# A validation study of phycocyanin sensors for monitoring cyanobacteria in cultures and field samples

A thesis submitted in partial fulfilment

of the requirements for the degree

of

**Masters of Science (Research) in Biological Sciences**

at

**The University of Waikato**

by

**Caroline Hodges**

---

The University of Waikato
2016


THE UNIVERSITY OF
WAIKATO
*Te Whare Wānanga o Waikato*

# Abstract

Cyanobacteria are photosynthetic prokaryotes present in almost all terrestrial and aquatic environments. Under favourable conditions of high nutrient inputs, high water temperature and adequate light, they can rapidly multiply and form blooms. Cyanobacterial blooms are harmful to aquatic processes as they reduce the amount of irradiance within the water column and some have the ability to produce toxins which pose a risk to human and animal health. Cyanobacterial blooms are becoming prevalent worldwide as a result of increased anthropogenic influences. To enable accurate risk assessment of drinking water reservoirs, water bodies for recreational use and to assist in the understanding of bloom dynamics, cyanobacterial biomass must be quantified. Traditionally this was done via grab sampling, the use of microscopy and measurements of chlorophyll-*a* concentration using spectrophotometry. These methods do not allow samples to be collected and analysed at a frequency that provides meaningful spatial and temporal resolution. Additionally, they can be time consuming, expensive and require taxonomic expertise.

Measurements of chlorophyll-*a* concentration can provide an estimate of overall phytoplankton biomass but cannot differentiate between eukaryotic cells and cyanobacteria. However, the fluorescent pigment phycocyanin is specific to cyanobacteria and has a unique fluorescence signature which can enable the estimation of cyanobacteria biomass. Methods to detect phycocyanin fluorescence have been integrated into handheld sensors which can be used as a proxy for estimation of cyanobacterial biomass *in situ*. However, robust validation of these sensors is rarely undertaken. Previous use of fluorescence sensors suggests that there may be multiple sources of interference of the optical signal. The aim of this study was to assess the performance of commercially-available phycocyanin sensors using a combination of culture-based laboratory experiments and a field investigation.

The laboratory studies showed strong linear relationships between phycocyanin and chlorophyll-*a* fluorescence from sensors and cyanobacterial biovolume obtained by microscopy. This was observed across a range of cell concentrations for solitary

(e.g. *Microcystis* sp.) and filamentous species (e.g. *Aphanizomenon* sp.). However this linear relationship was not observed with colonial and some filamentous cyanobacteria (*Microcystis* sp., *Nodularia spumigena*, and *Dolichospermum* sp.). Further investigation suggested that the morphology of densely aggregated species inhibited the penetration of light into the colony, resulting in an underestimation of phycocyanin. These results highlight a potential limitation for the use of phycocyanin sensors *in situ* as colonial and filamentous cyanobacteria are often observed. Other potential limitations for the use of phycocyanin sensors include natural variations in light intensity which can cause photobleaching; reducing fluorescence, as well as increased variability of phycocyanin fluorescence with changes in temperature.

Field experiments during this study showed that extracellular phycocyanin from lysed cells could in some instances account for more than 20% of the total phycocyanin fluorescence. The field study also showed a strong linear relationship between measurements of phycocyanin fluorescence and cyanobacterial biovolume. However measurements of chlorophyll-*a* were poorly correlated to cyanobacterial biovolume. The results from the field experiments indicate that phycocyanin sensors may be a valuable tool to monitor cyanobacteria biomass in the field. Collectively, this data highlights the potential for the use of phycocyanin sensors to obtain high frequency data on cyanobacterial biomass, while also demonstrating limitations for their use *in situ*. Further study is needed to determine more definitively the effects of these potential limitations on phycocyanin measurements and which supplementary parameters could be measured in order for the data collected to be more robust and informative.

# Acknowledgements

I wish to thank my supervisors. Susie Wood, who provided countless opportunities to me throughout the years. Thank you for your guidance, support and ideas. It has been a privilege to be your student. To David Hamilton, thank you for sharing your time and knowledge with me. What I have learnt from this project and others that you have been kind enough to help me with has been invaluable. Your critiques and meticulous editing are much appreciated.

I would like to extend my warmest thanks to the Cawthron community who offered an incredible working environment and especially to Jonathan Puddick for never being too busy to help, console, give advice, fix my equations and graphs and just being an all-round amazing person. Many thanks to the team of students that I've had the privilege of getting to know for their help in the field; Hugo, Oonagh, Emily and Konstanze.

Thank you to the staff at the University of Waikato for helping me find my way around when I needed to do last minute experiments and to the organisations who have helped fund my study; Marsden Fund and the University of Waikato Masters Research Scholarship.

Thank you Kyle for your support and encouragement and the daily reminders that I should be writing my thesis. To my wonderful parents who have been critical to the completion of this undertaking; thank you for your support and encouragement, even if you didn't quite understand why it was taking me so long to write this thesis.

# Table of Contents

# List of Figures

# List of Tables

# 1 Introduction

Cyanobacteria are photosynthetic prokaryotes which are abundant worldwide. Cyanobacteria have a long lineage dating back 3.5 billion years and as such, are present in almost every terrestrial and aquatic habitat, including geothermal areas and polar regions [1]. There are several features of cyanobacteria that have enabled them to out-compete other organisms in a multitude of environments. Including tolerance to desiccation and high levels of ultra-violet irradiation, utilization of light for photosynthesis at low photon flux densities and the ability to fix nitrogen [1]. Cyanobacteria are an integral component of most aquatic ecosystems. However, under favourable conditions such as high inputs of nitrogen, phosphorus, organic compounds and increased temperature, cyanobacteria cells can multiply rapidly and form dense blooms [2; 3]. Increases in the frequency of cyanobacterial blooms have been attributed to anthropogenic factors such as land clearance and runoff of nutrients [4], as well as increases in water temperature [5].

Some bloom forming species have gas vacuoles which allow buoyancy in response to changes in environmental conditions and thus a competitive advantage [1]. For example, when a lake stratifies and the mixing ability is insufficient to keep phytoplankton in suspension, buoyant species are able to migrate to the epilimnion where there is access to light. While non-buoyant species remain in the hypolimnion where there is less light and nutrients available [6]. Blooms disrupt ecological processes by encouraging a shift in phytoplankton community composition which can alter the energy flow paths and nutrients as well as trophic functionality of the aquatic ecosystem [7]. Irradiance is reduced in the water column, meaning growth of other primary producers which are typically attached (epiphyton and benthic algae) are reduced due to lack of sunlight [8]. Oxygen in the water column is depleted and toxic by-products such as ammonia are often released, which leads to fish kills and mortality for macroinvertebrates and other aquatic organisms [8; 9].

Some cyanobacteria species are capable of producing toxins, which are of risk to human health [10]. The cyclic peptide toxins microcystin and nodularin are regarded as the most common globally [11; 12]. These pose risks to water bodies used for drinking water or recreational activities, as they are known to cause liver

disease [11]. The worst reported incident occurred in Brazil where 52 people died due to intravenous treatment of microcystin contaminated water [13]. Deaths in animals have also been recorded [14-20]. Adverse health consequences for humans coming into contact with cyanobacteria blooms via bathing include, skin irritation [21] and increased risk of gastrointestinal symptoms [22].

In order to protect public health, improve knowledge related to cyanobacteria issues and improve water quality, cyanobacterial blooms must be identified, enumerated and analyzed for cyanotoxins [23]. Traditionally, cyanobacterial blooms were monitored using microscopy methods to identify species and determine cell concentrations and biovolume. Estimates of chlorophyll-*a* concentration are also commonly used as proxy for overall phytoplankton biomass [24]. These methods are time consuming, labour intensive and require specialist skills [25; 26]. The lengthy analysis process limits the number of samples that can be collected and analysed, reducing the spatial and temporal resolution of results. Slow processing also creates challenges when using these methodologies to inform management decisions as changes can occur over short time periods [23; 27]. Chlorophyll-*a* is also not a reliable indicator of cyanobacteria as it does not differentiate the relative pigment concentration from eukaryotic algae and cyanobacteria [28]. Phycocyanin is present in predominantly cyanobacteria and therefore presents a more accurate measure of cyanobacteria biomass [23; 25; 29].

## 1.1 Phycocyanin

Phycocyanin is a highly fluorescent, water soluble pigment belonging to the group of phycobiliproteins from blue-green and red algae. It is contained in the phycobilisomes which harvest light and transfer energy to chlorophyll for use in photosynthesis. Phycocyanin is present in cyanobacteria and cryptophyceae and rhodophyceae in smaller amounts [23; 27]. The characteristic fluorescence signature of phycocyanin is absorption of red and orange light between 610 to 630 nm with a maximum absorption peak of 620 nm, and emission of light between 600 to 700 nm with a maximum fluorescence at 647 nm [23]. This fluorescence signature makes it possible to specifically detect cyanobacteria within a mixed phytoplankton assemblage and estimate their biomass [25]. Measurement of phycocyanin fluorescence is a selective method based on measurement of photon

emissions by the pigment. Until recently, water resource managers lacked adequate monitoring tools capable of providing information about the spatial distribution and composition of blooms. Field probes or sensors and remote sensing tools measuring *in vivo* fluorescence of phycocyanin, represent an effective way to assess cyanobacteria biomass, especially in a changing environment where toxic blooms may be highly transient [30]. Measurement of phycocyanin fluorescence also offers the opportunity for use as a proxy to decide whether or not a sample should be taken for identification, i.e., if the measurements are close to prescribed management or health thresholds [23].

Phycocyanin sensors can be used to estimate cyanobacteria biomass as strong linear correlations have been observed between phycocyanin fluorescence and cell numbers for a number of different cyanobacteria species [23; 26; 31; 32]. For example, Brient et al. [31] observed strong correlations between phycocyanin fluorescence measured from the TriOS microFlu-blue sensor (TriOS Optical Sensor, Germany) and the cell concentration of *Planktothrix agardhii* ($R^2$ =0.9) and *Lemmermanniella* sp. ($R^2$ =0.9). Kong et al. [26] also showed strong linear relationships between phycocyanin fluorescence and cell concentration ($R^2$ =0.9), as well as cell biovolume ($R^2$ =0.9) for a number of species; *Microcystis aeruginosa*, *Cylindrospermopsis raciboskii*, *Pseudanabaena* sp. and *Dolichospermum* sp. While there appears to be a strong linear relationship between phycocyanin fluorescence and cell concentration for cultured species, cyanobacteria can vary in size by several orders of magnitude both within and across taxa. Therefore algal density may provide little information about the actual biomass [33]. Zamyadi et al. [25] suggests that the correlation between fluorescence readings and cell counts is almost meaningless and that correlation with biovolume is more suitable for management purposes.

## 1.2 *In vivo* fluorescence

*In vivo* fluorescence is based on the theory of Stokes fluorescence; the molecular absorption of light energy at one wavelength and the re-emission at another wavelength, which is often longer [34]. These two wavelengths are commonly referred to as a fluorescence signature and make up a fluorescence compound. As few compounds have the same fluorescence signature, *in vivo* fluorescence is

regarded as a highly specific analytical technique [34]. Measurements of phytoplankton biomass using light emitting diodes (LED) and spectral signature differentiation algorithms are used to measure photosynthetic pigment fluorescence. The excited pigments transfer photons to the chlorophyll-*a* terminal receptor which emitting a wavelength specific fluorescence which can then be measured [25]. *In vivo* fluorescence has been used to monitor phytoplankton in oceanography and limnology for a number of years [35]. It allows direct measurement of the fluorescence emission from photosynthetic cells [28] using the differences in pigmentation to discriminate phytoplankton taxonomic groups. Key advantages of using fluorescence are that it is very sensitive with limits of detection in the parts per billion (ppb) range for most analytes, meaning that even low sample concentrations can be detected. Fluorescence measures both emitted and absorbed light, as opposed to spectrophotometers which measure absorbed light only and are prone to interference as many materials can absorb light [36]. *In vivo* fluorescence is a simple analytical technique which does not necessarily require any sample preparation, reducing cost and saving time.

More recently the use of *in vivo* fluorescence has been integrated into handheld devices [28] to allow extension to the measurement of phycocyanin, a common photosynthetic pigment specific to cyanobacteria [27]. While *in vivo* fluorescence can successfully distinguish between phytoplankton groups, it is not able to distinguish between genera or species, therefore taxonomic or genomic analysis may be required to determine whether cyanobacteria taxa present have the ability to produce toxins [31]. Similarly, *in vivo* fluorescence gives no indication of the concentration of toxins present. Izydorczyk et al. [37] attempted to apply phycocyanin fluorescence to estimate microcystin concentrations and found a moderate correlation ($R^2$ =0.51), however it was lower than the correlation observed between cyanobacteria biomass and microcystin concentration ($R^2$ =0.74). Bastien et al. [23] also determined no relationship between the concentrations of cyanotoxins and cell density ($R^2$ =0.2) or total cyanobacterial biovolume ($R^2$ =0.2). The concentration of microcystins per cyanobacterial cell is not constant and depends on a range of parameters such as temperature and availability of nutrients, therefore *in vivo* fluorescence cannot estimate toxin concentration [37].

Other limitations of the use of *in vivo* fluorescence include variability of measurements under differing light regimes [25; 31] and at differing depths above the sediment boundary (boundary effects) [26; 31; 36], variability of measurements as a result of changes in temperature [33; 34; 38], the influence of co-existing cyanobacteria [25; 26], variability of measurements when species with complex morphology such as filaments and colonies are present [28; 39] and the influence of extracellular phycocyanin [23]. Other limitations (not discussed in this study) which are of importance, include the influence of chlorophyll-*a* and turbidity on measurements on phycocyanin fluorescence. The presence of other phytoplankton species containing chlorophyll-*a* is thought be a source of interference to phycocyanin fluorescence measurements. For example, Brient et al. [31] showed that the presence of populations other than cyanobacteria at high densities can produce a phycocyanin fluorescence signal between 0 to 10 µg/L. Gregor et al. [40] also observed that 'false positives' may occur when high densities of eukaryotic species are present. This is thought to be due to similar emission wavelengths for phycocyanin and chlorophyll-*a* (640-680 nm, and 660-680 nm respectively) which can cause an overlap in measurements [28].

Studies have also shown that the presence of turbidity can result in a loss in phycocyanin fluorescence signal proportional to the number of particles within the water column and also their grain size [31; 41]. This is particularly important when taking measurements from vertical profiling as a reduction in signal may be attributed to the absorption of particles as opposed to a reduction in cyanobacteria biomass. It is recommended that phycocyanin, chlorophyll-*a*, and turbidity measurements be taken simultaneously to avoid false readings [40].

## 1.3 Influence of light on measurements of fluorescence

Many cyanobacteria are adapted to low irradiance and can be impaired by light intensity which exceeds this threshold. Photosynthetic pigments such as phycocyanin and chlorophyll-*a* are thought to be affected by prolonged exposure to irradiance as the efficiency of phycocyanin to transfer energy to the photosynthetic reaction centre is decreased and as a result, energy is wasted in the form of fluorescence [42]. In several studies, the exposure of cyanobacteria to natural light has been shown to have no significant effect on the fluorescence signal [25; 31].

However under artificial light, Brient et al. [31] demonstrated that the fluorescence response at low light levels (50 µmol $m^{-2}$ $s^{-1}$) was nearly three times less than the response at high light levels (150 µmol $m^{-2}$ $s^{-1}$).

Some studies suggest that prior light exposure may result in a reduction in fluorescence measurements [42; 43]. Although several laboratory studies have not been able to validate this phenomenon. Zamyadi et al. [25] showed that prior light exposure up to 7 h had no effect on cyanobacterial fluorescence signatures and *in vivo* fluorescence measurements. These results were supported by previous work from Brient et al. [31]. The effects of light are likely to be more prominent in field samples than laboratory experiments as laboratory cultures are usually kept under controlled light conditions and the natural light field can be highly variable.

## 1.4 Boundary effects

The fluorescence signal in the water column is a function of the amount of pigments present within the first few centimeters below the sensor, as well as the distance of the sensor to the recipient boundary [31]. Some phycocyanin manufacturers suggest that the sensors should be held at least 7 cm from the recipient boundary [36]. This is supported by studies from Kong et al. [26] who observed a strong decrease in fluorescence signal under agitation, 7 cm from the boundary during laboratory experiments. Similarly, Brient et al. [31] observed a decrease in fluorescence signal below 5 cm from the recipient boundary.

## 1.5 Influences of temperature on fluorescence

The relative fluorescence of protein pigments can be affected by changes in temperature; as temperature increases, fluorescence decreases [34]. The loss of fluorescence with temperature increase is due to increased molecular motion resulting in higher collision rates and a loss of energy [44]. Increased temperatures are also thought to degrade phycobilin pigments, resulting in a reduction of fluorescence [38]. Manufacturer guidelines stress the importance of measuring blanks and standards alongside samples to be able to account for the changes in fluorescence. Correction equations have also been suggested as a means to adjust for the changes in fluorescence, especially in a field environment where the use of blanks and standards may not be feasible. Some sensors are currently on the market

which automatically correct for changes in temperature, which may be useful when deploying sensors on monitoring buoys [34].

## 1.6 Relationship between fluorescence and species biovolume

Strong linear relationships have been observed throughout many studies when comparing phycocyanin and chlorophyll-*a* fluorescence measurements from a sensor to the biovolume of a single cultured cyanobacterial species in a laboratory setting, as previously highlighted [26; 31]. This is because the amount of phycocyanin or chlorophyll-*a* is proportional to the volume of the cell [31]. Therefore if only one species is present, the relationship should be linear. However, in the field more than one species will usually be present, and in varying quantities. Cyanobacteria can vary in size by several orders of magnitude across taxa, thus when multiple species are present it may be hard to determine the actual biomass from the fluorescence measurement if the species composition is unknown. Studies have tried to demonstrate what the effect on fluorescence might be when multiple species are present in varying biovolume proportions. For example, Kong et al. [26] demonstrated that when two cultures of cyanobacteria; *Microcystis* sp. and *Dolichospermum* sp., were mixed at different ratios based on biovolume, phycocyanin measurements and cyanobacterial biovolume were still strongly correlated ($R^2 = 0.99$). However this was not representative of samples collected in the field as only two species are present in this experimental study.

Some cyanobacterial species form colonial aggregates, while filamentous species can become entangled forming complex dense assemblages. Dense aggregations of cells can prohibit the penetration of light into the colony or assemblage, as the cells on the outer perimeter absorb the light, leaving little to penetrate through to the inner portions which can lead to an underestimation of biomass [28]. Chang et al. [28] demonstrated that the morphology of colonial cyanobacteria may be causing an underestimation. They did this by measuring phycocyanin fluorescence prior to and following colony disaggregation. The results showed that greater increases in phycocyanin measurements were observed following disaggregation of larger colonies compared to the smaller colony sizes. To date, there has been little research into the effects of colonial cyanobacteria on fluorescence measurements, although Chang et al. [28] developed a model to account for the effect of aggregation on

measurements of cell number, but not biovolume. They acknowledged that the model developed for spherical colonies such as *Microcystis*, should be modified for other cyanobacteria morphologies such as mats or filaments. They also indicated that more rigorous testing is needed to confirm how applicable these modifications would be to the accuracy of the model.

## 1.7 Field validation

Physical, chemical and biological conditions in the laboratory are very different to those in the natural environment. Lower correlations between cyanobacteria biovolume in environmental samples compared to cultures, and the difference in phycocyanin concentration per unit cell number, suggest that factors other than those considered in the culture-based studies have an influence on measurements of phycocyanin fluorescence [26]. While phycocyanin sensors provide a rapid means of detecting cyanobacterial biomass, their accuracy in environmental conditions due to the disadvantages listed above, is questioned. This poses concern for their use as a management tool to trigger health advisory warnings and interventions [41].

Studies have shown correlations between cyanobacterial biovolume and field measurements of *in vivo* fluorescence are relatively strong. For example, Kong et al. [26] showed a strong linear relationship ($R^2$ =0.8), as did Brient et al. [31] ($R^2$ =0.7) and Bastien et al. [23] ($R^2$ =0.7). Kong et al. [26] also investigated the correlations between biovolume and phycocyanin fluorescence before and during an algal bloom. The results showed that prior to a bloom, when phytoplankton biomass was low, a strong correlation was observed ($R^2$ = 0.9). However when a change in species composition and structure indicative of bloom formation occurred, the correlation coefficient decreased ($R^2$ =0.8). Further validation of phycocyanin sensors is needed across a wider range of water bodies with varying species composition to determine more conclusively the response of sensors to changing phytoplankton assemblages.

## 1.8 Influences of extracellular phycocyanin

In some cases, phycocyanin fluorescence values from field samples are not correlated with the number of cells or cyanobacteria biovolume. It is thought that

this could be due to a large biomass of picoplankton which is not always able to be identified by microscopy, or else the presence of phycocyanin in solution from lysed cells [31]. Studies have shown that 15 to 21%, and in some cases up to 100%, of the total fluorescence signal can be related to extracellular phycocyanin most likely from lysed cells [23; 31]. This raises concern regarding the expression of results, as extracellular phycocyanin will be included in the overall fluorescence measurement to approximate cyanobacteria biomass. It has been suggested that to avoid an overestimation of phycocyanin concentration, measurement of a field blank from filtrated samples should be subtracted from the total measurement to gain an estimate of the level of cellular phycocyanin [23].

## 1.9 Research aims

The aim of this thesis was to undertake a validation study to evaluate the performance of different phycocyanin and chlorophyll-*a* sensors commonly used in the field. The outcomes of this study are intended to provide knowledge about sensor limitations, and how these limitations might impact the use of fluorescence sensors in the field as a tool in a management setting. The main objectives were to:

(1) Evaluate the performance of five commercially available phycocyanin and chlorophyll-*a* sensors in controlled laboratory conditions using single celled, filamentous and colonial species as single-species cultures and mixed cultures.

(2) Investigate sources of interference such as light, boundary effects, temperature, presence of colonial species and mixed community composition.

(3) Evaluate the performance of commonly used phycocyanin and chlorophyll-*a* fluorescence sensors in the field, where the phytoplankton composition consists of multiple cyanobacterial species and green algae.

# 2 Methodology

## 2.1 Description of phycocyanin and chlorophyll-*a* sensors tested

Five different models of phycocyanin and chlorophyll-*a* sensors were assessed in this study (Table 1). The Turner CYCLOPS-7 submersible fluorometer (Turner Designs, Sunnyvale, CA, USA) is a single channel detector and can be used to measure a variety of parameters including phycocyanin and chlorophyll-*a*. It uses a photodiode for detection of fluorescence. In this study, the two phycocyanin sensors from Turner are referred to as Turner 926 (T926) and Turner 927 (T927). Similarly, the chlorophyll-*a* sensor from Turner is referred to as Turner 928 (T928). The Manta II (Eureka, Austin, TX, USA) is a multi-probe which uses Turner sensors. The Seapoint fluorometer (Seapoint Sensors Inc. Kingston, NH, USA) is a low-power instrument which measures the red fluorescence of chlorophyll-*a* by excitation with blue light. The YSI EXO Sonde (YSI Inc., Yellow springs, OH, USA) uses a total algae dual-channel fluorescence sensor which generates two independent data sets; one from orange excitation for phycocyanin, and one from blue excitation for chlorophyll-*a* which are both detected at 685 nm. The TriLux (Chelsea Technologies Group Ltd, Surrey, England) is a multi-wave fluorometer which comes with a chlorophyll-*a* channel as a standard, and the option of turbidity, phycoerythrin and phycocyanin.

During this study only the phycocyanin and chlorophyll-*a* sensors were assessed, all sensors were handheld and each output was recorded from a handheld device or a laptop. Electronic outputs from the sensors were normalised to relevant units (µg phycocyanin/L or µg chlorophyll-*a*/L). All sensors were used to measure parameters in laboratory conditions. The YSI, Turner and Seapoint sensors were also assessed in the field.

**Table 1. Description and manufacturer specification of *in vivo* fluorescence sensors used in this study. Excitation and emission wavelength (nano metres; nm), standard error (SE), and range (cell/mL or µg/L).**

| Sensor | Excitation Wavelength (nm) | Emission Wavelength (nm) | Range (as specified by the manufacturer) |
|---|---|---|---|
| **Turner CYCLOPS – 7** | | | |
| Phycocyanin | 595 | 300-1,100 | 0-2,000,000 cell/mL |
| Chlorophyll-*a* | 460 | | 0-500 µg/L |
| **Eureka Manta II** | | | |
| Phycocyanin | 595 | 300-1,100 | 150-150,000 cell/mL |
| Chlorophyll-*a* | 460 | | 0.03-500 µg/L |
| **Seapoint** | 470 | 685 | 0.02-150 µg/L |
| **YSI EXO Sonde** | | | |
| Phycocyanin | 590 ± 15 | 685 ± 20 | 0-100 µg/L |
| Chlorophyll-*a* | 470 ± 15 | | 1-400 µg/L |
| **Chelsea TriLux** | | | |
| Phycocyanin | 610 | 685 | 0-100 µg/L |
| Chlorophyll-*a* | 470 | | |

## 2.2 Cyanobacteria Cultures

Cyanobacteria cultures were sourced from the Cawthron Institute Culture Collection of Micro-algae (CICCM; www.cultures.cawthron.org.nz, Figure 1). These species were selected to represent a range of morphologies, and single versus colonial strains. The filamentous cultures were; *Aphanizomenon* sp. (CAWBG595), *Dolichospermum* sp. (CAWBG567), *Nodularia spumigena* (CAWBG21), the unicellular species were *Microcystis* sp. (CAWRotoA, CAWBG617), and one colonial species was assessed, *Microcystis* sp. (CAWBG563). Cultures were maintained in a growth medium (MLA) [45] under a light regime of 92 µmol $s^{-1}$ $m^{-2}$ with a 12:12 light: dark cycle, at a temperature of 17˚C ± 1.

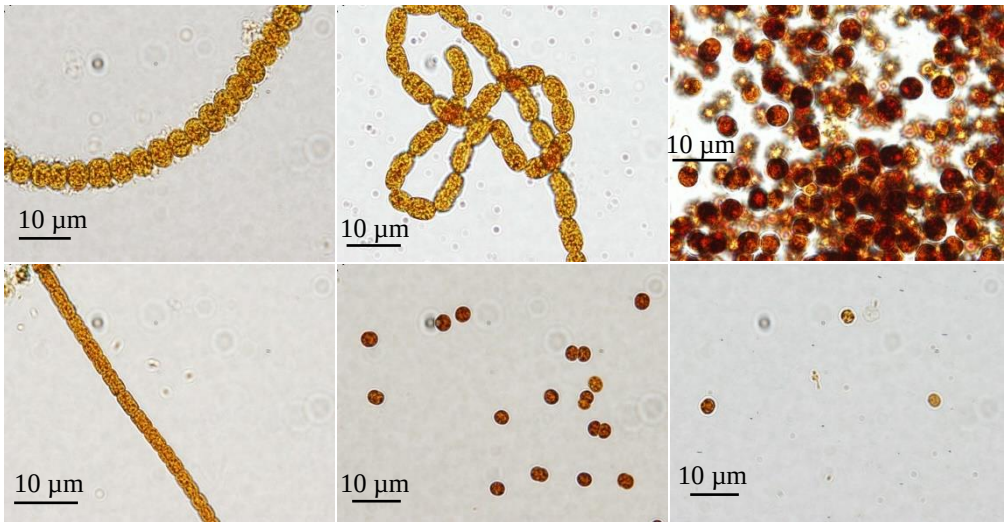



**Figure 1. Microscopic images of the cyanobacterial cultures used in this study (stained with Lugols iodine solution). Clockwise from left; *Nodularia spumigena* (CAWBG21), *Dolichospermum* sp. (CAWBG567), *Microcystis* sp. (CAWBG563), *Aphanizomenon* sp. (CAWBG595), *Microcystis* sp. (CAWRotoA), *Microcystis* sp. (CAWBG617).**

## 2.3 Cell enumeration and identification

For each of the experiments described below, samples (1-5 mL) were preserved in Lugols iodine solution and kept in the dark prior to cell enumeration and biovolume calculations. Subsamples (500 µL) were added to 12-well plates (ThermoFisher Scientific) and allowed to settle for at least 3 h. Cell enumeration was conducted using an inverted microscope (Olympus CK41, or Olympus CK2) using a magnification between 200-800×. Dense samples were diluted (1/10) and enumerated by counting cells in 10 fields of view, and less dense samples (or those containing filamentous algae) were counted along 2 transects. Biovolume for each species were estimated by measuring the cell dimensions of 30-50 cells at 1,000× (Olympus BX51 inverted microscope) and using volumetric equations of geometric shapes closest to each cell shape to calculate cell volumes [46; 47]. Taxonomic identification was carried out using identification guides [48; 49], as well as online resources [50] for field samples.

## 2.4 Laboratory setup for sensor validation

Experiments were performed in 1-L beakers which were 'blacked out' (see Section 2.10.1 for experiment regarding light experiment) using poly vinyl chloride (PVC) tape. All experiments were carried out under a low light regime (3 µmol $s^{-1}$ $m^{-2}$)

and at constant temperature (17 °C ± 1 °C with the exception of the temperature experiment). Unless stated, all measurements were made in triplicate, and culture volumes of 400 mL were used. Each sensor was individually tested in all experiments unless otherwise stated. During measurements, sensors were held 1 cm beneath the surface of the sample and at least 7.8 cm from the bottom of the sample vessel (see Section 2.10.2 for optimisation of depth measurements). Readings were taken once the sensor had stabilised (ca. 10 seconds). All experimental beakers were placed on a non-reflective black surface as suggested by a manufacturer [36]. Each probe was rinsed and dried thoroughly when moving between control and test samples.

## 2.5 Extraction and analysis of phycocyanin and chlorophyll-*a*

Grab samples from each field site were filtered (2-38 mL) in triplicates using GF/C filters (Whatman). Phycocyanin and chlorophyll-*a* was extracted from one set of triplicate filters. Phycocyanin was extracted in sodium phosphate buffer (1 mL, pH 7 50mM). The samples were sonicated (30 min, 60 kHz) and subjected to three freeze/thaw cycles (-20 °C, 2 h) before being clarified by centrifugation (5 min, 17,000 ×*g;* Thermo Scientific Haereus Pico 17 centrifuge*)*. Chlorophyll-*a* samples were extracted in acetone (1 mL; 30 min, 60 kHz) and clarified by centrifugation (1 min, 17,000 ×*g*). Phycocyanin and chlorophyll-*a* extracts were measured by spectrophotometry as described in Section 2.6.

## 2.6 Fluorescence measurements using spectrophotometry

Spectrophotometry was used to determine the concentration of phycocyanin and chlorophyll-*a* from filtered field samples (Thermo Scientific Helios Omega UV-VisSpectrophotometer). Samples of phycocyanin (750 µL) were diluted in plastic cuvettes with sodium phosphate buffer (50 mM; 750 µL) and measured at 615 and 652 nm. Concentrations of phycocyanin were calculated using equation 1.1 [51]:

$$\text{Phycocyanin (µg/L)} = \left(\frac{[A_{615} - (0.474 \times A_{652}) \times DF \times EV]}{5.34 \times VF}\right) \times 1000 \qquad (1.1)$$

where $A_{615}$ is the maximum absorbance of phycocyanin in a path length of 1 cm, $A_{652}$ is the maximum absorbance of allophycocyanin in a path length of 1 cm, *0.474* is the value to compensate for the absorbance of

allophycocyanin, $DF$ is the dilution factor, $EV$ is the extraction volume in L, *5.34* is the extinction coefficient at 652 nm and $VF$ is the volume filtered in L.

Chlorophyll-*a* samples (750 µL) were diluted in glass cuvettes with acetone (750 µL) and measured at 665 and 750 nm. Hydrochloric acid (1 N HCI; 20 µL) was added to the sample and measured again at 665 and 750 nm to account for phaeopigment [35]. Chlorophyll-*a* concentrations were determined using equation 1.2 [35]:

$$\text{Chlorophyll-}a\ (\mu g/L) = \left(\frac{\mathrm{DF} \times 11 \times 2.43 \times Ev(\mathrm{B_{Abs}} - \mathrm{A_{Abs}})}{Vf \times l}\right) \quad (1.2)$$

where *DF* is the dilution factor, *11* is the absorption coefficient of chlorophyll-*a*, *2.43* is the factor to equate the reduction in absorbance, *EV* is the extraction volume in mL, $B_{Abs}$ is the absorbance at 665 nm minus the absorbance at 750 nm before the addition of hydrochloric acid, $A_{Abs}$ is the absorbance at 665 nm minus the absorbance at 750 nm after the addition of hydrochloric acid, *Vf* is the volume filtered in L, *l* is the path length of the cuvette in cm.

## 2.7 Range, sensitivity and calibration of sensors

Standard curves were obtained determine the sensitivity and range of each of the sensors, and to calibrate raw fluorescence readings. For the phycocyanin sensors, C-phycocyanin sourced from *Spirulina* sp. (Sigma-Aldrich P2172) was dissolved in sodium phosphate buffer (30 mL) and diluted to 300 mL with reverse osmosis (RO) water. The phycocyanin concentration was determined by spectrophotometry (Section 2.6) using equation 1.1. A standard curve was obtained through sample dilutions (0-1,200 µg/L) which were measured using each phycocyanin sensor. Rhodamine B (Sigma-Aldrich R6626) was used to obtain a calibration curve for the chlorophyll-*a* sensors. A stock solution of Rhodamine B was produced by dissolving 0.05 g in Milli-Q water (MQ; 500 mL). This was diluted (1/200) to obtain a working solution of 0.5 mg/L. Chlorophyll-*a* concentration was determined using the suggested temperature-dependent chlorophyll-*a* co-efficient (86.4 µg/L) at the sample temperature (17 °C) [52]. This working solution was diluted to obtain an eleven point standard curve (0-86.4 µg/L) which was measured using each

chlorophyll-*a* sensor. The standard phycocyanin and chlorophyll-*a* curves were used to determine the working range of the sensors, and to calibrate raw fluorescence values to units of phycocyanin and chlorophyll-*a* (µg/L).

## 2.8 Background interference ('noise')

The background interference (i.e., 'noise') of each sensor was determined using daily measurements of MLA media [45]. The background 'noise' values were subsequently subtracted from all final measurements. When experiments were undertaken over multiple days, MLA media was measured each day and the values subtracted from the measurement on the corresponding day.

## 2.9 Preparation of phycocyanin and chlorophyll-*a* solutions

'In-house' phycocyanin and chlorophyll-*a* solutions were prepared to determine the short-term and intermediate precision of the sensors. Lyophilized *Microcystis* sp. CAWBG11 (0.65 g) [53] was extracted in sodium phosphate buffer (200 mL; 50 mM, pH 7) for phycocyanin, or acetone (200 mL) for chlorophyll-*a*. The sample was sonicated (30 min, 60 kHz) in a sonication bath (Kudos Ultrasonic Cleaner), and homogenized using an ultra-turrex (IKA Ultra turrex T25 Basic, 1 min, 12,000 rpm). The extract was clarified by centrifugation (10 min, 3200 × *g*).

## 2.10 Repeatability of sensor measurements

### *2.10.1 Short term precision*

An aliquot (15 mL) of the prepared 'in house' phycocyanin and chlorophyll-*a* solutions were individually diluted to a final volume of 300 mL in RO water. The solutions were measured with the appropriate sensors six times over a 1 h period. Means and standard deviations for each sensor were used to determine the short term precision for each sensor expressed as a relative standard deviation ($RSD_r$, Equation 1.3):

$$\text{RSD (\%)} = \left(\frac{SD}{x}\right) \times 100 \qquad (1.3)$$

where $x$ is the average fluorescence measurement from the sensor and *SD* is the standard deviation

### *2.10.2 Intermediate*

Aliquots (15 mL) of 'in house' phycocyanin and chlorophyll-*a* solutions were stored in the dark at -20 °C. A single aliquot of phycocyanin and chlorophyll-*a* solution was defrosted each day and individually diluted to a final volume of 300 mL with RO water, and measured with the appropriate sensors. Measurements were taken over a total of seven days. The standard deviations and means for these measurements were used to determine the intermediate relative standard deviation ($RSD_R$) for each sensor (Equation 1.3).

## 2.11 Robustness of sensor measurements

*Aphanizomenon* sp. (CAWBG595) was used to test for light and boundary effects, and *Microcystis* sp. (CAWRotoA) was used to test for temperature effects. Milli-Q water was used as a blank for the light, boundary and temperature effect experiments.

### *2.11.1 Light effects*

Six 1-L glass beakers were used to assess the effects of light on fluorescence measurements from sensors. Two were covered with a black plastic shield ('dark plastic'), two were 'blacked out' using black PVC tape ('dark tape') and the remaining two beakers were unmodified ('light'). No sub-samples were taken for cell enumeration during this experiment as a single homogenous culture was used. Differences between treatments were determined using analysis of variance (ANOVA) and Fishers post-hoc test.

### *2.11.2 Boundary effects*

Two 1-L glass beakers were 'blacked out' as described above, leaving a small space to view the height at which the sensors was being held. Measurements were taken at 4, 8 and 12 cm from the bottom of the beaker. One beaker was used to measure the sample and the second to measure the blank. MLA media was added to the culture (CAWBG595) to obtain a volume of 1.2 L. The beaker was filled to just above 4 cm mark to do the first measurement and so on up to 12 cm, to avoid spillage due to water displacement by the larger sensors. The YSI Sonde was only tested at the 8 and 12 cm marks. No sub-samples were taken for cell enumeration.

Differences between treatments were determined using analysis of variance (ANOVA) and Fishers post-hoc test

### *2.11.3 Temperature effects*

*Microcystis* sp. (CAWRotoA) was prepared at three cell concentrations using MLA media; high (828,000 cell/mL), medium (562,400 cell/mL) and low (180,600 cell/mL). Samples and sensors were placed into temperature controlled cabinets (Polar Cool); 4, 13.8, 17 and 23.5 °C, and left for 2 h to equilibrate before measurements were taken with all sensors except the YSI Sonde. The Chelsea TriLux was used to measure samples up to 17 °C. The experiment started at 4 °C and moved progressively up to 23.5 °C with 2 h equilibration periods between each temperature tested. The experiment was conducted over 10 h. Differences between treatments were determined using analysis of variance (ANOVA) and Fishers post-hoc test.

A correction equation from Turner Designs [54] was used following the experiment to assess its success in normalizing measurements of chlorophyll-*a* fluorescence from the T928 sensor:

$$Fr = Fs^{(n(Ts-Tr))} \tag{1.4}$$

where *Fr* is the calculated fluorescence reading at the reference temperature, *Fs* is the observed fluorescence reading at the sample temperature, *n* is the temperature coefficient, *Ts* is the sample temperature, and *Tr* is the reference temperature [54].

## 2.12 Assessing the linearity of sensor measurements

### *2.12.1 Single species*

A 1.3 fold dilution series of each cyanobacterial culture (Section 2.2) was prepared where 8 dilutions were measured with all sensors for each culture. A sub sample (1 mL) from each dilution was preserved in Lugols iodine solution and stored in the dark for cell enumeration and biovolume calculations (see Section 2.3). Correlations between fluorescence measurements from sensors and cell biovolume were determined using linear regression.

### *2.12.2 Mixed Culture*

Three cyanobacterial strains were used in a mixed culture experiment; *Microcystis* sp. (CAWBG617), *Dolichospermum* sp. (CAWBG567), and *Aphanizomenon* sp. (CAWBG595). A cell count was initially undertaken to determine the concentration of each individual culture, which was then converted to biovolume (as described above). Each strain was diluted with MLA media prior to the experiment to obtain equal biovolume. Four samples of differing biovolume ratios were prepared (Figure 2). Sub samples (1 mL) from each mixture were preserved in Lugols iodine solution and stored in the dark for cell enumeration and biovolume calculations (see Section 2.3). These were used to confirm the cell concentrations, biovolume concentrations and biovolume ratios. The total biovolume differed across the four samples despite attempts to ensure these were equal (Figure 2). To enable comparison of the data, the results were normalised by dividing the sensor measurement by the total biovolume. Differences between treatments were determined using analysis of variance (ANOVA) and Fishers post-hoc test.

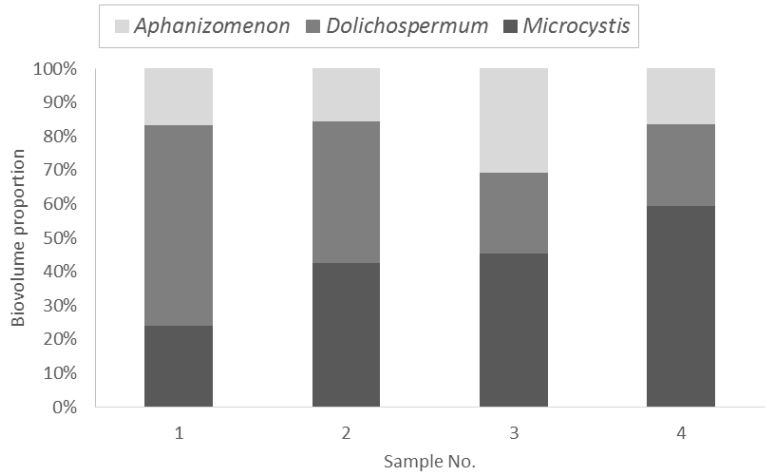



**Figure 2. Relative proportion of species biovolume (*Aphanizomenon* sp., *Dolichospermum* sp., *Microcystis* sp.) across four samples with similar total biovolume. (Sample 1 =10.3 mm3/L, Sample 2 =9.1 mm3/L, Sample 3 =5.3 mm3/L, Sample 4 =7.4 mm3/L).**

### *2.12.3 Colonial species*

A colony forming strain of *Microcystis*, CAWBG11 (see Section 2.2 for culture conditions) was used as a model cyanobacterium for fluorescence detection. Samples were sieved with different sized micrometer meshes (50, 60, 75, 102, 125,

150 and 250 µm) to produce sub-samples (50 mL) of differing colony sizes. Each colony size represents an individual sample i.e., samples sizes were not continuous. Each sub-sample was re-suspended in MQ water (150 mL) and measured in a 250 mL beaker using the Turner T927 and TriLux phycocyanin sensors. Each sample was then disaggregated using a manual grinder (10 mL Tissue Grinder, Wheaton, USA) and measured again with the same sensors. Sub-samples were taken from the disaggregated samples and preserved in Lugols iodine solution for cell enumeration (see Section 2.3) presented in Table 2. Visual observation of these samples showed no significant damage to the cells, and therefore the potential for lysing of cells was deemed to be insignificant.

To quantify the effect of disaggregation on phycocyanin fluorescence, the relative error was determined [28]:

$$RE_C(\%) = \left[\frac{N-N_O}{N_O}\right] \times 100 \quad (1.5)$$

where $N$ is the phycocyanin fluorescence from aggregated colonies and $N_O$ is the phycocyanin fluorescence following disaggregation.

**Table 2. Cell density of the colonial species CAWBG11 following the disaggregation of varying colony sizes.**

| Colony size (µm) | Cell concentration (cell/mL) |
|---|---|
| <50 | 29,310 |
| <60 | 11,380 |
| <75 | 18,830 |
| <102 | 32,900 |
| <125 | 27,100 |
| <150 | 74,370 |
| <250 | 23,700 |
| >250 | 28,760 |

## 2.13 Validation of fluorescence sensors in the field

A field validation of commonly used phycocyanin and chlorophyll-*a* sensors (YSI, T926 and T927, T928 and Seapoint) 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. Thirty sites were selected based on visual assessment to obtain a range of samples of varying cell density (Figure 3). 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 also collected using a 1-L ‘blacked out’ glass beaker, which was measured immediately with each sensor. These samples were transferred to 500-mL sample pottles and taken to shore within 1 h. On-shore, sub-samples (5 mL) were preserved in Lugols iodine solution for cell enumeration, taxonomic identification and biovolume calculations (see Section 2.3) and stored in the dark at ambient temperature. Triplicate samples (2-38 mL) for both phycocyanin and chlorophyll-*a* were collected on GF/C filters (Whatman), and placed in 1.8 mL tubes and stored on ice whilst in the field. Upon returning to the laboratory, the filters were stored in the dark at -20 °C until extraction and spectrophotometric analysis was undertaken.


**Figure 3. Locations of the 30 sites selected for sampling on Lake Rotorua (42.4081° S, 173.5814° E). Inset: location of Lake Rotorua in South Island, New Zealand.**

## 2.14 Determination of extracellular phycocyanin

Sub-samples (100 mL) from the 50 mL field samples (Section 2.13) were pre-filtered (mesh size 20-µm) then GF/C filtered (Whatman) and measured for extracellular phycocyanin with the Turner sensors (T926 and T927) in a 200-mL 'blacked out' beaker. The proportion of extracellular phycocyanin was determined:

$$\%E = (E \div T) \times 100 \qquad (1.6)$$

where $\%E$ is the proportion of extracellular phycocyanin expressed as a percentage of the total, $E$ is the measured extracellular phycocyanin (µg/L), and $T$ is the measured total phycocyanin (µg/L).

## 2.15 Accuracy of fluorescence sensors in the field

Phycocyanin and chlorophyll-*a* fluorescence measurements from the field were compared against spectrophotometry readings of the same samples, to determine the accuracy of the sensors. The variances of each sample set was determined. The variance of chlorophyll-*a* measurements from sensors and spectrophotometry were unequal. Comparisons were made using a t-test assuming equal variances for phycocyanin, and unequal variances for chlorophyll-*a*. The corresponding Pearson correlation coefficient ($R^2$) from regression analysis, and level of significance (P) from the t-test are presented.

# 3 Results

## 3.1 Range and sensitivity of sensors

The range of each sensor was determined using a known standard for phycocyanin (C-phycocyanin from Spirulina sp.) and Rhodamine B (in lieu of a chlorophyll-*a* standard). The upper end of the working range was a set concentration based on these known standards (1,200µg/L and 86.4 µg/L respectively). Phycocyanin concentrations were measured up to 12,000 µg/L. however, as these exceed the manufacturers recommended working range, value above 1,200 µg/L are not presented. Minimum concentrations for phycocyanin and chlorophyll-*a* fluorescence using the standard curve were measured at 1% of the highest concentration (120 µg/L and 0.86 µg/L respectively). No standard curve was able to be created for chlorophyll-*a* fluorescence with the TriLux sensor due to sensor malfunction. All sensors showed a good working range, responding linearly at the maximum concentrations.

## 3.2 Repeatability of sensor measurements

Measurements of fluorescence from phycocyanin and chlorophyll-*a* sensors were taken over a period of 1 h to determine the short term precision of each sensor. Average fluorescence values (µg/L), from three consecutive measurements are presented for phycocyanin in Table 3, and chlorophyll-*a* in Table 4. The relative standard deviation (RSD) for each sensor was also determined. A low RSD (<10%) indicates high precision. A high RSD (>10%) indicates low precision. The Manta, T926, T927 and TriLux phycocyanin sensors all exhibited good short-term precision (<5% RSD), however, the precision of the YSI sensor was poor (13.2% RSD; Table 3). Measures of phycocyanin fluorescence were similar for the two Turner sensors (T926; 393 µg/L, T927; 387 µg/L), however the T926 sensor had a higher relative standard deviation, and therefore a lower precision than T927 (4.3% RSD). RSD of chlorophyll-*a* fluorescence (Table 4) was more variable between sensors than phycocyanin, but showed good short-term precision (<10% RSD), with the YSI sensor the most variable (9.82% RSD).

Fluorescence measurements were taken over a period of 7 days to determine intermediate precision (%$RSD_R$) for phycocyanin and chlorophyll-*a* sensors. Intermediate RSD of phycocyanin fluorescence was more variable than those from the shorter time period. Therefore the precision of phycocyanin sensors was lower (>5% RSD; Table 3). The YSI sensor again showed the lowest precision of all the sensors (14% RSD). While the T926 and T927 sensors showed no difference in precision (9.5% RSD respectively). Chlorophyll-*a* fluorescence was more variable across the intermediate time period (Table 4) and the intermediate precision of all sensors was low (>12% RSD), with the YSI sensor displaying the lowest precision of all the sensors (>46% RSD).

**Table 3. Average phycocyanin fluorescence (µg/L) from sensors (n=3) and standard deviation (SD) for short term (RSDr, 1 h) and intermediate (RSDR, 7 days) precision.**

| | **Phycocyanin** | | | **Phycocyanin** | | |
|---|---|---|---|---|---|---|
| **Sensor** | **Short term precision** | | | **Intermediate precision** | | |
| | **Average** | **SD** | **$RSD_r$** | **Average** | **SD** | **$RSD_R$** |
| **YSI** | 372 | 48.9 | 13.2% | 395 | 55.3 | 14% |
| **Manta** | 445 | 1.4 | 0.31% | 420 | 51.5 | 12.2% |
| **T926** | 393 | 16.8 | 4.3% | 400 | 37.8 | 9.4% |
| **T927** | 387 | 3.7 | 0.95% | 430 | 41.5 | 9.6% |
| **TriLux** | 435 | 7.0 | 1.6% | 434 | 49.5 | 11.4% |

**Table 4. Average chlorophyll-*a* fluorescence (µg/L) from sensors (n=3) and standard deviation (SD) for short term (RSDr, 1 h) and intermediate (RSDR, 7 days) precision.**

| Sensor | Chlorophyll-*a* Short term precision | | | Chlorophyll-*a* Intermediate precision | | |
|---|---|---|---|---|---|---|
| | **Average** | **SD** | **$RSD_r$** | **Average** | **SD** | **$RSD_R$** |
| **YSI** | 0.9 | 0.1 | 9.8% | 1.3 | 0.61 | 46% |
| **Manta** | 2.0 | 0.01 | 0.49% | 2.2 | 0.43 | 19.8% |
| **T928** | 2.1 | 0.09 | 0.7% | 2.0 | 0.39 | 19.2% |
| **Seapoint** | 2.5 | 0.2 | 6.7% | 2.7 | 0.43 | 15.6% |
| **TriLux** | 3.8 | 0.2 | 6.2% | 4.5 | 0.5 | 12.1% |

## 3.3 Robustness of sensor measurements

### *3.3.1 Influences of light on measurements of fluorescence*

Phycocyanin and chlorophyll-*a* fluorescence was measured under different light treatments to determine whether the presence of light has an effect on the fluorescence measurements from sensors (Figure 4). ANOVA analysis with Fishers post-hoc test showed that the fluorescence measurements from the Manta and T926 sensor under the 'light' treatment were significantly different from the 'dark tape' treatment ($P < 0.05$) and the 'dark plastic' treatment ($P < 0.001$). No difference was observed between the two dark treatments ($P > 0.05$). There were no significant differences in measurements of chlorophyll-*a* fluorescence under the different light treatments.

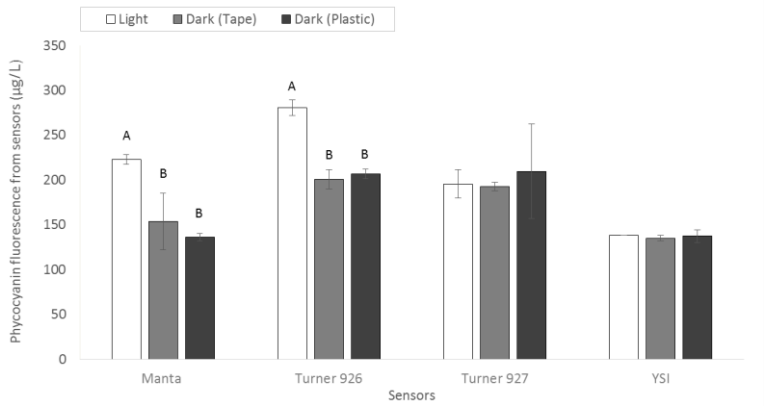
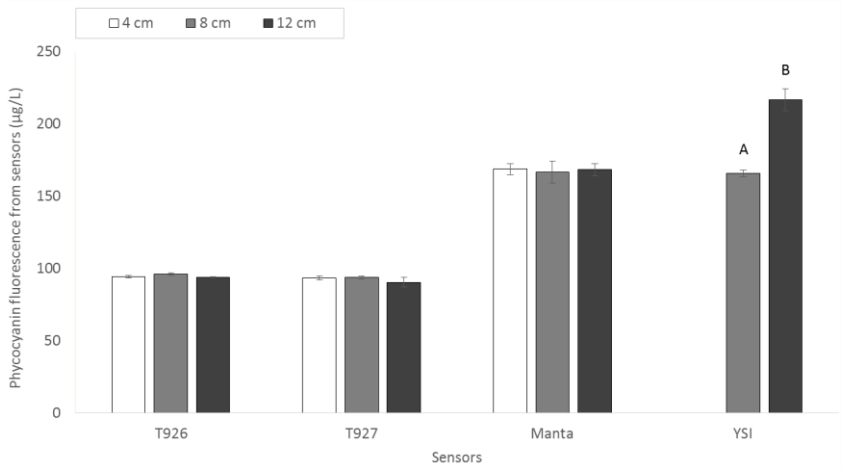



**Figure 4. Effects of different light treatments on average phycocyanin fluorescence from sensors (n=3). Error bars show one standard deviation. Letters indicate where significant differences occur between treatments (one way ANOVA with Fishers post-hoc test).**

### *3.3.2 Boundary effects*

Phycocyanin and chlorophyll-*a* fluorescence was measured using the sensors at different heights above the bottom of the sample beaker to determine whether boundary effects may be a source of interference. ANOVA analysis with Fishers post-hoc test showed that there significant differences in phycocyanin measurements between 8 and 12 cm above the bottom of the beaker for the YSI sensor ($P < 0.001$, Figure 5a). However no difference in sensor measurements were observed for other phycocyanin sensors. A significant difference was also observed for chlorophyll-*a* measurements for the YSI sensor between 8 and 12 cm ($P < 0.001$), and the T928 sensor between 4 and 12 cm ($P < 0.001$) and 8 and 12 cm ($P < 0.01$ Figure 5b). No significant differences in sensor measurements observed for the other chlorophyll-*a* sensors

a)




b)

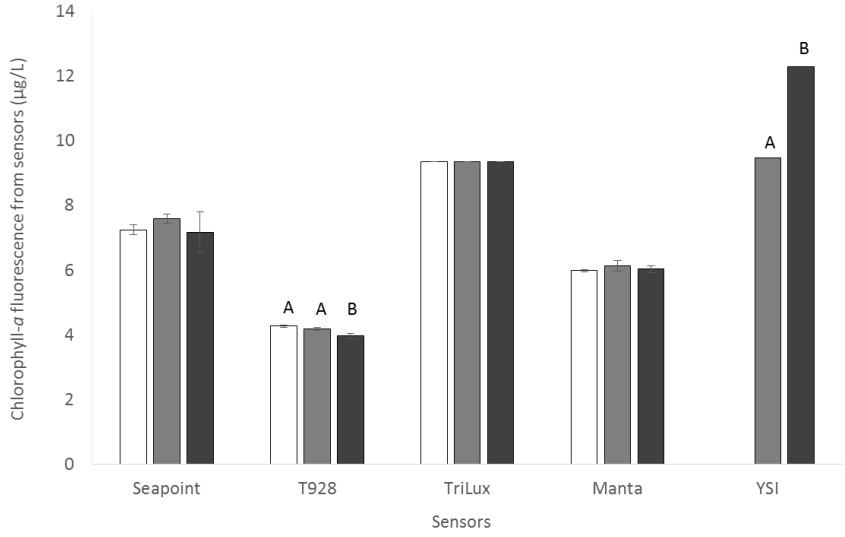



**Figure 5. Effect of sensor height on the average (n=3) (a) phycocyanin and (b) chlorophyll-*a* fluorescence measurements from sensors. Error bars show one standard deviation. Letters indicate where significant differences occur between treatments (one way ANOVA with Fishers post-hoc test). A measurement at 4cm was not possible with YSI due to the size of the probe.**

### *3.3.3 Effects of water temperature on fluorescence measurements*

Phycocyanin and chlorophyll-*a* fluorescence was measured across four temperature treatments, and three cell concentrations to determine what the influence of temperature is on fluorescence measurements, and whether this effect differs with cell concentration. Fluorescence measurements at high cell density for treatments at 17 °C were not carried out with the TriLux sensor, and also not at 23.5 °C for all cell concentrations due to sensor malfunction. Measurements of phycocyanin

fluorescence from sensors were significantly higher at 4 °C ($P < 0.01$) for the three cell concentrations high, medium and low (Figure 6a, b and c) for all sensors except the TriLux. A general pattern for all three cell densities was observed where, as water temperature increased, phycocyanin measurements decreased, however this result was not always statistically significant ($P > 0.05$). Fluorescence measurements in the medium cell density appeared to be the most stable, with no significant difference in fluorescence between 13.8, 17 and 23.5 °C ($P > 0.05$, Figure 6b). There was no statistically significant difference in fluorescence with change in temperature for the TriLux sensor in the high and low cell density treatments. Fluorescence at 23.5 °C were significantly different from all other temperature treatments in the low cell density experiment for the Manta and Turner sensors (T926, T927; $P < 0.01$, Figure 6c).

Chlorophyll-*a* fluorescence at 4 °C was significantly higher than measurements at 13.8 and 17 °C for the high and low cell density experiments ($P < 0.05$, $P < 0.01$ respectively). Fluorescence increased significantly at 23.5 °C in the high and medium cell densities for all sensors ($P < 0.05$), but not 4 °C (Figure 7a and c). Chlorophyll-*a* fluorescence did not follow the same pattern as phycocyanin. Fluorescence decreased at 13.8 °C across all sensors and densities, although the difference was not significant in the low cell density experiment (Figure 7c). A pattern similar to that observed in the phycocyanin fluorescence was observed for the Seapoint sensor in the low cell density experiment. Temperature had no statistically significant effect on the chlorophyll-*a* measurements for the TriLux sensor.

## 3.4 Relationship between fluorescence and species biovolume

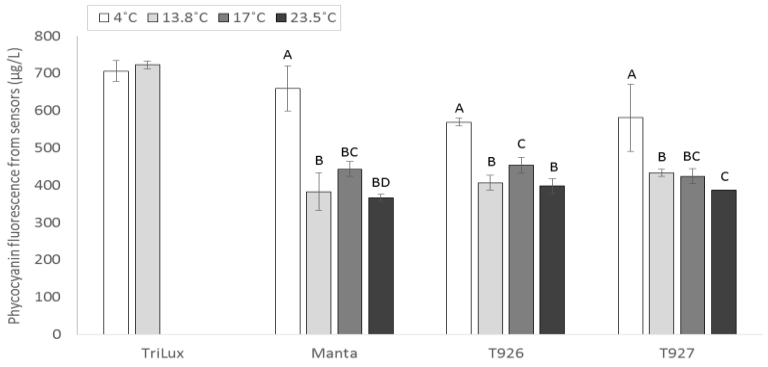
### *3.4.1 Fluorescence measurements and biovolume of individual cultures*

Strong correlations were observed between cell biovolume and phycocyanin measurements within the single celled *Microcystis* sp. CAWBG617 ($R^2 > 0.8$, $P < 0.01$) and CAWRotoA ($R^2 > 0.6$, $P < 0.01$), as well as the filamentous species *Aphanizomenon* ($R^2 > 0.9$, $P < 0.01$, Table 5). There was also a strong correlation between biovolume of *N. spumigena* and phycocyanin fluorescence from all sensors ($R^2 > 0.8$, $P < 0.01$), except the YSI which showed a poor correlation ($R^2 < 0.01$, $P > 0.05$). Strong correlations between biovolume of *Dolichospermum*

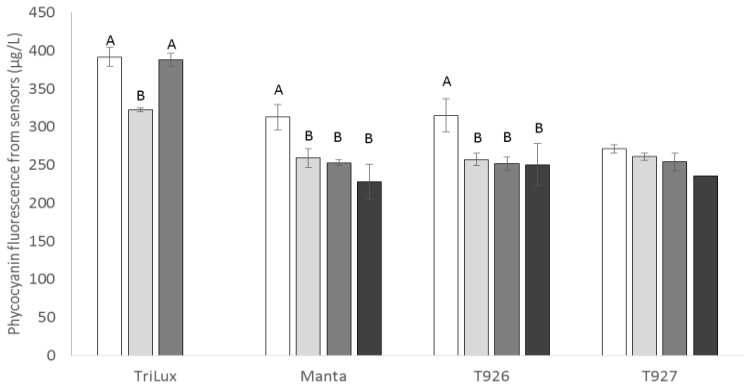
sp. and phycocyanin fluorescence were observed for all sensors ($R^2$ >0.9, P <0.01), except the TriLux, which showed a weak correlation ($R^2$ = 0.17, P >0.05). Weak correlations were observed for biovolume of the colonial *Microcystis* species and phycocyanin for all sensors except the TriLux, which showed a strong correlation ($R^2$ = 0.75, P <0.01).

Excellent linearity between cell biovolume and chlorophyll-*a* fluorescence was observed within the single celled *Microcystis* sp. CAWBG617 ($R^2$ >0.9, P <0.01) and CAWRotoA ($R^2$ >0.8, P <0.01) and the filamentous species *Aphanizomenon* ($R^2$ >0.6, P <0.01) and *Dolichospermum* ($R^2$ >0.9, P <0.01; Table 6). Biovolume of *N. spumigena* biovolume had a strong linear relationship with chlorophyll-*a* fluorescence from the T928 ($R^2$ = 0.95, P <0.01), and Seapoint sensors ($R^2$ >0.6, P <0.05,) but not with the YSI and Manta sensors ($R^2$ <0.1, P >0.05). A weak correlation was observed between biovolume of the colonial *Microcystis* and chlorophyll-*a* fluorescence using the YSI ($R^2$ <0.01, P >0.05), Manta ($R^2$ <0.01, P >0.05), T928 ($R^2$ = 0.34, P >0.05), and Seapoint ($R^2$ = 0.16, P >0.05). However a strong correlation was observed using the TriLux sensor ($R^2$ = 0.68, P <0.01).

a)




b)




c)

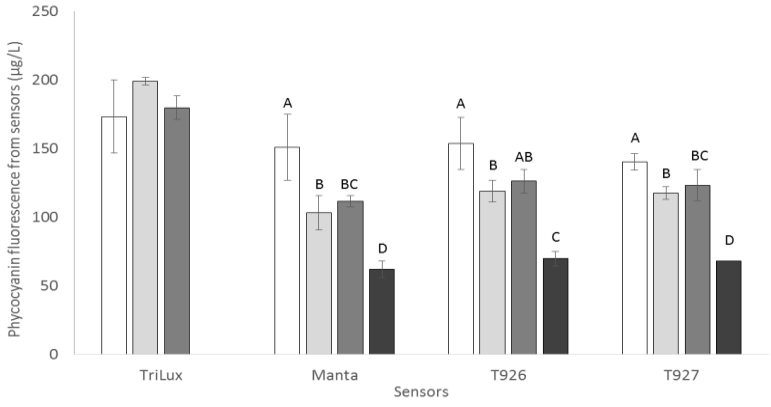
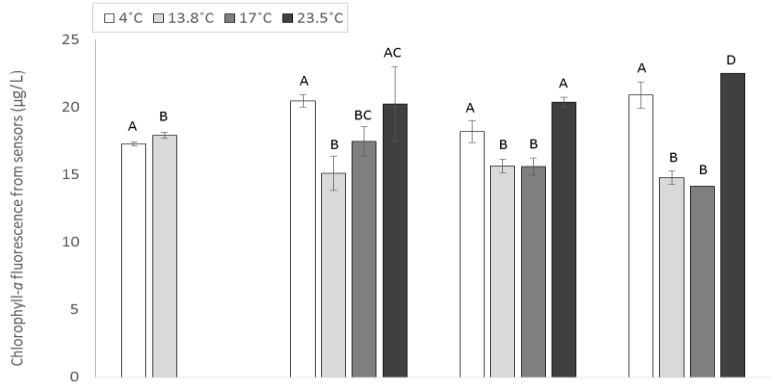
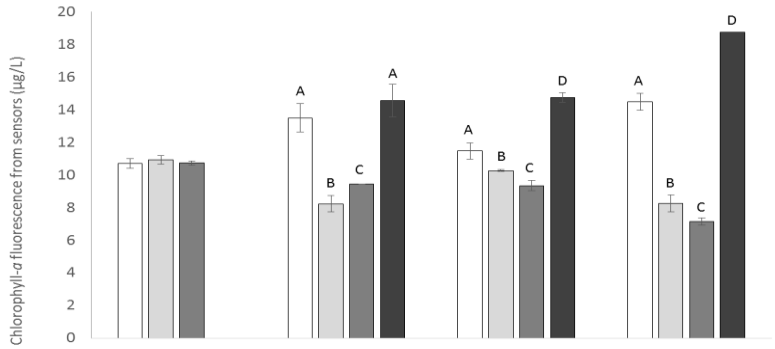



**Figure 6. Average phycocyanin fluorescence from sensors (n=3) at (a) high, (b) medium and (c) low cell density (cell/mL) across four temperatures. Error bars show one standard deviation. Letters indicate where significant differences occur between treatments (one way ANOVA with Fishers post-hoc test).**

a)




b)




c)

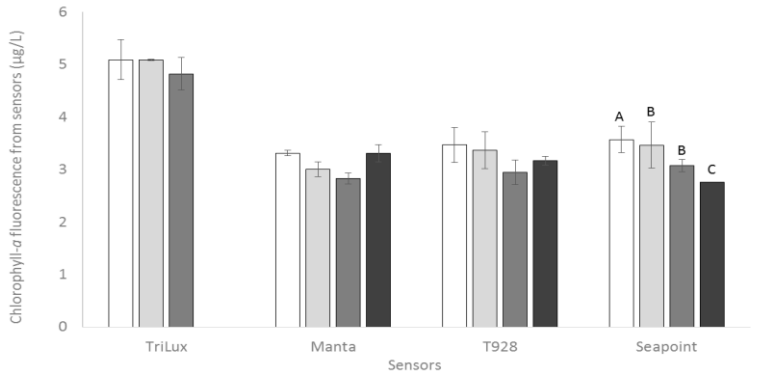



**Figure 7. Average chlorophyll-*a* fluorescence from sensors (n=3) at (a) high, (b) medium and (c) low cell density (cell/mL). Error bars show one standard deviation. Letters indicate where significant differences occur between treatments (one way ANOVA with Fishers post-hoc test).**

**Table 5. Linear regression between cell biovolume (0-196 µm³) and phycocyanin fluorescence from sensors. Correlation coefficients ($R^2$) and significance of the relationship (P) are presented. (P <0.05 taken as significant).**

| Sensor | YSI | | Manta | | T926 | | T927 | | TriLux | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Genera/Species (culture code)** | **$R^2$** | **P** | **$R^2$** | **P** | **$R^2$** | **P** | **$R^2$** | **P** | **$R^2$** | **P** |
| **Single celled** | | | | | | | | | | |
| *Microcystis* sp. (CAWBG621) | 0.97 | <0.01 | 0.96 | <0.01 | 0.98 | <0.01 | 0.98 | <0.01 | 0.85 | <0.01 |
| *Microcystis* sp. (CAWRotoA) | 0.85 | <0.01 | 0.84 | <0.01 | 0.68 | <0.01 | 0.83 | <0.01 | 0.92 | <0.01 |
| **Colonial** | | | | | | | | | | |
| *Microcystis* sp. (CAWBG563) | <0.01 | 0.52 | 0.34 | 0.08 | 0.37 | 0.3 | 0.18 | 0.33 | 0.75 | <0.01 |
| **Filamentous** | | | | | | | | | | |
| *Nodularia spumigena* (CAWBG21) | <0.01 | 0.4 | 0.80 | <0.01 | 0.80 | <0.01 | 0.89 | <0.01 | NA | NA |
| *Aphanizomenon* sp. (CAWBG595) | 0.90 | <0.01 | 0.92 | <0.01 | 0.95 | <0.01 | 0.97 | <0.01 | 0.94 | <0.01 |
| *Dolichospermum* sp.(CAWBG567) | 0.95 | <0.01 | 0.95 | <0.01 | 0.94 | <0.01 | 0.94 | <0.01 | 0.17 | 0.2 |

**Table 6. Linear regression between cell biovolume (0-196 µm³) and chlorophyll-*a* fluorescence from sensors. Correlation coefficient ($R^2$) and significance of the relationship (P) are presented. (P <0.05 taken as significant).**

| Sensor | YSI | | Manta | | T928 | | Seapoint | | TriLux | |
|---|---|---|---|---|---|---|---|---|---|---|
| **Genera/Species (culture code)** | **$R^2$** | **P value** | **$R^2$** | **P value** | **$R^2$** | **P value** | **$R^2$** | **P value** | **$R^2$** | **P value** |
| **Single celled** | | | | | | | | | | |
| *Microcystis* sp. (CAWBG617) | 0.96 | 0.01 | 0.97 | <0.01 | 0.97 | <0.01 | 0.92 | <0.01 | 0.92 | <0.01 |
| *Microcystis* sp. (CAWRotoA) | 0.96 | <0.01 | 0.85 | <0.01 | 0.91 | <0.01 | 0.92 | <0.01 | 0.95 | <0.01 |
| **Colonial** | | | | | | | | | | |
| *Microcystis* sp. (CAWBG563) | <0.01 | 0.808 | <0.01 | 0.52 | 0.34 | 0.08 | 0.16 | 0.18 | 0.68 | <0.01 |
| **Filamentous** | | | | | | | | | | |
| *Nodularia spumigena* (CAWBG21) | 0.11 | 0.218 | <0.01 | 0.42 | 0.95 | <0.01 | 0.63 | 0.04 | 0.62 | <0.01 |
| *Aphanizomenon* sp. (CAWBG595) | 0.90 | <0.01 | 0.90 | <0.01 | 0.64 | <0.01 | 0.95 | <0.01 | 0.95 | <0.01 |
| *Dolichospermum* sp. (CAWBG567) | 0.96 | <0.01 | 0.95 | <0.01 | 0.95 | <0.01 | 0.95 | <0.01 | 0.95 | <0.01 |

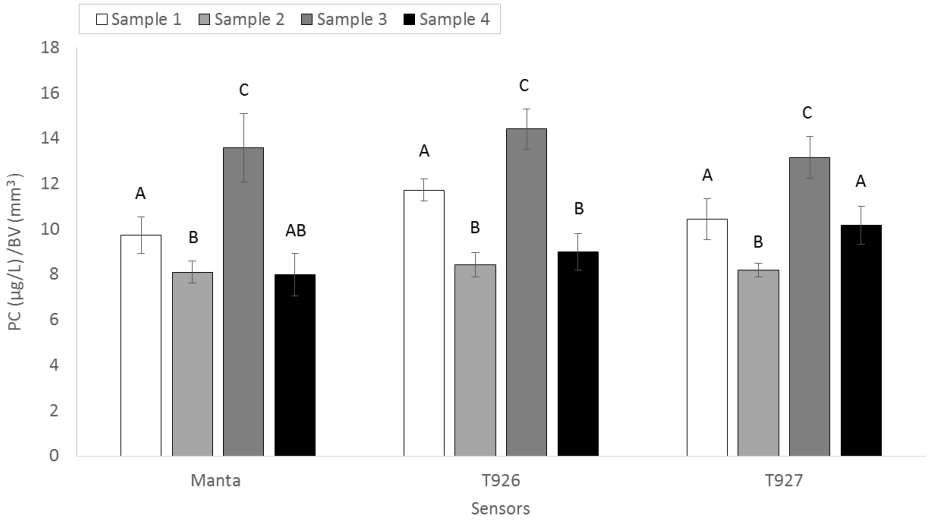
### *3.4.2 Fluorescence measurements and mixed species assemblage*

Measurements of phycocyanin and chlorophyll-*a* fluorescence from four mixed assemblages containing three cyanobacterial species (*Aphanizomenon* sp., *Dolichospermum* sp. and *Microcystis* sp.) were taken to determine the effect of a mixture of cyanobacteria species on the relationship of biovolume to fluorescence. Phycocyanin fluorescence normalised to biovolume (Figure 8a) showed statistically significant differences between samples; Sample 3 had a significantly higher phycocyanin to biovolume ratio (PC/BV) than other samples for all sensors ($P < 0.05$). This sample had the greatest proportion of *Aphanizomenon* sp. (Figure 2).

Chlorophyll-*a* fluorescence measurements from sample 3 were significantly higher than those from Sample 2 for the T928 sensor ($P < 0.01$; Figure 8b), and Sample 1 and 2 for the Seapoint sensor ($P < 0.01$). Measurements from the Seapoint chlorophyll-*a* sensor also increased in Sample 4 where the largest proportion of *Microcystis* sp. was present (Figure 2). This was significantly different from Samples 1 and 2 ($P < 0.01$) but not for Sample 3 ($P > 0.05$). Chlorophyll-*a* to biovolume trends were similar to phycocyanin to biovolume trends in the four samples. However the chlorophyll-*a* measurements were more variable and therefore the changes chlorophyll-*a* between samples was not always statistically significant.

Linear regression equations from previous measurements of phycocyanin and chlorophyll-*a* from pure single cultures (Table 5 and 6) were used to calculate phycocyanin and chlorophyll-*a* concentration in the mixed culture. The results in Figure 9a show predicted phycocyanin from the individual regression of each of the three species, compared to the observed values from the mixed culture. The result was statistically significant ($P < 0.01$). Figure 9b shows the predicted chlorophyll-*a* from the individual regression of each of the three species, and observed chlorophyll-*a* values from the mixed culture. These results show that chlorophyll-*a* fluorescence in a mixed assemblage was not predicted using the combination of linear regression from a single culture.

a)




b)

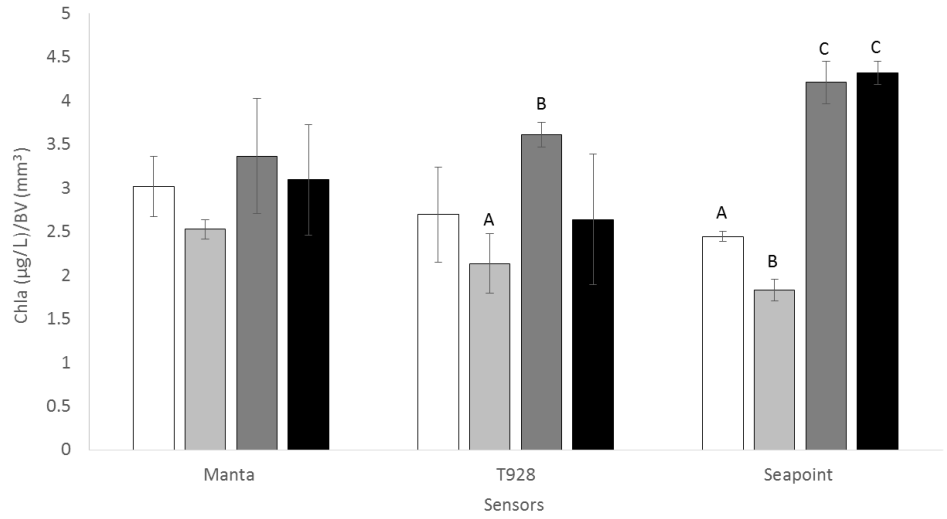
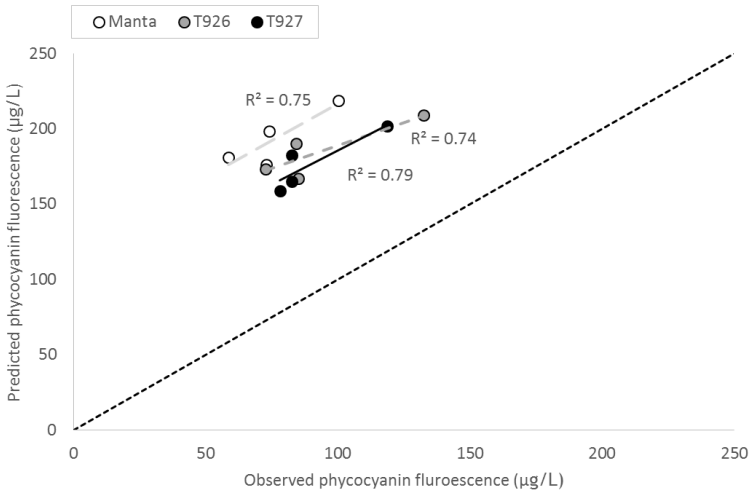



**Figure 8. Average fluorescence from sensors (n=3) from four mixed species assemblages for (a) phycocyanin/biovolume and (b) chlorophyll-*a*/biovolume. Error bars show one standard deviation. Letters indicate where significant differences occur between treatments (one way ANOVA with Fishers post-hoc test).**

a)




b)

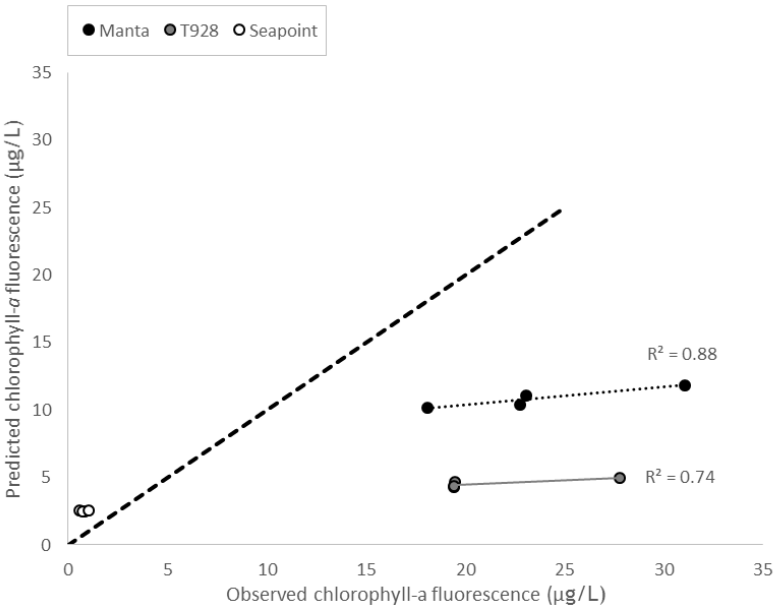



**Figure 9. Observed and predicted (a) phycocyanin and (b) chlorophyll-*a* fluorescence measurements for each mixed assemblage, based on regression equations of fluorescence measurements and biovolume of laboratory cultures (see Table 6 and 7 for correlation coefficients and statistical relationships, regression equations not shown). Significant regression lines and correlation coefficients are shown. The dashed line represents a 1:1 relationship.**

### *3.4.3 Phycocyanin fluorescence and colonial cyanobacteria*

Phycocyanin fluorescence of the colonial *Microcystis* sp. (CAWBG11) was measured using the T927 and TriLux sensors. Samples were measured twice; once when colonies were aggregated and again following disaggregation. Figure 10 shows measurements of phycocyanin fluorescence from sensors of aggregated and disaggregated samples. Each size represents an individual sample i.e. size classes are not continuous. Increases in phycocyanin measurements based on the relative error following colony disaggregation were observed using the Turner sensor for colony sizes <50 µm, <125 µm and >250 µm (4%, 3.5% and 9.9% respectively). For this sensor, a decrease in phycocyanin measurements following disaggregation was observed for size classes <60 µm and <102 µm (13.2% and 2% respectively), and no change was observed for size classes <75 µm, <150 µm and <250 µm.

Increases in phycocyanin fluorescence following colony disaggregation were observed for the TriLux sensor for colony size classes <75 µm, <125 µm, <250 µm and >250 µm (0.4%, 0.6%, 3.9% and 10.4% respectively). A decrease in phycocyanin fluorescence following disaggregation was observed for size classes <50 µm, <60 µm and <102 µm (3.3%, 3.5%, and 2.6% respectively). No change was observed for size classes <150 µm. The largest increase in phycocyanin fluorescence for both sensors was observed for colony sizes >250 µm.

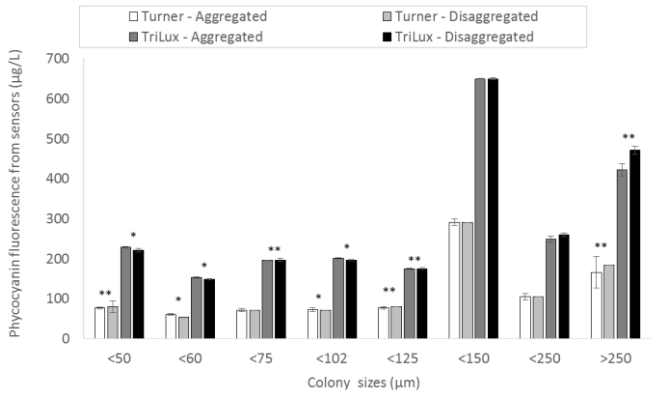



**Figure 10. Average phycocyanin fluorescence from sensors (n=3) for different colony sizes in aggregated and dis-aggregated samples. Size ranges represent individual samples. Error bars represent one standard deviation (SD). Size ranges represent individual samples. Double asterisk represents an increase in fluorescence following disaggregation, a single asterisk represents a decrease in fluorescence following disaggregation (relative error; equation 1.5).**

## 3.5 Validation of fluorescence sensors in the field

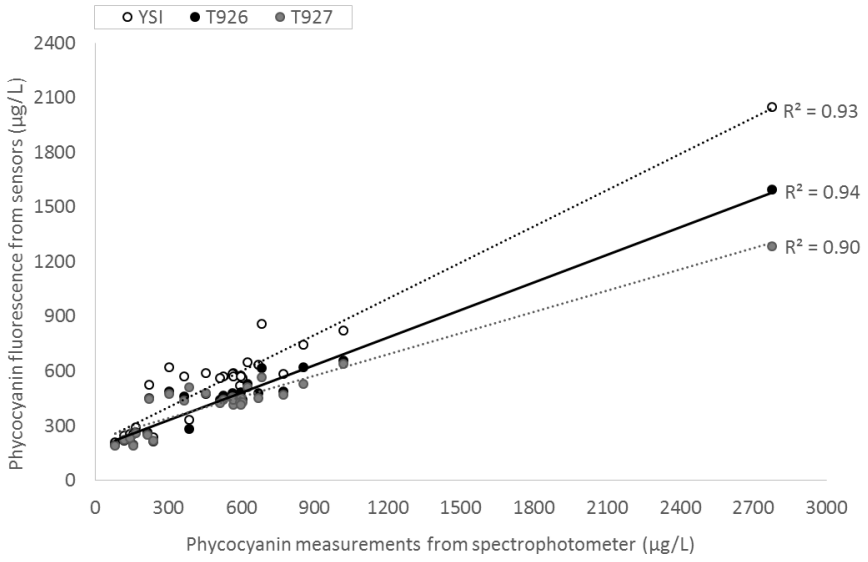
Phycocyanin and chlorophyll-*a* fluorescence was measured at 30 sites in Lake Rotorua, Kaikoura using common fluorescence sensors (YSI, T926, T927, T928 and Seapoint). At the time of sampling, *Dolichospermum* sp. was the dominant species accounting for more than 90% of the total cyanobacterial biovolume (Appendix 2). Phytoplankton biomass at site 20 was significantly higher than other sites (44 $mm^3$/L). However it was important to include measurements from this site in the analysis despite it being somewhat of an outlier, as it represents the variability present in field samples. Fluorescence readings were taken at each site *in situ*, and from a grab sample. There was no difference in phycocyanin fluorescence between these two methods ($P > 0.05$, Appendix 1a). No difference was observed between chlorophyll-*a* fluorescence from *in situ* or grab samples ($P > 0.05$), except for the T928 sensor where the grab sample measurements were higher than the *in situ* sample ($P < 0.01$ Appendix 1b).

The accuracy of phycocyanin and chlorophyll-*a* sensors was assessed by comparing sensor measurements of phycocyanin fluorescence from field samples (calibrated to units of μg/L), to extracted phycocyanin concentrations from field samples using spectrophotometry. A strong linear relationship between the sensor measurements and values from spectrophotometry was observed for all sensors ($R^2 > 0.9$, Figure 11a) and the relationship was statistically significant ($P < 0.01$). The relationship between chlorophyll-*a* fluorescence from sensors, and chlorophyll-*a* concentration determined by spectrophotometry was statistically significant ($P < 0.01$, Figure 11b), however the Pearson correlation coefficient was low ($R^2 < 0.4$).

The performance of phycocyanin and chlorophyll-*a* sensors *in situ* was assessed. A statistically significant linear relationship between phycocyanin fluorescence from sensors and total phytoplankton biovolume from field samples was observed for all sensors ($R^2 > 0.4$, $P < 0.01$, Figure 12a). The correlation coefficient improved when sensor measurements were plotted against cyanobacterial biovolume ($R^2 > 0.5$, $P < 0.01$; Figure 12b). By contrast, there was a weak linear relationship between

chlorophyll-*a* fluorescence and total phytoplankton biovolume and cyanobacterial biovolume from field samples ($R^2$ <0.15, Figure 13a and b).

a)




b)

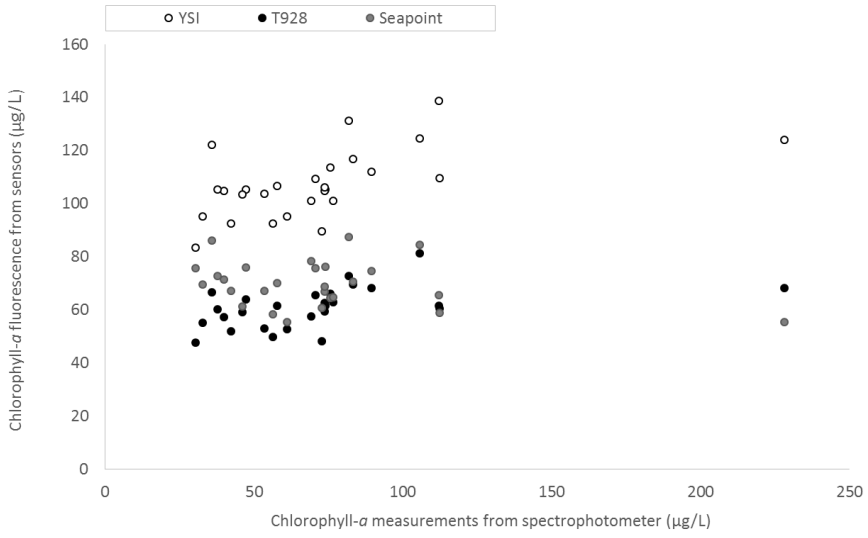
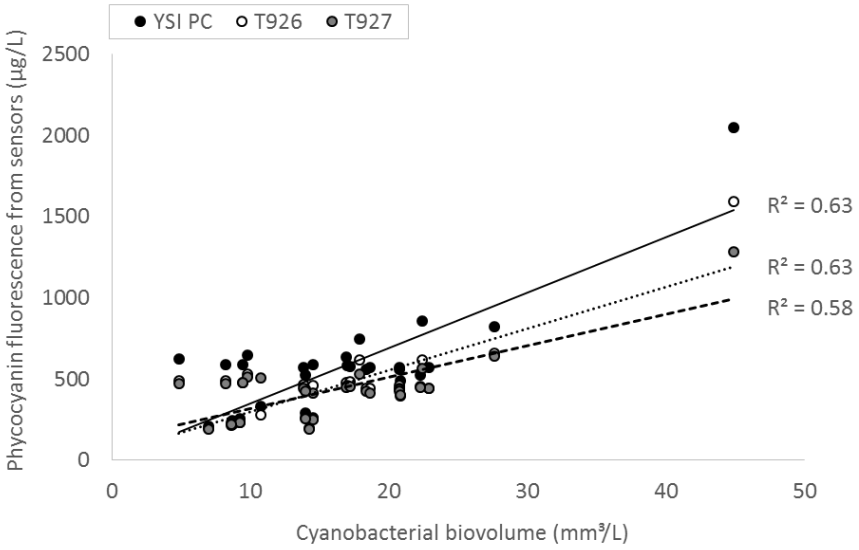



**Figure 11. Fluorescence measurements from sensors and extracted measurements from spectrophotometry for (a) phycocyanin and (b) chlorophyll-*a* from 30 field samples in Lake Rotorua, Kaikoura. Regression lines and correlation coefficients are shown for each phycocyanin sensor, correlation coefficients for chlorophyll-*a* sensors were not significant and are not shown.**

a)




b)

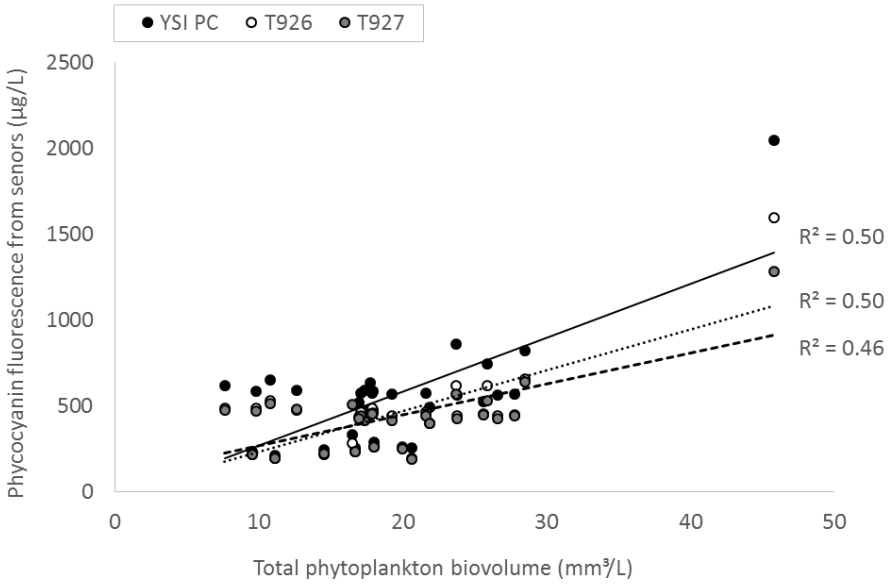
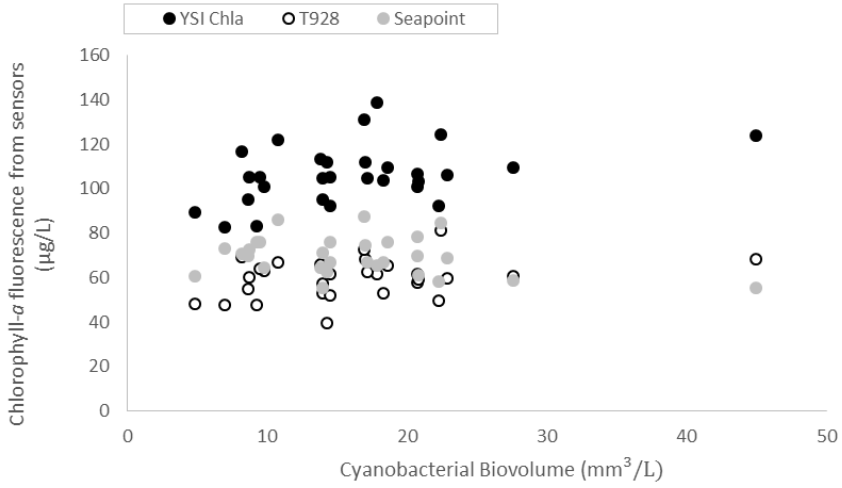



**Figure 12. Phycocyanin fluorescence from sensors and (a) cyanobacterial biovolume and (b) total phytoplankton biovolume from 30 field samples in Lake Rotorua, Kaikoura. Regression lines and correlation coefficients are shown for each sensor.**

a)




b)

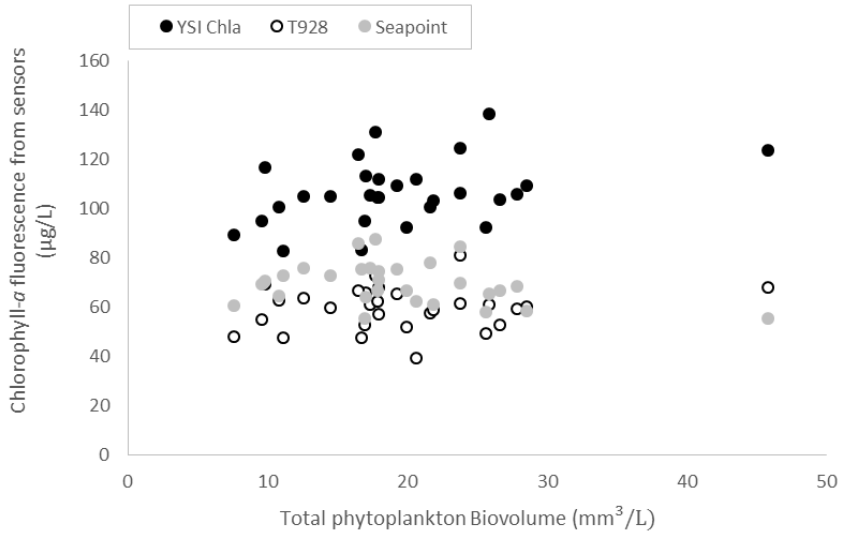



**Figure 13. Chlorophyll-*a* fluorescence from sensors and (a) cyanobacterial and (b) total phytoplankton biovolume from 30 field samples in Lake Rotorua, Kaikoura. Correlation coefficients ($R^2$ <0.3) were not significant and are not shown.**

## 3.6 Influence of extracellular phycocyanin on fluorescence measurements

Grab samples from the field study (Section 3.5, Figure 14a) were filtered through Whatman GF/C filters to separate intra and extracellular phycocyanin. The filtered sample was then measured using the Turner sensors (T926 and T927) to determine whether extracellular phycocyanin was present. The average proportion of phycocyanin fluorescence from 30 sites attributed to extracellular phycocyanin was 6-8% (Figure 14a and b). At sites 4, 5 and 6 the proportion of extracellular phycocyanin exceeded 20% for both of the Turner sensors. The biovolume at these sites was higher (17-27 $mm^3/L$) than the biovolume at most other sites (4-17 $mm^3/L$).

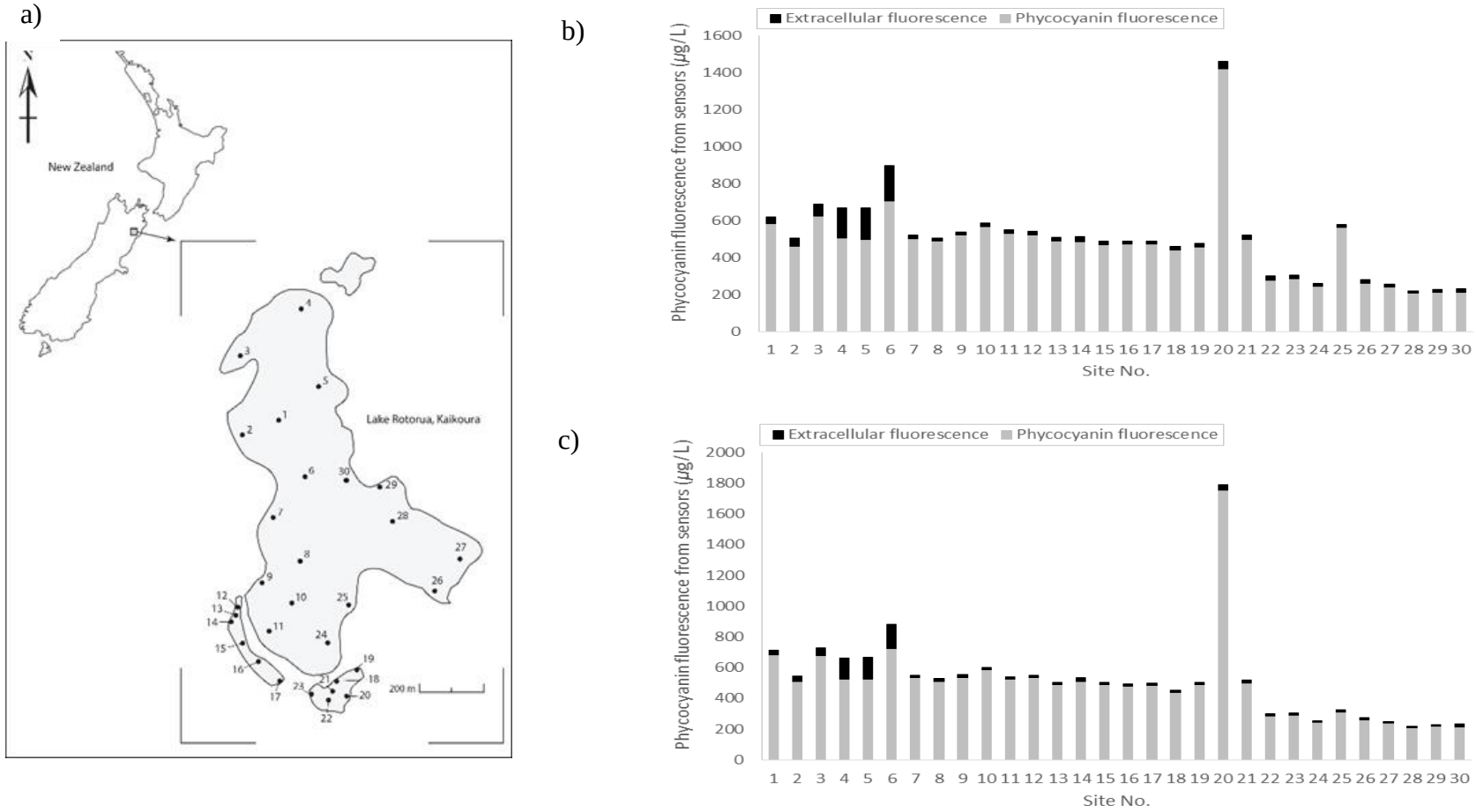



**Figure 14. Proportion of extracellular phycocyanin attributed to the total phycocyanin measurements from (a) 30 sites on Lake Rotorua, Kaikoura for (b) T927 and (c) T926 sensors. Refer to Figure 3 for larger site map.**

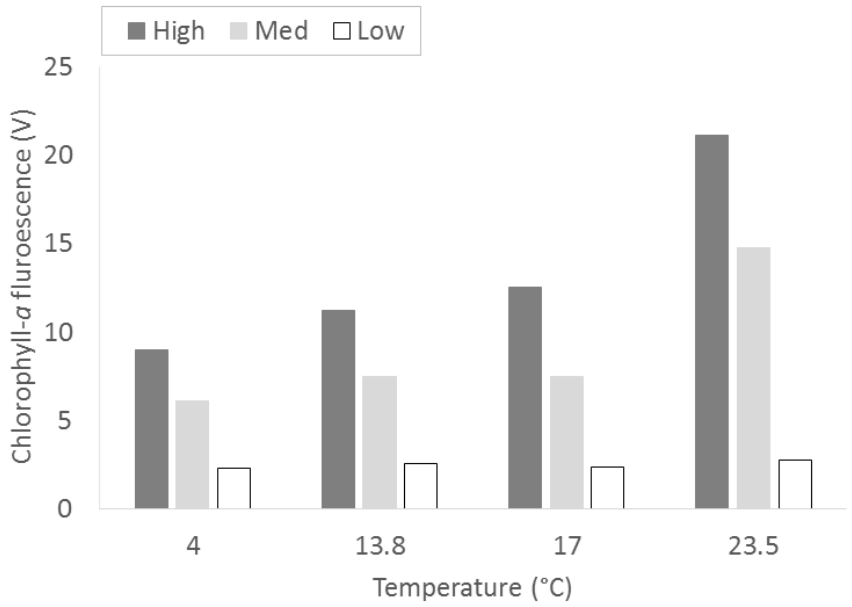



**Figure 15. Chlorophyll-*a* fluorescence at varying temperatures from the T928 sensor, corrected to a reference temperature (17 °C) using the temperature correction equation from Turner manufacturer (equation 1.4) at high (828,000 cell/mL), medium (562,400 cell/mL) and low cell densities (180,600 cell/mL).**

# 4 Discussion

## 4.1 Variability between sensors

The range of each sensor was determined using a known standard of phycocyanin and chlorophyll-*a*. All phycocyanin sensors showed a good working range, still responding linearly at the maximum concentrations (1,200 µg/L). Fluorescence was measured down to 1% of the original concentration (120 µg/L) however the lower detection limit was not determined. Fluorescence measurements lower than 120 µg/L were measured in the culture experiments by the T927, T926, Manta, TriLux and YSI sensors (8, 12, 15, 24 and 35 µg/L respectively) indicating good sensitivity and suitability for samples with low concentration of phycocyanin. Further work is needed to validate this. Chlorophyll-*a* sensors also showed a good working range, responding linearly at high concentrations (86.4 µg/L). However the YSI sensor was not able to detect fluorescence below 2.6 µg/L, indicating it may not be suitable for samples with low chlorophyll-*a* concentration. It is possible that the lower detection limits of these sensors could exceed the minimum concentration measured in this experiment (0.86 µg/L), however further study is needed to determine this.

Variations in sensor outputs are generally due to differences in working range, band widths and the specificity of the light source used in the sensor [26]. Turner sensors used in this study provide a 0 to 5 volt (V) output which is proportional to fluorescence (chlorophyll-*a* as µg/mL and phycocyanin as cell/mL). The Seapoint sensor uses a 0 to 1 V output proportional to chlorophyll-*a* fluorescence in µg/mL, the TriLux sensor output is given as relative fluorescence units (RFU), while the YSI and Manta sensors output readings as cell/mL. Direct output of some sensors in cell/mL may be interpreted incorrectly by a novice user. They are of little use to operators as the meaning is not absolutely clear, and will not be appropriate for all cyanobacteria species. For example, a relationship between RFU and number of cells has been obtained for some sensors, e.g. the YSI 6600 and YSI 606131 sensor (YSI Environmental, USA) which transforms phycocyanin fluorescence into an equivalent cell density for *Microcystis aeruginosa* [23]. The use of a single species to generalise phycocyanin fluorescence is not appropriate as specific absorption is

dependent on physiological conditions, structure of algal communities, changes in pigment concentration, growth conditions and most importantly species, of which cell size can vary [55-59]. In the current experiment, the Manta sensor used outputs in cell/mL, however the species with which the relationship has been determined is not stated. Linear regression between cell density measured by microscopy (cell/mL) and the density determined with the Manta sensor (cell/mL) using the manufacturer calibration was highly significant ($P < 0.01$, $R2 > 0.8$). However the Manta sensor underestimated cyanobacterial cell density by 87-99%.

Similarly, Bastien et al. [23] found that the YSI sensor consistently underestimated cell density when compared to measurements by microscopy using the manufacturers calibration (70-93%). Zamyadi et al. [25] observed that phycocyanin measurements from the YSI 606131 sensor underestimated cyanobacterial biomass in a field sample. This was likely due to the translation of RFU of phycocyanin to biovolume based on measurements of cultured *M. aeruginosa* which was not representative of the field sample consisting of multiple cyanobacteria species. Furthermore, Bastien et al. [23] observed good accuracy between cell density estimated by the YSI 6600 and those measured using microscopy when samples were dominated by *Microcystis* ($R2 = 0.7$), but found this decreased when other species were dominant ($R2 = 0.6$) as *M. aeruginosa* fluorescence would have been low compared to the more dominant species in the assemblage. Bastien et al. [23] also suggests using outputs in RFU instead of cell/mL as RFU is directly related to the quantity of phycocyanin. Results from the current study showed that phycocyanin to biovolume ratio differed between species, and as a result, one cyanobacterial species cannot be used to generalise fluorescence. The use of cell/mL could be useful if the user is well informed by the manufacturer of exactly the conditions used to obtain this relationship, e.g. the reference species, and the sample temperature, as well as the potential need to re-calibrate if there is drift.

Throughout the current study, phycocyanin sensors produced similar results when calibrated i.e. there was no significant difference between measurements from sensors ($P > 0.05$). However, chlorophyll-*a* measurements from field samples produced results which differed significantly in their range across sensors ($P < 0.01$), despite being calibrated (Figure 10b, Figure 12), reducing the ability to

compare results. This may be due to the chlorophyll-*a* sensors being more variable across time and therefore calibration may be required more frequently.

## 4.2 Repeatability of fluorescence measurements

The repeatability of phycocyanin and chlorophyll-*a* sensors was assessed using an in-house standard, over short term (1 h, $\%RSD_r$) and intermediate (7 days, $\%RSD_R$) periods. Measurements of phycocyanin were stable for both time periods suggesting that calibration of phycocyanin sensors is not needed on a week-to-week basis and sensors can be deployed for a longer period of time. Conversely, measurements from the chlorophyll-*a* sensors were more variable across both short term and intermediate periods, suggesting calibration of chlorophyll-*a* sensors is required regularly, and they may not be suitable for periods of prolonged deployment. Many sensors are not calibrated using a primary standard as it is considered impractical, and are often only 'checked 'using a secondary standard, to quantify sensor drift [56]. A primary standard contains a known concentration of phycocyanin or chlorophyll-*a* and is the initial calibration to which the secondary standard is compared. Primary standards are often overlooked, and instead the manufacturer calibration is used.

As mentioned above, the manufacturer calibration can significantly underestimate fluorescence values and therefore primary calibration of the sensor with a known standard is recommended. It is acceptable to calibrate sensors once, but the user may wish to do this annually to ensure accurate measurements. Additionally, calibration can be achieved by comparing sensor measurements with quantitative data collected from the field where the sensor is deployed. Stability and accuracy of fluorescence measurements are critical and can be checked using a secondary standard, often a substance in a synthetic form which fluoresces at wavelengths similar to the sample being measured. Secondary standards can be used to calibrate sensors in place of the primary standard once the initial calibration has been done.

In the current study, there were consistent differences in readings between the Turner phycocyanin sensors (T926 and T97) which had been calibrated by the manufacturer. The specifics of what this calibration involves are unclear. The differences in output results between these two identical sensors highlights the

importance of calibration with a known standard prior to use, and further calibration using a secondary standard before sensors are deployed.

## 4.3 Robustness of sensor measurements

### *4.3.1 Effects of light on fluorescence measurements*

Phycocyanin and chlorophyll-*a* fluorescence of *Aphanizomenon* sp. CAWBG595 was measured under three treatments; one light and two dark (consisting of different shading material) to determine whether the presence of light interfered with the phycocyanin and chlorophyll-*a* fluorescence readings. Phycocyanin fluorescence was higher in the 'light' treatments, i.e., when samples were not shaded from ambient light, than the two shaded treatments. Thus fluorescence may be overestimated when ambient light is present. Investigations into whether the presence of natural and artificial light affect fluorescence measurements have been undertaken by Brient et al. [31]. Measurements of natural light ranged from 0 to 1900 µmol $m^{-2}$ $s^{-1}$ and 0 to 150 µ$m^{-2}$ $s^{-1}$ for artificial light. The fluorescence signal of phycocyanin in both an algal culture and a commercial standard was not modified by the presence or absence of natural light, however under artificial light fluorescence increased with increasing light intensity [31]. While the samples in the present study were not exposed to varying levels of light intensity, the results from the shading experiment are consistent with those of Brient et al. [31]. These results show that shading of the measurement vessel and sensor is required in the laboratory under artificial light. This is easily achieved by using a darkened container; covered with non-reflective black tape, black paint, or a non-reflective black plastic.

Studies also suggest that prior prolonged light exposure can result in photobleaching of phycobilin pigments reducing fluorescence and affecting absorption and fluorescence spectra [42; 43]. Zamyadi et al. [25] investigated the effects of prior light exposure on phycocyanin fluorescence using the cyanobacterial species *M. aeruginosa*. They determined that prior light exposure of up to 7 h had no effect on the *in vivo* measurements for their probe. Brient et al. [31] also showed prolonged natural light exposure of *Planktothrix agardhii*, of up to 1,900 µmol $m^{-2}$ $s^{-1}$ for 30 min had no effect on fluorescence. Contrary to this, Ma et al. [60] showed an increase in cellular phycocyanin and chlorophyll-*a*

occurred in the cyanobacterium *Nostoc sphaeroides* with higher light intensity (90 µmol $m^{-2}$ $s^{-1}$). Increases in cellular phycocyanin and chlorophyll-*a* content cause an increase in fluorescence, potentially leading to an overestimation of cyanobacteria biomass [61].

In the current experiment, light exposure was constant for 12 h of the day (90 µmol $m^{-2}$ $s^{-1}$) under a diurnal light regime, which may have increased the cellular pigment content, increasing fluorescence measurements. However changes in cellular content due to changes in irradiance are species specific, as Jones and Myers [62] showed that chlorophyll-*a* content decreased significantly under elevated irradiance in the species *Anacystis nidulans*. It is not possible to know in this experiment whether the increases in fluorescence were a result of changes to cellular content, as this was not extracted and measured. Despite some studies suggesting prior light exposure had no effect on fluorescence under laboratory conditions, light history may be an important parameter to consider in the field as some cyanobacteria have the ability to control buoyancy through gas vacuoles [1], which provides a competitive advantage over other photosynthetic organisms. This can allow exposure to sunlight for longer periods during the day [6] and thus there may be variations in fluorescence for a given biovolume between the morning and afternoon.

These data suggest that shading may be required when using phycocyanin sensors in the field to reduce ambient light. This is achievable by using a shade cap or shield which are included for some sensors (Turner CYCLOPS-7), or by taking measurements from a dark container. Shading of sensors may present more of an issue when fixed to monitoring buoys as additions such as shade caps may alter or prevent the natural flow of phytoplankton past the sensor. Additionally, more studies into the effect of varying light history on fluorescence readings should be undertaken in order for this to be accounted for.

No significant differences amongst light treatments were observed for chlorophyll-*a* measurements despite studies showing that chlorophyll-*a* fluorescence declines rapidly in bright light [63]. This is a result of non-

photochemical quenching where excess excitation energy is dissipated as heat [43]. This is particularly common in the field where non-photochemical quenching in response to ambient changes in light can present an apparent diurnal cycle showing less fluorescence during the day when oxygen is being produced, and more at night during the organisms resting phase [56]. This results in variation in fluorescence measurements, even if the actual phytoplankton content of the water is stable. Laboratory conditions were likely inadequate to mimic the conditions required for a response from chlorophyll-*a* similar to that expected in the field, particularly with respect to the bright light required to non-photochemical quenching [43; 63].

### *4.3.2 Boundary effects*

Changes in absorbance when light emission comes into contact with properties other than the sample i.e. the recipient boundary, can cause shading, scattering or re-absorption of light resulting in a non-linear response [39]. This was demonstrated by Kong et al. [26] and Brient et al. [31] who both showed decreases in fluorescence signal when sensors were held below 7 cm from the recipient boundary under agitated conditions for a number of species (*Dolichospermum* sp., *Cylindrospermopsis raciborskii, M. aeruginosa, Planktothrix agardhii,* and *Pseudanabaena* spp.) [26; 31]. Most manufacturers recommend holding the sensors a minimum of 8 cm from the boundary although reasoning for this is not given [36]. Contrary to this, with the exception of the YSI, there was no significant difference in phycocyanin fluorescence signal at 4 cm or 8 cm from the recipient boundary ($P > 0.05$). There was some variability in the YSI measurements for both phycocyanin and chlorophyll-*a* at distances between 8 and 12 cm; measurements at 4 cm above the boundary were not possible for the YSI due to the size of the probe. In previous experiments (see above; Section 4.2 repeatability) the YSI sensor was consistently more variable than other sensors, thus it is unclear whether these differences are legitimate, or whether it is due to expected variation in sensor measurements. The T928 sensor also showed a difference in chlorophyll-*a* measurements between 8 and 12 cm ($P < 0.05$). Although the difference was significant statistically, measurements differed by only 0.2 µg/L and would therefore not have a marked effect on the sensor measurements.

The cultures used in this experiment were not agitated as has been done in other studies [26; 31]. Agitation of the sample would prevent cells from settling and thus maintain a homogenous distribution. The lack of agitation in our experiments may have meant that the cells within the sample settled on the bottom of the beaker creating a dense layer. Although it is unclear how this would have reduced any boundary effects. In the current experiment a non-reflective surface was used as recommended by the manufacturer, and the beaker was shaded from ambient light following results from the previous light experiment (Section 4.3.1) [36]. This could have reduced the amount of residual light resulting in less variable fluorescence readings. A reflective surface would have otherwise created light scatter and fluorescence readings may have increased [34]. In the current experiment three distances were used to examine boundary effect, whereas in other studies measurements have been made at up to 20 distances of up to 35 cm. Manufacturer recommendations should be followed whilst awaiting more conclusive data and recommendations. Future work should include a wider range of distances under varying levels of agitation to develop a more definitive understanding of boundary effects on fluorescence.

### *4.3.3 Effects of temperature on fluorescence measurements*

A general trend of decreasing fluorescence with increasing temperature was observed in the present study. Phycocyanin fluorescence measurements from sensors were higher at 4 °C which is consistent with other studies. Kasinak et al. [33] found that extracting phycocyanin at 4 °C gave higher values than at warmer temperatures (23.5 °C), suggesting phycocyanin measurements are highly variable across a range of temperatures. However fluorescence values from the medium cell density experiment were relatively stable across temperature 13.8, 17 and 23.5 °C which may suggest an optimum sample density at which temperature changes have minimal effect on fluorescence. The fluorescence of protein pigments including phycocyanin and chlorophyll-*a* can be affected by changes in temperature; as temperature increases, fluorescence decreases [34]. This loss of fluorescence is due to an increase in molecular motion resulting in more collisions and a loss of energy [44]. Phycobilin pigments may degrade at elevated temperatures, reducing fluorescence [38]. In the New Zealand environment, most lake temperatures would not reach 4 °C, except for some inland, alpine South Island lakes [64]. At this

temperature, fluorescence readings are significantly higher than readings at warmer temperatures. This is likely to produce and overestimation of cyanobacteria biomass, especially since growth of cyanobacteria at this temperature would be low [65]. Average temperature in New Zealand for the period 1971-2000 are 10 °C in the South Islands, and 16 °C in the North Island, with these temperature varying throughout the year by up to 14 °C [66]. The temperature variability across time and space raises concerns regarding the accuracy of fluorescence measurements.

Ideally standards, blanks and samples should be measured at the same temperature to ensure accurate results across a study. This is possible in a laboratory setting and potentially with grab samples in the field being transported to controlled temperature conditions. However this would be challenging when sensors are deployed for long periods of time on monitoring buoys, and when results are required to be compared across studies where temperature is likely to vary. In these circumstances, the temperature at the time of sampling can be recorded and the sensor output corrected for changes in temperature at a later date. This would be feasible for monitoring buoys as they collect temperature data simultaneously.

Turner Designs [36] suggest an equation to correct samples to a standard temperature when chlorophyll-*a* fluorescence and temperature data are collected simultaneously. This correction factor was applied using the temperature data collected from the Turner chlorophyll-*a* sensor in the current study (Figure 13). The correction factor performed well at low and medium cell densities reducing the measured value to within the reference value (6.6 and 7.5 µg/mL respectively). The 17 °C treatment (Section 3.2.3) was used as the reference temperature, as this was the temperature that all other laboratory experiments were conducted under. The correction equation did not perform as well when adjusting for fluorescence measurements at higher temperatures (23.5 °C) and when cell density was high (828,000 cell/mL), as the measured values increased above the reference value (12.5 V). This suggests that adjusting values across various temperatures with differing cell concentration using one standard equation may not be applicable. The correction did not hold for higher cell concentrations and as such, more rigorous testing and further validation is required, especially if use is intended across species, as different species are likely to have varying temperature dependencies. Some field

fluorometers such as the Turner Designs (10-AU-005-CE), are capable of compensating for temperature automatically [34]. However based on the above results, caution should be taken when using this generalized approach and the use of blanks, standards and simultaneous temperature data is recommended. This will allow for sufficient data to be able to correct for changes in fluorescence with changes in temperature at a later date, using a correction that is not necessarily an inherent part of what the manufacturer does.

## 4.4 Relationship between fluorescence and species biovolume

Phycocyanin fluorescence from single-celled and filamentous cyanobacterial cultures grown in controlled laboratory conditions, showed strong linear relationships with total biovolume. This is consistent with previous studies which have also shown highly significant linear regressions ($R^2$ >0.9) between sensor measurements and cell density [23] or biovolume of monocultures ($R^2$ >0.7) [31]. These linear relationships confirm *in vivo* measurement of phycocyanin fluorescence as a useful indicator of cyanobacterial biovolume. A strong linear relationship was also observed for chlorophyll-*a* biovolume from single-celled and filamentous cyanobacterial cultures. Chlorophyll-*a* is a proxy for total phytoplankton biomass, and can also be a useful indicator of cyanobacteria biomass when this groups dominates the community assemblage [67]. Since only cyanobacteria were used in the laboratory we would expect a strong correlation between chlorophyll-*a* fluorescence and cyanobacteria biovolume. However in a typical field environment, measurement of chlorophyll-*a* fluorescence cannot distinguish between eukaryotic and prokaryotic cells. As well as this, in the current experiment, linear relationships between chlorophyll-*a* fluorescence and cyanobacteria biovolume were low, suggesting the sensitivity of this method is too low, and therefore phycocyanin will provide a better approach to determine cyanobacterial biomass.

Although strong relationships between sensor measurements and total biovolume were observed for most of the cyanobacteria species in the single culture experiment, poor relationships occurred when measuring fluorescence of the filamentous species *N. spumigena* with the YSI sensor, and filamentous species *Dolichospermum* sp. with the TriLux sensor. This may be related to the tendency

of filamentous species to form dense clumps especially in culture conditions. Dense samples or clumping can produce non-linear responses due to the surface cells in the sample absorbing the majority of the light, and leaving only a small amount available for cells in the inner parts of the sample [34]. This should apply to all sensors, however there may be differences in response due to variations in the light sources used between the sensors.

Field samples generally contain a diverse array of cyanobacteria species with varying cell biovolume as well as different chlorophyll-*a* and phycocyanin cellular content which influence the relative fluorescence [26]. While the single species in laboratory conditions in the present study generally showed a strong relationship between fluorescence measurements from sensors, and species biovolume, the mixed species assemblage did not. For a mixed assemblage, differing biovolume proportions, each with different phycocyanin yields, appear to influence the sensor measurements. Factors such as differing cell morphology and pigment content are likely to influence fluorescence also. This suggests that in the field, an understanding of the composition of the sample prior to measurements may be required in order for a correction to be made to compensate for the variations in phycocyanin. Calibrating the sensor with a known field sample may help to reduce the variability.

Contrary to these results, Brient et al. [31] demonstrated that in a suspension consisting of two cyanobacterial species (*Planktothrix agardhii* and *Lemmermanniella* sp.), fluorescence measurements were proportional to biovolume. In contrast to my study, this experiment, which was limited to only two species may have had similar phycocyanin yields in relation to biovolume, therefore returning a linear relationship. Kong et al. [26] tested a total of three cyanobacteria species, but only used a combination of *Cylindrospermopsis* and *Microcystis* or *Dolichospermum* and *Microcystis* at any one time. They found that regardless of mixed culture composition, phycocyanin concentration and biovolume were strongly correlated. A community assemblage consisting of two cyanobacterial species is likely to be an unrealistically simple situation compared with the field situation, and as such, the current experiment used three species. Although this also represents a simplified case study.

Phycocyanin was predicted from linear species regression equations summed, and compared to observed phycocyanin measured in the four mixed assemblages. The results showed that the predicted phycocyanin was overestimated compared to the observed values from the sensors. In a similar experiment, Kong et al. [26] showed that the observed phycocyanin measurements from a mixed culture of *Microcystis* and *Dolichospermum* sp. almost perfectly matched the predicted phycocyanin from pure culture ($R^2$ =0.99). In the current study, the predicted phycocyanin was based on linear regression from single species culture which were measured several weeks prior to this experiment being undertaken. Factors such as growth phase may affect the phycocyanin content of cells [28] and this may partly explain the differences in predicted and observed values. Further study should include measurements of phycocyanin content at each stage of the experiment and with careful control of the light exposure history.

Correlations between biovolume and phycocyanin or chlorophyll-*a* were poor for the colonial *Microcystis* for all sensors excluding the TriLux. Aggregations of cells into colonies likely reduce the penetration of light into the colonies and reduce the emission of fluorescence relative to biovolume. Chang et al. (2012) suggest light will not penetrate into the inner part of colonies larger than 13-18 µm. Thus cells deeper in the colony will not be excited, or if light is able to penetrate, the fluorescence may be substantially reduced, resulting in underestimation of phycocyanin and thus cyanobacterial biomass. Personal observations during the experiments showed that when large colonial aggregates drifted past the light source, fluorescence spiked. So while the lack of correlation between phycocyanin fluorescence and biovolume may be a result of the light being unable to penetrate the colony, it may also be due to the non-homogenous distribution of cells throughout the sample as a result of the species morphology.

Chang et al. (2012) performed a simple experiment to determine whether the disaggregation of colonies had any effect on phycocyanin measurements. They found that phycocyanin fluorescence increased after colonies were dispersed and the larger the colony, the greater the increase in phycocyanin fluorescence. This experiment was recreated in the present study with a similar range of colony sizes (50-250 µm diameter). There was an increase in phycocyanin when colonies were

disaggregated, but only in some of the colony size classes. Similar to Chang et al. [28], the largest colony size class (250 µm) yielded the greatest relative increase in phycocyanin measurements when disaggregated (9.9% and 10.4%). A different colonial Microcystis sp. was used for this particular experiment (CAWBG11) as opposed to the initial colonial species used in all other experiments in the laboratory (CAWBG563). The species used in the current experiment (CAWBG11) forms much smaller aggregations or colonies which are dispersed more homogenously throughout the water column. Therefore, the results of this experiment may have offered more insight into the understanding of the impacts of colony disaggregation, had the original species (CAWBG563) been used.

During the experiment, it was clear that the lower size classes (50 -102 µm) were too small for any colonies to pass through, and that the majority of the colonies present were larger than 100 µm. Due to the lack of mesh sizes, there was a smaller spread of colony size classes. This experiment should be repeated with the original colonial species across a wider range of colony size classes to obtain a better understanding of the impact of colony disaggregation on measurements of phycocyanin fluorescence. There is much evidence to suggest that the presence of colonies has an effect on fluorescence readings [26; 28; 39; 65] and causes underestimation of cyanobacterial biomass in the field. Chang et al. [28] proposed a model to correct for the underestimation of biovolume by sensor measurements due to the aggregation of spherical colonies. The model had inputs of intercellular porosity of a colony and the penetration distance of the emitting light. However, this model yields cell number and not biovolume, which as discussed previously is not ideal. The authors suggest that the model can be used in the field for determining cell numbers in *Microcystis* blooms.

It has also been suggested that to avoid potential variability of fluorescence as a result of influences of morphology, an ultrasonic device be coupled to fluorometers to disaggregate colonies and filaments and even cause cell lysis [23]. However, if not all cells lysed i.e., if colonial morphology was simply disaggregated but cells remained intact, the fluorescence would consist of both intra and extracellular phycocyanin. This could cause overestimations in the field, as extracellular phycocyanin has been shown to account for a significant proportion of total

phycocyanin fluorescence (34%; Figure 15b), and there would be no way of distinguishing extracellular phycocyanin from naturally lysed cells, and extracellular phycocyanin from manual lysing of cells.

The margin of error associated with cyanobacterial cell counts is considered to be ± 20-30% [68]. Obtaining reliable estimates of abundance of colonial cyanobacteria such as *Microcystis* is problematic due to their morphology and tendency to form dense three dimensional aggregates of cells (i.e. colonies). Estimates of abundance for some filamentous species such as *Aphanizomenon*, *Cylindrospermopsis* and *Planktothrix* can also be compromised as cells in trichomes can be poorly defined i.e. it can be difficult to determine the boundaries of a cell, making accurate counting difficult [46]. In my study, errors in cell counts are likely to have occurred in samples containing filamentous and colonial species, and this may have contributed to non-linearity or variability in relationships between phycocyanin fluorescence from sensors, and species biovolume.

## 4.5 Validation of fluorescence sensors in the field

The accuracy of phycocyanin and chlorophyll-*a* sensors in the field was assessed by comparing the fluorescence measurements from sensors (converted via calibration to units of phycocyanin or chlorophyll-*a* concentration µg/L), to spectrophotometer measurements of extracted phycocyanin and chlorophyll-*a* (µg/L). A strong relationship was observed between spectrophotometer and *in vivo* fluorescence measurements of phycocyanin in this study, which is consistent with findings of previous studies [67; 69]. The precision and accuracy of *in vivo* fluorescence measurements can be influenced by sources of interference, such as turbidity and the presence of other algae, which are less likely to influence the spectroscopy method. Spectrophotometry also has issues of interference as many materials can absorb light making it difficult to target the specific pigment in a sample. Fluorometric methods can achieve 1000 to 500,000 times more accurate detection than spectrophotometers [34]. While no significant differences were found between the two methods, the use of the *in vivo* fluorescence is a highly practical and robust alternative to the standard spectroscopy method.

Measurements of chlorophyll-*a* concentration from spectrophotometry were significantly higher than measurements of chlorophyll-*a* from the sensors, and no significant relationship was observed. Zamyadi et al. [67] observed high concentrations of chlorophyll-*a* extracted in laboratory cultures of *M. aeruginosa* (2-102 µg $L^{-1}$), but low RFU values were measured using the chlorophyll-*a* sensor (0.1-1.2 RFU). They determined that this could be due to the fact that the sensor targeted a specific photosynthetic pathway within the phytoplankton; photosystem II, where only around 10-20% of the total pigment is located, as opposed to photosystem I which is highly efficient and holds 80-90% of the chlorophyll-*a* pigment but is weakly fluorescent. Therefore the total cyanobacterial chlorophyll-*a* content was underestimated by the sensor as only the pigments in photosystem II were excited and detected [67]. Zamyadi et al. [67] observed a similar relationship between high chlorophyll-*a* concentrations and low measurements of RFU in field samples, as did Seppala et al. [55]. In the current study, fluorescence measurements were calibrated to concentrations of chlorophyll-*a* to be directly compare to spectrophotometer readings. This demonstrated that the use of chlorophyll-*a* sensors significantly underestimated the total chlorophyll-*a* concentration.

Field measurements from the phycocyanin sensors in the present study were highly correlated with cyanobacterial biovolume as has been demonstrated in previous research [23; 26; 31; 67]. Kong et al. [26] demonstrated that during periods of low phytoplankton biomass and stable community structure (dominated by *Pseudanabaena*, 80-99%), there was a strong correlation between phycocyanin fluorescence and cyanobacterial biovolume. A decrease in *Pseudanabaena* from 94% biomass to 24% and an increase in *Cylindrospermopsis* from 4-72%, decreased the correlation (R2 =0.9, 0.8 respectively). In the current study, samples were taken within one day (9 h) as opposed to the study by Kong et al. [26] which was conducted over one year. The phytoplankton assemblage was dominated by *Dolichospermum* (>90%). The field validation from this study suggests that phycocyanin sensors are a suitable method for monitoring cyanobacterial biomass, although future work should evaluate the sensors over a wider range of water bodies with varying proportions and densities of species, and across varying periods of time. It is important to note that while phycocyanin sensors can distinguish

eukaryotic from prokaryotic algae, it is not possible to provide any information on which species are present, nor the presence of cyanotoxins [23; 37]. However the use of phycocyanin fluorescence can be used to inform decisions on whether analysis for the presence of cyanotoxins should be under taken, for example if cyanobacterial biomass is high.

### *4.5.1 Influences of extracellular phycocyanin on fluorescence measurements*

Previous studies have shown that high fluorescence values do not always correlate with cyanobacterial cell counts, likely due to the presence of phycocyanin in solution as a result of cell lysis. Phycocyanin sensors do not differentiate between intra and extracellular phycocyanin [23; 31]. In the present study, the average proportion of extracellular phycocyanin across 30 sites was 6-8%, however at some sites where the total biovolume was higher (17-27 $mm^3/L$), extracellular phycocyanin accounted for more than 20% of the sensor measurement. Natural lysis of cells occurs due to the breakdown of the cell wall as a result of high light intensity [70], natural breakdown of cyanobacterial cells, and contact and parasitism with other phytoplankton [71]. Bastien et al. [23] demonstrated that 21-25% of the measured fluorescence signal from phycocyanin sensors was related to extracellular phycocyanin. In some samples, the entire fluorescence signal was attributed to phycocyanin lysed from cells. The presence of phycocyanin in solution can result in overestimation of cyanobacterial biomass, in particular when actual biomass is high such as in the breakdown of a bloom. To avoid an overestimation in results, sensor measurements should be treated as total phycocyanin concentration and a field blank obtained from filtered samples can be measured for fluorescence and this reading subtracted from the total to determine intracellular phycocyanin [23].

# 5 Conclusions and recommendations for further research

This study demonstrated that phycocyanin sensors are a suitable method for monitoring cyanobacteria biomass in the field, however there are sources of interference. This study also highlighted the importance of calibration of sensors with a known standard to ensure that sensor repeatability is within an acceptable range and to ensure robust data through time, between sites and across studies. Outputs of relative fluorescence units (RFU) are recommended and direct sensor output units of cell/mL and µg/L should not be relied upon for representative concentrations of phycocyanin or chlorophyll-*a*. However, these outputs can be transformed to informative measures through calibration using a known standard.

One of the sources of interference was the presence of ambient light which influenced sensor measurements in the laboratory. It is therefore recommended that shading be used when measuring samples in the field. Further study on the effects of natural light and the influence of light history on fluorescence should be undertaken. In this study, boundary effects were not a source of interference, however manufacturer recommendations that sensors be held at least 7 cm from a recipient boundary should be followed.

This study indicated that increases in temperature reduced fluorescence measurement. However it did not have the same effect on all cell concentrations which may suggest an optimum sample density at which temperature changes have minimal effect on fluorescence. However more study is required to determine this. As a result of the variation in fluorescence with temperature, blanks and standards should be measured at the same temperature alongside sensor measurements. Measurements of phycocyanin fluorescence from monitoring buoys could be corrected by taking simultaneous measurements of temperature. However caution must be taken when using correction equations from manufacturers, and more rigorous studies and validation are needed.

Phycocyanin sensors showed a strong linear relationship with cyanobacterial biomass for solitary cultured species, while poor relationships were observed

between phycocyanin fluorescence of colonial and filamentous species and biovolume for some sensors, resulting in an underestimation of cyanobacterial biomass. To account for the loss of fluorescence in filamentous and colonial assemblages, a correction equation may be applied, or a method of manual disaggregation of filaments or colonies could be undertaken prior to measurement. However, this is unlikely to be feasible when sensors are deployed in the field on buoys for example. Further study is needed to determine the effects of species morphology, including spherical colonies, filaments and mats, on fluorescence.

Results from the mixed species experiment indicate that fluorescence measurements in a mixed assemblage may not be proportional to cyanobacterial biomass due to differences in phycocyanin yield between species. An understanding of the composition of the sample may be required to compensate for the variations in phycocyanin. It is recommended that this study be repeated to further determine the effects of multiple cyanobacteria species. Fluorescence of individual species should be measured prior to the samples being mixed, and the phycocyanin content of each species should also be determine. Total biovolume for each sample should be equal, but the proportion of each species should differ.

Phycocyanin fluorescence measurements from field samples were strongly correlated with total phytoplankton and cyanobacterial biovolume, indicating their potential for field applications. However, at the time of sampling the community assemblage was dominated by *Dolichospermum* sp. and as such, further studies are required to validate sensor response in the field using a wider range of lakes, or identical sites at different times of the year, with differing species assemblages as well as sampling before and during a bloom event. Finally, this study showed that extracellular phycocyanin can account for more than 20% of the total fluorescence signal, which if not corrected for would result in overestimations of cyanobacterial biomass. Therefore a second phycocyanin reading from filtered samples should be taken and subtracted from the original sample to correct for extracellular phycocyanin.

Overall the use of phycocyanin sensors presents a valid approach to monitoring cyanobacterial biomass in natural environments and the relationships observed

between cyanobacterial biomass and phycocyanin fluorescence were significant. However laboratory studies showed that interferences do occur from biological parameters causing variations in sensor measurements. These sources can be mitigated by shading sensors in the field, measuring samples and standards at the same temperature, or taking sample and temperature measurements simultaneously and following manufacturer recommendations. It is important that interferences are understood by the user and it is recommended that experimental work be undertaken to determine responses to temperature, species, morphology, as well as regular field calibration to account for changes in species assemblages.

# 6 References


[1] Whitton, B., & Potts, M. (2012). *Ecology of cyanobacteria II: Their diversity in space and time*. Introduction to the cyanobacteria (B.A Whitton ed., Vol. 2). New York: Springer.

[2] Merel, S., Walker, D., Chicana, R., Snyder, S., Baurès, E., & Thomas, O. (2013). State of knowledge and concerns on cyanobacterial blooms and cyanotoxins. *Environment International, 59*, 303-327.

[3] Drikas, M., Chow, C. W. K., House, J., & Burch, M. D. (2001). Using coagulation, flocculation, and settling to remove toxic cyanobacteria. *Journal of American Water Works Association, 93*, 100-111.

[4] Paerl, H. W., & Fulton III, R. S. (2006). Ecology of harmful cyanobacteria. In E. Graneli & J. Turner (Eds.), *Ecology of Harmful Marine Algae* (pp. 95-107). Berlin: Springer.

[5] Paerl, H. W., & Huisman, J. (2008). Climate - blooms like it hot. *Science, 320*, 57-58.

[6] Oliver, R. L., & Gnaf, G. G. (2000). Freshwater blooms. In B. A. Whitton & M. Potts, *The ecology of cyanobacteria: their diversity in time and space* (pp. 149-194). Netherlands: Kluwer Academic.

[7] Rastogi, R. P., Sinha, R. P., & Incharoensakdi, A. (2014). The cyanotoxin-microcystins: current overview. *Reviews in Environmental Science and Biotechnology, 13*, 215-249.

[8] Havens, K. E. (2008). Cyanobacteria blooms: effects on aquatic ecosystems. In N. Back, A. Lajtha & R. Paoletti, *Cyanobacterial Harmful Algal Blooms: State of the Science and Research Needs.* (Vol. 619, pp. 733-747). Berlin, Germany: Springer-Verlag.

[9] Jones, B. I. (1987). Lake Okeechobee eutrophication research and management. *Aquatics, 9*, 21-26.

[10] Codd, G., Morrison, A., Louise, F., & Metcalf, J. S. (2005). Cyanobacterial toxins: risk management for health protection. *Toxicology and Applied Pharmacology, 203*, 264-272.

[11] Sivonen, K., & Jones, G. (1999). Cyanobacterial toxins. In I. Chorus & J. Bartram (Eds.), *Toxic cyanobacteria in water* (pp. 55-124). London: E & FN Spon.

[12] Tillett, D., Dittmann, E., Erhard, M., von Dohren, H., Borner, T., & Neilan, B. A. (2000). Structural organisation of microcystin biosynthesis in *Microcystis aeruginosa* PCC7806: an integrated peptide-polyketide synthetase system. *Chemistry & Biology, 7*, 753-764.

[13] Jochimsen, E. M., Carmichael, W. W., An, J., Cardo, D. M., Cookson, S. T., Holmes, C. E. M., Antunes, M. B., de Melo Filho, D. A., Lyra, T. M., Barreto, V. S. T., Azevedo, S. M. F. O., & Jarvis, W. R. (1998). Liver failure and death after exposure to Microcystins at a hemodialysis center in Brazil. *New England Journal of Medicine, 338*, 873-878.

[14] Codd, G. A., Edwards, C., Beattie, K. A., Barr, W. M., & Gunn, G. J. (1992). Fatal attraction to cyanobacteria? *Nature, 359*, 110-111.

[15] Francis, G. (1878). Poisonous Australian lake. *Nature, 18*, 11-12.

[16] Thomas, A. D., Saker, M. L., Norton, J. H., & Olsen, R. D. (1998). Cyanobacterium *Cylindrospermopsis raciborskii* as a probable cause of death in cattle in northern Queensland. *Australian Veterinary Journal*, 76, 592-594.

[17] Falconer, I. (1998). Algal Toxins and Human Health. In J. Hrubec, *Quality and Treatment of Drinking Water II* (Vol. 5, pp. 53-82). Springer Berlin Heidelberg.

[18] Edwards, C., Beattie, K. A., Scrimgeour, C. M., & Codd, G. A. (1992). Identification of anatoxin-A in benthic cyanobacteria (blue-green algae) and in associated dog poisonings at Loch Insh, Scotland. *Toxicon, 30*, 1165-1175.

[19] Gunn, G. J., Rafferty, A. G., Rafferty, G. C., Cockburn, N., Edwards, C., Beattie, K. A., & Codd, G. A. (1992). Fatal canine neurotoxicosis attributed to blue-green algae (cyanobacteria). *Veterinary Record, 130*, 301-302.

[20] Nehring, S. (1993). Mortality of dogs associated with a mass development of *Nodularia spumigena* (Cyanophyceae) in a brackish lake at the German North Sea coast. *Journal of Plankton Research, 15*, 867-872.

[21] Grauer, F. H., Arnold, H. L., & Jr. (1961). Seaweed dermatitis: First report of a dermatitis-producing marine alga. *Archives of Dermatology, 84*, 720-732.

[22] Pilotto, L. S., Douglas, R. M., Cameron, S., Rouch, G. J., Cowie, C. T., Beers, M., Robinson, P., Kirk, M., Burch, M. D., Hardiman, S., Moore, C., & Attewell, R. G. (1996). Health effects of exposure to cyanobacteria (blue-green algae) during recreational water-related activities. *Australia, New Zealand Journal of Public Health, 6*, 562-566

[23] Bastien, C., Cardin, R., Veilleux, E., Deblois, C., Warren, A., & Laurion, I. (2011). Performance evaluation of phycocyanin probes for the monitoring of cyanobacteria. *Journal of Environmental Monitoring, 13*, 110-118.

[24] Chorus, I., & Bartram, J. (Eds.). (1999). *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management* (1 ed.). London: E & FN Spon.

[25] Zamyadi, A., McQuaid, N., Dorner, S., Bird, D. F., Burch, M., Baker, P., Hobson, P., & Prevost, M. (2012). Cyanobacterial detection using *in vivo* fluorescence probes: Managing interferences for improved decision-making. *American Water Works Association*, 104, E466-E479

[26] Kong, Y., Lou, I., Zhang, Y., Lou, C. U., & Mok, K. M. (2014). Using an online phycocyanin fluorescence probe for rapid monitoring of cyanobacteria in Macau freshwater reservoir. *Hydrobiologia*, *741*, 33-49.

[27] Beutler, M., Wiltshire, K. H., Meyer, B., Moldaenke, C., Lüring, C., Meyerhöfer, M., Hansen, U. P., & Dau, H. (2002). A fluorometric method for the differentiation of algal populations *in vivo* and *in situ*. *Photosynthesis Research, 72*, 39-53.

[28] Chang, D.-W., Hobson, P., Burch, M., & Lin, T.-F. (2012). Measurement of cyanobacteria using *in-vivo* fluoroscopy - Effect of cyanobacterial species, pigments, and colonies. *Water Research, 46*, 5037-5048.

[29] Lee, T., Tsuzuki, M., Takeuchi, T., Yokoyama, K., & Karube, I. (1994). *In vivo* fluorometric method for early detection of cyanobacterial waterblooms. *Journal of Applied Phycology*, *6*, 489-495.

[30] Gobler, C. J., Davis, T. W., Coyne, K. J., & Boyer, G. L. (2007). The interactive influences of nutrient loading and zooplankton grazing on the growth and toxicity of cyanobacteria blooms in a eutrophic lake. *Harmful Algae, 6*, 119-133.

[31] Brient, L., Lengronne, M., Bertrand, E., Rolland, D., Sipel, A., Steinmann, D., Baudin, I., Legeas, M., Le Rouzie, B., & Bormans, M. (2008). A phycocyanin probe as a tool for monitoring cyanobacteria in freshwater bodies. *Journal of Environmental Monitoring, 10*, 248-255.

[32] Song, K., Li, L., Tedesco, L., Clercin, N., Hall, B., Li, S., Liu, D., & Sun, Y. (2013). Remote estimation of phycocyanin (PC) for inland waters coupled with YSI PC fluorescence probe. *Environmental Scientific Pollution Research, 20*, 5330-5340.

[33] Kasinak, J.-M., Holt, B. M., Chislock, M. E., & Wilson, A. E. (2015). Benchtop fluorometry of phycocyanin as a rapid approach for estimating cyanobacterial biovolume. *Journal of Plankton Research, 37*, 248-257.

[34] Turner Designs. (2015). *Technical note: An introduction to fluorescence measurements*. Retrieved from http://www.turnerdesigns.com.

[35] Lorenzen, C. J. (1967). Determination of chlorophyll and pheopigments: spectrophotometric equations. *Limnology and Oceanography, 12*, 343-346.

[36] Turner Deisgns. (2007). *CYCLOPS-7 Submersible sensors user's manual*. Sunnyvale, California: Turner Designs.

[37] Izydorczyk, K., Tarczynska, M., Jurczak, T., Mrowczynski, J., & Zalewski, M. (2005). Measurement of phycocyanin fluorescence as an online early

warning system for cyanobacteria in reservoir intake water. *Environmental Toxicology, 20*, 425-430.

[38] Moreth, C. M., & Yentsch, C. S. (1970). A sensitive method for the determination of open ocean phytoplankton and phycoerythrin pigments by fluorescence. *Limnology and Oceanography, 15*, 313-317.

[39] Gregor, J., & Marsalek, B. (2004). Freshwater phytoplankton quantification by chlorophyll *a*: a comparative study of *in vitro*, *in vivo* and *in situ* methods. *Water Research, 38*, 517-522.

[40] Gregor, J., Marsalek, B., & Sipkova, H. (2007). Detection and estimation of potentially toxic cyanobacteria in raw water at the drinking water treatment plant by *in vivo* fluorescence method. *Water Research, 41*, 228-234.

[41] McQuaid, N., Zamyadi, A., Prevost, M., Bird, D. F., & Dorner, S. (2011). Use of *in vivo* phycocyanin fluorescence to monitor potential microcystin-producing cyanobacterial biovolume in a drinking water source. *Journal of Environmental Monitoring, 13*, 455-463.

[42] Donkor, V. A., & Hader, D. P. (1996). Effects of ultraviolet irradiation on photosynthetic pigments in some filamentous cyanobacteria. *Aquatic Microbial Ecology, 11*, 143-149.

[43] Muller, P., LI, X. P., & Niyogi, K. K. (2001). Non-Photochemical quenching. A response to excess light energy. *Plant Physiology, 125*, 1558-1566.

[44] Guilbault, G. G. (1990). General aspects of luminescene spectroscopy. In G. G. Guilbault (Ed.), *Practical Fluorescence, Second Edition*. New York: Marcel Dekker Inc.

[45] Bolch, C. S., & Blackburn, S. (1996). Isolation and purification of Australian isolates of the toxic cyanobacterium *Microcystis aeruginosa* Kutz. *Journal of Applied Phycology, 8*, 5-13.

[46] Newcombe, G. (2009). Development and implementation of a monitoring program. In G. Newcombe (Ed.), *International guidance manual for the management of toxic cyanobacteria*. London, United Kingdom: Global Water Research Coalition.

[47] Olenina, I., Hadju, S., Edler, L., Anderson, S., Wasmund, N., Busch, S., Gobel, J., Gromisz, S., Huseby, S., Huttunen, M., Jaanus, A., Kokkonen, P., Ledaine, I., & Niemkiewicz, E. (2006) Biovolumes and size-classes of phytoplankton in the Baltic Sea. *HELCOM Balt. Sea Environ. Proc. No. 106* (pp. 144).

[48] Wood, S. A., Hamilton, D. P., Paul, W. J., Safi, K. A., & Williamson, W. M. (2009). *New Zealand guidelines for managing cyanobacteria in recreational fresh waters - interim guidelines.* . Wellington: Ministry for the Environment.

[49] Entwisle, T. J., Sonneman, J. A., & Lewis, S. H. (1997). *Freshwater algae in Australia: a guide to conspicuous genera.* Potts Point, NSW, Australia: Sainty & Associates.

[50] Landcare Research. (2015). *Identification Guide*. Retrieved from http://www.landcareresearch.co.nz/resources/identification/algae/identification-guide/identify.

[51] Bennett, A., & Bogorad, L. (1973). Complementary chromatic adaptation in a filamentous blue-green alga. *The Journal of Cell Biology, 58*, 419-435.

[52] YSI Environmental. (2005). *Alternate calibration methods for the YSI 6025 chlorophyll sensor.* Retrieved from http://www.ysi.com

[53] Puddick, J., Prinsep, M. R., Wood, S. A., Kaufononga, S. A., Cary, S. C., & Hamilton, D. P. (2014). High levels of structural diversity observed in microcystins from *Microcystis* CAWBG11 and characterization of six new microcystin congeners. *Marine Drugs, 12*, 5372-95.

[54] Turner Designs. (2016). Retrieved from www.turnerdesigns.com/component/content/article/155-general-fluorometer-faqs/718-fluorescence-temperature-sensitive.

[55] Seppala, J., Ylostalo, P., Kaitala, S., Hallfors, S., Raateoja, M., & Maunula, P. (2007). Ship-of-opportunity based phycocyanin fluorescence monitoring of the filamentous cyanobacteria bloom dynamics in the Baltic Sea. *Estuarine, Coastal and Shelf Science, 73*, 489-500.

[56] Hambrook Berkman, J. A., & Canova, M. G. (2007). *Algal biomass indicators.* U.S. Geological Survey.

[57] Richardson, T. L., Lawrenz, E., Pinckney, J. L., Guajardo, R. C., Walker, E. A., Paerl, H. W., & MacIntyre, H. L. (2010). Spectral fluorometric characterization of phytoplankton community composition using the Algae Online Analyser ®. *Water Research, 44*, 2461-2472.

[58] Alpine, A. E., & Cloern, J. E. (1985). Differences in *in vivo* fluorescence yield between three phytoplankton size classes. *Journal of Plankton Research, 7*, 381-390.

[59] Zimba, P. V., & Gitelson, A. A. (2006). Remote estimation of chlorophyll concentration in hyper-eutrophic aquatic systems: Model tuning and accuracy optimization. *Aquaculture, 256*, 272-286.

[60] Ma, R., Lu, F., Bi, Y., & Hu, Z. (2015). Efects of light intensity and qualilty on phycobiliprotein accumulations in the cyanobacterium *Nostoc sphaeroides* Kützing. *Biotechnology Letters, 37*.

[61] Campbell, D., Hurry, V., Clarke, A. K., Gustafsson, P., & Öquist, G. (1998). Chlrophyll fluorescence analysis of cyanobacterial photosynthesis and acclimation. *Microbiology and Molecular Biology Reviews, 62*, 667-683.

[62] Jones, L. W., & Myers, J. (1965). Pigment variations in *Anacystis nidulans* induced by light of selected wavelengths. *Journal of Phycology, 1*, 7-14.

[63] Cullen, J. J., & Lewis, M. R. (1995). Biological processes and optical measurements near the sea surface: some issues relevant to remote sensing. *Journal of Geophysical Research, 100*, 13,55-13,266.

[64] Zink, K.-G., Vandergoes, M. J., Mangelsdorf, K., Dieffenbacher-Krall, A. C., & Schwark, L. (2010). Application of bacterial glycerol dialkyl glycerol tetraethers (GDGTs) to develop modern and past temperature estimates from New Zealand lakes. *Organic Geochemistry, 41*, 1060-1066.

[65] Robarts, R. D., & Zohary, T. (1984). *Microcystis aeruginosa* and underwater light attenuation in a hypereutrophic lake (Hartbeespoort Dam, South Africa). *Journal of Ecology, 72*, 1001-1017.

[66] NIWA. (2013). *Overview of New Zealand climate*. Retrieved from https://www.niwa.co.nz/education-and-training/schools/resources/climate/overview.

[67] Zamyadi, A., McQuaid, N., Prevost, M., & Dorner, S. (2012). Monitoring of potentially toxic cyanobacteria using an online multi-probe in drinking water sources. *Journal of Environmental Monitoring, 14*, 579-588.

[68] Lawton, L., Marsalek, B., Padisák, J., & Chorus, I. (1999). Determination of cyanobacteria in the laboratory. In I. Chorus & J. Bartram (Eds.), *Toxic cyanobacteria in water: A guide to their public health consequences, monitoring and management*. London: E & FN Spon.

[69] Ahn, C.-Y., Joung, S.-H., Yoon, S.-K., & Oh, H.-M. (2007). Alternative alert system for cyanobacterial bloom, using phycocyanin as a level determinant. *The Journal of Microbiology, 45*(2), 98-104.

[70] Fallon, R. D., & Brock, T. D. (1979). Lytic organisms and photooxidative effects: influence on blue-green algae (cyanobacteria) in Lakw Mendota, Wisconsin. *Applied and Environmental Microbiology, 38*, 499-505.

[71] Gumbo, J. R., & Cloete, T. E. (2011). Light and electron microscope assessment of the lytic activity of *Bacillus* on *Microcystis aeruginosa*. *African Journal of Biotechnology, 10*, 8054-8063.

# 7 Appendices

**Appendix 1. Phycocyanin fluorescence values from sensors (µg/L) from field samples for *in situ* and grab samples.**

| **Sensor** | **YSI** | | **T926** | | **T927** | |
|---|---|---|---|---|---|---|
| **Site #** | In situ | Grab | In situ | Grab | In situ | Grab |
| **1** | 550 | 744 | 541 | 620 | 461 | 529 |
| **2** | 570 | 591 | 570 | 463 | 474 | 415 |
| **3** | 552 | 857 | 547 | 616 | 479 | 566 |
| **4** | 566 | 564 | 512 | 477 | 451 | 457 |
| **5** | 578 | 635 | 544 | 478 | 447 | 450 |
| **6** | 581 | 820 | 525 | 656 | 456 | 639 |
| **7** | 564 | 575 | 543 | 485 | 427 | 453 |
| **8** | 582 | 572 | 532 | 464 | 457 | 441 |
| **9** | 559 | 586 | 562 | 487 | 473 | 470 |
| **10** | 592 | 649 | 574 | 531 | 490 | 511 |
| **11** | 581 | 591 | 546 | 476 | 452 | 479 |
| **12** | 613 | 621 | 526 | 484 | 465 | 473 |
| **13** | 594 | 570 | 527 | 445 | 437 | 442 |
| **14** | 580 | 571 | 5.0 | 463 | 436 | 439 |
| **15** | 550 | 561 | 475 | 442 | 431 | 423 |
| **16** | 565 | 522 | 492 | 435 | 441 | 427 |
| **17** | 561 | 560 | 481 | 440 | 428 | 427 |
| **18** | 527 | 491 | 465 | 397 | 402 | 399 |
| **19** | 595 | 569 | 458 | 443 | 382 | 414 |
| **20** | 712 | 2,048 | 787 | 1,594 | 858 | 1,284 |
| **21** | 496 | 523 | 461 | 453 | 375 | 448 |
| **22** | 311 | 262 | 354 | 257 | 275 | 250 |
| **23** | 303 | 290 | 209 | 263 | 261 | 258 |
| **24** | 216 | 245 | 219 | 219 | 191 | 221 |
| **25** | 392 | 334 | 284 | 281 | 249 | 509 |
| **26** | 280 | 254 | 224 | 233 | 191 | 233 |
| **27** | 354 | 235 | 272 | 215 | 225 | 218 |
| **28** | 335 | 214 | 232 | 190 | 195 | 187 |
| **29** | 536 | 209 | 208 | 196 | 179 | 192 |
| **30** | 256 | 255 | 275 | 195 | 221 | 191 |

**Appendix 2. Chlorophyll-*a* fluorescence values from sensors (µg/L) from field samples for *in situ* and grab samples.**

| Sensor | YSI | | T928 | | Seapoint | |
|---|---|---|---|---|---|---|
| **Site #** | In situ | Grab | In situ | Grab | In situ | Grab |
| **1** | 105.7 | 138.6 | 55.2 | 61.4 | 81.7 | 65.5 |
| **2** | 106.5 | 105.3 | 61.4 | 61.4 | 76.4 | 76.1 |
| **3** | 138.5 | 124.5 | 74.5 | 81.3 | 74.6 | 84.6 |
| **4** | 118.0 | 112.0 | 56.0 | 68.2 | 83.1 | 74.5 |
| **5** | 175.2 | 131.2 | 70.4 | 72.8 | 95.0 | 87.5 |
| **6** | 43.8 | 109.6 | 58.7 | 60.4 | 62.2 | 58.7 |
| **7** | 120.3 | 104.7 | 67.5 | 62.4 | 84.2 | 66.7 |
| **8** | 106.8 | 113.5 | 52.8 | 66.1 | 84.1 | 64.3 |
| **9** | 89.1 | 116.9 | 46.9 | 69.4 | 65.8 | 70.6 |
| **10** | 101.0 | 100.9 | 65.3 | 62.9 | 73.5 | 64.6 |
| **11** | 44.6 | 105.2 | 59.8 | 63.9 | 95.0 | 75.8 |
| **12** | 98.1 | 89.5 | 40.0 | 48.2 | 48.1 | 60.7 |
| **13** | 99.6 | 106.1 | 43.1 | 59.4 | 66.8 | 68.7 |
| **14** | 110.2 | 100.9 | 50.1 | 57.6 | 63.4 | 78.3 |
| **15** | 104.4 | 106.6 | 48.2 | 61.4 | 69.9 | 69.9 |
| **16** | 104.3 | 95.1 | 47.0 | 52.7 | 77.1 | 55.3 |
| **17** | 96.3 | 103.7 | 44.5 | 52.8 | 61.1 | 67.0 |
| **18** | 104.5 | 103.4 | 41.7 | 59.1 | 74.3 | 61.1 |
| **19** | 100.9 | 109.4 | 53.0 | 65.6 | 72.8 | 75.7 |
| **20** | 139.6 | 124.0 | 70.4 | 68.2 | 69.9 | 55.3 |
| **21** | 109.4 | 92.4 | 43.2 | 49.6 | 74.3 | 58.2 |
| **22** | 114.5 | 92.4 | 47.5 | 52.0 | 84.4 | 67.0 |
| **23** | 107.5 | 104.7 | 40.1 | 57.3 | 80.1 | 71.3 |
| **24** | 124.0 | 105.2 | 54.6 | 60.1 | 65.5 | 72.8 |
| **25** | 181.8 | 122.2 | 56.7 | 66.7 | 101.9 | 85.9 |
| **26** | 85.4 | 83.3 | 35.5 | 47.6 | 74.2 | 75.7 |
| **27** | 425.9 | 95.0 | 43.1 | 55.0 | 49.9 | 69.6 |
| **28** | 155.4 | 116.6 | 47.7 | 61.8 | 11.4 | 64.5 |
| **29** | 211.7 | 82.9 | 39.1 | 47.7 | 59.8 | 72.9 |
| **30** | 112.1 | 112.0 | 39.4 | 39.4 | 58.4 | 62.6 |

**Appendix 3. Biovolume proportion of cyanobacterial species in field samples.**