells/mL</b> ; Alert level 2: <b>2,000–15,000 cells/mL</b> ; Alert level 3: <b>≥ 15,000 cells/mL</b>   | <i>M. aeruginosa</i>               | LoD <sub>2</sub> – Alert level 1  |                                  |                                  |                                  |
| <i>D. circinale</i>  |   |  | LoD <sub>3</sub> – Alert level 3   |   |                                  |                                  |                                  |
| <i>D. circinale</i>  |   |  | Yes                                |   |                                  |                                  |                                  |
| <i>D. circinale</i>  |   |  | LoD <sub>2</sub> – Alert level 2   | LoD <sub>2</sub> – Alert level 1  | LoD <sub>2</sub> – Alert level 2 | LoD <sub>2</sub> – Alert level 2 |                                  |
| <i>C. raciborskii</i>  |   |  | LoD <sub>3</sub> – Alert level 2   | LoD <sub>3</sub> – Alert level 3  | LoD <sub>3</sub> – Alert level 2 | LoD <sub>3</sub> – Alert level 2 |                                  |
| <i>C. raciborskii</i>  |   |  | Yes                                |   |                                  |                                  |                                  |
| Draft national protocol for the monitoring of cyanobacteria and cyanotoxins (Burch <i>et al.</i> 2003) | Detect level: <b>500 cells/mL</b> ; Alert level 1: <b>&gt; 2,000 cells/mL</b> ; Alert level 2: <b>&gt; 5,000 cells/mL</b> ; Alert level 3: <b>&gt; 50,000 cells/mL</b>          | <i>M. aeruginosa</i>   | LoD <sub>2</sub> – Alert level 1   | LoD <sub>2</sub> – Alert level 2  | LoD <sub>2</sub> – Alert level 2 | LoD <sub>2</sub> – Alert level 1 |                                  |
|  |   | <i>D. circinale</i>  | LoD <sub>3</sub> – Alert level 3   |   |                                  |                                  |                                  |
|  |   | <i>D. circinale</i>  | Yes                                |   |                                  |                                  |                                  |
|  |   | <i>D. circinale</i>  | LoD <sub>2</sub> – Detect level    | LoD <sub>2</sub> – Alert level 2  | LoD <sub>2</sub> – Detect level  | LoD <sub>2</sub> – Detect level  |                                  |
|  |   | <i>D. circinale</i>  | LoD <sub>3</sub> – Alert level 2   |   |                                  |                                  |                                  |
|  |   | <i>C. raciborskii</i>  | Yes                                |   |                                  |                                  |                                  |
| Fluorometer accuracy in the presence of interference sources   | Measurement inaccuracy tendency<br>Measurement inaccuracy <sup>a</sup> (in terms of equivalent biovolume)<br>Can potentially apply corrections to reduce measurement inaccuracy |  | Mixed: over- and underestimation   | Overestimation  | Mixed: over- and underestimation | Overestimation                   |                                  |
|  |   |  | 2                                  | 4   | 1                                | 3                                |                                  |
|  |   |  | Yes                                | Yes   | Yes                              | Yes                              |                                  |
|  |   |  |                                    |   |                                  |                                  |                                  |

<sup>a</sup>Relative magnitude of measurement inaccuracy where 1 refers to the fluorometer which was the most accurate and 4 refers to the fluorometer that was the least accurate.

- The linear regressions between the fluorometer output and cyanobacteria biovolume showed that for a specific fluorometer model, the output value was dependent on the species tested. That is, for a certain cell density, the fluorometer output would vary depending on the species tested.
- The presence of green algae and turbidity can adversely impact the fluorometer output with most fluorometers tending to overestimate the cell biovolume in mixed suspensions.
- Certain conditions trigger the underestimation of cyanobacteria biovolume for certain fluorometers: high levels of turbidity can contribute to the underestimation of cell biovolume. In terms of the measurement inaccuracy exhibited by the fluorometers, the measurement error is inherently systematic.

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