Sensor manufacturer, temperature, and cyanobacteria morphology affect phycocyanin fluorescence measurements

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Abstract

Sensors to measure phycocyanin fluorescence *in situ* are becoming widely used as they may provide useful proxies for cyanobacterial biomass. In this study we assessed five phycocyanin sensors from three different manufacturers. A combination of culture-based experiments and a 30 sample field study were used to examine the effect of temperature and cyanobacteria morphology on phycocyanin fluorescence. Phycocyanin fluorescence increased with decrease in temperature, although this varied with manufacturer and cyanobacterial density. Phycocyanin fluorescence and cyanobacterial biovolume were strongly correlated ($R^2 > 0.83$, $P < 0.05$) for single-celled and filamentous species. The relationship was generally weak for a colonial strain of *Microcystis aeruginosa*. The colonial culture was divided into different colony sizes classes and phycocyanin measured before and after manual disaggregation. No differences were measured, and the observation that fluorescence spiked when large colonial aggregates drifted past the light source suggests that sample heterogeneity, rather than lack of light penetration into the colonies, was the main cause of the poor relationship. Analysis of field samples showed a strong relationship between *in situ* phycocyanin fluorescence and spectrophotometrically-measured phycocyanin ($R^2 > 0.7$, $P < 0.001$). However, there was only a weak relationship between phycocyanin fluorescence and cyanobacterial biovolume for two sensors ($R^2 = 0.22\text{-}0.29$, $P < 0.001$), and a non-significant relationship for the third sensor ($R^2 = 0.29$, $P < 0.4$). The five sensors tested in our study differed in their output of phycocyanin fluorescence, upper working limits (1,200 to >12,000 µg/L) and responses to temperature, highlighting the need for comprehensive sensor calibration and knowledge on the limitations of specific sensors prior to deployment.

Keywords: *Aphanizomenon*; cyanobacterial blooms; environmental monitoring; *Dolichospermum*; *Microcystis*; *Nodularia*

Introduction

A global increase in the frequency, intensity and duration of cyanobacterial blooms is raising concerns as many bloom-forming species produce skin irritants and harmful compounds that pose a risk to human and animal health (Codd et al. 2005, Harke et al. 2016). In many countries, the occurrence of cyanobacterial blooms is most pronounced during summer, which coincides with the period of highest water demand and recreational use. To evaluate the risk posed to drinking water supplies and recreational water bodies, and to assist in the understanding of bloom dynamics, cyanobacterial biomass is often quantified. Traditionally, biomass is assessed via grab samples analyzed by microscopy or chlorophyll *a* measurements using filtration and spectrophotometry (Hötzel and Croome 1999). Whilst these methods provide accurate and robust data, they are time-consuming and expensive. This limits the frequency with which samples can be collected and analyzed, preventing real-time risk assessments and limiting our ability to understand and predict cyanobacterial bloom formation.

Phycocyanin is a phycobiliprotein that is primarily associated with cyanobacteria and cryptophytes, and is a trace pigment in rhodophytes (Bastien et al. 2011). Phycocyanin absorbs light at 620 nm, and emits fluorescence at about 650 nm (Gregor and Marsalek 2004). In freshwater systems, phycocyanin is generally associated with cyanobacteria, which enables their biomass to be estimated within a mixed assemblage of phytoplankton. A number of sensors measure phycocyanin fluorescence and therefore enable phycocyanin concentrations to be assessed *in situ*. These sensors show potential for assessment of cyanobacterial biomass spatially and temporally in reservoirs and lakes, and they have now been incorporated into *in situ* monitoring systems across the globe (Gregor et al. 2007; Brient et al. 2011; McQuaid et al. 2011; Hamilton et al. 2014). Some studies have highlighted caveats to sensor use, including interference from turbidity and other phytoplankton (Zamyadi et al. 2011), and differing effects

of cyanobacteria community composition on phycocyanin concentrations (e.g., Bowling et al. 2012; Kong et al. 2014; Kasinak et al. 2015). With rapid expansion in the use of phyocyanin sensors *in situ*, it is evident that there is only rudimentary information on how sensors from the same and different manufacturers perform, and how environmental conditions (such as temperature) affect the performance of phycocyanin sensors.

In this study we evaluated the performance of five commercially-available phycocyanin sensors (from three different manufacturers). A combination of culture-based laboratory experiments and a field study was used to investigate the effect of temperature and cyanobacterial morphology on the performance of the sensors. Our objective was to elucidate the advantages and limitations of use of phycocyanin sensors, compared to conventional cyanobacterial monitoring approaches.

Methods

Sensors, cyanobacterial cultures and laboratory experiments

Phycocyanin sensors from three different manufacturers were evaluated. Two matching stand-alone CYCLOPS-7 probes (hereafter referred to as T926 and T927) from Turner Designs (USA) were assessed, as well as one of these probes incorporated into a Manta II water quality sonde from Eureka (USA; hereafter referred to as Manta). A TriLux sensor (Chelsea Technologies Group Ltd, England), and a YSI phycocyanin blue-green algae sensor (incorporated into a EXO Sonde; YSI Inc., USA) were also assessed. All sensors were used in the laboratory experiments unless stated. The T926, T927 and YSI sensors were also assessed in the field.

To convert the fluorescence readings from each sensor into units of phycocyanin (µg/L), calibration curves were constructed. C-phycocyanin standard (*Spirulina* - P2172; Sigma-

Aldrich, USA) was dissolved in sodium phosphate buffer (30 mL; 50 mM, pH 7) and diluted to 300 mL with Milli-Q water. The phycocyanin concentration was determined by spectrophotometry (see below) and the solution was diluted to obtain an eleven point standard curve (120-12,000 µg/L) which was measured using each sensor.

Cyanobacteria cultures for the laboratory experiments were sourced from the Cawthron Institute Culture Collection of Micro-algae (CICCM; www.cultures.cawthron.org.nz; Rhodes et al. 2016). The species were selected to represent a range of morphologies; filamentous *Aphanizomenon* gracile (CAWBG595), *Dolichospermum lemmermannii* (CAWBG567) and *Nodularia spumigena* (CAWBG21); unicellular *Microcystis aeruginosa* (CAWBG617 and CAWBG624); and colonial *Microcystis aeruginosa* (CAWBG563). Cultures were maintained in MLA medium (Bolch and Blackburn 1996) under a light regime of 90 µmol m^{-2} s^{-1} with a 12 h:12 h light:dark cycle, at 17°C ± 1°C.

Experiments were performed in 1-L beakers which were wrapped with black polyvinyl chloride tape and placed on a non-reflective black surface. Unless otherwise stated, all experiments were carried out under low light (<3 µmol m^{-2} s^{-1}) and at a controlled temperature (17°C ± 1°C). Measurements were made in triplicate, and culture volumes of 400 mL used. During measurements, sensors were held 1 cm beneath the surface of the sample. Readings were taken once the sensor reading had stabilized (ca. 10 s). Each sensor was rinsed and dried thoroughly when moving between control and test samples. The background reading of each sensor was determined daily using measurements of Milli-Q water or MLA media, which were subtracted from the final measurements.

For each of the experiments described below, samples (1-5 mL) from cultures or environmental samples were preserved in Lugol's iodine solution and kept in the dark for later cell enumeration and biovolume calculations. Subsamples (500 µL) were added to 12-well plates (ThermoFisher Scientific, USA) and allowed to settle for at least 3 h. Cell enumeration was conducted using an inverted light microscope (CK41 or CK2; Olympus, Japan) using a magnification between 200-800×. Biovolumes for each species were estimated by measuring the cell dimensions of 30-50 cells at 1,000× (BX51; Olympus, Japan) and using volumetric equations of geometric shapes closest to each cell shape to calculate cell volumes (Olenina et al. 2006, Wood et al. 2009). Statistical analyses were performed using Minitab 15, and statistically significant relationships were given by $P < 0.05$.

Short-term and intermediate precision

A phycocyanin extract was prepared to determine the intermediate and short-term precision of each sensor. Lyophilized *Microcystis* sp. CAWBG11 (0.65 g; Puddick et al. 2014) was extracted in sodium phosphate buffer (200 mL; 50 mM, pH 7), sonicated (30 min, 60 kHz; Kudos Ultrasonic sonication bath), homogenized using an ultra-turrax (IKA Ultra T25 Basic, 1 min, 12,000 rpm) and clarified by centrifugation (10 min, 3,200 × g).

To determine the short-term precision, the phycocyanin extract was diluted in Milli-Q water and measured with each sensor six times over a 1 h period. The mean and standard deviation readings from each sensor were used to determine the short-term precision expressed as a relative standard deviation (RSD, Equation 1.1):

$$\%RSD = \left(\frac{SD}{x}\right) \times 100\% \qquad (1.1)$$

where *SD* is the standard deviation, and x is the mean of the replicate measurements.

To assess the intermediate-term precision, aliquots of the phycocyanin extract were stored in the dark at −20°C. Each day for seven days, a single aliquot of phycocyanin was defrosted, diluted in Milli-Q water and measured using each sensor. The standard deviations and means over these seven days were used to determine the intermediate precision expressed as the RSD (Equation 1.1).

Effect of temperature

Only *M. aeruginosa* (CAWBG624) was used to assess for temperature effects. No sub-samples were taken for cell enumeration during these experiments as a single homogenous culture was used. CAWBG624 was prepared at three cell concentrations; high (828,000 cells/mL), medium (562,000 cells/mL) and low (181,000 cells/mL). These values were selected to represent the approximate range of biovolumes observed during our field study. Samples and sensors were placed into temperature controlled cabinets (4, 13.8, 17 and 23.5°C; Polar Cool, New Zealand) and left to equilibrate for 2 h, before phycocyanin measurements were made using the sensors. The experiment was conducted over a 10 h period, beginning at 4°C and increasing at the prescribed temperature steps to 23.5°C, with 2 h equilibration periods between each temperature tested. Due to its large size, the YSI sensor was not used for this experiment. For the TriLux sensor, measurements at the high cell density for the 17°C treatment, and for all cell concentrations at 23.5°C, were not undertaken due to a sensor malfunction which could not be repaired in time to repeat the experiments. Differences between treatments were assessed using analysis of variance (ANOVA) and Fisher's post-hoc test.

Relationships between cyanobacterial biovolume and phycocyanin

A 1.3-fold dilution series of each cyanobacterial culture was prepared (8 dilutions) and each of the cell dilutions was measured with each sensor. Sub-samples of each cell dilution for each culture were taken to measure cyanobacterial biovolumes by microscopy. Relationships between the cyanobacterial biovolumes and phycocyanin measurements were determined using linear regression.

Effect of colony size on fluorescence detection

The colony-forming strain of *M. aeruginosa* (CAWBG563) was used to investigate the effect of colony size on phycocyanin fluorescence measurement made using *in situ* sensors. Samples were sieved with different sized meshes (50, 60, 75, 102, 125, 150 and 250 µm) to produce sub-samples (50 mL) of differing colony bin sizes. Each sub-sample was re-suspended in Milli-Q water (150 mL) and measured in a 250 mL beaker using the T927 and TriLux sensors. Following the initial sensor examination, each sample was disaggregated using a manual grinder (10 mL tissue grinder; Wheaton, USA) and measured again with the sensors. Microscopic observation of these samples showed no significant damage to the cells, and cellular disruption/lysis due to the manual disaggregation was deemed to be insignificant. Two-tailed paired t-tests were conducted to compare phycocyanin concentrations between aggregated and disaggregated samples for each colony bin size.

Field validation of sensors

A field validation of the T926, T927 and YSI sensors was undertaken over a 9 h period on 27 March 2015 at Lake Rotorua (Kaikoura, New Zealand; 42°24’05 S, 173°34’57 E). Lake Rotorua is a small (0.55 km^2) and shallow (3 m) eutrophic lake known to be dominated by cyanobacteria (Wood et al. 2012, 2016). Thirty sites were selected based on visual assessment,

to obtain a range of samples of varying cell density (Supplementary Information, Fig. 1). At each site an *in situ* measurement was taken by holding each sensor ca. 1 cm below the water surface. A surface grab sample (500 mL) was collected using a 1-L opaque glass beaker and was immediately measured using the three sensors. These samples were transferred to 500-mL sample containers and taken to shore for processing within 1 h. Surface temperature varied from 17.6 to 18.8°C over the sampling period (data from Wood et al 2016).

On-shore, the samples were processed for phytoplankton enumeration and biovolume assessment by microscopy, phycocyanin measurement by spectrophotometry and extracellular phycocyanin assessment. For the microscopy analysis, a subsample (5 mL) was preserved in Lugol's iodine solution and stored in the dark at ambient temperature. For the spectrophotometry analysis, triplicate subsamples (2-38 mL) were collected on GF/C filters (Whatman, United Kingdom), placed in 1.8 mL tubes and stored on ice whilst in the field and later stored in the dark at −20°C until extraction and spectrophotometric analysis. To assess the level of extracellular phycocyanin, 100 mL of sample was pre-filtered (mesh size 20 µm) then syringe-filtered (GF/C; Whatman) and measured for phycocyanin concentration using the T926 and T927 sensors.

In the laboratory, phycocyanin was extracted in sodium phosphate buffer (1 mL; 50 mM, pH 7). The samples were sonicated (30 min, 60 kHz, on ice) and subjected to three freeze/thaw cycles (−20°C, 2 h) before being clarified by centrifugation (5 min, 17,000 × *g*). The phycocyanin concentrations of the extracts were determined using spectrophotometry (Helios Omega UV-Vis Spectrophotometer; Thermo Scientific, USA) as described in Bennett and Bogorad (1973).

Differences between *in situ* phycocyanin readings and those in the grab sample were tested for using a two-tailed paired t-test. Linear regression was used to assess the relationships between phycocyanin measured using spectrophotometry and the fluorescence sensors, and between cyanobacterial biovolumes and phycocyanin measured using the sensors. Sample 20 (Supplementary Information, Figure 1) was markedly higher than the other values, and was regarded as an outlier which was excluded from the linear regression.

Results

General sensor performance

Using a standard curve of phycocyanin solution, the five sensors were tested for their linear operating ranges and closeness of fit (assessed using the coefficient of determination for the standard curves). The linear operating range of the sensors differed (Table 1). The T926, T927 and Manta sensors (all using Turner probes) yielded a linear response up to the highest phycocyanin concentration tested (12,000 µg/L). The Trilux and YSI sensors did not produce a linear response at phycocyanin concentrations above 1,200 and 2,400 µg/L, respectively (Table 1). However, within their linear operating ranges, all of the sensors yielded very high coefficients of determination ($R^2 > 0.99$).

The T926, T927, Manta and TriLux phycocyanin sensors all exhibited good short-term precision ($RSD_r \leq 4.3\%$), however, the precision of the YSI sensor was low ($RSD_r = 13.1\%$; Table 1). Measures of phycocyanin fluorescence were similar for the two matching Turner sensors (T926 and T927), however the T926 sensor demonstrated lower short-term precision than the T927 sensor. As expected, the variability for all of the sensors was higher when the intermediate precision was assessed ($RSD_R = 9.5\text{-}14\%$; Table 1). The YSI sensor again showed the lowest precision of all sensors ($RSD_R = 14\%$) although the intermediate precision for this sensor was similar to its level of short-term precision.

Temperature effects

A general pattern of sensor response for all three cell densities was observed where, as water temperature increased, phycocyanin measurements decreased, however this result was not always statistically significant (Fig. 1). Measurements of phycocyanin fluorescence from sensors were significantly higher at 4°C ($P < 0.01$) for the three cell concentrations for all sensors except the TriLux (Fig. 1A). Phycocyanin for the medium cell density (Fig. 1B) followed this pattern most clearly, however only the fluorescence at 4°C for the T926 and Manta sensors was significantly different from any other temperature treatment ($P < 0.01$). Measurements for the high and low cell density experiments (Fig. 1A and 1C) increased at 17°C, although this was only statistically significant for T926 ($P < 0.05$). There was no statistically significant difference in fluorescence with change in temperature for the TriLux sensor in the high and low cell density treatments. Measurements from the TriLux at 4°C in the medium cell density treatment were significantly different from those at 13.8°C, and measurements at 17°C were significantly different from those at 13.8°C but not from 4°C (Fig. 1B). Fluorescence at 23.5°C was significantly different from all other temperature treatments in the low cell density experiment for the T926, T927 and Manta sensors ($P < 0.01$, Fig. 1C).

Relationships between cyanobacterial biovolume and measured phycocyanin concentration

Strong relationships were observed between biovolume and the measured phycocyanin concentration using various dilutions for each of the six cyanobacterial strains assessed (Table 2). The single-celled *M. aeruginosa* strains (CAWBG617 and CAWBG624) and the filamentous cyanobacterial strains (*N. spumigena* CAWBG021, *A. gracile* CAWBG595 and *D. lemmermannii* CAW567) generally showed strong relationships ($R^2 \geq 0.83$, $P \leq 0.042$). However, the correlation between *D. lemmermannii* biovolume and phycocyanin using the TriLux sensor was not statistically significant ($R^2 = 0.86$, $P = 0.074$) probably because only four observations were possible. Weaker correlations were observed between the biovolume of colonial *M. aeruginosa* and phycocyanin measured using the various sensors ($R^2 = 0.09$-0.79, $P = 0.003$-0.526). Whilst significant correlations were observed using the Manta and Trilux sensors ($P \leq 0.024$), the relationships observed using the other sensors were weak ($R^2 \leq 0.17$, $P \geq 0.303$).

Of the cultures which demonstrated good relationships between cyanobacterial biovolume and phycocyanin concentration (*M. aeruginosa* CAWBG617, *M. aeruginosa* CAWBG624, *N. spumigena* CAWBG021, *A. gracile* CAWBG595 and *D. lemmermannii* CAWBG567), the slopes of their linear regression equations were relatively consistent between the five sensors (Table 2). The slopes for each of the cyanobacterial cultures varied between the strains assessed, with an average between sensors of 3 µg/mm^3 for *M. aeruginosa* CAWBG6 17 to 19 µg/mm^3 for *N. spumigena* CAWBG021. The y-intercepts of the regression equations were low for two of the strains (*M. aeruginosa* CAWBG617 and *A. gracile* CAWBG595; −4 to 28 µg/L),

but were large for the others (50 to 217 µg/L), suggesting some background interference in these cultures.

Effect of colony size on fluorescence detection

To assess whether fluorescence was affected by colony size in *M. aeruginosa* (CAWBG563), the culture was divided into different colony sizes classes and *in situ* phycocyanin fluorescence was measured before and after manual disaggregation of the colonies. The >150 to <250 µm size class contained the greatest number of colonies (thus much higher phycocyanin values shown in Fig. 2). No statistically significant differences were observed within each colony size class ($P > 0.05$, t-test) for T926 and TriLux sensors (Fig. 2). For both sensors the standard errors were markedly higher for the aggregated measurements in the > 250 µm size class (Fig. 2).

Field validation of sensors

At the time of sampling of the field site of Lake Rotorua, Kaikoura, *Dolichospermum* sp. was the dominant species, accounting for more than 90% of the total cyanobacterial biovolume (Supplementary Fig. 2). No significant differences were evident between *in situ* phycocyanin measurements and those measured in grab samples ($P > 0.05$, t-test). Furthermore, sensor measurements from the grab samples were strongly linearly related to phycocyanin concentrations determined by spectrophotometry for each sensor ($R^2 \geq 0.7$, $P < 0.001$; Fig. 3A). The slope for each sensor was $\leq 0.66x$, indicating that the sensors under-estimated the phycocyanin concentration compared with measurements made by spectrometry. The YSI sensor measurements were closer to the spectrophotometry values than the two Turner sensors (T926 = $0.48x$ and T927 = $0.42x$). The y-intercepts for each regression equation were ca. 200 µg/L, which indicated that a similar level of background noise was evident for each of the

three sensors. The level of extracellular phycocyanin present in the grab samples ranged from 3-34% of the total phycocyanin, with an average of 8% (Supplementary Table 1). This may account for some of the background observed in the sensor measurements as the extracellular phycocyanin would not have been assessed in the spectrophotometer measurements where filter-bound samples were extracted.

Significant relationships were observed between phycocyanin measurements and total cyanobacterial biovolume of the grab samples for the two Turner sensors ($P < 0.001$), but not the YSI ($P = 0.4$; Fig. 3B). The strength of the relationship was reasonably consistent, but weak for each sensor ($R^2 = 0.22$ to 0.29, Fig. 3B). Additionally, the phycocyanin reading per unit of cyanobacterial biovolume was not consistent between the three sensors. When total phytoplankton (as opposed to just cyanobacteria) biovolumes were compared to the phycocyanin sensor measurements (data not shown), the relationship was weaker for each sensor ($R^2 = 0.12$ for T926, $R^2 = 0.10$ for T927, $R^2 = 0.13$ for YSI) indicating that cyanobacterial biovolume is a better predictor of phycocyanin concentration in the water samples).

Discussion

In this study five *in situ* phycocyanin fluorescence sensors produced by three different manufacturers were assessed for their performance in estimating cyanobacterial biomass. Of the five sensors the three Turner sensors (T926, T927 and Manta) had the highest operating range, producing linear responses above 12,000 µg/L. The upper limits of the Trilux and YSI sensors were lower (ca. 1,200 and 2,400 µg/L respectively) and may not be suitable at very high concentrations of phycocyanin corresponding to intense cyanobacterial blooms. Calibration of the two matching Turner sensors using a phycocyanin standard gave a signal

response difference for the two sensors of 17%. This reinforces previous suggestions that sensors require calibration prior to deployment (and preferably after also) to ensure accurate phycocyanin measurements (Bastien et al. 2011).

The short-term precision for four of the sensors was good ($RSD_r \leq 4.3\%$) but the YSI sensor showed higher variability ($RSD_r = 13.1\%$). In addition to differences in short-term precision, there were also notable differences in the phycocyanin fluorescence (with the TriLux and YSI sensors generally estimating higher concentrations). The intermediate precision for all of the sensors varied (9.5-14% RSD_R) over a seven-day period of assessment. If better precision was desired, daily calibration of the sensors might increase the repeatability of measurements, however, this would be unlikely to be practical for sensors installed on monitoring buoys/stations.

A general trend of decreasing phycocyanin fluorescence was observed with increasing temperature during the present study. The fluorescence of protein pigments including phycocyanin can be affected by changes in temperature; as temperature increases, fluorescence decreases (Turner Designs, 2015). This loss of fluorescence is due to an increase in molecular motion resulting in more collisions and a loss of energy (Guibalt 1990). In temperate lakes, particularly those in inland continental areas of the Northern Hemisphere, temperature can decrease to < 4°C, in winter. Under this scenario, phycocyanin sensors might overestimate cyanobacterial biomass in the colder months or underestimate biomass in the warmer months, unless compensations in the sensor calibration are made. Ideally standards, blanks and samples should be measured at the same temperature to ensure accurate results across a study. While this is possible in a laboratory setting and potentially with grab samples in the field being transported to controlled temperature in the laboratory, it would be challenging when sensors

are deployed *in situ*. Some manufacturers provide algorithms to account for the influence of temperature for chlorophyll-*a* fluorometers. To our knowledge, no such algorithms exist for phycocyanin sensors and more data is required before these could be developed. In this study we only investigated the response of *Microcystis* to temperature. Although we anticipate similar responses among other species, further studies should be undertaken to confirm this.

The relationships between cyanobacterial biovolume and phycocyanin florescence were significant for the unicellular and filamentous cyanobacterial strains assessed during the present study. This is consistent with previous research that has shown strong linear relationships between phycocyanin measurements from single-celled and filamentous cyanobacterial cultures and total biovolumes (e.g., Bastien et al. 2011, Brient et al 2008). However, correlations between biovolume and phycocyanin fluorescence were poor for the colonial *Microcystis* for three of the five sensors. Chang et al. (2012) suggested that light would not penetrate the inner part of colonies beyond 13-18 µm. To assess whether this was the cause for the poor relationship for colonial *Microcystis*, colonies were categorized by size class bins and were disaggregated. There was no significant difference in phycocyanin fluorescence between aggregated and disaggregated colonies, suggesting that limited light penetration to inner cells in colonies was not the cause for the weak correlations. During the experiments we observed that when large colonial aggregates drifted past the light source, fluorescence spiked, suggesting poor sample heterogeneity was the main cause of the weak relationship. This is particular apparent for the large (>250 µm) size class, where the standard deviations of aggregated samples were markedly highly than disaggregated samples (Fig. 2). Potential background interference was observed in some cultures (demonstrated by large y- intercepts). Although all cultures were healthy and in their exponential growth phase this could have been

due to some cell lysis (particularly in the more concentrated samples) and therefore the detection of extra-cellular phycocyanin.

The accuracy of phycocyanin sensors in the field was assessed by comparing the sensor phycocyanin measurements in the field to phycocyanin measurements obtained by spectrophotometry on filter-extracted subsamples. Consistent with previous studies (Ahn et al. 2007, Zamyadi et al. 2012), a strong relationship was observed between the spectrophotometer measurements and the *in situ* phycocyanin fluorescence measurements made in the field. Whilst there was a strong correlation between the two methods of analysis, the relationship was not equal with the sensors under-estimating phycocyanin content in the field samples. The phycocyanin fluorescence measurements underestimated the phycocyanin concentration and displayed background interference. The accuracy of the measurements can be influenced by factors such as turbidity and the presence of other algae (Zamyadi et al. 2011, Zamyadi et al. 2016). As the spectrophotometry measurements were performed on extracted samples and not *in situ*, they were less prone to these interferences.

Field measurements from the phycocyanin sensors in the present study were only weakly correlated with cyanobacterial biovolume, which is in contrast to many previous studies that have shown strong relationships (Leboulanger et al. 2002, Brient et al. 2008, Bastien et al., 2011, Kong et al. 2014, Zamyadi et al 2012). Reasons for this weak relationship are unclear. The presence of phycocyanin in solution as a result of cell lysis is a possible cause (Bastien et al. 2011). Phycocyanin sensors do not differentiate between intra- and extra-cellular phycocyanin (Brient et al. 2008). In the present study, the average extracellular phycocyanin across 30 sites was 8%, however at some sites where the total biovolume was higher (17-27 mm^3/L), extracellular phycocyanin accounted for > 20% of the phycocyanin measured. Natural

lysis of cells occurs due to the breakdown of the cell wall as a result of high light intensity (Fallon and Brock 1970), attrition of cyanobacterial cells, and contact and parasitism with other phytoplankton (Gumbo and Cloete 2011). Bastien et al. (2011) demonstrated that, on average, 21-25% of the measured fluorescence signal from phycocyanin sensors was related to extracellular phycocyanin. A field blank can be obtained from filtered samples and this reading subtracted from the total to determine cellular phycocyanin (Bastien et al. 2011). However, this scenario is not feasible if sensors are deployed on monitoring buoys/stations.

The weak relationship between phycocyanin and biovolume in the present study may also be caused by interference from other algal groups which were also present in high abundance in Lake Rotorua (7.6 - 45.8 mm^3/L). Beutler et al. (2002) demonstrated that light emitted from different algal groups overlaps with the phycocyanin signature from cyanobacteria and that when present in high densities can reduce the accuracy of phycocyanin readings. The cyanobacterial assemblage in Lake Rotorua was dominated by one *Dolichospermum* (> 90%) which grows in relatively tight coiled filaments. Sample heterogeneity, i.e., whether a filament passes under the light source when a reading is being taken, or penetration of the light source into the filament are further possibilities for the weak phycocyanin to biovolume relationship and require further investigation.

In summary, our study supports the finding of others (e.g., Brient et al. 2008, Bastien et al. 2011, Bowling et al. 2012, Zamyadi et al 2012), that *in situ* phycocyanin fluorometry has the potential to be a useful tool for providing cost-effective, high-frequency estimates of cyanobacterial biovolume. However, there are caveats, and in the present study we showed that

temperature and cyanobacterial morphology significantly affect the relationship between cyanobacterial biovolumes and *in situ* phycocyanin fluorescence. Such differences will likely vary spatially and temporally, and local and regular determination of the relationship between microscopically determined biovolumes and *in situ* phycocyanin fluorescence is required. We also demonstrated differences between sensors from the same and different manufacturers. Based on these data we recommend calibration prior to (and preferably following) deployment, and encourage users to test the working limits of their sensors using cultures or samples containing species present in the systems they plan to monitor. *In situ* fluorometry does not provide any information on which species are present, nor the presence of cyanotoxins (Izydorczyk et al. 2005). Where this information is needed, sampling and laboratory-based analysis is required to complement the *in situ* data. Although previous studies have demonstrated a wide applicability to a range of cyanobacterial species there is an ongoing need to evaluate the sensors over a wider range of water bodies with varying proportions and densities of species, and across varying periods of time when physicochemical conditions vary.

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Table 1 General performance of five phycocyanin sensors which were assessed for their upper range, closeness of fit (R^2), short-term precision (RSD_r, over 1 h) and intermediate precision (RSD_R, over 7 days)

Table 2 Linear regression results assessing the relationships between cyanobacterial biovolume (mm^3/L) and phycocyanin measurements (µg/L) from five sensors. Significant relationships are bolded ($P \leq 0.05$)

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Fig. 1 Average phycocyanin fluorescence based on three consecutive measurements at; (A) high, (B) medium, and (C) low cell densities for sensors T926, T927, Manta and TriLux across four temperatures. Letters indicate where significant differences ($P < 0.05$) occur between treatments (one way ANOVA with Fishers post-hoc test)

Fig. 2 Average phycocyanin fluorescence (n=3) from (A) T926, and (B) TriLux sensors, for different colony sizes in aggregated and dis-aggregated samples. Error bars represent one standard deviation

Fig. 3 Relationships between phycocyanin measurements from 29 grab samples Lake Rotorua (Kaikoura) using the T926, T927 and YSI sensors (left to right), and spectrophotometry after phycocyanin extraction by filtration (A), and total cyanobacterial biovolumes (B)