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DETERMINING THE EFFICACY OF A SUBMERSIBLE *IN SITU* FLUOROMETRIC DEVICE FOR CYANOBACTERIA MONITORING COALESCED WITH TOTAL SUSPENDED SOLIDS CHARACTERISTIC OF LOWLAND RESERVOIRS

E. SYMES* AND F. VAN OGTROP

Faculty of Agriculture and Environment, The University of Sydney, Eveleigh, New South Wales, Australia

ABSTRACT

The purpose of this study was to ascertain the effect of turbid water on a fluourometric device designed to detect phycocyanin and chlorophyll *a* in cyanobacteria cells *in vivo*. Cell densities corresponding to the Blue Green Algae Alert levels endorsed by the Australian National Health and Medical Research Council and adopted by numerous water resource managers were coalesced with a range of total suspended solids at defined gradients characteristic of lowland freshwater ecosystems. The parameters of interest were phycocyanin and chlorophyll *a*. *Microcystis aeruginosa* was the experimental organism used to establish cell densities consistent with the three-stage alert level framework. We found phycocyanin to be an effective measure for detecting *M. aeruginosa* at concentrations prescribed within the cyanobacterial alert levels (Green, Amber and Red) in turbid waters up to 200 Nephelometric Turbidity Units. Copyright © 2016 John Wiley & Sons, Ltd.

KEY WORDS: fluorometry; freshwater cyanobacteria; turbidity; alert level framework

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INTRODUCTION

The proliferation of undesirable cyanobacteria within eutrophic freshwaters is now reported on a global scale. Therefore, both accurate and early detection methods are critical to the activation of public warnings and mitigation measures because of the potential ecological and public health impacts of the toxins produced in association with harmful algal blooms (Bastien *et al.,* 2011; Paerl *et al.,* 2011; Steffen *et al.,* 2014). If the escalating trend of harmful algal blooms continues as predicted, advanced capabilities such as *in vivo* fluorescence may counteract weaknesses often ascribed to the field sampling and microscopy methods while also serving as a rapid assessment tool (Izydorczyk *et al.,* 2005; Dall'Olmo, 2006; Bastien *et al.,* 2011; Sosik *et al.,* 2014).

Limited studies have been undertaken to assess the performance of fluorometric probes where emphasis has been placed on measuring cyanobacteria cell densities aligned to alert levels, in conjunction with a range turbidity gradients typical of many inland aquatic ecosystems (Randolph *et al.,* 2008; Bastien *et al.*, 2010; Song *et al.,* 2013). In part, this is as a result of the emerging and transitional nature of the technology and also the challenges associated with measuring fluorescence in optically complex turbid waters (Palmer *et al.,* 2014). The presence of inorganic and organic particles may cause shifts in mixing, light scattering and attenuation that can affect fluorescence emission spectra (Grobbelaar, 1989,1990).

Phycocyanin (PC) pigment is universally present in cyanobacteria and contained in photosystem II (PSII) absorbing light in the orange to red (590–630 nm) spectrum, with a fluorescence emission of 640–660 nm (Bryant, 1994; Gregor and Maršálek, 2005; Allen, 2008; Whitton, 2012; Sun *et al.,* 2013). The excitation spectra of chlorophyll *a* (Chl *a*) are approximately 430–480 nm and the fluorescence emission wavelength band between 675 to 690 nm (Bryant, 1999; Beutler *et al*., 2002; Gregor and Maršálek, 2004; Whitton, 2012; Simis *et al*., 2012). Studies have shown the distribution of Chl *a* in PSI is variable due to weakly fluorescing long wavelengths, but the small amount present in PSII (~12%) is thought to remain constant (Simis *et al*., 2012). Chl *a* however is mostly present in PSI (~78%) (Anttila *et al*., 2012; Middepogu *et al.,* 2012; Johnsen and Sakshaug, 2007).

Surprisingly, fluorometry, a developing technology, and its effectiveness in measuring PC were found to lack detailed laboratory examination (Madrid and Zayas, 2007; Kasinak *et al.,* 2014). A field-based study has cast uncertainty regarding instrument precision within turbid waters as findings suggested the fluorometric instrument examined measured false positives in waters that were characterized by turbidity greater than 50 Nephelometric Turbidity Units

*Correspondence to: E. Symes, Faculty of Agriculture and Environment, The University of Sydney, Biomedical Building, 1 Central Avenue, Australian Technology Park, Eveleigh New South Wales, Australia.
E-mail: elizabeth.symes@sydney.edu.au

(NTUs). Also, a cyanobacterial abundance of 0.4 mm^3/L at a minimum was essential for detection purposes (Bowling *et al*., 2013).

While fluorometry holds enormous promise for advancing scientific monitoring of aquatic ecosystems (Perrillin *et al.,* 2012; Zamyadi *et al*., 2014), pigment measurements may be questionable if rudimentary calibration is not performed, and implicit to optimum performance is systematic instrument maintenance (Song *et al.*, 2013; Kasinak *et al.,* 2014). Furthermore, phytoplankton *in vivo* fluorescence has been found to be dependent on intrinsic factors such as hierarchical taxonomic position and photo adaptation state, and therefore, growth stages and different cyanobacteria species will have a significant influence on the fluorescence spectra (Zhang *et al.,* 2009; Kasinak *et al.,* 2014). Regardless of the limitations indicated, as a stand-alone method *in vivo*, fluorescence has the potential to measure bulk biomass continuously, and it could also enhance the traditional spot sampling system if found to be reliable (Matthews *et al.,* 2010; Groetsch *et al.*, 2014).

The spot sampling and laboratory analysis method may be subject to error at any phase of the process (e.g. heterogeneity of the sample, no representative statistical population, limited replication, seasonal changes, contamination, volatilization and misidentification) (Ahn *et al.,* 2007; Madrid and Zayas, 2007). Moreover, thriving cyanobacteria may be inconspicuous because of size suspended in the water column as a result of buoyancy or their particular lifecycle phase, and consequently, the visual observational approach to ascertain the presence of cyanobacteria is patently incompatible with early detection standards (Bastien *et al.,* 2011). The delay of several days between sample collection and the analytical data becoming available to water resource managers or health authorities is especially concerning as response frameworks (Alert Levels) are triggered when cell densities (cells/mL) are quantified and reached (Beutler *et al.,* 2002; National Health and Medical Research Council (NHMRC), 2008). The results of toxin analysis from disparate laboratories may also lead to misinterpretation, as the data extrapolated for correlation between biovolume and the alert level criterion may in effect be guesstimates (AL-Tebrineh *et al.,* 2011). When cyanobacteria enumeration and identification to genera level reaches or exceeds Blue Green Algae Alert levels notifications advising the general public of risks associated with ingestion, aspiration or dermal contact are triggered additional to risk management measures at key water assets (Newcombe *et al.,* 2010). Table I shows the putative cyanobacteria alert level construct that triggers intensified monitoring and implementation of risk compliant actions in Australia (NHMRC, 2008).

There is a paucity of knowledge concerning the reliability of *in vivo* fluorescence measurement specific to turbid waters and directly relatable to the alert level framework.

Table I. Cyanobacteria alert levels (NHMRC, 2008)

Alert level	Total cell count (cells mL_L)	Biovolume (mm^3 L)
Green	500 to <5000	>0.04 to <0.4
Amber	≥5000 to <50 000	≥0.4 to <4
Red	≥50 000	≥4

Therefore, the objectives of this study were to (i) investigate the accuracy and capability of fluorescence to quantify the cyanobacteria species *Microcystis aeruginosa* in turbid conditions corresponding to inland waterways of Australia in a controlled experimental setting and (ii) evaluate the efficacy of *in vivo* probe readings concomitant with the alert level framework recommended by the Australian National Health and Medical Research Council (NHMRC, 2008) for cyanobacteria monitoring. The cyanobacterium *M. aeruginosa* was selected for the study because it is one of the most common species proliferating and dominating phytoplankton communities in freshwater eutrophic waters (WHO, 2003; NHMRC, 2008; Humbert *et al.,* 2013; Oberholster *et al.,* 2003), and moreover, *M. aeruginosa* cell equivalents (cells/mL) are often used as the benchmark cyanobacterium for probe standardization (Zamyadi, 2011).

Our paper reports on the performance of a fluorometric device, as practical information is needed to give water resource managers' greater understanding and confidence in emergent technologies designed to detect cyanobacteria *in vivo*. It is our understanding that this is an original study as ostensibly limited scrutiny existed addressing *in vivo* fluorescence, gradient turbidity levels using natural turbidities representing Australian inland water bodies and alert level frameworks utilized by several water authorities in an experimental setting (Zamyadi, 2011, pp. 21). We tested our experimental results against the null hypothesis, that is, *in vivo* fluorescence correctly differentiates cyanobacteria (cells/mL) at turbidity levels below 50 NTUs.

MATERIALS AND METHODS

Measuring pigments analogous to the alert level criterion in conjunction with discrete turbidity gradients formed the basis for this experiment. The experimental design comprised a combination of four discrete total suspended solid (TSS) gradients (including a control with no TSS) and three cyanobacteria concentrations consistent with guidelines for cyanobacteria monitoring within a freshwater body (Level 1 = green surveillance mode, Level 2 = amber alert mode and Level 3 = red alert mode) (NHMRC 2008 pp. 107). The replicates included a control (no cells) and alert levels aligned to recommended guideline concentrations of ≥500–

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<5000, ≥5000–<50 000 and ≥50 000 cells/mL (NHMRC 2008). The experiment was performed in triplicate with a total of 16 replicates per group (48 in total) and a Eureka Manta 2 multi-parameter *in situ;* fluorometric submersible instrument was used to measure the parameters of interest, Chl *a*, PC and turbidity.

Multi parameter submersible fluorometer

The Manta 2 probe is equipped with light-emitting diodes (LEDs) for excitation of pigment complexes, emitting orange light at (peak 610 nm) wavelength and scanning for a specific, different wavelength (600–700 nm) in return, and consequently, the level of the return luminosity is relatable to the amount of analyte present. The Manta 2 sensors [blue green algae (BGA), Chl *a* and turbidity] were calibrated prior to the spectral analysis.

The turbidity sensor has a 0–3000 NTU range with linearity of ± 1% below 1000 NTUs. The Sigma-Aldrich certified turbidity 100 NTU calibration standard (polymer microbeads) was used to calibrate the instrument, and calibration was performed using a two-point calibration (zero point with distilled water and 100 point) consistent with the manufacturer guidelines (Eureka Environmental Engineering, 2008). The Chl *a* and BGA sensors fitted to the instrument were manufactured by Turner Designs and were calibrated to *M. aeruginosa* cultures at log growth phase cultured at the University of Sydney's ATP laboratory. Initial enumeration of the stock culture was equivalent to 750 000 cells/mL, and a sub sample dilution with DI water using a standard dilution was estimated to be 86 000 cells/mL. Importantly, prior to calibration, the instrument was modified to permit aggregate values beyond the factory default settings as the upper value of 100 000 cells/mL meant that the analytic range of 100–200 000 cells/mL ± 3% accuracy could not be applied. Following instrument calibration PC readings were 104 000 cells/mL following repeated measurement; thus, a 20% variation from estimated dilution to the fluoroscence readings for *M. aeruginosa* was found. To reduce error in flourometric readings, calibration using cell densities beyond 3000 cells/mL is recommended for instrument calibration (Macário *et al.,* 2015). The Chl *a* sensor measures in a range 0–500 µg/Land; it has a resolution of 0.1 µg/L and linearity r^2 of 0.9999. In order to measure the norm spectra and to carry out the laboratory *in vivo* experiment, the Manta 2 was set-up as a bench-top fluorometer and mounted for stability using hardware (retort stand and clamps).

Experimental organisms

The experimental microorganism selected was a pure (monoalgal) culture of cyanobacterium *M. aeruginosa* (Kützing), grown in sterilized MLA media (Gorham *et al.,* 1964) and maintained at 24 ± 1 °C, a 16/8 D: N photoperiod and a sub culturing timeframe of 14 days with a light intensity of 200 µmol m^{-2} s^{-1}.

Cell concentrations

The population density was estimated by subsampling (1 mL aliquot) prior to commencement of the laboratory work. Enumeration of the *M. aeruginosa* stock culture (2) for inoculum and also *M. aeruginosa* culture material used for instrument calibration was calculated using a Fuch's Rosenthal haemocytometer counting chamber and an Olympus binocular microscope adjusted to 100x magnification. On average, 20 fields were counted for each stock culture in order to achieve an error of 10–20% (Lawton *et al.,* 1999). Cell densities of the stock cultures were equal to 1×10^6 cells/mL and 2×10^5 cells/mL (100 mL each). These cultures were diluted using the standard dilution equation ($C_1V_1 = C_2V_2$ where C_1 is the concentration of the stock solution, V_1 is the volume removed from the concentrated stock solution, C_2 is the final concentration of the diluted solution and V_2 is the final volume of the diluted solution) equivalent to categories of cyanobacteria alert levels (Green, Amber and Red). In addition, a Control (nil cell) replicate group was also prepared. The total volume for each replicate was 70 mL, and concentrations were formulated by dispensing suspended culture material with a single channel volumetric pipette (1–10 mL) with a disposable tip. The diluted cell concentrations used in the experiment were 0 cells/mL (no alert level and Control), 4000 cells/mL (Green), 48 000 cells/mL (Amber) and 104 000 cells/mL (Red). Biovolume was calculated based on the geometric shape (sphere) for *Microcystis* (Hillebrand *et al*., 1999).

Chlorophyll a concentrations using spectrophotometry

Chlorophyll *a* content was determined using a method adapted from Wasmund *et al*. (2006). A hot ethanol extraction procedure was performed and spectrophotometric absorbency wavelength analysis undertaken using a Synergy H1 Hybrid Multi-Mode Microplate Reader (0.2 mL aliquot of supernatant). Absorbency readings were measured at 665 and 750 nm wavelengths, and replicate samples were acidified with the addition of 0.2 µ/L of hydrogen chloride at 0.3mole/L concentration and measured at the same wavelengths for turbidity correction. For corrected absorbance and turbidity correction, absorbance at 750 nm was subtracted from 665 nm pre and post addition of hydrogen chloride according to Lawton *et al.* (1999).

Total suspended solids

The preparation and calculation of TSS was consistent with established procedures (APHA, 1998). Alluvial sediment collected during routine fieldwork in 2014 from an

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impoundment located in the riverine plains bioregion of New South Wales and classified as an unconsolidated dispersive soil type was used to establish the representative TSS gradients (Kirk, 2011). The sediment was dried in an oven at 104° until no weight change was observed and stored in a desiccator. The dried sediment was sieved (200 micron mesh aperture) to ensure the heterogeneous mixture of fine silt and clay would remain suspended for a prolonged period of time when added to the standards and consequent media for each treatment. The TSS standards were initially prepared using the following TSS estimates: 300 mg/L = 60 NTUs, 600 mg/L = 120 NTUs and 1200 mg/L = 240 NTUs. To prepare the individual stock standards, 2000 mL of DI filtered water was measured in a graduated glass cylinder and added to 4000 mL screw top glass bottles (4), and following the addition of dried and weighed sediment, each bottle was vigorously shaken for 60 s. Turbidity values were then derived using the Manta 2 calibration cup with sufficient volume of each standard covering the turbidity sensor, and a magnetic stirrer was inserted. Before the standards were measured, the turbidity sensor was stabilized (90s), and logged values were then transcribed to each standard bottle with the turbidity values (0, 54, 112 and 220 NTUs) recorded. The difference in the values measured for the turbidity standards from the experimental estimates mentioned earlier was encountered when the sensor was immersed in the aqueous solution, as dissolved constituents in the calibration cup absorbed or scattered light, thus introducing an adjusted fluorescence measurement. The size of particles will affect fluorescence measurement (Brient *et al.,* 2008), and the interactions between light and conjugated chemical moieties in media, especially dissolved or suspended material, affects optical measurements (Downing *et al.,* 2012). Therefore, precise replication was unlikely given the sediment (derived from the lake bed) used to establish TSS standards.

Culture inoculant

One hundred milliliters wide mouth polypropylene bottles with screw top lids were used for the individual replicates, and each replicate was prepared by measuring a precise volume (total 70 mL) of the TSS standards in a graduated glass cylinder and dispensed to each bottle following 60 s of agitation. The subculture inoculant to establish the replicates was as follows: Red treatment, 6 mL (stock culture 10^6 cells/mL) added to 64 mL of TSS solution; Amber, 1.75 mL (stock culture 10^6 cells/mL) added to 68.25 mL of TSS solution; and Green, 1.5 mL (stock culture 2×10^5 cells/mL added to 68.5 mL TSS solution).

Procedure for analysis

All 48 replicates were established at the same time, and each was labelled and acclimated to laboratory conditions following the addition of the measured inoculum. For the spectral analysis, each replicate was decanted to a 100 mL opaque plastic container, and a magnetic stirrer inserted to ensure the media remained homogenous. The container was placed on a raised platform that had a black base consistent with required instrument calibration procedures. Before each spectral analysis, the samples were exposed to each sensor light for 90s, and three readings logged for each parameter of interest. The PC (cells/mL), Chl *a* (μg/L) and turbidity (NTUs) data were annotated with the date, time and the treatment label using the (Manta 2) software and the data saved as a text (.csv) file pending statistical analysis.

Statistical analysis

The statistical analysis was performed using the software Prism version 6.00 for Mac OS X, (GraphPad). A significance level of $p < 0.05$ was used for all tests, and unless otherwise stated, analyses including analysis of variance (ANOVA) was performed on normalized data.

RESULTS

Phycocyanin (PC) spectral data

A two-way ANOVA test considering the interactions between the TSS groups (T1, T2, T3 and T4) and the Alert Levels (no Cells, Green, Amber and Red) was performed using the PC (cells/mL) response data that were normalized prior to analysis. The ANOVA interaction was found to be extremely significant ($F = 21.32$, DFn = 9, DFd = 30, $p < 0.0001$).

The Alert Level or measured PC accounted for 98.33% of the variation ($p < 0.0001$) and TSS accounted for 0.31% of the total variation. A post hoc Dunnett's multiple comparisons test examining the interactions for the individual TSS groups versus Alert levels calculated significant *p*-values for all interactions (Table II). The only exception was the T1 (no cells vs Green) comparison where no significant difference was indicated. Overall TSS was found to have negligible effect on the PC measurements and the corresponding alert levels.

The family interactions were examined using a single step Dunnett's multiple comparisons test for the Alert Levels versus the nil TSS control group. No statistically significant difference was found for the six family comparisons (no Cells, Green and Amber) versus each of the T1 (no TSS), T2 (300 mg/L), T3 (600 mg/L) or T4 (1200 mg/L) groups. In contrast, significant *p*-values (<0.001) for all Red (T2, T3 and T4) versus T1 (nil TSS) comparisons were indicated (Table III).

A simple linear regression was calculated to predict PC values based on the TSS variable, and overall, the

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Table II. Dunnett's multiple comparison's test showing within group analysis

Within group response	Mean difference	95% CI of difference	Adjusted *p*-value	Summary
T1–Nil TSS				
No Cells vs. Green	−4195	−9325 to 935.5	0.1285	ns
No Cells vs. Amber	−40 581	−45 510 to −35 651	<0.0001	****
No Cells vs. Red	−137 262	−142 191 to −132 332	<0.0001	****
T2 - 300 mg/L				
No Cells vs. Green	−6145	−11 656 to −633.2	0.0262	*
No Cells vs. Amber	−40 898	−46 409 to −35 387	<0.0001	****
No Cells vs. Red	−110 467	−115 978 to −104 956	<0.0001	****
T3 - 600 mg/L				
No Cells vs. Green	−5380	−10 309 to −450.1	0.0300	*
No Cells vs. Amber	−38 038	−42 968 to −33 109	<0.0001	****
No Cells vs. Red	−110 616	−115 545 to −105 686	<0.0001	****
T4 - 1200 mgnL				
No Cells vs. Green	−5818	−10 747 to −888.2	0.0178	*
No Cells vs. Amber	−37 792	−42 721 to −32 863	<0.0001	****
No Cells vs. Red	−109 843	−114 773 to −104 914	<0.0001	****

ns = not significant,
*= $p \leq 0.05$,
****= $p \leq 0.0001$
CI, confidence interval.

differences between the slopes were noteworthy. A significant relationship between TSS gradients and PC for the Control (No cells) (Figure 1) F (1,10) = 6.794, $p = 0.0262$ was found. The 95% confidence interval around the slope parameter for PC values in the no cells group showed an increase anywhere from 653–1545 cells/mL is likely when TSS reaches 1200 mg/L despite the absence of cellular material. Also, a significant relationship was found for the Red group F (1,10) = 8.015, $p = 0.0173$, (r^2 value 0.447) between TSS and PC. The 95% confidence interval showed PC values may increase anywhere from 94 478 to 117 036 cells/mL when TSS is 1200 mg/L. At the lower end of the TSS spectrum (300 mg/L), the 95% confidence interval for PC values in the Control group showed PC is likely to

Table III. Dunnett's multiple comparisons test showing the comparisons to the control Nil total suspended solids group

Group comparisons with the control	Mean difference	95% CI of difference	Adjusted *p*-value	Summary
No Cells				
0 TSS vs 300 mg/L	−583.7	−5714 to 4546	0.9848	ns
0 TSS vs 600 mg/L	−579.2	−5709 to 4551	0.9851	ns
0 TSS vs 1200 mg/L	−885.0	−6015 to 4245	0.9510	ns
Green				
0 TSS vs 300 mg/L	−2900	−8030 to 2230	0.3782	ns
0 TSS vs 600 mg/L	−1764	−6894 to 3366	0.7326	ns
0 TSS vs 1200 mg/L	−2508	−7638 to 2622	0.4922	ns
Amber				
0 TSS vs 300 mg/L	−1268	−6398 to 3862	0.8747	ns
0 TSS vs 600 mg/L	1963	−3167 to 7093	0.6682	ns
0 TSS vs 1200 mg/L	1904	−3226 to 7034	0.6876	ns
Red				
0 TSS vs 300 mg/L	25 844	20 714 to 30 974	<0.0001	****
0 TSS vs 600 mg/L	26 067	20 937 to 31 197	<0.0001	****
0 TSS vs 1200 mg/L	26 533	21 403 to 31 663	<0.0001	****

ns = not significant, and
****= $p \leq 0.0001$
CI, confidence interval.

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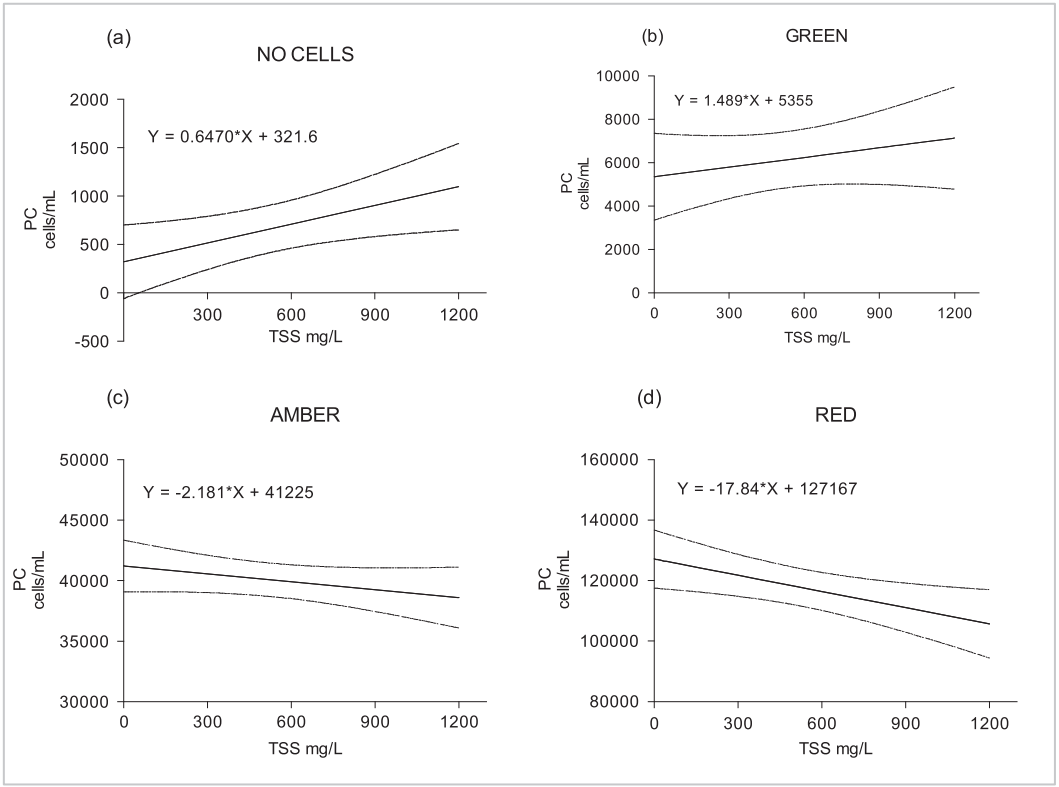

Figure 1. Linear regression model for the measured phycocyanin (PC) data showing No Cells, Green (top), Amber and Red (lower) treatments versus Total Suspended Solids shown (±95% CL bands $n = 3$)

increase from 240 to 790 cells/mL, and in the Red group, PC values may range between 114 868 to 128 761.

In contrast, the Amber $F\ (1,10) = 2.44$, $p = 0.1491$, (r^2 value 0.196), and Green $F\ (1,10) = 1.297$, $p = 0.2814$ (r^2 value 0.114) levels did not show a statistically significant relationship between PC and TSS values at 1200 mg/L or at the lower TSS gradients. For the Green and Amber groups, the 95% confidence interval showed PC values may increase from 4789 to 9496 and 36 079 to 41 119 cells/mL when TSS is 1200 mg/L, respectively. At the lower end of the TSS spectrum (300 mg/L), the 95% confidence interval for PC values in the Green and Amber Groups showed an increase from 4352 to 7525 and 39 025 to 42 117 cells/mL, respectively.

Cell enumeration and phycocyanin data

A simple linear regression analysis was calculated to examine the relationship between the microscopic enumeration (cells/mL) and pigment-based (PC) measurements (cells/mL) for all treatments (excluding the no cells group). A significant relationship was found between enumeration and PC data $F\ (1,9) = 171$, $p < 0.0001$, (r^2 value 0.95) (Figure 2). Thus, PC data characterized cell counts accurately.

Biovolume and phycocyanin data

To test for a relationship between the measured biovolume and PC (log) data, a linear regression was performed. The

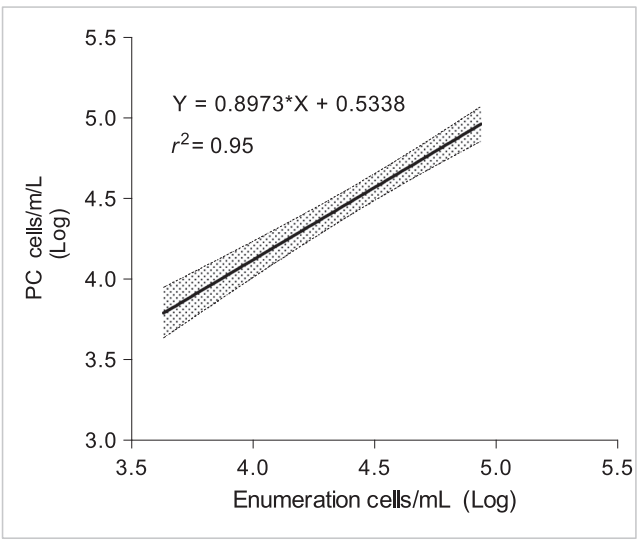

Figure 2. Linear regression showing the relationship between the cell counts versus phycocyanin data (log transformed)

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relationship was found to be statistically significant F (DFn = 1 DFd = 14) = 21.78, $p = 0.0004$ (Figure 3).

Chlorophyll a spectral data

Chlorophyll *a* concentrations for the *in vivo* fluorescence method and the spectrophotometry versus TSS concentrations are shown in Figure 4. The separation in the values was particularly evident at TSS 600 mg/L where the spectrophotometric Chl *a* measurement was 19.41 ± 1.732 µg/L, while the fluorescence Chl *a* was 8.962 ± 0.4677. Similarly at the 1200 mg/L level, the separation in Chl *a* values was significant between the spectrophotometric result (42.98 ± 3.109) and measured fluorescence Chl *a* (16.78 ± 0.8396).

Chlorophyll a, spectrophotometry versus fluorometry relationship

Linear regression was performed to examine Chl *a in vivo* fluorometry results versus spectrophotometry data (Figure 5). The results showed a significant relationship F (1,14) = 157.3, $p < 0.0001$ (r^2 value 0.918), despite observable spectrophotometry values being more than double at the elevated TSS levels. While fluorescence underestimates Chl *a* in cyanobacteria, the linear relationship found between the extracted and fluorometric data indicated prediction and estimation from PC data was feasible.

Biovolume and chlorophyll a relationship

Correlation was performed to examine spectrophotometric Chl *a* data versus fluorometric PC data (biovolume mm^3/L) (not shown in graphical format) to examine the relationship between the corresponding treatments. The Red (Chl *a*) versus Red biovolume ($r^2 = 0.48$, $p = 0.38$ $n = 4$), Amber (Chl *a*) versus Amber biovolume ($r^2 = 0.00$, $p = 0.91$ $n = 4$) and Green (Chl *a*) versus biovolume ($r^2 = 0.30$, $p = 0.44$, $n = 4$) indicated a weak or no relationship found between the compared treatments. The fluorometric Chl *a* data versus biovolume (mm^3/L) were examined, and the results similarly showed weak relationships for all comparisons, Red ($r^2 = 0.11$, $p = 0.66$ $n = 4$), Amber ($r^2 = 0.39$, $p = 0.37$ $n = 4$) and Green ($r^2 = 0.34$, $p = 0.41$ $n = 4$). Thus, the observed spectrophotometric and fluorometric Chl *a* and PC comparisons were weakly correlated.

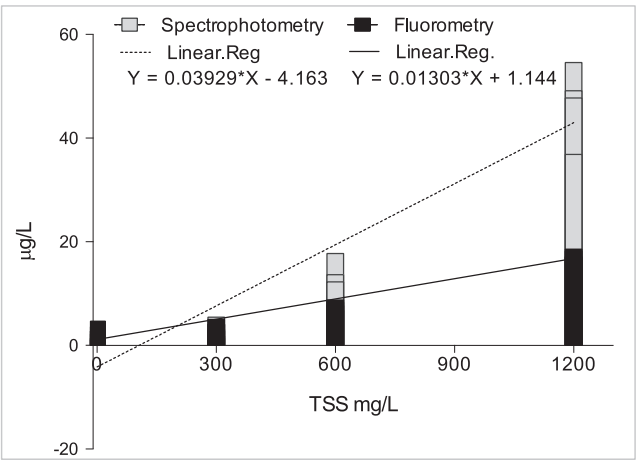

Figure 4. Fluorometric and Spectrophometric combined plot showing chlorophyll *a* versus Total Suspended Solids and Linear Regresssion (x¯values)

DISCUSSION

The two indicative measures of cyanobacteria abundance, PC and Chl *a*, were determined by *in vivo* fluorescence in

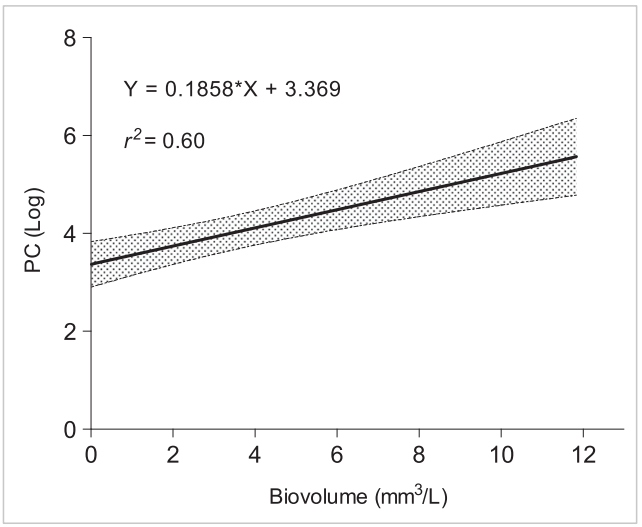

Figure 3. Linear regression plot showing biovolume versus phycocyanin (PC) (Log) data

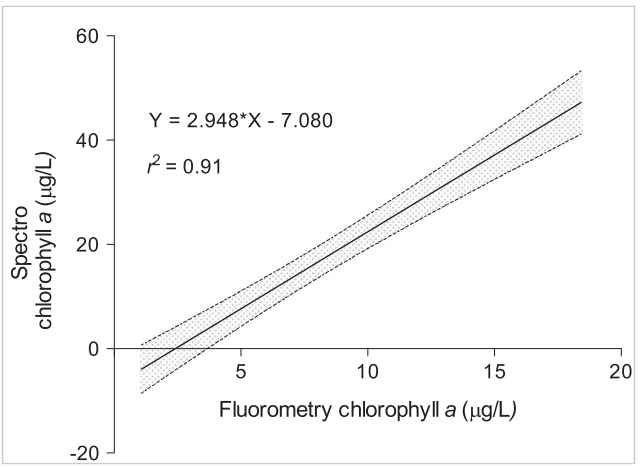

Figure 5. Chlorophyll a *in vivo* fluorometry versus spectrophotometry data Green, Amber and Red. (x¯values, $n = 16$)

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the current study, with the addition of TSS gradients. Previous studies have shown poor correlations between *in vivo* fluorescence PC and cyanobacterial cell densities (Chang *et al.,* 2012; Kasinak *et al.,* 2014), whereas significant relationships have been found between *in vivo* PC fluorescence and cyanobacterial biovolume (Gregor and Maršálek, 2004; McQuaid *et al.,* 2011; Bowling *et al.,* 2012). We report a statistically significant relationship between PC and cell densities (enumeration), and this may be attributed to the exploitation of living cellular material for instrument (sensor) specific calibration, the use of a dispersed mono cyanobacteria suspension at log growth phase, and also PC cell content was potentially maximized under the constant light conditions in the laboratory (Chang *et al*., 2012; Zamyadi *et al*., 2012). In natural systems where cyanobacteria assemblages have wide biovolume ranges (colonial and filamentous) correlations similar to those reported for cell densities in the current study may not be replicated using fluorometry (PC). Nevertheless, our results were consistent with those reported by Brient *et al.* (2008) where a number of freshwater lakes were considered, as we also found a significant correlation ($r^2 = 0.95$) between the PC content and the cyanobacteria abundance (cells/mL). Also, our results show consistency with Bowling *et al.* (2012), McQuaid *et al.* (2011) and Gregor and Maršálek (2004) as a significant relationship was found between biovolume (mm^3/L) and PC data ($r^2 = 0.60$).

In the controlled experiment, the single species culture used for calibration and analytical purposes proved to be a sound approach. However, previous research suggests calibrating submersible *in situ* probes with one taxon, such as *M. aeruginosa* may be questionable because of the phytoplankton composition existing in natural aquatic ecosystems (Bastien *et al.,* 2011). While instrument calibration using living cells is optimal, mixed assemblages (prokaryotic and eukaryotic), as well as spatial and temporal heterogeneity, may influence readings that could lead to an under or overestimation of PC (McQuaid *et al*., 2011; Zamyadi *et al*., 2012). To limit equivocality and complexity when considering natural populations, selecting a single but invariant calibration standard, that is, a profiler particular to the water body being considered, may be preferable (Zamyadi, 2011; Chang *et al*., 2012). Erroneous data output has been attributed to inadequate calibration measures; thus, refinement of factory default settings, implicit to the current study, may enhance signal resolution (Song *et al.*, 2013; Kasinak *et al*., 2014).

The absence of information regarding commercially available probes performances in conditions analogous to those reported in this study makes it difficult to compare experimental findings currently. Furthermore, the range of fluorometric tools available for field-based or laboratory research means invariably data show inconsistencies because of, for example, quality, factory settings, maintenance, experimental design and specific site or species characteristics. Indeed, inherent sources of discrepancy affecting wavelength sensitivity and cyanobacteria measurement have been documented from evaluations considering commercially available fluorescence instruments (Ahn *et al.,* 2007; Brient *et al.,* 2008, Zamyadi *et al.,* 2012). Unsurprisingly, anomalous results are likely to be attained between different probes because of quality, standards, maintenance and calibration (Bastien *et al*., 2011).

The results of our study suggest a pragmatic approach must be applied in turbid waters above 300 mg/L TSS (or 50 NTUs), as fluorescence (PC) results in the simulated Control (no cells) replicates were equivalent to the lower range of the Green alert trigger values, despite the absence of the inoculant. Thus, a false positive value towards the lower range of the alert level threshold (Green) was indicated. However, when cell densities increased beyond the Red alert level and TSS was elevated (1200 mg/L), lower PC values were recorded when compared with the nil TSS replicates. The PC values in the upper TSS and Red alert treatments were likely to be influenced by a reduced signal, and absorption of particles implied consistent with Brient *et al*. (2008). Nevertheless, the values recorded were within an acceptable error of ± 20%. Zamyadi *et al.* (2011) examined the main interference factors that could limit *in vivo* fluorescence and found readings applied to laboratory cultures of *M. aeruginosa* remained quasi-constant with increasing turbidity, up to 6000 NTUs, whereas concurrent field experiments found a correction factor was needed for the BGA data using a simultaneous Chl *a* measurement.

Previous field-based studies have reported TSS affected Chl *a in vivo* fluorescent measurement, and as turbidity increased, the spectral signal of the water was misinterpreted by the sensor as fluorescence (665 nm), and as a consequence, Chl *a* values increased (Ferreira *et al.,* 2012). We found that spectrophotometric concentration was 100% greater than fluorescence Chl *a* values in the elevated TSS (600 and 1200 mg/L) treatments. Gregor and Maršálek (2004) argue a key characteristic of Chl *a* is its PSII fluorescence, and while spectrophotometric values may be highly correlated, significantly lower concentrations will be found using fluorometry, as the majority of cyanobacteria's Chl *a* is located in weakly fluorescing PSI (Zamyadi, 2011). Therefore, optical proxies used to develop coupled hydrodynamic models where Chl *a* is a key parameter may need careful consideration and corrections to reduce bias as proposed by Zamyadi *et al.* (2012). Importantly, Chl *a* may not be the most appropriate measure of cyanobacterial abundance, and indeed could even be a misleading indicator as it is also found in eukaryotic algae (Ahn *et al.,* 2007; Williams and Burghdoff, 2010; Zamyadi *et al.,* 2012). Pronounced variations in the PSII/PSI stoichiometry are influential in changes to the ratio between PSII and Chl *a* (Beutler *et al.,* 2002). Thus, the potential exists for the

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generation of inconsistent results. Chl *a* as an indicator of cyanobacteria abundance to determine alert level status using *in vivo* fluorometry or spectrophotometry may be limited or in the least a poor measure for lowland aquatic ecosystems characterized by elevated turbidity and high densities of cyanobacteria (Gregor and Maršálek, 2005).

The three-stage alert level and response framework in Australia has been described as the Burch Model (Burch *et al.,* 2003), and in many respects, the current national protocols for monitoring cyanobacteria in freshwaters still resemble the original procedures (Preez *et al.,* 2007). The World Health Organization (WHO, 2003) has similar procedures to the Burch model, but different alert level concentrations trigger actions (Bartram and Chorus, 2014). Risk-based alert systems in the future may be based on PC measurements particularly as PC has been shown to exhibit a strong correlation with a variety of bloom-related factors (Ahn *et al*., 2007), and this current study provides empirical data to support the use of PC fluorescence in optically complex waters.

CONCLUSION

The results indicate *in vivo* fluorometry using the Manta 2 probe was useful for detecting *M. aeruginosa* consistent with the alert framework (Green, Amber and Red) in turbid conditions. PC, in particular, shows considerable potential for use in scientific studies as a less onerous alternative to cell enumeration, and the prospect of reliable field data acquisition has palpable benefits for water resource managers. However, when cellular material is present in the media and TSS above 300 mg/L it is likely that PC values at the lower end of the Green alert level will be recorded.

Currently, pigment fluorescence measurement is embedded within the scientific domain, and an adoption shift to the responsible entities (water resource managers) is needed. Indeed, wider acceptance could improve monitoring programmes more generally as PC specific fluorescence as a function of cyanobacteria biomass could complement the standard field surveillance and analysis procedures. It is evident submersible *in vivo* fluorometry has the capability for measuring horizontal and vertical heterogeneities, and also, continuous on line monitoring is entirely feasible as an antecedent strategy. Yet, *in vivo* fluorometry cannot solve all monitoring requirements consequently microscopy for the characterization of taxa, quantitative enumeration, community structure and topology examinations of cyanobacteria are requisite in terms of ecology, morphology and physiology descriptions of the communities they constitute.

We used a monoculture population to constrain a number of variables, and therefore, extrapolation to natural populations where freshwater algae and cyanobacteria (colonial and filamentous) species coexist would simply be conjecture. We recommend further study to test the efficacy of *in situ* fluorometry within a field-based eutrophic setting where elevated turbidity and eukaryotic photosynthetic organisms and cyanobacteria co-occur. This would permit *in vivo* fluorometry to be assessed with a combination of factors such as spatial heterogeneity, population dynamics and the interaction of a range of environmental factors. Given the evidence that *in vivo* fluorescence differentiated cyanobacteria abundance measured as PC at turbidity up to 220 NTUs, we were inclined to reject the null hypothesis.

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