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College of Engineering and Science

Improvement of spectral analysis sensitivity for detecting cyanobacteria in low concentrations in freshwater containing interfering NOM and other microorganisms

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ABSTRACT

Phycocyanin is an accessory pigment that aids photosynthesis by capturing photons of light in cyanobacteria cultures. Cyanobacteria cultures, often referred to incorrectly as “blue-green algae”, are aqueous bacteria that can produce toxins based on the strain type and gene properties. The toxins produced have reportedly killed livestock and are potentially carcinogenic. Detectable by fluorescence along with chlorophyll-a and some fractions of natural organic matter, phycocyanin is specific to cyanobacteria cell cultures. Detection of phycocyanin in water sources can specifically relate to the presence of cyanobacteria cultures. To determine the concentration of cyanobacteria at up to 2,000 cells/mL in sampled waters, a device was required to isolate cyanobacteria cells, extract the phycocyanin pigment and concentrate phycocyanin to produce a more detectable positive emission signal. This device was known as the Isolated Pigment Analyser (IPA).

Cyanobacteria cultures used in this study were *Microcystis aeruginosa, Anabaena circinalis* and *Cylindrospermopsis raciborskii*. Green algae cultures used in this study were *Chlorella vulgaris* and *Chlamydomonas reinhardtii*.

Cyanobacteria isolation was performed by the filtration of intact cyanobacteria cells on 0.45 μm membrane filters. Tubular membranes with a pore size of 0.45 μm were used to collect cyanobacteria cells to enable pre-concentration and separation from dissolved organic matter interferences.

Cell disruption by use of a sonication probe liberated phycocyanin from the cell. Phycocyanin was evaluated to be most stable in aqueous solutions; water for a brief period of time or sodium phosphate buffers up to 100mM. The addition of organic solvents into the extraction solution precipitated phycocyanin out of solution. Temperatures should be maintained below 20 degrees Celsius and at optimum pH of 6.0. Increased temperatures above twenty degrees resulted in decreased emission sensitivity.

Anionic exchange resin DEAE Sephadex A25 was utilised to separate phycocyanin from natural organic matter and chlorophyll-a. Phycocyanin was captured with DEAE-Sephadex A25 resin and eluted with 0.16-0.27 M NaCl solution. Chlorophyll-a was not retained on the resin at all. Humic acid saturated the column and was retained on the resin. The resin was not able to be regenerated using 1 to 4 M NaCl, even when 1 M NaOH was used in the NaCl regenerated solution.

The IPA was able to take an emission signal and measure a phycocyanin concentration without false positives or interference from chlorophyll-a and natural organic matter, and estimated cyanobacteria concentration in cells/mL. The IPA was 99.2 % accurate at cell concentrations <350,000 cells/mL and 85.8 % at cell concentrations <2,000 cells/mL.

Doctor of Philosophy Student Declaration

Doctor of Philosophy Declaration

"I, Aaron Jay Scard, declare that the PhD thesis entitled *Improvement of spectral analysis sensitivity for detecting cyanobacteria in low concentrations in freshwater containing interfering NOM and other microorganisms* is no more than 100,000 words in length including quotes and exclusive of tables, figures, appendices, bibliography, references and footnotes. This thesis contains no material that has been submitted previously, in whole or in part, for the award of any other academic degree or diploma. Except where otherwise indicated, this thesis is my own work".

Signature

Date

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Chapter One

Introduction and Summary

As a decreasing resource, water has been the subject of many publications. Its reduced availability is a result of population increase, altered weather patterns due to climate change, and water pollution and contamination. The estimated available drinkable freshwater is 2.5 % of the total water available on the planet. This estimate includes ice caps, permanent snow and glaciers, and of these sources, only 1% of drinkable water is readily available (Capelli and Perlman, 2012, US Department of Interior and US Geological Survey, 2014). Therefore, the need to preserve and maintain vital water sources across the globe and ensure these sources are free from potential chemical and biological hazards is a high priority.

The World Health Organisation (WHO) estimates that 2 million children die each year from diarrheal diseases from contaminated water sources, and that a further 1.1 billion people do not have access to safe drinking water in developing countries (World Health Organisation, 2008). Microbiological hazards such as *Escherichia coli*, the cause of diarrheal diseases, are hard to detect without appropriate monitoring protocols in place.

The danger of a biological hazard is their ability to infect. Even low numbers of microorganisms can present a significant danger without any visual indication, taste or odour. Among the many potentially fatal public health hazards commonly found in marine and fresh waters across the globe is cyanobacteria.

Cyanobacteria are aquatic photosynthetic bacteria that inhabit freshwater, coastal and marine waters. Commonly referred to as Blue-Green Algae, cyanobacteria exhibit the characteristics of other algae species and aquatic plants. However, some cyanobacteria species or strains of the same species can produce harmful toxins that are released into the water sources they inhabit and different concentrations of toxin (Keränen et al., 2009, Warren et al., 2002, Yilmaz et al., 2008). Under ideal conditions, cyanobacteria cultures can increase to excessive levels, known as a bloom.

The toxins produced by cyanobacteria are harmful to humans and can be easily mistaken for other causes, like food poisoning. Misdiagnosed symptoms and the effect of particular strains of cyanobacteria, may result in an under reported representation of the potential seriousness of this bacteria in drinking water for human and livestock consumption. It is important to acknowledge that there are resource constraints and complexities endured by community officials and water authorities in preventing and intervening on cyanobacteria blooms. Therefore, testing is necessary to analyse, identify and determine the presence and risk of cyanobacteria. With rapid analysis techniques, the greater the opportunity for reducing the potential of a bloom event and increase the effectiveness of management countermeasures.

There are a number of techniques capable of detecting cyanobacteria. However, spectral detection techniques provide rapid analysis of samples with little purification. There is also minimum sample preparation/purification that allows for more robust sample analysis without decreasing the selectivity. Although the presence of natural organic matter (NOM) such as proteins, polysaccharides and humic substances and, other micro-organic cultures commonly found in water systems, can inhibit the detection of cyanobacteria by spectral techniques.

The focus of this research is on the early detection of cyanobacteria from fresh water sources in the presence of NOM and other microscopic cultures such as green algae.

This was achieved by assisting;

- Several spectral detection techniques such as UV-VIS spectrophotometry to identify and characterise pigments and interfering compounds with the use of synthetic and environmental waters.
- Various membranes types for the isolation and accumulation of cell cultures from source water. Assessments of the membranes practically, durability and cell recovery were assisted.

- Various pigment extraction techniques such as freeze-thaw, sonication and chemical extraction were undertaken to optimising conditions to suitability extract pigments specific to cyanobacteria.
- Various resin types were evaluated for the optimal isolation and concentration of pigments specific to cyanobacteria. The resin types used to investigate pigment properties and characteristics were size exclusion, anionic and cationic exchange. The properties determined from this study led to the isolation and controlled elution of phycocyanin.
- The accumulation of all ideal conditions into a functional prototype.

Chapter Two

Review of Literatures

2.1 Cyanobacteria

Cyanobacteria are a morphologically and physiologically diverse group of micro-organisms capable of aerobic respiration and photosynthesis that are found in marine, freshwater and terrestrial environments worldwide.

Cyanobacteria are unique aquatic micro-organisms with certain characteristics, traits and features similar to algae despite being bacteria. They are oxygenic phototrophic, able to perform aerobic respiration and photosynthesis (Batzing, 2002). They can have similar cell structures, compositions and phenotypes similar to algae, maintain buoyancy, and cause bloom events when environmental conditions are conducive. Similar to micro algae, cyanobacteria vary in cell dimension and shape depending on the species. *Microcystis aeruginosa,* with an average cell diameter of 2-5 μm, is one of the smallest species, while *Anabaena circinalis* has a significantly larger average cell diameter of approximately 20 μm (Allpike et al., 2005, Henderson et al., 2008a, Henderson et al., 2008b, Lee et al., 2006, Stork et al., 2009).

Cyanobacteria are green-blue in appearance. This coloured appearance is due to the presence of two pigments working in unison; phycocyanin, the accessory pigment with protein structure, which assists in the capture of light photons to be used for photosynthesis, and chlorophyll-a that captures photons of light for photosynthesis and cell respiration. Chlorophyll-a is the key component for undertaking photosynthesis and is common in all plants and species able to undertake photosynthesis. Therefore, detection of chlorophyll-a cannot be used to uniquely identify one species or another. Phycocyanin, on the other hand, is specific to cyanobacteria and detection of this pigment is specific to the presence of cyanobacteria.

As mentioned, there are different species and different strains of the same cyanobacteria, which might have identical or similar phenotype characteristics but have slightly different genetics. Some species or strains provide a great threat to public health and livestock due to the production and release of toxins into water systems. Yilmaz *et al* (2008) conducted a study of Florida strains of *Cylindrospermopsis raciborskii* for cylindrospermopsin production. In that study, eight different strains of *Cylindrospermopsis raciborskii* were analysed with no signs of cylindrospermopsin toxin being produced. However, Orr *et al*. (2004) investigated Australian strains of *Cylindrospermopsis raciborskii, which* produced two types of toxins; cylindrospermopsin and eoxycylindrospermopsin. Hence, identification of a cyanobacteria species or even a strain of species is not sufficient to identify if toxins will be produced, and testing for toxins are specific to the type of toxins and concentration within the source of water.

2.1.1 **Toxins**

Cyanobacterial toxins are naturally excreted from the cell or liberated upon cell death. There are three classifications of cyanobacteria toxins; hepatotoxins, alkaloids and lipopolysaccharides (Aráoz et al., 2009). Hepatotoxins are cyclic peptides comprising of: microcystins, cylindrospermopsin, deoxy-cylindrospermopsin and nodularins that inhibit different protein syntheses and have led to liver tumours forming in laboratory mice and livestock (Aráoz et al., 2009, Orr et al., 2004, Sangolkar et al., 2006). Alkaloids are neurotoxins (such as anatoxin-a and anatoxin-a(s)) which act on neurotransmitters of acetylcholine inhibiting the activity of acetylcholinesterase that exist where the nerve endings meet the muscle and aid in muscle contraction (Aráoz et al., 2009, Everson et al., 2009). Lipopolysaccharides, like saxitoxin, inhibit conduction in nerves by blocking sodium channels, which disrupt nerve messages (Aráoz et al., 2009, Bitterncourt-Oliveira, 2003, Hobson et al., 2006, Hoegar et al., 2004).

A study by Oudra *et al* (2001) evaluated the concentration of microcystin toxin production by a *Microcystis aeruginosa* bloom in a reservoir. This study measured microcystin released during cyanobacteria blooms of between 10^2 cells to 10^6 cells/ml of *Microcystis aeruginosa* with a predicted range of toxin released into drinking water between 0.7 to 8.8 μg/mg dry weight of cyanobacteria.

Cyanobacteria toxicity depends on factors such as the environmental conditions and the strain of cyanobacteria, and because of this potential toxicity. The WHO has recommended guidelines to safeguard the public from this threat in relation to cyanobacteria and presence of toxins.

Due to the toxicity and frequency of cyanobacteria blooms, the National Health and Medical Research Council of Australia (NHMRC) (2011) and the Victorian Government Department of Environment and Primary Industries (DEPI) (2008) introduced standards that coincide with WHO standards for cyanobacteria management. The NHMRC and DEPI standards state that water supplies for drinking may represent a public health risk if one or more of these factors exist in a water source for drinking;

- Microcystin toxin level is ≥1.3 μg/L (expressed as microcystin-LR toxicity equivalents);
- *Microcystis aeruginosa* is present at ≥5,000 cells/mL;

For Reservoir the presence of cyanotoxins the NHMRC (2011) guidelines state;

- Target limit is 1,000 cells/mL
- Critical limit is 5,000 cells/mL

Water sources require continuous monitoring for temperature and dissolved oxygen through water column and in addition cynaobacteria during summer period.

The concentration of 1.3μg/L for microcystin was calculated by the following equation, which estimates the concentration of toxin required to affect an average person:

Equation 2.1 $1.3\mu g/L = 40\mu g/kg\ bodyweight\ per\ day * 70\ kg * 0.92 L/day * 1000$

The calculation assumes a limit of 40 μg/kg body weight/day intake with no observed adverse effect predicted from 13 weeks of ingestion of microcystin-LR in mice. This also assumes an average adult weight of 70 kg, the proportional intake of microcystin from water (efficiency of uptake) was estimated

to be 0.9, the average daily water consumption to be 2 L per day for an adult and a safety factor for extrapolation of an animal study to humans was set at 1000 (Newcombe et al., 2010). Whilst Canada maintains a guideline value of 1.5 μg/L microcystin-LR, other countries such as Czech Republic, China, France, Italy, Japan, Korea and New Zealand have adopted the WHO recommended guideline standard of 1 μg/L microcystin-LR (Newcombe et al., 2010).

2.1.2 **Management**

Current operations for the management of cyanobacteria blooms in reservoirs and lakes mainly consists of changing or adapting the environment and water chemistry to inhibit plankton growth (Brookes et al., 2008). This is achieved by artificial destratification, algicide addition, or removal of nutrients that support cyanobacteria growth (NHMRC/NRMMC, 2011). Treatment strategies include intact cell removal by membrane filtration, pre-oxidation, coagulation/flocculation and, dissolved air flotation. These processes are typically integrated into a water treatment plant and can be used either as a continuous process or seasonal depending of the location of the source water and requirement for treatment. These techniques are water purification and not detection techniques.

Each of these strategies improve water quality regardless of the presence of additional chemical and/or biologicals through different functions and can be used continuously or when necessary.

Other strategies include dissolved metabolite removal such as removal of toxins and geosmin and 2-methylisoborneol responsible for taste and odour by adsorption with activated carbon or their oxidation by chemical processes such as chlorination (NHMRC/NRMMC, 2011). Chlorination is used for biological control in water systems, however, the formation of disinfection by-products from reaction with DOC can also pose a risk to public health (Mergen et al., 2008).

Although, the occurrence of cyanobacteria is becoming more problematic with climate change, as cyanobacteria is blooming at unpredictable and unseasonal periods of the year compared to previous bloom periods (CSIRO, 1993, Department of Environment and Primary Industries, 2008).

2.2 Impact

Seasonal blooms of toxic cyanobacteria have affected the health and economies of communities. Reported health effects of "blue-green algae" related illness include; conjunctivitis without discharge, blisters on the lips or in the mouth, asthma, myalgai with fever, allergic respiratory symptoms, hayfever skin rash with or without itch and gastroenteritis, nausea and/or diarrhea and/or vomiting and/or abdominal cramps (CSIRO, 1993). Deaths of domestic and wild animals and livestock by cyanotoxins have been recorded, although human deaths have not been reported within Australia. In Brazil 1996, 100 people were exposed to cyanobacterial toxins, specifically hepatotoxic cyclic peptide microcystin and alkaloid cylindrospermopsin (Carmichael et al., 2011). Of the 100 people, 76 people died. Of these deaths, 56 were contributed to the present of cyanobacteria toxins in the water source.

While not all people exposed to cyanobacteria demonstrate such symptoms (Hobson et al., 2006), these effects are nevertheless serious enough to warrant considerable effort being expended to prevent exposure. Historically, cyanobacteria commonly referred to as "blue-green algae" has been reported as an ever present problem for the Australian public.

Table 2.1 lists selected news reports related to cyanobacteria covered by ABC broadcasts. These few news articles do not represent all reported cyanobacteria events within Australian history but do provide insight into the impact of cyanobacteria in a public eye. The list of articles below identify disruption to the community related to fishing and tourism, loss of drinking water availability, and direct health effects, with the impact occurring over a period of weeks or months.

Table 2.1: Brief examples of cyanobacteria blooms and impact on the public in Australian waters

Source	Date	Article
(MDBA, 2020)	1878	A bloom of Nodularis spumigena caused the deaths of animals that drank from Lake Alexandrina.
(Hawkins, et al. 1985)	November 1979	An outbreak of hepatoenteritis occurred at Palm Island, Northern Queensland involving 148 people, including children of which the majority were hospitised.
(Hoegar et al., 2004, Orr et al., 2004, CSIRO, 1993)	November/ December 1991	Cyanobacteria bloom stretching for 1000 km along Darling River, New South Wales, cost a one million people days of drinking water and hospitalised two people with cyanobacteria toxin related illnesses.
(ABC, 2011a, ABC, 2011d)	October, 2011	Cyanobacteria blooms have been reported in Lake Eppalock, near Bendigo.
(ABC, 2011b, Australian Broadcasting Corporation News, 2011)	Summer, 2011-2012	Gippsland area, a bloom of cyanobacteria placed major concern on tourism in the region with the Department of Sustainability and Environment warning to avoid the affected areas and lakes where the blooms had occurred and also affected the local fishing industry.
(ABC, 2011c)	19th December, 2011	Article aired concerns of local fishermen over the ban set by the Health Department on consumption of fish in the cyanobacteria affected region. A similar ban back in 1998 was maintained for sixteen weeks and affected the fishing industry.
Gus Gowell (2012)-ABC News	27th April, 2012	Article related to fish in Gippsland that were given the all clear for consumption, five months after the individual reports of cyanobacteria outbreaks in the region.
(Dunlevie, 2014)	17th November, 2014	Northern Territory's Environmental Protection Authority (NTPA) issued alerts to residents and visitors. Quoted to say "Don't' go padding, don't go swimming. Swimming would be a high-risk activity".
(Australian Broadcasting Corporation News, 2015a)	Summer 2015	Marine cyanobacteria detected at Carnden Haven River, Googly's Lagoon and Hastings River. Alerts issued to residents and visitors of region. Public asked to avoid water with discolouration.

Media reports in March of 2015 linked cyanobacteria to public concerns in Broome and Lake Torrens. In one report, Broome residents were warned of a toxic bloom of *Lyngbya* that had washed up on the beaches and interfered with the fishing industry within the region (Australian Broadcasting Corporation News, 2015c). The second media report described the South Australian Government, in an attempt to remove a cyanobacteria bloom from Torrens Lake in the centre of Adelaide, used numerous methods to eradicate the algae without success and resulted in discoloured water stretching 2.5 km from the Torrens outlet and into the Gulf of St. Vincent. Authorities warned people to stay away from the discoloured water at the popular beach and Torrens Lake. The South Australian government then tried

treatments of hydrogen peroxide, which have been successful in smaller pools along the lake, to reduce the cyanobacteria concentrations below the threat level (Australian Broadcasting Corporation News, 2015b).

The threat of cyanobacteria is simply not just to health, but also to lifestyle and the economy. Therefore, government water authorities' invest considerably in safeguarding public health via monitoring, prevention and treatment programs for control of cyanobacteria.

2.3 Monitoring

Monitoring of cyanobacteria is not currently effective for the prevention of cyanobacteria blooms and the implementation of treatment processes seem to be the most common action once a bloom has occurred (Newcombe et al., 2010).

Monitoring of freshwater reservoirs for cyanobacteria is usually the responsibility of the local water provider and local water agencies in rural Victoria. Monitoring can be costly to both the region's water agency, who are required to monitor for cyanobacteria and take action in the presence of blooms, and to the community.

Current monitoring involves a visual site observation, sampling for determination of cell concentrations and speciation by laboratory analysis. Often there is considerable time lost resulting from travel distances to and from sample sites and, sample analysis, which may take over a number of days. With this potential time lost, a bloom event can easily occur (Fu et al., 1979, Mendiola et al., 2005, Richardson et al., 2010). Therefore, early detection of cyanobacteria by sensor technology to allow rapid determination of blue green algae concentrations would provide the possibility for early intervention to limit the formation of a full bloom occurring and its subsequent impacts. Thereby reducing the risk to the public and the region's water agency. Blooms are likely to occur if the conditions of climate, nutrient and light support their growth, which is usually during late spring, summer and early autumn

The Australian government has adopted the World Health Organisation (WHO) recommended standards for cyanobacteria limits within freshwater and recreational water systems with some variation to the response to a cyanobacteria bloom (Department of Environment and Primary Industries, 2008, World Health Organisation, 2008). The National Health and Medical Research Council/National Resource Management Ministerial Council (2011) issued recommended "Notification" (Alert Level 1) and "Alert" (Alert Level 2) guidelines for known common cyanobacteria species.

Table 2.2: Threshold definitions – lower threshold or trigger level "Notification" (Alert Level 1) and "Alert" (Alert Level 2) for the range of known toxic cyanobacteria given in the Australian Drinking Water Guidelines; (NHMRC/NRMMC, 2011).

Species/Type	**Notification (Alert Level 1)**		**Alert (Alert Level 2)**	
	Cell Numbers (cells/mL)	Biovolume (mm^3/L)	Cell Numbers (cells/mL)	Biovolume (mm^3/L)
Microcystis aeruginosa	2,000	0.2	6,500	0.6
Anabaena circinalis	6,000	1.5	20,000	5
Cylindrospermopsis raciborskii	4,500	0.18	15,000	0.6
Nodularia spumigena	12,000	2.7	40,000	9.1

Alert Level 1 may require notification and consultation with health authorities and other agencies on the bloom status and progress. Variable factors that need to be determined are whether the cyanobacteria are known toxigenic species, location the of bloom event relative to water supply, nature of supply and associated water treatment.

Alert Level 2 indicates the threat has increased with or without knowing if the bloom is toxic. Cell concentrations of 6,500 cells/mL for *Microcystis aeruginosa* are equivalent to the guideline of 1.3 µg/L of microcystin (World Health Organisation, 2008). This level is determined by monitoring upward trending increasing cell concentration measurements over at least two weeks with at least two samples taken during the week.

2.3.1 **Monitoring by toxins**

Cyanobacteria toxins pose a very serious threat to public and wildlife health as mentioned previously in this chapter. Symptoms of cyanotoxins range from mild hayfever-like symptoms and rash to tumours and even death, there have been multiple methods of analysis developed for measuring various cyanotoxins. Table 2.3 contains a summary of various methods of analysis for three freshwater cyanotoxins; anatoxins, cylindrospermopsins and microcystins.

Table 2.3: Methods available for cyanobacteria toxin analysis (United Stated Environmental Protection Agency, 2012).

Freshwater Cyanotoxins			
Methods	**Anatoxins**	**Cylindrospermoposins**	**Microcystins**
Biological Assays			
Mouse	Yes	Yes	Yes
Protein Phosphate Inhibition Assays (PPIA)	No	No	Yes
Neurochemical	Yes	No	No
Enzyme Linked Immunosorbent Assay (ELIZA)	Yes	Yes	Yes
Gas Chromatography (GC)			
GC with Flame Ionisation Detector (FID)	Yes	No	No
GC with Mass Spectrometry (MS)	Yes	No	No
Liquid Chromatography (LC)			
LC with Ultra-Violet (UV/Vis)	Yes	Yes	Yes
LC with Fluorescence (LC-FL)	Yes	Yes	Yes
LC with Ion Trap Mass Spectrometry (LC/IT MS)	Yes	Yes	Yes
LC with single Quadrupole Mass Spectrometry (LC/MS)	Yes	Yes	Yes
LC with Triple Quadrupole Mass Spectrometry (LC/MS/MS)	Yes	Yes	Yes

ELIZA (Enzyme-Linked Immunosorbent Assays) kits and strip test field kits do exist for a range of toxins from numerous suppliers and manufacturers across the globe. Toxin detection in ELIZA kits and strip test field kits is accurate and sensitive and, specific to the individual toxins present being tested for. Detection of cyanobacteria toxins is not only reliant on the species of cyanobacteria but also the

strain present. Cyanobacteria toxin determination is usually used when a cyanobacteria species is identified and the correct kit/strip is applied to determine toxicity (Bitterncourt-Oliveira, 2003, Hoegar et al., 2004, Msagati et al., 2006, Saker et al., 2007, Sangolkar et al., 2006, United Stated Environmental Protection Agency, 2012).

Liquid chromatography (LC) is another form of analysis for cyanotoxin detection. Compared to ELIZA kits, liquid chromatography is just as, if not more, accurate and sensitive. Further, LC is able to quantify multiple analytes (various cyanotoxins) from a single analysis compared to just one for ELIZA kits (Anjos et al., 2006, Drinovec et al., 2011, Garrido and Zapata, 2006, Hoegar et al., 2004, Matilainen et al., 2011, Mergen et al., 2008, P'erez and Aga, 2005, Simis et al., 2007, Suzuki et al., 2005, United Stated Environmental Protection Agency, 2012). However, the disadvantages of LC analysis is the length of time for results (requiring hours when taking into account sample preparation and purification), cost of instrumentation, reagents and solvents and sample purification required to detect and quantify the concentration of various cyanobacteria toxins, if present. Due to the running cost and operational requirements of LC analysis, it would be impractical to set up an in-field analysis procedure involving this technique.

After a cyanobacteria presence confirmation within a water source, toxin analysis techniques can be employed to determine toxicity within the water. Due to toxin analysis being specific to individual toxins, the right technique for the specific toxin must be implemented every time.

2.3.2 **Cyanobacteria Pigments**

Fluorescence is a well-adapted analysis technique for water samples. As a qualitative and quantitative application of measurement, fluorescence applies light to excite electrons in molecules in certain compounds by the adsorption, excitation and emission of energy on compounds. This energy is in the form of light is adsorbed by the compound, which causes excitation of atoms to a higher state, and the emission of that energy in the form of light as the compound reverts back to

its original state. The adsorption, excitation and emission of energy are instantaneous and this excitation/emission is measured at different wavelengths between 190-800 nm generally. This unique process of excitation and emission of different electron energy states allows emission to be measured on the right angle, 90°, to the direction of light being used to excite the compound. This allows for more sensitive detection than absorption by UV-Visible light spectrometry which is measured in a single direction from light source to detector.

The sensitivity of fluorescence techniques can suffer interferences from natural organic matter and other compounds in water, and the fluorescence response can be affected by pH, ionic strength and multivalent cations concentration. Some current techniques and detection devices for monitoring coloured dissolved organic matter and/or cyanobacteria blooms use fluorescence (Bastien et al., 2011, Burlage, 2002, Del Castillo et al., 1999, Echenique-Subiabre et al., 2016, Flynn, 2010, Izydorczyk et al., 2009, Li et al., 2015, Odriozola et al., 2007, Richardson et al., 2010, Siswanto et al., 2013, Villagarcía et al., 2002, Zamyadi et al., 2012, Zhang et al., 2016). The devices that monitor or detect cyanobacteria blooms focus on the detection cyanobacteria pigments.

Cyanobacteria have two specific pigments, chlorophyll-a and phycocyanin. Chlorophyll-a provides energy to the cell for photosynthesis by capturing violet-blue and red-orange light while green/yellow light is reflected. Phycocyanin is an accessory pigment which captures light at a different wavelength to chlorophyll-a and passes the energy on to chlorophyll-a (Batzing, 2002). Phycocyanin is a phycobiliprotein. Chlorophyll-a is able to use this extra energy for photosynthesis. Carotenoids are also accessory pigments but exist in the cell membrane rather than adhering to the cell membrane. Table 2.4 outlines the variation in accessory pigments between different kingdoms of organisms capable of photosynthesis (Garrido and Zapata, 2006, Thain and Hickman, 2000).

Table 2.4: Layout of pigments, accessory pigments and habitats of various types of aquatic plankton

Organisms	***Pigment***	***Accessory Pigments***	***Habitat***
Green algae	chlorophyll-a	Chlorophyll-c, Carotenoids	Mostly aquatic (Marine and freshwater)
Plants	chlorophyll-a	Chlorophyll-c, Carotenoids	Land, and some aquatic
Dinoflagellates	chlorophyll-a	Chlorophyll-c, Carotenoids	Marine and freshwater
Euglenoids	chlorophyll-a	Chlorophyll-c, Carotenoids	Mostly freshwater, some marine
Haptophyte algae	chlorophyll-a	Chlorophyll-c, carotenoids	Mostly marine, some freshwater
Diatoms	chlorophyll-a	Chlorophyll-c, Carotenoids	Marine and freshwater
Brown algae	chlorophyll-a	Chlorophyll-c, Carotenoids	Mostly marine
Cryptophytes	chlorophyll-a	Chlorophyll-b, phycobiliprotein	Marine and freshwater
Rhodophyta	chlorophyll-a	Chlorophyll-b, Phycobiliprotein	Mostly marine, some freshwater
Cyanobacteria	chlorophyll-a	Chlorophyll-b, Phycobiliprotein	Marine and freshwater

As seen in Table 2.4, phycobiliprotein are present in Cyanobacteria, Rhodophyta and Crytophytes. The phycocyanin pigment is found in cyanobacteria and crytophytes.

The accessory pigment for Rhodophyta is classified as a phycobiliprotein with the specific type being phycoerythrin, which reflects red light and absorbs in the blue region giving its red appearance (Thain and Hickman, 2000). Hence, cyanobacteria species can be identified and separated from Rhodophta, which contain phycoerythrin and excite at a different wavelength to that of phycocyanin. As fluorescence detection of phycoerthrin occurs by excitation at 496, 545 and 565 nm and has a maximum fluorescence emission at 578 nm, it can be easily identified from species containing phycocyanin that excites at 609 nm with a maximum fluorescence emission at 647 nm.

The accessory pigment excites in the orange-red region of the light spectrum at approximately 590-630 nm, and passes the captured light onto chlorophyll-a as outlined by Gregor *et al.* (2007). Phycobiliproteins are found on the thylakoids floating free in the cyptoplasm of the cell. The structure of phycocyanin is defined as a tetrapyrrole structure tightly linked by covalent bonds to a chromoprotein, also known as a coloured protein, that as a whole is known as a phycocyanin (Colyer et al., 2005, Thain and Hickman, 2000). The main chromoprotein of phycocyanin and allophycocyanin is phycocyanobilin. Figure 2.1 shows the tetrapyrrole structure of a phycocyanobilin (Fu et al., 1979, Isailovic et al., 2004, Colyer et al., 2005).

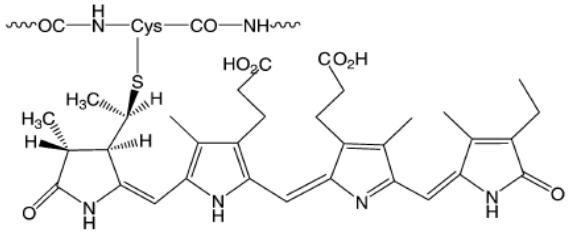


Figure 2.1: Chemical structure of the phycocyanin linked to peptide (Kathiravan et al., 2009, Colyer et al., 2005)

Chlorophyll-a is located within the free floating thylakoids within the cyanobacterial cell. The separation of phycocyanin from chlorophyll-a via the thylakoid membrane is due to the chemical differences between the pigment and accessory pigment. Whilst phycocyanin is a water soluble compound, chlorophyll-a is not and general standard methods for extraction of chlorophyll-a require organic solvents, mainly 80-90% methanol or acetone, to extract the pigment. Chlorophyll-a is generic to all plants and organisms that perform photosynthesis, whereas phycocyanin are specific to cyanobacteria and crytophytes (Batzing, 2002, Thain and Hickman, 2000, Wildman and Bowen, 1974).

Detection of cyanobacteria by fluorescence is commonly undertaken at 480-650nm for chlorophyll-a detection for cyanobacteria in high concentrations (≥x10$_6$cells/mL) (Gregor et al., 2007, Hoegar et al., 2004, Hunter et al., 2010, Izydorczyk et al., 2009, Richardson et al., 2010). These concentrations, however, are much higher than the concentrations recommended by the NHMRC, WHO and DEPI guidelines for early intervention of cyanobacteria blooms, and hence does not allow management interventions at a sufficiently early stage in the bloom.

A culture of *Microcystis aeruginosa* was characterised by Ziegmann *et al.* (2010) and marine *Synechoccus* and *Nodularia* species by Keränen *et al.* (2009). Ziegmann *et al.* (2010) used 5 nm width excitation from 235-700nm with 1nm width emission between 235-700 nm to measure the fluorescence absorbance of *Microcystis aeruginosa* as a function of excitation wavelength. The approach has also been successfully applied to the identification of eukaryotic algae from cyanobacteria (Keränen et al., 2009, Larson and Passy, 2005). Keränen *et al.* (2009) identified the difference between strains of toxic and non-toxic *Nodularia* compared to eukaryotic green algae and diatoms through fluorescence mapping between a 594-857nm from 580nm excitation for their measurements of marine *Nodularia* and *Synechoccus* species. After normalising, the spectra were divided by the intensity at 724 nm. In order to remove the instrument noise and any organic matter from the measurements of cyanobacteria, they excited samples at 580 nm and measuring emission at 430 nm, 35 data points, which taken as baseline. As their research was more qualitative than quantitative, no cell concentrations were specified.

The sensitivity of fluorescence spectrometry sensor techniques in surface waters can be influenced and subject to a wide variety of interference. The benefit, however, of having little or no sample preparation for these techniques, compared to other techniques, is a considerable advantage.

2.3.3 **Monitoring by pigment**

It is well established there are commercial fluorescence devices capable of detecting cyanobacteria via their pigments. These commercial devices provide rapid real-time information and water monitoring even in waters containing dissolve organic matter and mixed algal/cyanobacterical cultures (Bastien et al., 2011, Burlage, 2002, Del Castillo et al., 1999, Echenique-Subiabre et al., 2016, Flynn, 2010, Izydorczyk et al., 2009, Li et al., 2015, Odriozola et al., 2007, Richardson et al., 2010, Siswanto et al., 2013, ThermoFisher Scientific, 2015d, ThermoFisher Scientific, 2015b, ThermoFisher Scientific, 2015a, ThermoFisher Scientific, 2015e, ThermoFisher Scientific, 2015c, Villagarcía et al., 2002, YSI, 2015, Zamyadi et al., 2012, Zhang et al., 2016).

The study by Del Castillo et al. (1999) monitored, measured coloured dissolved organic maater (CDOM) in water, and characterised CDOM excitation and emission wavelengths in marine waters. Whilst salinity concentration in marine water did have some effect on CDOM measurements, excitation was conducted at 250 nm and measured at 450 nm.

Elliott et al. (2006) characterised fresh water to measured amino acids of *P. aeruginosa* in the presence of humic acid by fluorescence spectrometry, generating Excitation-Emission Matrices (EEM). Fluorescence Excitation-Emission Matrix (EEM) plotted the growth of serial diluted cell concentrations at three different temperature ranges, 11°C, 25°C and 37°C. The EEM region of measurement was between emission wavelengths of 280-500 nm, and excitation between 200-400 nm. Humic acid additions produced a final concentration of 5 mg/L of humic acid. In this study, humic acid did not present any interference with amino acid detection, namely tyrosine- and tryptophan-like fluorescence compounds, which could be characterised by EEM and measured between 280-500 nm.

In a later study, a study by Villacorte et al. (2015) measured and characterised algal organic matter (AOM) with fluorescence EEM between excitation 240-450 nm and emission between 290-500 nm. In this study, the emission profiles from three different cyanobacteria species identified cell concentrations, cell growth and cellular extractions (AOM).

Florescence measurements can be applied to any water source and can be measured using the commercial devices listed in Table 2.5. Zero-point corrections for dissolved organic matter can be applied by taking spectral measurements for CDOC based on SUVA, Gelbstoff measurements or humicification index (Villagarcía et al., 2002), and applying a correction to account for the contribution of organic matter to the detection wavelength This correction allows for zero-point/blank determination. The humicification index can also be referred to as the Gelbstoff measurement. Gelbstoff measurements are measurements of CDOM, generally yellow in appearance. It is an important optical constituent in water often dominating absorption in the blue region and able to excite under fluorescence light (Villagarcía et al., 2002, Zhang et al., 2016). Highly coloured humic substances have excitation wavelengths between 300-370 nm, 320-340 nm and 370-390 nm with emission wavelengths between 400-500 nm, 410-430 nm and 460-480 nm.

Table 2.5: Commercial devices for cyanobacteria determination

Device	**Pigment measured**	**Detection specification**
bbe-Algae Torch	Chlorophyll-a	0-200 µg/L
bbe-Bentho Torch	Chlorophyll-a	0-10 µg/cm^3
bbe-Fluoro Torch	Chlorophyll-a	0-200 µg/L
bbe-Algae Online Analyser	Chlorophyll-a	0-200 µg/L
YSI EX01/02 Probes	Chlorophyll-a Phycocyanin	0-100 FRU, 0-400 µg/L Chlorophyll-a 0-100FRU, 0-100 µg/L Phycocyanin
TriOS: microFlu	Not specified	(No other specified data) FRU

2.3.3.1 bbe-Algae Torch

The bbe-Algae torch is a hand held device that is capable of measuring chlorophyll-a between 0-200 ± 0.2 µg/L in algae (ThermoFisher Scientific, 2015c). The measurements incorporate zero-

point correction which allows the bbe-Algae Torch to estimate a concentration based on emission from chlorophyll-a in the presence of coloured dissolved organic matter. The device uses three coloured light emitting diodes (LED) to excite pigments within algae and cyanobacteria cells with the intensity of emitted light from pigments measured and an internal algorithm used to determine the concentration of chlorophyll-a. The device measures a spectrum of light and zeroes the device allowing any additional fluorescence signal at the target wavelengths for pigments to be counted as a positive presence. The torch is placed into the water below the surface and measurements are taken in real time. Species concentrations are calculated as chlorophyll-a μg/L. To determine speciation, excitation is conducted at three different wavelengths to excite different accessory proteins to determine speciation; the presence or absence of different accessory pigments determines which species is within that water source; though the detection limit or sensitivity of measuring accessory pigments was not specified.

2.3.3.2 bbe-Bentho Torch

The bbe-Bentho Torch is a hand-held device that is capable of measuring chlorophyll-a in algae and determining the total algae in algal biofilms. It is capable of measuring between 0-10 ± 0.2 μg/cm3of chlorophyll-a (ThermoFisher Scientific, 2015d). The bbe-Bentho Torch is able to distinguish between diatoms, algae and cyanobacteria based on their fluorescence profile. The device uses three coloured LEDs to excite pigments within the cells of algae and cyanobacteria with the intensity of emitted light from pigments measured applied with an internal algorithm to determine relative concentration of chlorophyll-a (Echenique-Subiabre et al., 2016). The three wavelengths used are 470, 525 and 610 nm. The bbe-Bentho Torch calibration is based on algal biofilms and uses an algorithm to correlate the fluorescence emission signal from biomass into μg of chlorophyll-a/L. Echeique-Subiabre at al., (2016) evaluated the bbe-Bentho Torch on the waters taken from New Zealand and France. Of the waters analysed, there were two types of biomass thickness analysed, <2 mm and >2 mm. Echeique-Subiabre at al., (2016) determined that for the

thicker biomass > 2 mm. This device reports the chlorophyll-a concentration in the relative biomass or biofilm measured. As the device requires biofilms for measurement, this type of measurement would not be not suitable for individual cell detection before a bloom event.

2.3.3.3 bbe-Fluoro Probe

The bbe-FluoroProbe utilses the same florescence technology as the bbe Algae torch and the bbe – Bentho torch, with excitation at three separate wavelengths. Much like the bbe-Algae Torch, this probe uses fluorescence spectrometry to quantify chlorophyll-a between 0-200 μg/L with the aid of an internal algorithm and applies Gelbsotff measurements for zero point correction. It can be submerged into freshwater and marine waters for real-time measurement and analysis (ThermoFisher Scientific, 2015e).

2.3.3.4 bbe-Algae Online Analyser®

The bbe-Algae Online Analyser® (AOA) applies excitation wavelengths 370, 430, 470, 525 and 610 nm and measures the emission between 680 and 700 nm. These emission wavelengths allow for detection of five classes of algae including cyanobacteria. From these excitation and emission wavelengths, the AOA is able to determine chlorophyll-a between 0-200 ± 0.01 μg/L of chlorophyll-a (ThermoFisher Scientific, 2015b). Izydorczyk et al., (2009) and Richardson et al., (2010) conducted independent studies conducted on environmental samples were analysed by the AOA. Being environmental samples, the Algae Online Analyser® (AOA) was able to remove the CDOM interference through Gelbstoff measurements. However, applying these correlations can lead to potential miscalculations including overestimations which was found by Richardson et al., (2010) investigating the AOA. There was a 1.2 to 3.4 times over estimation of plankton biomasses. Izydorzyk et al., (2009) attempted to implement an early detection Alert Framework for early detection of cyanobacteria by measuring biovolumes. The correlation between biovolume and chlorophyll-a concentration achieved at R2 = 0.68 (n=46). The detectable limits for AOA® for this

study were between 2.5-71 μg/L of chlorophyll-a at three observation stations. A fourth station measured between 0.3-11.7 μg/L of chlorophyll-a.

2.3.3.5 YSI - EXO Probes

The YSI EXO Probes are submergible probes that can be fitted to buoys or fitted as a free probe. The EXO1 and EXO2 probes are capable of detecting pigment ranges between 0-400 μg/L ± 0.1 ug/L for chlorophyll-a and 0-100 μg/L ± 0.1 ug/L for phycocyanin. Linearity between these ranges was estimated to be R^2 = 0.999 for both chlorophyll-a and phycocyanin (YSI, 2015). However, linearity was not specified if completed within environmental or laboratory conditions. Bastien et al., (2011) evaluated the YSI 6600 and TriOS probes for detecting *Microcystis aeruginosa* in field and laboratory water samples. In this study, the background noise of the YSI probe was equivalent to 310 cells/mL regardless of the calibration applied. Cell concentrations were determined by fluorescence spectrometry with detectable limits at 1500 cells/mL for the YSI probe (R^2= 0.71) and 0.69 μg/L of phycocyanin for the TriOS probe (R^2= 0.75) for this research. Samples were conducted on 91 environmental samples with zero-point correction applied to remove CDOC influence when measuring the samples. A study by Zamyadi et al., (2012) used the YSI 6600 probe to measure *Microcystis aeruginosa* concentrations. Under environmental conditions, in vivo fluorescence for phycocyanin had a correlation of R^2 = 0.79 and a weaker correlation of R^2 = 0.23 for in vivo fluorescence of chlorophyll-a. This weaker correlation for chlorophyll-a suggests there was interfering material which led to miscalculation in the correlation applied to determine the concentration of chlorophyll-a.

2.3.3.6 Summary of Commercial Probes

The common features that are applied to the commercial devices for detecting algae and cyanobacteria are the detection by fluorescence, correlations used for biomass estimation, and detection and quantitation by chlorophyll-a. The YSI/EXO Probes also used florescence of

phycocyanin for detection of cyanobacteria. The technologies that measure different species of algae, diatom and cyanobacteria all measure chlorophyll-a and various other wavelengths were accessory pigments are excited. This allows for presence/absence and various in species concentration by the level of accessory pigment. As all cyanobacteria, diatom and green algae species contain chlorophyll-a, no distinction of different species can be made from measuring only chlorophyll-a.

In order to account for the interference of dissolved organic matter, fluorescence intensities excited at 254 with emission measured at 300-345 nm and 435-480 nm are used to correct for NOM interferences. From the emission intensity in these specific ranges, a correlation for zero-point correction is determined mathematically and applied as a baseline correction for the measurement of phycocyanin at 647 nm. The application of Gelbstoff measurements to apply zero-point/blank correction allows for rapid spectral analysis of water systems without sample preparation, however, taking into account the spectral interferences it may lead to over-estimations of cyanobacteria concentrations. Cyanobacteria estimation errors are a result of calculation errors. Overestimations occur when the chlorophyll-a concentration is determined to be higher for cyanobacteria when it was actually present in the sampled water. This may be due to additional chlorophyll-a from other sources being applied to cyanobacteria concentration, or if the sample is not homogenous and does not represent the correct CDOM value. The chemical nature may change with the flow of water or other environmental conditions, such as rain or heat, which will lead to an error applied baseline correction.

2.3.3.7 Spectral Interferences

The chemistry of a source of water can be different to the next due to environmental conditions in various regions, states and countries. The environmental conditions that change the chemistry in surface water include; temperature, season, rainfall, wind patterns, soil content, and the presence

of pollutants in air, soil or/and water (Filella, 2009, World Health Organisation, 2008). Water chemistry such as the level of turbidity and concentration of natural organic matter, can change over a period of a few hours to a day or week depending on the environmental conditions (Filella, 2009, Mergen et al., 2008). These water chemistry parameters can influence detection of cyanobacteria by fluorescence spectrometry.

In studies by Shorrock et al., (2006) and Rubia et al., (2008), natural organic matter (NOM) was described as a complex mixture of organic compounds in natural surface water which influences odour, colour and the taste of water. NOM is a heterogeneous mixture containing various fractions of organic matters and is almost impossible to categorise precisely; NOM is typically categorised by size, functional groups or origin of the organic matter. Research by Lee et al., (2006) and Henderson et al., (2008b) suggested NOM is the combination of two natural sources of organic matter; pedogenic and aquogenic matter.

Aquogenic organic matter, or autochthonous matter, contains organic substances from microorganisms, such as algal organic matter (AOM) (Henderson et al., 2008b, Her et al., 2004). This contribution influences the taste and odour of water sources, promotes ecological events such as red tides, and may contain algal toxins such as neurotoxins and cyanotoxins. AOM has two forms; the extracellular organic matter, which contribute to normal metabolic excretions of waste, proteins and other compounds, and intercellular organic matter released upon cell death. Research by Bolto et al., (2002b) identified the compounds present in autochthonous matter to include amino acids, fatty acids, phenols, sterols, sugars, hydrocarbons, urea, lipids, polysaccharides, and humic substances. These substances are released upon autolysis of the cell if they are not released normally through metabolic excretions.

Pedogenic organic matter, or allochthonous matter, inputs arise from the degradation of higher plants, bacteria and fungi and surface runoff adding organic colloids, hydrophobic, transphilic, and hydrophilic fractions in surface water. Hence, the environmental conditions and surrounding ecosystem influence the makeup of pedogenic organic matter (Borisover et al., 2011, Filella, 2009, Lee et al., 2006, Matilainen et al., 2011, Rodriguez and Nunez, 2011, Sharma et al., 2011).

The composition of aquogenic and pedogenic organic matter are very different. Pedogenic organic matter generally has a large carbon to nitrogen ratio close to 100:1, has a significant presence of aromatic carbon and is highly coloured. Aquogenic organic matter on the other hand is almost colourless, has a low carbon to nitrogen ratio of close to 10:1 and has a low presence of aromatic carbon structured compounds (Sharma et al., 2011).

By definition, dissolved organic matter is organic matter that can pass through 0.45μm filtration (Mergen et al., 2008, Sentana et al., 2009). Humic substances are also commonly referred to as humic and fulvic-like acid substances in the literature. Approximately 50% of dissolved organic carbon has hydrophobic properties (Lee et al., 2006). Similar to this finding, Kabsch-Korbutoxicz & Majewska-Nowak (2008) found that aquatic humic substances generally comprise one-third to one-half of the dissolved carbon in water, and therefore are the dominant fraction of natural organic matter in surface water.

In studies by Bridgeman et al (2011) and Sharma *et al* (2011), they outlined three dominant fluorescence peaks of natural organic matter. These peaks are humic/fulvic like organic matter with an excitation of 330-350 nm and emission of 420-480 nm, humic-like organic peak with an excitation of 250-260 nm and emission of 380-480nm and protein-like organic matter with an excitation of 250-280 nm and emission of 280-350 nm. Most work involving cyanobacteria is focused on excitation of the photo-pigments where the emission peaks may be subject to

interference from natural organic matter. By taking into account the presence of natural organic matter in a body of water or removing it from the detection process, it will allow more sensitive and reliable detection of cyanobacteria, and hence potentially provide an earlier warning for management of an impending cyanobacteria bloom.

Similarly, interference of UV detection of pigments by natural organic matter is also possible because of its absorbance at various wavelengths. NOM may contain coloured dissolved organic matter (CDOC) that absorbs light in the visible region, and the presence of highly coloured allochthonous organic matter could possibly interfere with the detection of cyanobacteria pigments by overlapping absorbance bands. When phycocyanin is excited at 610 nm, phycocyanin's emission wavelength was measured at 647 nm (Moberg et al., 2001, Simis et al., 2007). CDOC can have a wide emission band between 450-700 nm even when excited at 610 nm (Bastien et al., 2011, Del Castillo et al., 1999, Izydorczyk et al., 2009, Richardson et al., 2010). This increases the baseline upon which further fluorescence from phycocyanin may be added and so it either needs to be corrected for, as current commercial devices do, or removed before measuring phycocyanin by fluorescence spectrometry.

Commercial devices apply algorithms for calculating CDOC interference, measure chlorophyll-a and apply corrections based on assumptions regarding the presence or absence of accessory pigments. No sample preparation allows for low maintenance and real-time results, however, not providing sample purification can limit detection sensitivity or accuracy at lower detection limits. Seen through studies, the YSI probe has a background noise of less than 310cell/mL for any calibration used (Bastien et al., 2011), over-estimations of the cell concentrations recorded in the AOA (Richardson et al., 2010), most devices apply Gelfstoff measurement for baseline or zero-point correction, and most devices measure and report in total chlorophyll-a.

2.4 Objectives of this research

This research aims to establish a proof of concept technique for on-line measurement of cyanobacteria cell concentrations below 2,000 cells/mL in fresh water in the presence of green algae, turbidity and natural organic matter. The novelty of this research compared to current techniques and commercial devices available, is this concept will remove the need for zero-point or Gelbstoff measurements. Instead, the technique will aim to concentrate, purify and quantitate the cyanobacteria pigment, phycocyanin.

The following specific objectives were set to address this overall outcome of early detection of cynaobacteria within a water source:

- Evaluate various spectral measurement techniques to determine optimum sensitivity and minimise spectral interferences in the detection of cynaobacteria.
- Evaluate various membrane types for direct filtration in order to collect cyanobacteria cells. Further, concentration of the phycocyanin pigment was developed and evaluated through the extraction of the pigment from the cell and concentrating the pigment through ion exchange chromatography.
- The optimal conditions from each stage from measurement technique, appropriate filter, pigment extraction method and method for pigment concentration developed the proof of concept and prototype. This prototype and proof of concept was evaluated.

Chapter Three

Materials and Methods

The experimental process can be separated into various sections; cultures, filtration, various synthetic and natural water samples, prototype hardware and instrumental analysis. Pure and natural cultures were used in this research, and conditions for culture growth along with safety procedures and methods for determining culture concentration are outlined in Section 3.3. The amount of pigment contained in cyanobacteria and green algae varies throughout their growth cycle (Madigan and Martinko, 2006, Solomon et al., 2005). The filtration setup and procedures used to evaluate the efficiency of membranes for repeated concentration of algae, along with pigment extraction and concentration procedures and, the prototype cyanobacteria analysis instrument are outlined.

3.1 Materials

3.1.1 **Chemicals**

All chemicals used during the study were analytical reagent (AR) grade and were supplied by Sigma-Aldrich (Australia). All water used during this study was Milli-Q water with the resistance higher than 18.2MΩ and was prepared by using a Milli-Q Ultrapure Water System (Millipore) unless specified otherwise.

3.1.1.1 C-Phycocyanin

Purified c-phycocyanin (52468) was purchased from Sigma Aldrich. C-phycocyanin was dissolved in Milli-Q water or various phosphate buffer. For purified c-phycocyanin dissolved into Milli-Q water, the standard stock solution was stable for seven days when stored at 4°C before there were visible signs of protein degradation. Any further diluted standards were used within 24 hours. The stability of solutions was determined by fluorescence measurements twice a day for 10 days for stock solutions and 5 days for diluted standards.

For purified c-phycocyanin dissolved into sodium phosphate buffer (pH 6.0), the stock solution was stable for one week and any further dilutions stable for 48 hours.

3.1.1.2 Chlorophyll-a

Purified chlorophyll-a from *Anacystis nidulans* algae (C1644) was purchased from Sigma Aldrich. Chlorophyll-a was dissolved into 80/20 methanol:Milli Q water solution or 80/20 acetone:Milli Q water solution. Stock solutions were able to be stored at 4°C for one month. Diluted standards were stored for no longer than ten days. Any prolonged time out of this 10-day period would potentially result in cell concentrations changes of the diluted stocks.

3.1.1.3 Humic Acid Sodium Salt

Humic acid sodium salt (H16752) was purchased from Sigma-Aldrich. Approximately 27.9 mg/L of humic acid sodium salt was weighed and dissolved with Milli-Q water. The solution was filtered through 0.45 μm filters/membranes in order to remove any undissolved particulate matter and to create an approximately 10mg/L coloured dissolved organic matter (CDOC) solution which was also confirmed by TOC analysis.

3.1.1.4 Bovine Serum Albumin

Bovine serum albumin (BSA) (A2153) was purchased from Sigma-Aldrich. Approximately 10 mg was dissolved into 1L of Milli Q water, or the required amounts dissolved to achieve the various concentrations required. Solutions were stored at 2-8 ºC for no more than 2 weeks.

3.1.1.5 Alginic Acid

Alginic acid calcium salt from brown algae (21054) was purchased from Sigma-Aldrich. Approximately 10 mg was dissolved into 1L of Milli Q water, or the required amounts dissolved

to achieve the various concentrations specified. Solutions were stored at 2-8 ºC for no more than 2 weeks.

3.1.2 **Water**

3.1.2.1 Synthetic laboratory test waters

Blank sample solutions were Milli-Q water and MLA growth medium prepared in Milli-Q filtered through 0.45µm.

Synthetic CDOC samples were primary dissolved and filtered humic acid sodium salt to produce approximately 10 mg/L of CDOC and 5 mg/L of BSA. Variations to these ratios were also used through experimentation.

Aliquots of green algae and/or cyanobacteria cell culture of known cell concentration were added to various waters during reliability studies to assess the effect of water composition on cyanobacteria analysis. The various types of waters containing known concentrations of cyanobacteria and/or green algae were used for control test waters and water containing variations of different concentrations and ratios of synthetic CDOC waters.

3.1.2.2 Natural Organic Matter (NOM)

The influence of NOM was measured using humic acid sodium salt, alginic acid calcium salt from brown algae and bovine serum albumin (BSA) as surrogates for NOM. Alginic acid was used to simulate polysaccharides. Bovine serum albumin was utilised as a model compound for proteins. Humic acid as sodium salt was used to simulate the presence of humic-substances or humic-like compounds present in water systems. Purified compounds were dissolved in Milli-Q water and filtered through 0.45µm were classified as DOC or CDOC matter. The use of this synthetic CDOC/NOM enabled reproducible test waters to be used and eliminated potential variations in

water quality that would naturally occur if using several samples of natural water. The use of these synthetic waters also removed variation in the water to minimise experimental variation in results.

3.1.2.3 Mixed waters of NOM

Mixed organic solutions were comprised of BSA, alginic acid and humic acid. The DOC content of the final solution was 10 mg-C/L for each control compound added for a total of 30 mg-C/L of NOM. Measurement of dissolved organic carbon in previous works has been carried out using UV at 254 nm (Henderson et al., 2008a, Henderson et al., 2008b, Her et al., 2004, Humber et al., 2007, Lee et al., 2006, Sentana et al., 2009). Dissolved organic carbon absorbance were measured on a Shimadzu Model UV-1800 Spectrophotometer in quartz cuvettes at 254 nm, as well as at other wavelengths including 618, 647 and 680 nm for measurement of interference on the detection of cyanobacteria and green algae species.

3.2 Environmental test waters

Environmental samples were taken from Lake Fyans in March 2012 and June 2013 by GWM Water, then in May 2014 from Deans Marsh. During this period, there was an unseasonable lack of cyanobacteria and algae. Macro-size debris was removed from the solution, with material larger than 1.0 mm in diameter removed via screening and further filtered to remove particulate matter above 0.45 μm in size. Waters were stored at 4 °C for 1 month or -18°C for 8-12 months. If additional solution was required, one of the frozen aliquots of sample was thawed and used for analysis. Turbidity was measured by a turbidity tube to be 4 ± 1 NTU, and DOC was analysed by TOC analyser with measurements recorded between 6.6 to 7.8 mg/L of dissolved TOC for all waters tested.

The waters contained low-level concentrations of natural organic matter. There was no cyanobacteria or green algae were detected by spectrometry methods. Analysis by UV- Visible

spectrometry and florescence spectrophotometry, including fluorescence EEM, demonstrated the presence of proteins and humic substances, as well as other unidentified compounds.

No visible presence of cyanobacteria or green algae after filtration in test waters was confirmed by a visual check under a light microscope, fluorescence analysis for pigments and the addition of MLA growth medium to a subsample for culture under the same conditions as all green algae and cyanobacteria monocultures.

3.3 Cyanobacteria and Algae

3.3.1 **Preservation of cultures and safety pre-treatments**

3.3.1.1 Biosafety Cabinet pre- and post-treatment

A biosafety cabinet was used when handling of cyanobacteria and algae cultures. Whilst the cultures were either non-toxic or potentially capable of producing toxins, there was precaution taken on handling, culturing and sample preparation of all cultures assuming that toxins could be produced. The use of the biosafety cabinet also prevented sample contamination. Initially, the UV light on the bio-safety cabinet was turned on for 15 minutes, followed by turning the UV light off and venting and filtering of the cabinet by turning the airflow on for 15 minutes. The cabinet was then sprayed and wiped down with 70% ethanol. The cabinet was then ready for use to handle pure cultures of cyanobacteria and algae. On completion of the work in the bio-safety cupboard, it was again sprayed with 70% ethanol solution and left for 5 minutes. Paper towel with 70% ethanol was used to wipe down the bench in the fume cupboard and was discarded into the autoclave bin. The bio-safety cupboard fan remained on for another 30 minutes to an hour to vent and filter the air. If spillage occurred, 70% ethanol was sprayed into a paper towel and the wet paper towel was placed over the spill and remained in place for 10 minutes to clean the spill. After 10 minutes, using another dry paper towel, the wet paper towel was collected and any remaining liquid was wiped up and discarded into the autoclave bin.

All cyanobacteria were kept in a PC2 controlled environment facility and labelled as Risk Group 2. All cyanobacteria strains were used in the bio-safety cabinet in a PC2 facility or a bio-safety cabinet following the safety protocols at all times. Each strain and sub-culture of each strain was isolated to individual test tubes and sealed glass containers secured with parafilm around the cap. Before and after use of strains in the bio-safety cabinet or PC2 facility, all equipment, containers and cabinet benches were sprayed and wiped down with a 70% ethanol solution or hospital-grade bacterial disinfectant to prevent contamination or spread of cyanobacteria strains from isolated containers.

3.3.1.2 Cyanobacteria species and strains

Microcystis aeruginosa (CS-338/01 MABU01) and *Anabaena circinalis* (CS-538/01 ACRMR01) strains had a toxicity below <0.01 μg/mg dry of Total MCYST (microcystin-LR equivalent toxins), as specified by CSIRO. *Cylindrospermopsis raciborskii* CS-510 CRS/ANN was specified as a non-toxin producing strain.

From the purchased culture from CMAR, each cyanobacteria culture was sub-sampled into four separate centrifuge tubes and diluted with MLA growth medium. Photoperiods of approximately 12:12 hour light to darkness were used during growth periods using cool soft fluorescent lights (1.5 Watts per 4L). The temperature was maintained at 23°C ± 2°C. Growth cycles were monitored by UV absorbance of chlorophyll-a at 680 nm (Bridgeman et al., 2011, Randolph et al., 2008, Silveira et al., 2008). Growth curves and calibration data were developed from one tube per culture. Oxygen content was not measured as long as the cultures were within stable growth cycles and MLA media was changed routinely. Initial growth of each cyanobacteria culture was determined by UV/Visible spectrometry, on a Shimadzu UV-1800 UV Spectrophotometer. A one mL sample from each culture was taken and the absorbance measured at 680 nm over a period of 30 days. The volume of

the cyanobacteria suspensions was kept constant by addition of 1 mL of MLA growth medium to replace the 1 mL of culture taken daily. Growth curves for the three cyanobacteria, shown in Figure 3.1.

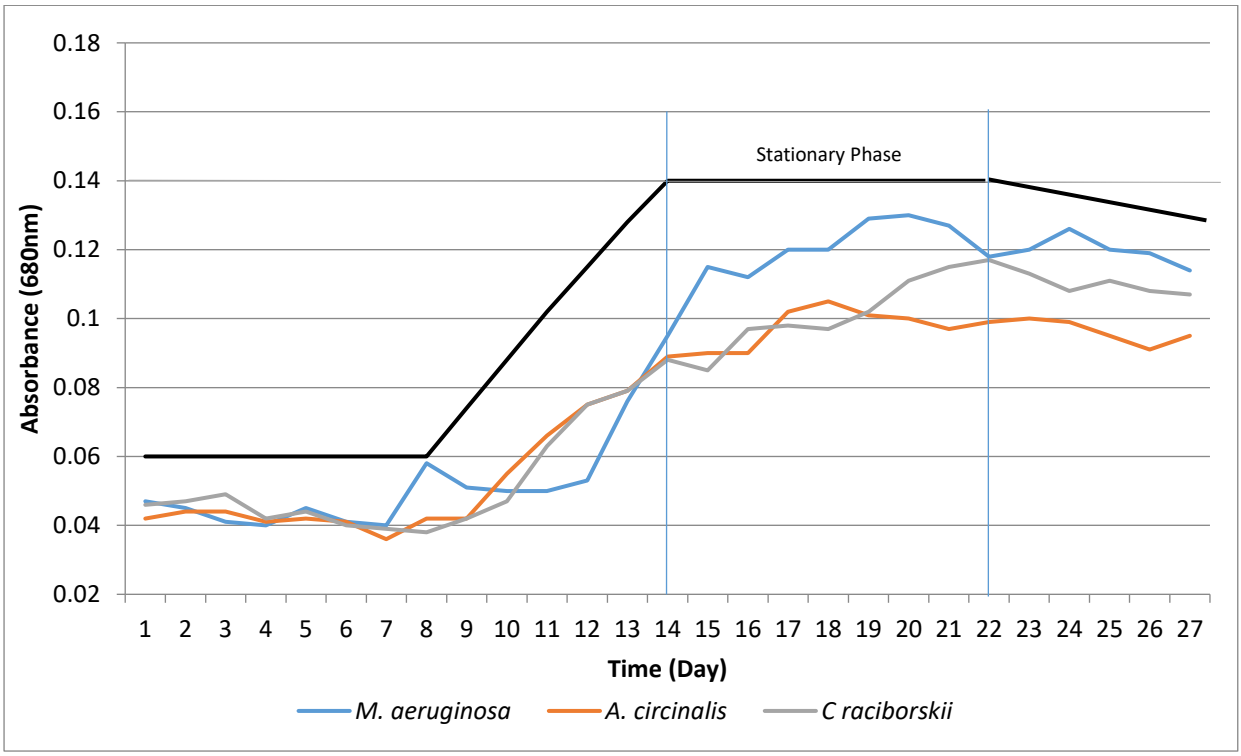


Figure 3.1: Initial growth curves for cyanobacteria species

All experimentation was conducted using cyanobacteria sampled during the stationary phase to avoid the cell concentration potentially increasing or decreasing too rapidly if experiments exceeded 12 hours. The stationary phase occurred between days 14-22 of the growth cycle, Figure 3.1 (Madigan and Martinko, 2006, Solomon et al., 2005). Two days were allowed for the stationary phase to stabilise.

3.3.1.3 Green algae species

Green algae species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, are non-toxic and classified under Risk Group 1. These species could be used in either a PC1 facility or biosafety

cabinet (i.e. Laminar flow cabinet). However, due to the availability of a PC2 facility and bio-safety cabinet for the strains of cyanobacteria, *C. reinhardtii* and *C. vulgaris* were also handled in the PC2 facility for convenience.

C. vulgaris and *C. reinhardtii* cultures were purchased from CMAR, split into four separate centrifuge tubes and then diluted with MLA growth medium. Growth curves and calibration data were developed from one tube of each culture. Initial growth of each cyanobacteria culture was determined by UV/Visible spectrometry, on a Shimadzu UV-1800 UV Spectrophotometer. A one mL sample was taken each day from each culture and the absorbance measured at 680 nm for a period of 30 days. To maintain a constant volume for the algae suspension, 1 mL of growth MLA medium replaced the 1 mL sample of culture each day, Figure 3.2. This method was adopted from research conducted by Jang et al., (2004) who used absorbance at 680 nm for monitoring the growth of their cultures as well as optical density measurements.

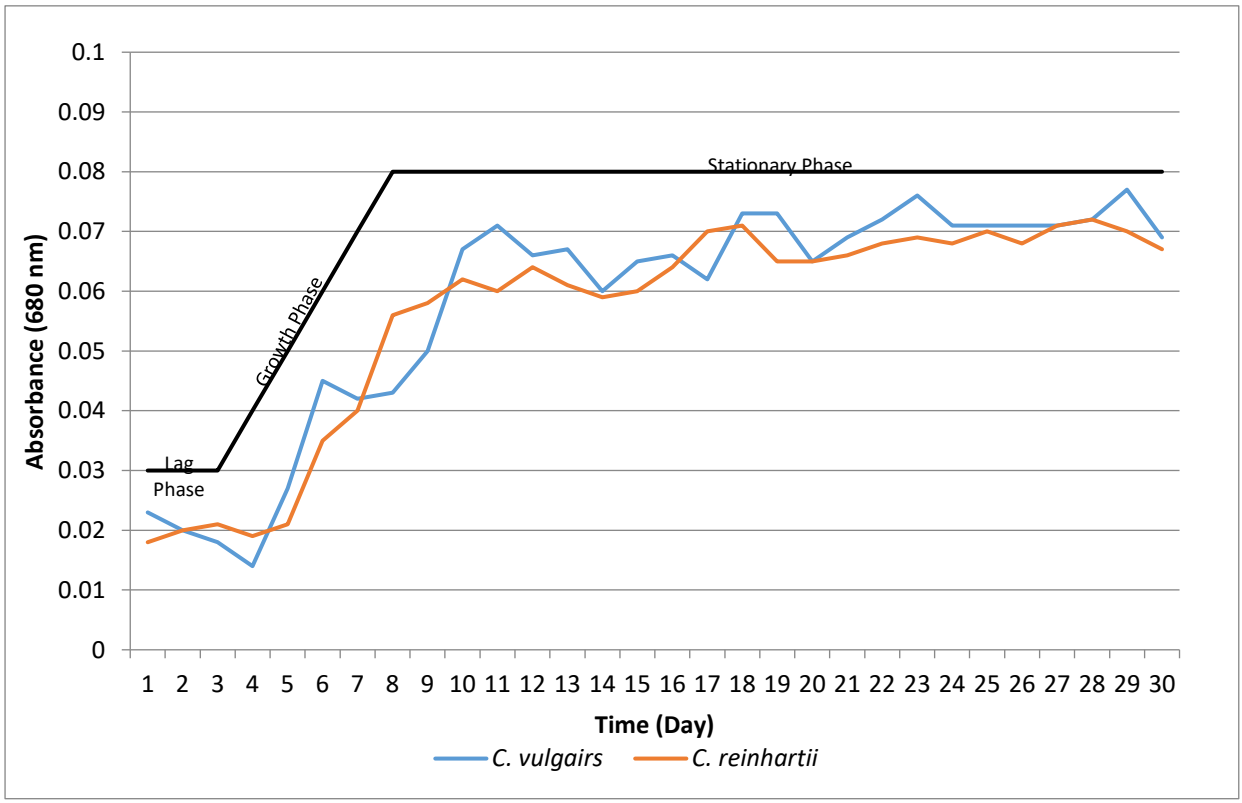


Figure 3.2: Growth curves for green algae species

Green algae samples for detection experiments were taken during the stationary phase between days 12-22 to avoid the cell concentration potentially increasing or decreasing too rapidly if experiments exceeded 12 hours duration. Samples were taken at day 12 despite the stationary phase beginning at approximately day 10; two days were allowed for the stationary phase to stabilise.

3.4 Microbiological methods

3.4.1 Media and growth procedures

All green algae species and cyanobacteria strains were single cell cultured in either MLA medium, slightly modified from ASM-1medium recommended by CSIRO Micro-Algae Research Centre (CMAR) (Bolch and Blackburn, 1996).

Table 3.1: Components of MLA media preparation

Stock Solution	Chemical	Amount
1	$MgSO_4.7H_2O$	49.9 gL^{-1}
2	$NaNO_3$	85.0 gL^{-1}
3	K_2HPO_4	6.96 gL^{-1}
4	H_3BO_3	2.47 gL^{-1}
5	H_2SeO_3	1.29 mgL^{-1}
6	Vitamin Stock Solution – 100 mL - Biotin - Cobalamin - Thiamine HCl	0.5 mL from Primary Stock – 10 mgL^{-1} 0.5 mL from Primary Stock – 10 gL^{-1} 10 mg
7	Micronutrient Stock Solution – 1 L - Na_2EDTA - $FeCl_3.6H_2O$ - $NaHCO_3$ - $MnCl_2.4H_2O$ - $CuSO_4.5H_2O$ - $ZnSO_4.7H_2O$ - $CoCl_2.6H_2O$ - $Na_2MoO_4.2H_2O$	4.36 g 1.58 g 0.60 g 0.36 g 10 mL from Primary Stock (1.0 gL^{-1}) 10 mL from Primary Stock (2.2 gL^{-1}) 10 mL from Primary Stock (1.0 gL^{-1}) 10 mL from Primary Stock (0.6 gL^{-1})
8	$NaHCO_3$	16.9 gL^{-1}
9	$CaCl_2.2H_2O$	29.4 gL^{-1}

From stock solutions 1-8, 10 mL was filtered through a 0.22 μm filter. Stock 9 required 1 mL to be filtered through a 0.22 μm filter. Green algae species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, were recommended to be grown in MBL or NB2 growth medium. However, it was possible to use MLA medium with no side effects for growth of *C. reinhardtii* and *C. vulgaris* as determined by the CSIRO micro-algae research centre. Hence, for simplicity, *C. reinhardtii* and *C. vulgaris* were cultivated in MLA medium using the same environmental conditions as each cyanobacteria species.

3.5 Concentration Analysis

3.5.1 **Cyanobacteria species and green algae**

Cell concentrations were determined by cell counting using a transparent field double Neubauer Counting Chamber and compound microscope. Species concentrations were serial diluted by 100 to 100,000 of their original concentrations. Cells in 0.1 μL of solution were inserted into the chamber and spread over the central grid, five grids in total, of 25 groups of 16 small squares each containing 0.00025 mm3. The calculation is outlined by Equation 3.1.

Equation 3.1

$$Cells\ concentration(cells/mL) = no.\ of\ cells\ (square\ mm)\ x\ dilution\ factor\ x\ 10{,}000$$

Cell counts were between 50-150 cells per grid or cultures were diluted accordingly to fit within this cell range.

3.5.2 **Error analysis**

Automatic plate readers were not available and all cell counts were conducted by visual inspection using a compound microscope. The error associated with each grid measurement was estimated to be 20-30% of the range dependant on dilution factors, auto pipette technique and calibration error, and homogeneity of the sample upon sampling (Willen, 1976). This error decreased with each

additional grid measured. To ensure a more accurate cell count measurement was taken, all cell counts were conducted over four grids and a duplicate measurement starting from a second serial dilution was performed. The resultant error was estimated to be approximately 15%.

3.5.3 **Effect of temperature on phycocyanin fluorescence**

The fluorescence intensity of pure 10 μg/L phycocyanin solution in Milli Q was measured at temperatures ranging from 2 °C to 50 ºC. The temperature was varied by first placing the solution in a slurry ice bath at 2 ºC and slowly increasing the temperature to 50 ºC on a hot plate. Ambient light exposure was limited, with the samples stored in 50 mL amber test tubes during the experiments. The temperature was recorded from a thermometer suspended in a vial similar to that containing the phycocyanin solution, so that the temperature of the solution and not the water bath was recorded. A second control was stored at 20 ºC and measured with each increase in temperature to record any potential drop in emission from pigment degradation in Milli Q water. Phycocyanin stability with regards to temperature was determined by plotting fluorescence intensity versus temperature. The fluorescence intensity was also measured when cool back to 20 and 4◦C.

3.5.4 **Filtration**

Filtration was utilised for this research as a tool for isolating intact cells and the removal of CDOC and other compounds that may interfere with the fluorescence emission of phycocyanin. Intact cells were able to retained on the membranes and were washed with Milli Q water to remove CDOC and other potential dissolved interfering compounds within the sample.

For consistency throughout experimentation, all disc membranes were evaluated using the same membrane housing under the same conditions, as presented in Figure 3.3 of Chapter 3. All sonication of membranes was performed by placing the disc membrane loaded with cell cultures into a flat round bottom container with 10 mL of Milli-Q and were sonicated for 5 minutes. The

only variation to this procedure was for the tubular membranes outlined later in this chapter. All stock solutions were cell cultures in weak aqueous based media diluted with Milli-Q water and all membrane washes were performed with fresh Milli-Q.

3.5.4.1 Filtration using flat disc membrane

Microfiltration has been used for the filtration and collection of algae, bacteria and other micro-organisms whilst ultrafiltration has been used for undissolved humic and fulvic compound collection in water purification (Fane et al., 2011, Watanabe and Kimura, 2011).

Ceramic, metal, nylon and glass fibre membranes were investigated for their capacity to capture cyanobacteria and green algae cells. The ceramic and nylon membranes were 0.45 μm in pore size, the glass fibre was 0.7 μm in pore size and the metal membranes were 1.0 and 5.0 μm in pore size. Filtration occurred under a slight vacuum; no more than -5 inches of Mercury Nylon and glass fibre membranes required a metal membrane as a supporting base to prevent them from breaking. To secure the membrane to the membrane housing and ensure filtration was directed through the membrane, parafilm was wrapped tightly around the sides of the membrane and membrane housing. This was done to ensure the vacuum passed through the membrane and was not bypassed allowing air to escape under the membrane in use.

The filtration setup is presented in Figure 3.3, and consisted of a conical flask with vacuum valve and a membrane holder suitable to hold a 0.45 μm membrane, or any pore size as long as the diameter of the membrane was suitable for the membrane housing (Bottino et al., 2001, Fane et al., 2011, Rossi et al., 2008, Watanabe and Kimura, 2011). The membrane was placed on top of the membrane housing and secured with parafilm around the edge.

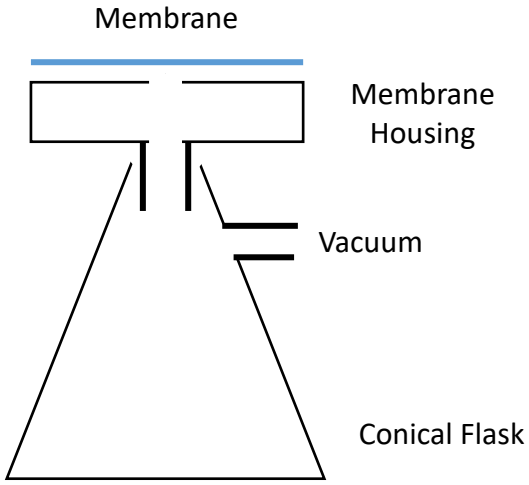


Figure 3.3: Membrane Filtration Investigation setup and preparation

3.5.4.2 Filtration Method

Large volumes of sample solution were not able to be filtered directly through the test membranes due to the vacuum drawing the solution to the edge of the membrane and underneath the membrane. In order to prevent sample solution bleed through under the membrane, 5 mL of suspension was loaded on the centre of the membrane and allowed to filter before another 5 mL was loaded on the membrane until the entire volume of suspension was filtered. Samples of the filtrate were collected in a conical flask to determine if there was cell bleed through the membrane. Cell concentrations in the backwash from the membrane and initial feed were also determined.

3.5.4.2.1 Prototype – Filtration preparation using flat disc membrane

Ceramic, metal, nylon and glass fibre membranes were investigated for their filtration capacity to capture cyanobacteria and green algae cells. Filtration occurred under water pressure; either by drawing sample solution through the housing (presented in Figure 3.4) by vacuum or pushing the sample through the membrane. There was little difference between vacuum or pressure filtration through the membrane.

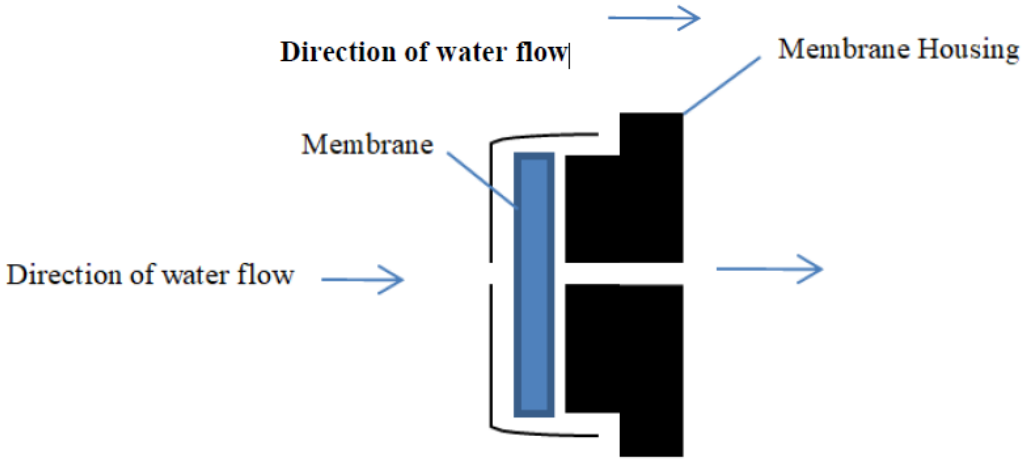


Figure 3.4: Schematic diagram of the experimental filtration setup utilised during prototype development.

The membrane housing was able to handle large volumes of sample water compared to the investigation setup in Figure 3.1. The filtration vessel was air tight and easily allowed 400 mL to filter for the initial investigation of samples up to 2 L in the trial l procedures. Ceramic membranes were unsuitable for use in the vessel due to the thickness of the membrane. The membrane thickness was approximately 0.3 mm compared to the thin membranes of nylon, metal and glass fibre.

3.5.4.2.2 Evaluation of two tubular membranes

The final design of the tubular filtration unit was the result of numerous trials and re-designs. Initial design concepts were used to evaluate the effectiveness of the tubular membranes with regard to filtration rate and bleed through of algae. Algae samples were pumped into the 20 mm diameter membranes and the direction of filtration was from the inside to the outside of the tubular membrane. Filtrate was collected in a 1L measuring cylinder. Samples were taken from the initial solution, during filtration, at the end of filtration, and from the backwashing water. Membrane backwashing was performed by removing the membranes from the container and submerging the membranes into a container of fresh Milli-Q water. The pump was reversed drawing fresh Milli-Q

water through the membrane pores in the reverse direction to filtration (outside – in). Cell bleed through porous membranes were measured by collecting the filtrate that passed through the porous membrane and analysed for chlorophyll-a and phycocyanin by fluorescence spectrometry.

Six mm diameter tubular membranes were also tested by pumping algae suspensions through the tube. The membranes was placed horizontally in a small plastic bag, and a second pump extracted the filtrate water into a 100 mL measuring cylinder to evaluate cell bleed through. Filtrate samples were taken from the initial solution, during filtration and the end of filtration, and the backwash water was also sampled.

Figure 3.5: Experimental bench set up of tubular membrane investigations

Figure 3.5 demonstrates the experimental setup of the tubular membrane with sample water from a 2 L container being pumped through the tubular membrane. One end of the tubular membrane was capped off and placed into a plastic bag. A slurry ice bath was used to maintain temperatures between 3 ± 1°C to maintain c-phycocyanin stability during sonication. The ice slurry bath was used to absorb any heat generated from sonication, which demonstrated degradation and loss of fluorescence intensity for phycocyanin.

Bench scale models used small tubular membranes approximately 6 mm in diameter with a 0.5 mm thickness and approximately 127 mm length. The external surface area of the membrane was calculated to be 2,394 mm^2. The total volume of the tubular membrane was calculated to contain 3,591 mm^3.

3.6 Chromatographic resin and anionic resin preparation

Adsorption of analytes were investigated by anionic and chromatographic resin. These types of resins were evaluated for their ability to isolate, retain and/or separate phycocyanin from CDOM and chlorophyll-a in order to reduce spectral interference during detection of phycocyanin. The resins utilised for this research project were;

- LH-20 Sephadex
- Sephadex G25
- Sephadex G75
- Activated Silica resin
- Activated Aluminia resin
- DEAE-Sephadex A25
- QAE Sephadex A25
- Amberlite IRA-416
- Amberlite IR-93

Each resin was prepared using the same procedure. Disposable 5 or 10 mL syringes were used as a resin columns following removal of the plunger. A 0.45 μm glass fibre filter or small ball of glass wool, approximately 4 mm in diameter, was shaped and pushed to the bottom of the syringe. In addition, 5 and 10 mL pipette tips were also utilised as small resin columns with glass wool placed into the bottom of the narrow head of the pipette tip to stop any loss of resin during adsorption. Gel

resins were suspended in Milli-Q water and poured into the syringe or pipette tip. The resin was left to settle and excess water was filtered through the glass fibre filter. The bed volume of the gel resin was approximately 3 mL in a 5 mL syringe or pipette tip and 5-6 mL of resin in a 10 mL syringe or pipette tip. Gel resin and bead resin were regenerated with 20 mL of 0.5 M NaCl and then flushed with 20 mL of Milli-Q water to restore the resins ability for ion exchange or to activate adsorption sites. Gel resin for size exclusion was treated with the same preparation as ionic exchange resin and flushed repeatedly with Milli-Q water. The syringe or pipette tip was secured vertically with a laboratory stand and grip. Flow rates for the loading of sample onto each resin and elusion flow rate were approximately 2 mL/min, maintained by peristatic pump. The assessment of each resin's performance was measured by the separation of phycocyanin from organic compounds for chromatographic resins and/or elusion solution phycocyanin concentration for anionic exchange resins.

A full list of chromatographic and anionic resin utilised in this research along with their specifications is located within Table 1, Appendix B.

3.7 Pigment Extraction Methods

Methods for chlorophyll-a extraction were based on work from Liao et al., (2011) adapted from Sarada et al., (1999) and Simis et al., (2007). All cyanobacteria species and green algae were handled in a bio-safety cabinet to maintain sample purity and for personal protection. Samples were taken from stock solutions or samples of various cell concentrations from 100,000 cells/mL to >1,000,000 cells/mL.

3.7.1 **Cell Disruption Methods**

3.7.1.1 Sonication

3.7.1.1.1 Disruption using Sonication Probe

Cell disruption by sonication was investigated for the purpose of phycocyanin extraction. Cell disruption was investigated from cyanobacteria monocultures using techniques from literature. The techniques utilised were cell disruption by sonication by bath and probe (Furuki et al., 2003, Rajasekhar et al., 2012a, Rajasekhar et al., 2012b, Wu et al., 2011, Wu et al., 2012b, Zhang et al., 2006a, Zhang et al., 2006b). The sonication probe (Branson Sonifier 450 with 400 Watts maximum capability) was maintained at 30% duty cycle every 1 second and a power output of 3. Of the 400 Watt maximum output of the Branson Sonifier 450, the conditions of the 30% duty and 3 Output setting meant 60 Watts was used during sonication.

Sample volumes were approximately 250 mL with various mono-cultured cell concentrations between 100,000 cells to a few million cells/mL. The tip of the 3 mm sonication probe was submerged into about 100 mL of cyanobacteria or green algae suspension in a 250 mL Schott bottle. To limit overheating of the sonication probe tip, samples were exposed for 1 minute to 2.5 minutes at a time, with a 1-2 minute break between sonication treatments. After sonication, the Schott bottle was closed, the solution mixed thoroughly before an aliquot of 8 mL was taken: 4 mL was dispensed directly into one cuvette and the remaining 4 mL was dispensed through a disposable 0.45 μm filter into another cuvette. To determine the effectiveness of sonication for release of pigments, comparisons were made between the concentrations of pigments in sonicated filtered samples and sonicated unfiltered samples.

The concentrations of sonicated filtered and unfiltered samples were converted into percentage pigment release upon sonication by assuming the filtered sample was representative of pigment concentrations released from the cyanobacteria cells and the unfiltered samples contained both

released and non-released pigment. These percentages were plotted as a percentage against time of sonication.

3.7.1.1.2 Disruption by sonication bath

Cell disruption by sonication bath was conducted by two methods. Test waters were transferred to 15 mL amber centrifuge tubes to reduce light exposure. Amber centrifuge tubes were used in order to minimise or reduce the effect of light degradation on c-phycocyanin. The first method consisted of alternating periods of 10 seconds sonication following 10 seconds of no sonication for a total of 10 minutes. The second method consisted of sonication for 10 minutes. The sonication bath had a 500 mL capacity and a sonication frequency of 50 MHz. Samples were centrifuged at 4,000 rpm for 5 minutes to allow any cell fragments to settle. The liquid was decanted into cuvettes and analysed by fluorescence spectrometry.

3.7.1.2 Disruption by the freeze/thaw technique

The method consisted of placing an algae or cyanobacteria sample into a 15 mL amber centrifuge tube, then freezing the sample at -20°C for at least 2 hours. The samples were left to thaw to ambient temperature, approximately 20°C, and the cycle repeated another three times. After the final thaw cycle, the amber tubes were centrifuged at 4,000 rpm for 5 minutes. The samples were then decanted into cuvettes and analysed by fluorescence spectrometry. This method was suitable for phycocyanin analysis, while the technique was nor suitable for chlorophyll-a analysis because chlorophyll-a is not water soluble (Benedetti et al., 2006, Chaiklahan et al., 2012, Chaiklahan et al., 2011, Lemasson et al., 1973, Moraes et al., 2011, Niu et al., 2007, Sarada et al., 1999, Silveira et al., 2008, Simis et al., 2007).

3.7.1.3 Chemical cell disruption

For extraction of chlorophyll-a, 80% methanol was used as the solvent for cell disruption. Concentrated 1 mL aliquots of cultures above two million cells per mL were transferred to a test tube along with 9 mL of methanol. The solutions were gently mixed by being inverting multiple times and then the test tubes were sonicated for 5 minutes in a sonication bath. This solution was filtered through a Whatman GF/F glass fibre filter that let the dissolved chlorophyll-a through while the disrupted cells remained on the filter paper, including the precipitated phycocyanin. The filter paper was then blown dry with nitrogen and the filter paper washed with Milli-Q water that dissolved the phycocyanin back into solution so it passed through the filter. For extraction of phycocyanin, the solvent used was Milli-Q water or 100 mM sodium di-hydrogen phosphate at pH 7. The solutions were gently mixed by being inverted multiple times and then the test tubes were sonicated for 5 minutes. Freshwater samples containing aqueous phycocyanin extracts were stored at 0°C in total darkness to prevent photo degradation and analysed within 48 hours of collection.

3.7.2 **Concentrations for cell disruption**

3.7.2.1 High cell concentration samples

Cell concentrations exceeding 1.0 x 10^5cells/mL were diluted to 1/10 of the original concentration with 1 mL of sample added to 1 mL of Milli-Q water and 8 mL concentrated methanol in an amber 15 mL centrifuge tube and mixed thoroughly for extraction of chlorophyll-a. For phycocyanin extractions, deionised water replaced methanol. The amber centrifuge tubes were sonicated for 10 minutes and mixed thoroughly. The solvent extraction solutions were centrifuged at 4,000 rpm for 5 minutes to settle organic matter of the disrupted cells. The solution was decanted into a fluorescence cuvette for measurement. Chlorophyll-a was eluted through all resin columns without adsorption onto the resin.

3.7.2.2 Low cell concentration samples

Lower cell concentration samples were decanted into an amber centrifuge tube along with the appropriate solvent. The samples were centrifuged at 4000 rpm for 5 minutes without sonication. The solvent was decanted leaving the intact cell cultures at the bottom of the amber centrifuge tube. Depending on the pigment being extracted, the appropriate solvent was added to the test tube. The amber test tubes were then sonicated and centrifuged for a second time under the same conditions. Analysis was by fluorescence spectrophotometry.

3.7.3 **Samples containing natural organic matter (NOM)**

3.7.3.1 NOM removal by centrifuge

Natural water samples containing NOM required sample purification before pigment extraction. Ten mL of sample solution was placed into an amber 15 mL test tube, capped and parafilm applied around the cap to prevent leakage. The samples were centrifuged at 4,000 rpm for 10 minutes. The liquid suspension was decanted and collected for analysis. Ten mL of 90% methanol solution was added to the sediment retained in the bottom of the amber test tube. Samples were gently mixed for 5-10 minutes to suspend the sediment from the bottom of the test tube and allowed to interact with the methanol solution. Samples were then sonicated for 10 minutes. Following sonication, the samples were moved to a fume cupboard, left to settle for 20 minutes as toxins from the cyanobacteria species may also have been released during extraction. Samples were then decanted into disposable plastic cuvettes, para-filmed and analysed by fluorescence spectrometry. If processes were delayed, samples were stored on ice or at 4°C and protected from exposure to light. Opaque bottles were used as brief exposure to light will alter chlorophyll-a analysis.

3.7.3.2 Separation of NOM from pigments

Sample solutions were filtered through a 0.45 μm glass fibre membrane. Dissolved NOM passed through the porous membrane filter while intact cell cultures were collected on the membrane

surface. The glass fibre membrane was placed into a centrifuge tube with the appropriate extraction solvent. The centrifuge tube was then sonicated for 10 minutes. After sonication, the sample tube was centrifuged for 5 minutes at 4,000 rpm. The clarified solution was decanted for analysis and the remaining sample was discarded with the centrifuge tube.

Tubular membranes were used in a similar fashion for filtration, with intact cell cultures captured within the tubular membrane and CDOC passing through the filter with the filtrate. The filtrate source water was collected for analysis. The tubular membrane filter was suspended in a sonication bath with Milli-Q water and sonicated for 10 minutes. The sonicated Milli-Q was collected for analysis to determine the phycocyanin concentration using fluorescence spectrometry. The phycocyanin concentration was determined by comparing the fluorescence response of the sample to the fluorescence response of known standards.

3.8 Extraction Solutions

3.8.1 **Organic solvent solutions**

Visual observation of the use of 90% methanol or 90% acetone as extraction solutions proposed by Moberg et al., (2001) and Parésys et al., (2005) presented a problem with the plastic cuvettes, as these solvents decreased the transparency of the plastic cuvettes. This was due to a chemical interaction between the plastic of the cuvettes and the 90% methanol or acetone solutions. To maintain the transparency of the cuvettes, the concentration of the extraction solution was lowered from 90% to 80% (Garrido and Zapata, 2006). This concentration did not alter the transparency of the cuvettes and maintained the capacity of the solution to extract the desired pigment, chlorophyll-a, from green algae and cyanobacteria. Extraction solutions of 80% methanol were used for chlorophyll-a measurements with fluorescence and UV-Visible spectrometry.

3.9 Prototype operational procedure

3.9.1 **Pigment Isolation Process by chemical purification**

A 50 mL syringe was suspended vertically with two Whatman GF/C glass fibre 0.7 micron filters secured to the bottom of the syringe. The plunger was removed to allow filtration to occur. One litre of synthetic laboratory water (Milli-Q containing *Microcystis aeruginosa*, approximately 100,000 cells/mL) were used for the following experimentations. Seven 20mL aliquots of filtered water were collected for analysis during the filtration of the 1L sample. These collected aliquots after filtration were analysed by fluorescence EEM, and for the presence of cyanobacteria via phycocyanin fluorescence. The filters were collected and placed into a centrifuge tube with 50:50 acetonitrile/Milli-Q solutions.

The collected cell culture on the filters in the centrifuge tube containing 50% acetonitrile solution was exposed to ultra-sonication for 20 minutes to disrupt and lyse the cells in order to release the target pigment, phycocyanin. The solvent and lysed cells were left to settle for 20 minutes allowing for solidification of phycocyanin in the organic solvent. After the time allocated for solidification of phycocyanin, the solvent was slowly drained out of the sonication vessel through a second glass filter. These samples were analysed by fluorescence EEM for profile analysis and identification.

To redissolve the precipitated phycocyanin pigment, the filter paper retaining the phycocyanin was dried under a light stream of nitrogen. Milli-Q water washed through the filter paper and washed onto a diethylaminoethyl Sephadex (DEAE Sephadex) A25 anion exchange resin column for pre-concentration (Patil et al., 2006, Silveira et al., 2008). After the deionised water had passed through the DEAE Sephadex A25 resin column at a flow rate of 2.0 mL/min, the column was eluted with 3 x 3 mL 0.5 M NaCl for fluorescence EEM analysis.

3.9.2 **Tubular Membrane Preparations**

Metal tubular membranes from Advanced Material Solutions were also utilised during this study. The pore size of the metal tubular membranes were approximately 0.5 μm. There were two sizes of tubular membranes considered, both with 0.5μm pore size; the large membrane was 20 mm in diameter and 370 mm in length and the smaller membrane was 6 mm in diameter and 127 mm in length. Both were fabricated from stainless steel. During operation of the Isolated Pigment Analyser (IPA), several tubular membranes were placed in parallel with each other. Water was pumped into both ends of the membranes and the outlets were combined via a manifold to produce dead-end filtration. Water was fed to the interior of the tubular membranes and the filtered water permeated through the walls. Figures 3.6 and 3.7 illustrate the flow of water through the tubular membranes.

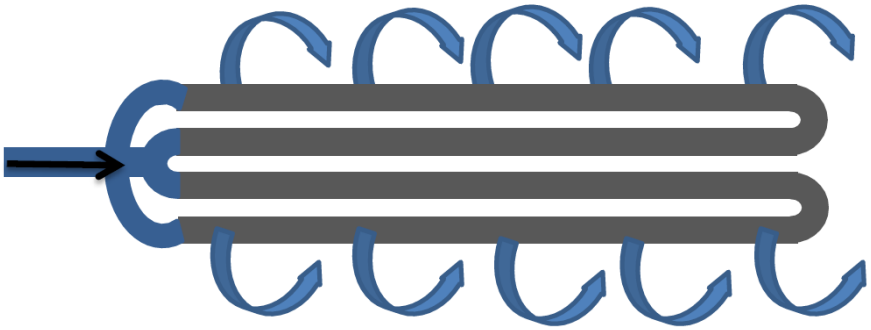


Figure 3.6: Experimental setup of metal tubular membranes in parallel

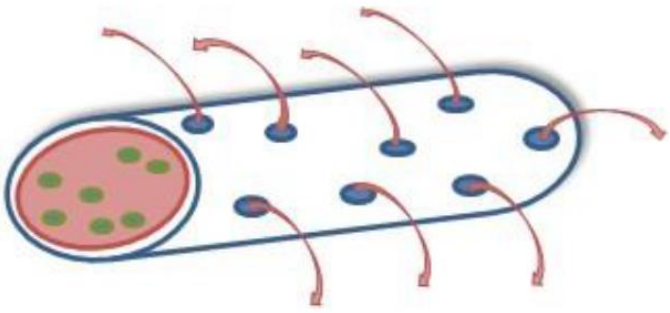


Figure 3.7: Filtration of source water and capture of cell cultures on tubular membrane

3.9.3 **Source Water Preparation for the pigment analyser**

Synthetic laboratory solutions with known concentrations of cyanobacteria and green algae cultures were prepared. To remove macro-particles or debris, the sample inlet was connected to a 1.0 mm filter with a debris net enclosed around the filter. This allowed cyanobacteria and algae cells to pass through without blocking the tubing, valves or membranes. After passing through the metal membranes, samples of the filtrate were collected for analysis for any sign of pigments by fluorescence spectrometry.

3.10 Analytical Methods

3.10.1 **Spectrofluorophotometry**

The detection of low concentrations of cyanobacteria was achieved by fluorescence spectroscopy using a Shimadzu RF-5301PC model spectrofluorophotometer. Measurements were taken in either one of two modes; single wavelength quantitation or wavelength range scans. Fluorescence intensity data was collected from the maximum peak height and corrected to baseline noise (FIU). Scans were performed for Excitation Emission Matrices (EEM) data, peak shift determination and spectral interferences. Wavelengths used in this research are stated in their relevant sections below; taken from literature values initially and confirmed by fluorescent applications on purified compounds and cultures.

Equation 3.2 represents the equation used for all fluorescence data measurements.

Equation 3.2 $c=kA=k\ (_{PoP})$

Where; (A) = Absorbance,

Po = initial photon intensity,

P = photon intensity after sample,

Є= molar intensity,

b = path length, and c = concentration.

The transparency of the plastic cuvettes did not add any spectral interference at the excitation or emission wavelengths for fluorescence for chlorophyll-a and phycocyanin or any other excited compound within the visible light region. Within the protein region, there were was additional interferences from the plastic cuvettes when compared to quartz cuvettes. Spectra scans were conducted at a "fast" speed setting with high sensitivity to optimise low limit detection of cyanobacteria cell cultures. Scans were conducted every 1 nm. All quantification for pigment concentration, spectral interference and conversions from pigment concentration to cell concentration were conducted by excel spreadsheet and SPSS software for statistical analysis using the spectral data.

Analysis of data was conducted using Microsoft Excel and SPSS PAWS Statistics 18. Microsoft Excel was useful for EEM data plotting, mass data plotting, mean and standard deviation. Mass data plotting PAWS Statistics 18 was used for single t-tests with box plot along with mean determination and ANOVA analysis. Linear curves for standard concentrations were plotted through Excel and by linear regression by SPSS.

3.10.1.1 Series Dilution

Cyanobacteria and green algae samples were serial diluted from concentrations of 1 to $x10^{-6}$ cell/100 ml using 1 mL of sample to 9 mL of Milli-Q water. Background measurements were made with Milli-Q water. Control samples were 9 mL of Milli-Q water to 1 mL of MLA cyanobacteria growth medium. For compounds in organic solvents, 90% methanol or acetone solutions were used instead of water in the control samples.

3.10.1.2 Cyanobacteria

Purified phycocyanin was used to identify the optimum excitation and emission wavelengths for cyanobacteria analysis by fluorescence spectrometry. Each individual species of cyanobacteria was scanned using an excitation wavelength of 610 nm with emission measured at 647 nm corresponding to the phycocyanin fluorescence peak. Determination of cyanobacteria concentrations used the spectral mode of the spectrofluorophotometer with were comparison to the background scans of the instrument; Milli-Q water was used as the blank solution and MLA medium as a control.

3.10.1.3 Green algae

Green algae species, *Chlorella vulgaris* and *Chlamydomonas reinhardtii*, were measured using excitation at 609 nm and emission at 680 nm. Determination of green algae concentrations used the spectral mode of the spectrofluorophotometer with comparison to background, blank solutions and control.

3.10.1.4 Chlorophyll-a analysis

Prepared serial dilution standards of pure chlorophyll-a (Sigma-Aldrich) solutions of approximately 2, 6, 20 and 60 µg chlorophyll-a/L were used to construct a chlorophyll-a concentration versus fluorescence calibration. Optimum sensitivity for chlorophyll-a extraction measurements was obtained at an excitation wavelength of 609 nm and emission peak of 680 nm. Then the prepared chlorophyll-a extract was analysed spectrophotometrically. Sample concentrations were calculated based on equation 3.3.

Equation 3.3 $c=k\ (_{PoP})$

Where; c = concentration

Po = initial photon intensity

P = photon intensity after sample

k = constant.

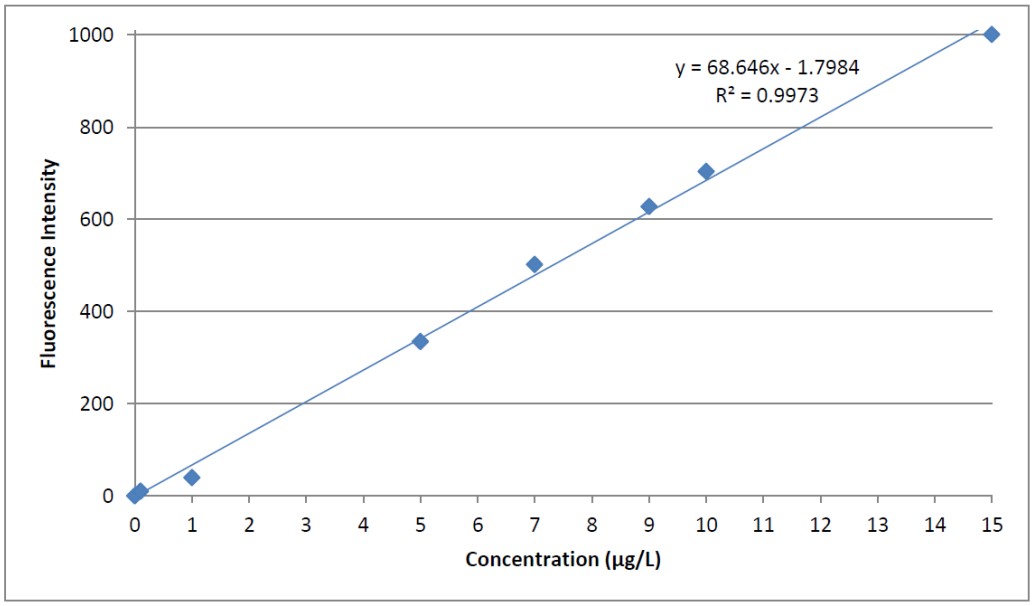


Figure 3.8: Linear relationship of chlorophyll-a concentration in 80 % methanol and fluorescence intensity (609 nm excitation / 680 nm emission)

3.10.1.5 Phycocyanin analysis

The method of analysis was based on research by Simis et al., (2007). Optimum sensitivity for phycocyanin extract measurements was obtained at an excitation wavelength of 610 nm and emission peak of 647 nm. Both excitation and emission bandwidths were set to 3 nm. Prepared phycocyanin extract was then analysed spectrophotometrically.

Prepared serial dilutions standards of pure phycocyanin were diluted into phosphate buffer with concentrations of 2, 10, 20, 60 and 100 µg phycocyanin/L for comparison with the sample extract. Samples readings were calculated based on equation 3.3. Elution of phycocyanin from DEAE-Sephadex A25 resin with 0.2-0.5 M sodium chloride solutions shifted the emission peak from 647 nm in Milli-Q water and other aqueous solutions to 637 nm.

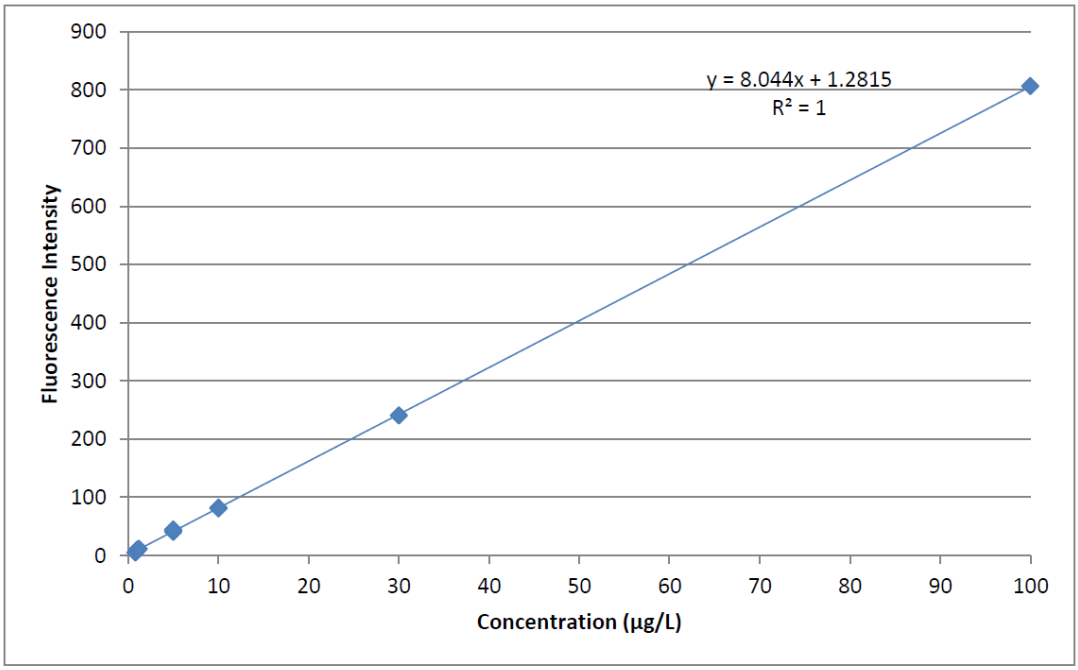


Figure 3.9: Calibration curve of c-phycocyanin in Milli-Q water versus fluorescence intensity 609 nm excitation/647 nm emission

3.10.1.6 Fluorescence Excitation Emission Matrices (EEM)

Fluorescence excitation emission matrices (EEM) analysis was performed between the ranges of 220 to 800 nm. A study by Moberg et al. (2001) investigated chlorophyll-a, b and c and accessory pigments, pheoporphyrin c and pheophytin a and b, within the excitation range of 350-500 nm and emission range of 600-720 nm. From this study, the EEM ranges for excitation and emission were extended to cover the region between 220-800 nm by 10 nm increments to include any spectral interference by NOM on chlorophyll-a and the accessory pigment, phycocyanin (Ludig et al., (2001)).

3.10.2 **Ultra-violet (UV) spectrophotometry**

Ultra-violet/Visible absorbance spectroscopy was performed on a Shimadzu UV-1800 UV Spectrophotometer. The detection of pigments employing UV-Vis was observed at different wavelengths. Chlorophyll-a was measured at 680 nm, phycocyanin at 647 nm (Bridgeman et al.,

2011, Randolph et al., 2008, Silveira et al., 2008). Standards for phycocyanin, dissolved into Milli-Q and/or weak phosphate buffer, and chlorophyll-a, dissolved into 80% Methanol solution, ranged between 10 to 50 mg/L.

Analysis of data was conducted using Microsoft Excel and SPSS PAWS Statistics 18. Microsoft Excel was useful for EEM data plotting, mass data plotting, mean and standard deviation. Mass data plotting PAWS Statistics 18 was used for single t-tests with box plot along with mean determination and ANOVA analysis. Linear curves for standard concentrations were plotted through Excel and by linear regression by SPSS. Linear curves were not determined through any instrumentation such as spectrometers.

3.10.3 **Total Organic Carbon (TOC) Analyser**

Total organic carbon (TOC) analyser is a useful and highly sensitive analytical technique to measure organic carbon in water. This technique was utilised in previous work by Fearing et al (2004), Mergen et al (2008), Zhang et al (2008), Stork et al (2009), Johnson et al (2004), (Henderson et al., 2008a, Henderson et al., 2008b), and Humbert et al (2007).

Dissolved organic carbon in this project was measured by a Shimadzu TOC-V Total Organic Carbon Analyser. The amount of total organic carbon present in each sample was measured by the non-purgeable organic carbon (NPOC method). The potassium hydrogen phthalate calibration curve used a NPOC method calibration ranging to 100ppm with an r^2 value of 0.9999 and a slope of 3.5 (Figure 3.10).

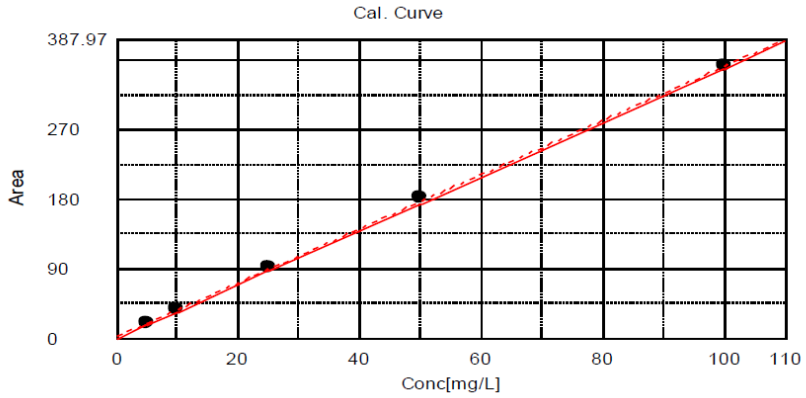


Figure 3.10: NPOC calibration curve used in TOC analysis.

Parameters for NPOC measurements were a manual dilution factor of 5, 50µL injection per sample with 3 replicate measurements for each sample, 2 clean water washes between samples, and 1:30min sparge time with a 10% addition of 2M hydrochloric acid. Zero air carrier gas (150mL/min flow rate) was used to minimise production of carbon dioxide production when samples were oxidised by catalyst in the combustion tube. The sample was then heated to 680°C in the combustion tube in the furnace. Units in mg/L of carbon. All measurements were analysed for mean and standard deviation determination.

3.10.3.1 Sample preparation

All samples for TOC analysis were filtered through a 0.45µm membrane before analysis.

3.10.3.2 Standard preparation

Reagent grade potassium hydrogen phthalate was weighed and dried at 105°-120°C for 1 hour and then allow to cool in a desiccator. Of the previously dried potassium hydrogen phthalate 2.125g was accurately weighed, transferred to a 1L volumetric flask and dissolved with water containing zero carbon. When full dissolved, additional water was added up to the 1L mark. This was retained as a standard stock solution. The carbon concentration of this stock solution corresponded to 1000mg/L or 1000ppm. The stock solution was diluted to concentrations within the calibration

range when calibration of the instrument was undertaken, and for preparing known TOC concentration samples for inclusion in each batch of samples for analysis (i.e. internal standard).

3.11 Limit of Detection (LOD)

The LOD of each species of cyanobacteria was determined by the standard deviation of the baseline measurements as outlined in Equation 3.4 using the approach established by the International Conference of Harmonisation (Shrivastava and Gupta, 2011).

Equation 3.4 $LOD = (3.3 * \sigma / s)$

Where; s = slope of the curve

$\boldsymbol{\sigma}$ = Standard Deviation of Lowest Detectable Standard compared to baseline

Chapter Four

Detection and detection limits of *Microcystis aeruginosa*, *Anabaena circinalis* and, *Cylindrospermopsis raciborskii*.

4.1 Introduction

Fluorescence analysis has been used as a technique for cyanobacteria detection as well as for characterisation of natural organic matter and cyanobacteria as previously discussed in Chapter 2. Three species of cyanobacteria, *Anabaena circinalis*, *Cylindrospermopsis raciborskii* and *Microcystis aeruginosa*, were analysed individually as monocultures to determine the individual species limit of detection by fluorescence and ultra-violet spectrometry. The use of purified pigment standards of phycocyanin and chlorophyll-a, obtained from Sigma-Aldrich, were used to determine the optimum absorbance, excitation and emission wavelengths and applied to cyanobacteria and green algae species detections.

As mentioned in Chapter 3, fluorescence wavelengths that had previously been used for phycocyanin and chlorophyll-a were excitation between 580-620 nm with emission between 620-690 nm, respectfully (Beutler et al., 2003, Dash et al., 2011, Eriksen, 2008, Gons, 2005, Gregor et al., 2007, Izydorczyk et al., 2009, Kana et al., 2009, Larson and Passy, 2005, Logan et al., 2007, Matilainen et al., 2011, Richardson et al., 2010, Simis et al., 2007, Ziegmann et al., 2010).

During the initial fluorescence scans of phycocyanin, other regions of excitation/emission wavelengths were investigated that may have increased sensitivity for phycocyanin or less interference from other compounds. One such alternative wavelength region was within the protein region (Bridgeman et al., 2011, Rodriguez and Nunez, 2011).

During the initial fluorescence scans of phycocyanin, other regions of excitation/emission wavelengths were investigated that may have increased sensitivity for phycocyanin or less interference from other compounds. One such alternative wavelength region was within the protein region (Bridgeman et al., 2011, Rodriguez and Nunez, 2011).

The use of ultra-violet/visible (UV/Vis) spectrophotometry was used for mapping cell growth and was also investigated for any potential benefit for the detection of cyanobacteria. UV/Visible spectrometry was utilised to determine detection limits, optimum absorbance wavelengths of various pigments and observe any associated interfering compounds. Further, UV/Visible spectrometry was investigated to determine if other characteristics, such as the potential separation of cyanobacteria pigments from other species of algae and NOM, and the separation of individual cyanobacteria species from one another, could be employed to aid detection and potentially be a beneficial alternative technique or in addition to fluorescence spectrometry.

The aim of this chapter was to detect phycocyanin, establish the optimum wavelengths for detection and evaluate detection limits on the fluorescence and UV-Visible spectrometers used for this research. This chapter also investigated the influence of interfering compounds that are commonly found in freshwater systems. By determining the various levels of spectral interference and their impact on cyanobacteria detection, the level of interference and influence from CDOC and other algae species present could be measured. Cultures were investigated as pure monocultures, mixed cultures and environmental samples.

4.2 Phycocyanin

Phycocyanins are part of the phycobilins group which are found in cyanophyta that also contain c-phycoerythrin and allophycocyanin. Whilst there are many pigments to aid or undertake photosynthesis, only phycocyanin is found in algae divisions of cyanophyta and glaucophyta

(Barsanti et al., 2008). Figure 4.1 is a representation of phycocyanin and the specific analyte for this research.

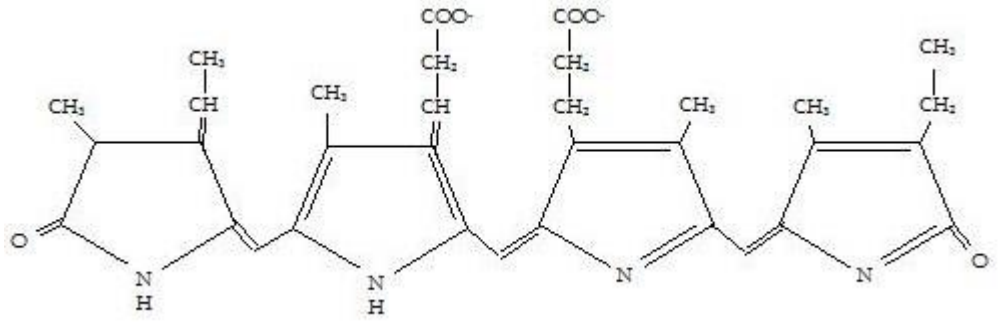


Figure 4.1: Chemical structures of phycocyanin (Colyer et al., 2005, Eriksen, 2008).

Figure 4.2 shows that corresponding to the phycocyanin excitation ranges from literature, the range of excitation wavelengths for *Microcystis aeruginosa* lay between 600-620 nm (Gregor et al., 2007, Hoegar et al., 2004, Hunter et al., 2010, Izydorczyk et al., 2009, Richardson et al., 2010). Any excitation in this range demonstrated a single and consistent emission peak at 647 nm. As the excitation wavelength increased from 600 nm to 620 nm, the emission intensity at peak 647 nm increased, as shown in Figure 4.2. However, due to the close proximity of the excitation and emission wavelengths, a suitable excitation wavelength of 609 nm was selected.

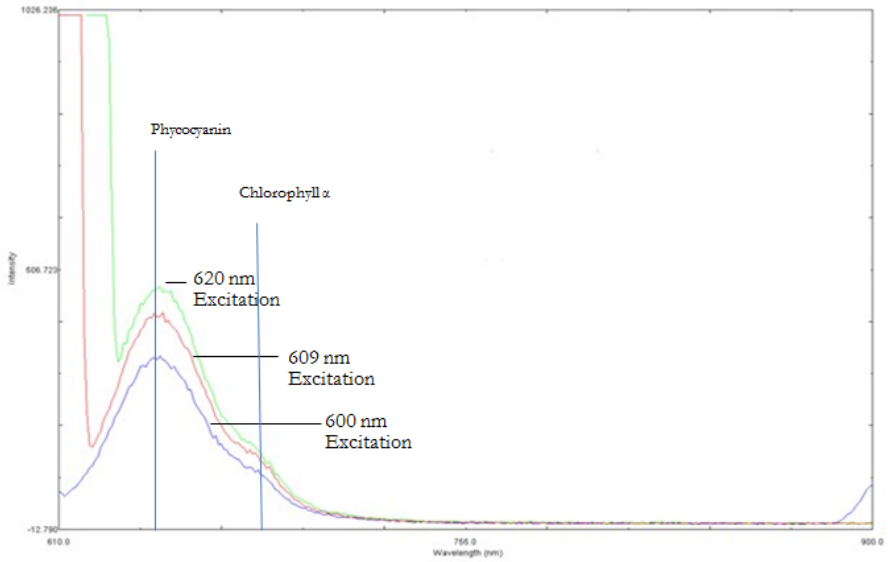


Figure 4.2: Emission of cyanobacterium, *Microcystis aeruginosa* at 647nm when excited at 600 nm (blue), 609nm (red) and 620 nm (green).

In Figure 4.2, the excitation of a culture of *Microcystis aeruginosa* cells had an emission consistent with phycocyanin. However, the smaller shoulder peak to the right of phycocyanin peak was consistent with the emission of chlorophyll-a, which also fluoresces in the same region. This excitation and emission region for measuring cyanobacteria demonstrated increased sensitivity for cell concentrations as the excitation wavelengths (600, 609, and 620 nm) approached the emission wavelength at 637 nm. Chlorophyll-a was a co-emitted peak next to phycocyanin from the same excitation wavelength and had a high potential for interference from other aquatic cultures, such as green algae

4.3 Detection and measurement of cyanobacteria

The detection and measurement of cyanobacteria was conducted by the analysis of phycocyanin at two separate excitation and emission wavelengths. The first excitation/emission region of phycocyanin analysis was the protein region; 291 nm excitation with 337 nm emission. The second was the visible light spectrum and pigment region; 609 nm with 647 nm emission.

Analysis of phycocyanin at two different excitation wavelengths is possible because of the dual nature of the accessory protein, phycocyanin. Measuring phycocyanin at two different regions would allow an alternative source of measurement if one wavelength is compromised by a form of interference. As a pigment with protein functionality, phycocyanin was detectable within the protein region. Figure 4.3 demonstrates the EEM of phycocyanin containing properties within the visible light region and between 280-360 nm. Emission within the visible light region allows phycocyanin to capture light and pass it to chlorophyll-a.

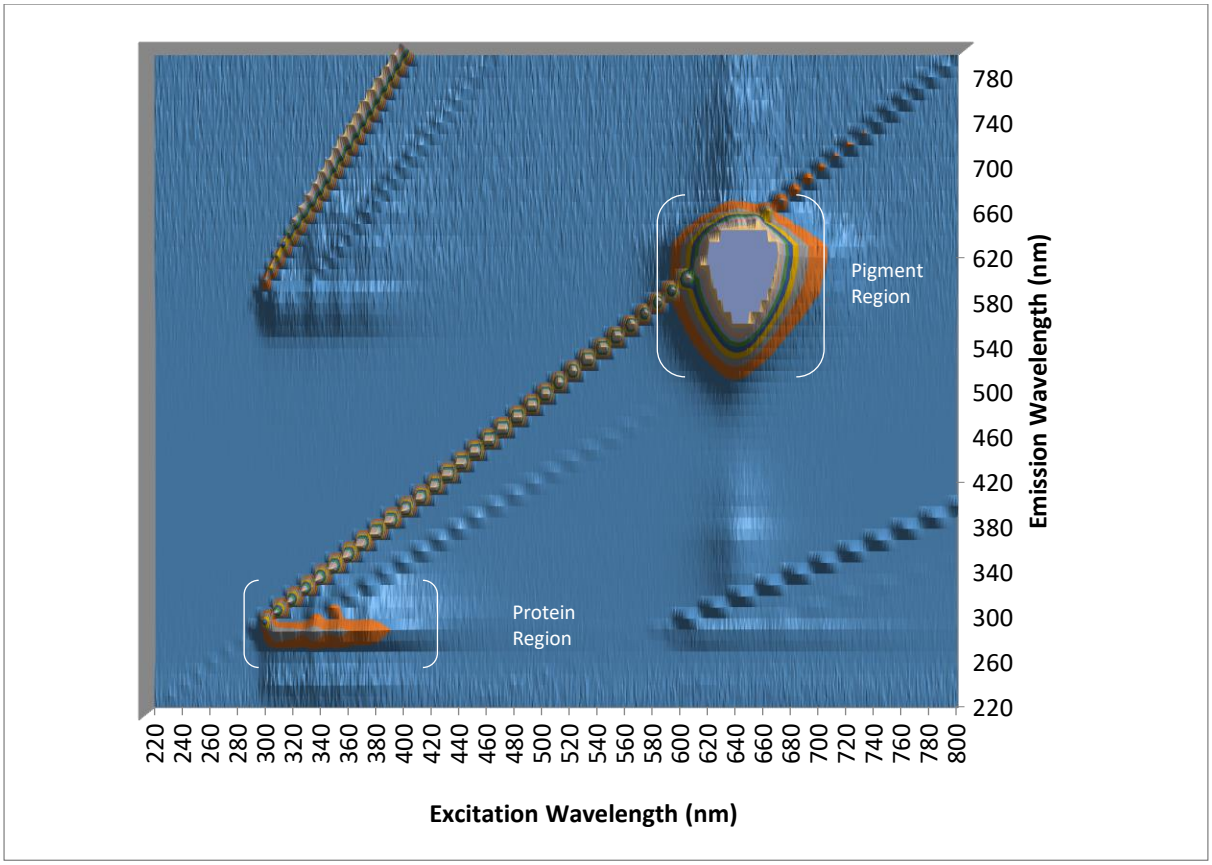


Figure 4.3: Fluorescence EEM matrix plot of 25 mg/L of phycocyanin in deionised water.

Figure 4.3 shows the emission profile of phycocyanin through excitation between 220- 800 nm at 10 nm increments. The multiple colours within the EEM indicate the fluorescence emission intensities between each 100 FIU. Emission was measured between 220-800 nm, which highlighted the main single peak within the visible light region. The purified phycocyanin had a strong fluorescence signal in the visible light region between 580-630 nm excitation with 620-670 nm emission. Mono-cultures of cyanobacteria demonstrated greater linearity within the visible light region associated with the accessory pigment phycocyanin compared to the pigment excitation wavelength of 291 nm.

To determine the effectiveness of the two analysis regions for detecting cyanobacteria, there were three factors taken into consideration; i) linearity of the concentration-cell curve, ii) the Limit of Detection (LOD), and iii) the level of interference from other sources at the wavelength being

investigated, namely NOM and green algae. The LOD is the limit of measurement at which the baseline is observed to establish the minimal limit of detection of an analyte. In this study, that is the detection of the minimum concentration of cyanobacteria. The fluorescence LOD analysis conducted on cyanobacteria required entering the LOD values, determined at emission 337 and 647 nm for all species, outlined in Chapter 3 by Equation 3.7.

The fluorescence spectra of three species of cyanobacteria were measured for a range of cyanobacteria concentrations to determine the linearity of the fluorescence response with cell concentrations, and for determining the LOD of each species. Table 4.1 shows summarised data for excitation at 291 and 609 nm and measuring emission at 337 and 647 nm, respectively. Linearity and detection limits were greatly improved with the detection of phycocyanin within the visible light region compared to detection in the protein region.

Table 4.1 shows summarised data for excitation at 291 and 609 nm and measuring emission at 337 and 647 nm, respectively. Linearity and detection limits were greatly improved with the detection of phycocyanin within the visible light region compared to detection in the protein region.

Table 4.1: Linear calibration of fluorescence at two sets of excitation/emission wavelengths for determination of all three cyanobacteria cultures

Cyanobacteria	Wavelength λ (nm)	(R^2)	No. of Data points (N)	LOD (Fluorescence Intensity Units)	LOD (cells/mL)
Microcystis aeruginosa	Excitation = 291 Emission = 337	0.370	35	58.454 ± 0.724	633,000
	Excitation = 609 Emission = 647	**0.985**	58	**0.521 ± 0.0321**	**22,864 ± 3,464**
Anabaena circinalis	Excitation = 291 Emission = 337	0.527	32	58.454 ± 0.735	105,000
	Excitation = 609 Emission = 647	**0.964**	59	**0.865 ± 0.028**	**43,274 ± 6,557**
Cylindrospermopsis raciborskii	Excitation = 291 Emission = 337	0.194	41	6.90 ± 0.018	379,000
	Excitation = 609 Emission = 647	**0.986**	59	**0.741 ± 0.023**	**49,394 ± 7,484**

Phycocyanin was more sensitively detected, with greater fluorescence emission intensity, when measured within the visible light region with excitation at 609 and emission at 647 nm, than within the protein region of 291 nm excitation with 337 nm emission. With a series of standards between 1-100 μg/L phycocyanin, emission measurement at 647 nm presented a more accurate linear response compared to the protein region as demonstrated by the R_2 values in Table 4.1. The lack of linearity within the protein region (291 nm excitation/ 337 nm emission) was a result of cultures producing proteins other than phycocyanin that contribute to the emission. The production and excretion of proteins at various stages in the cell life cycle expands the number of specific proteins within the samples, and the ratio between phycocyanin and the total protein present cannot be assumed constant (Henderson et al., 2008b, Her et al., 2004). Whilst analysis on live cell samples

were conducted on cultures within the stationary phase of the life cycle, fluorescence measurements at 291 nm excitation and 337 nm emission demonstrated the variable emission of proteins from the cultures.

In further comparison between the two regions of analysis, there was increased linearity of the calibration curves associated with the pigment region when excited at 609 nm. At the same excitation wavelength, the sensitivity of the cyanobacteria was increased.

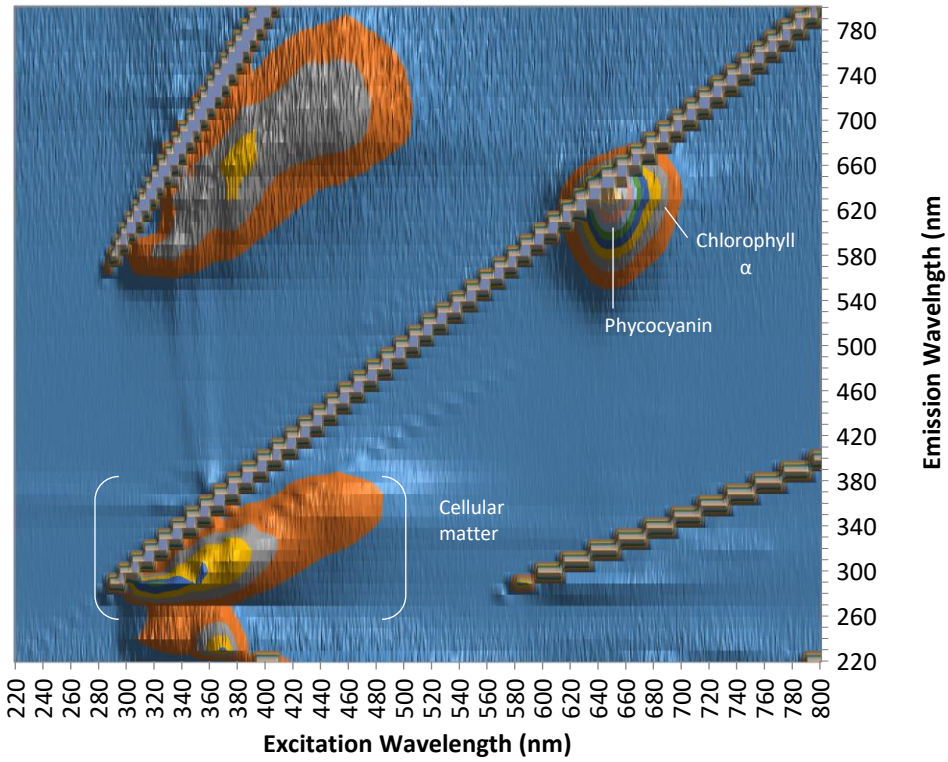


Figure 4.4: Fluorescence EEM of cyanobacterial cells, *Cylindrospermopsis raciborskii*, at cell concentration of $5.2x10^7 \pm 3,10^6$ cells/mL

In Figure 4.4, the emissions of phycocyanin and chlorophyll-a were clearly present in similar regions. However, the emission between 280-450 nm from excitations of 260-340 nm demonstrates the presence of other intercellular organic material. Further, the fluorescence EEM of

cyanobacteria, *Cylindrospermopsis raciborskii*, demonstrates the emission/excitation of phycocyanin along with other cell components within the protein region of excitation/emission, at 220-380 nm excitation wavelengths with emission between 300-480 nm. A decrease in the limit of detection for cell concentration and fluorescence intensity by 2-3 orders of magnitude depending on the species for each cyanobacteria.

Table 4.2: Ratios of phycocyanin excited at 610 nm and measured at 647 nm to chlorophyll-a measured at 680 nm emission for each species cyanobacteria: *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*

Species Blue-green	***Microcystis aeruginosa***	***Anabaena circinalis***	***Cylindrospermospsis raciborskii***
Ratio (610/680)	3.14 ± 0.79	2.15 ± 0.79	2.01 ± 0.49
Equation	y = 27.116x-7.38	y = 35.735x-23.44	y = 23.41x-4.4114
Linear curves (R^2)	0.965	0.860	0.934
Data Points	30 (3 outliers)	23 (2 outliers)	20 (3 outliers)

'*' = observed noise at wavelength 647 nm from green algae species.

Phycocyanin had greater fluorescent response than chlorophyll-a in cyanobacteria species, as demonstrated in Table 4.2. The ratio between phycocyanin and chlorophyll-a was approximately 3.14 for *M. aeruginosa* and approximately 2.15 and 2.01 for *C. raciborskii* and *A. circinalis*, respectfully.

The linear response for purified phycocyanin plotting fluorescence intensity versus cell concentration demonstrated varied R2 values for all three cyanobacteria cultures. Using purchased purified phycocyanin, a linear curve responses of R2 = 0.999, n=18 utilising concentrations between 1-100 μg/L was established. In comparing the linear responses of phycocyanin of each live monoculture to the response from purified compounds, the decrease of R2 in live cell cultures

was contributed to the error of the cell counting technique and the natural variation of pigments (phycocyanin) in living cyanobacteria cultures.

4.3.1 Interference from natural organic matter

The detection of chlorophyll-a and accessory pigment, phycocyanin, was subject to interference by coloured materials, matters and compounds from natural organic matter. The allochthonous fraction of NOM which arises from surface runoff, and degradation of plant and animal matter makes up the foul odour, taste and colour in raw water (Filella, 2009, Henderson et al., 2008a, Henderson et al., 2008b, Her et al., 2004, Lee et al., 2006). The allochthonous matter can interfere and add turbidity or pollute the water system with other colour to overwhelm the target wavelength of interest. This can limit detection of algae and cyanobacteria by masking the detection of pigments.

4.3.1.1 Interference from humic acid

Humic acid was analysed by fluorescence EEM and the results are presented in Figure 4.5. From this matrix plot of the data, the maximum excitation wavelengths for humic acid dissolved into deionised water occurs at an excitation wavelength of 470 nm with emission measured at 531 nm. Whilst this could be an emission shadow from the excitation wavelength running diagonally across the graph, the emission peak at 531 nm was wide spread across the light spectrum.

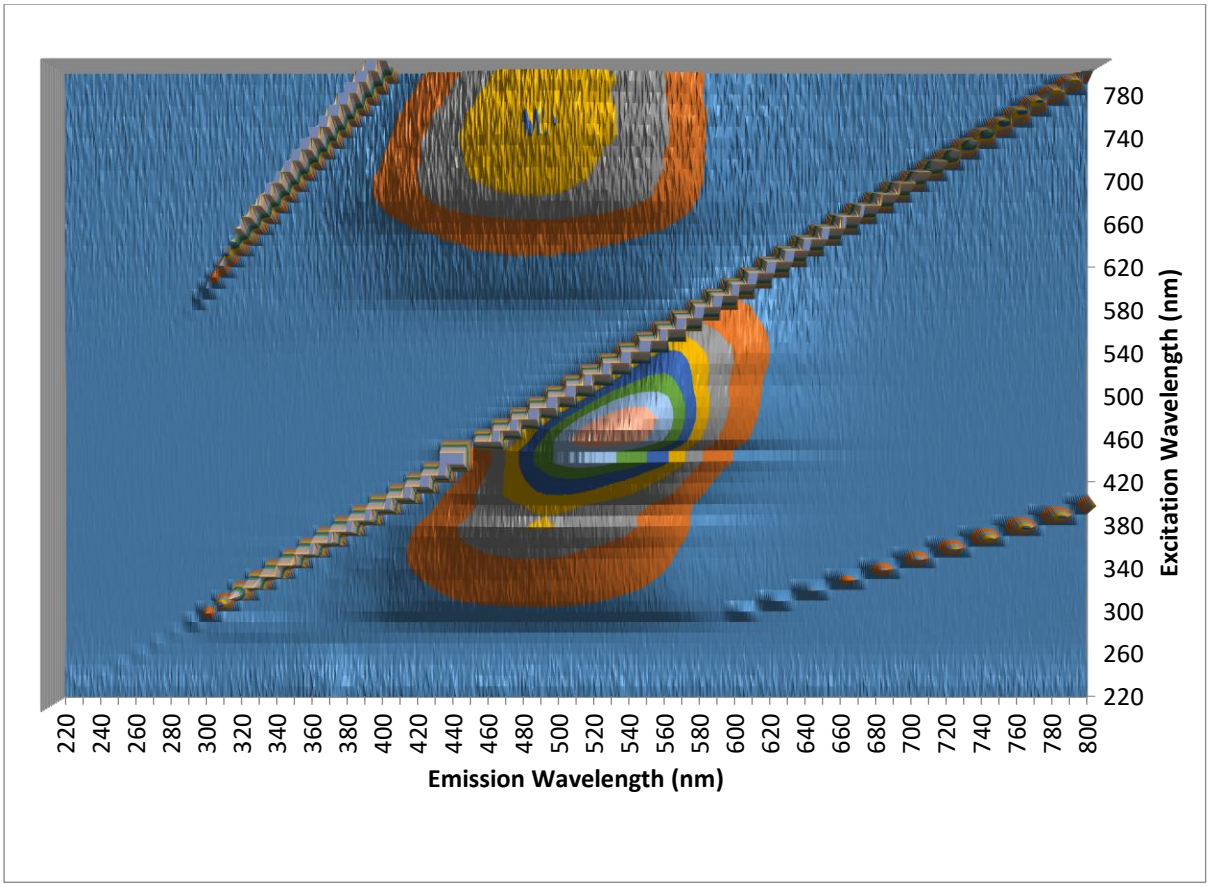


Figure 4.5a: Fluorescence EEM matrix plot of 50mg/L of humic acid sodium salt dissolved into deionised water.

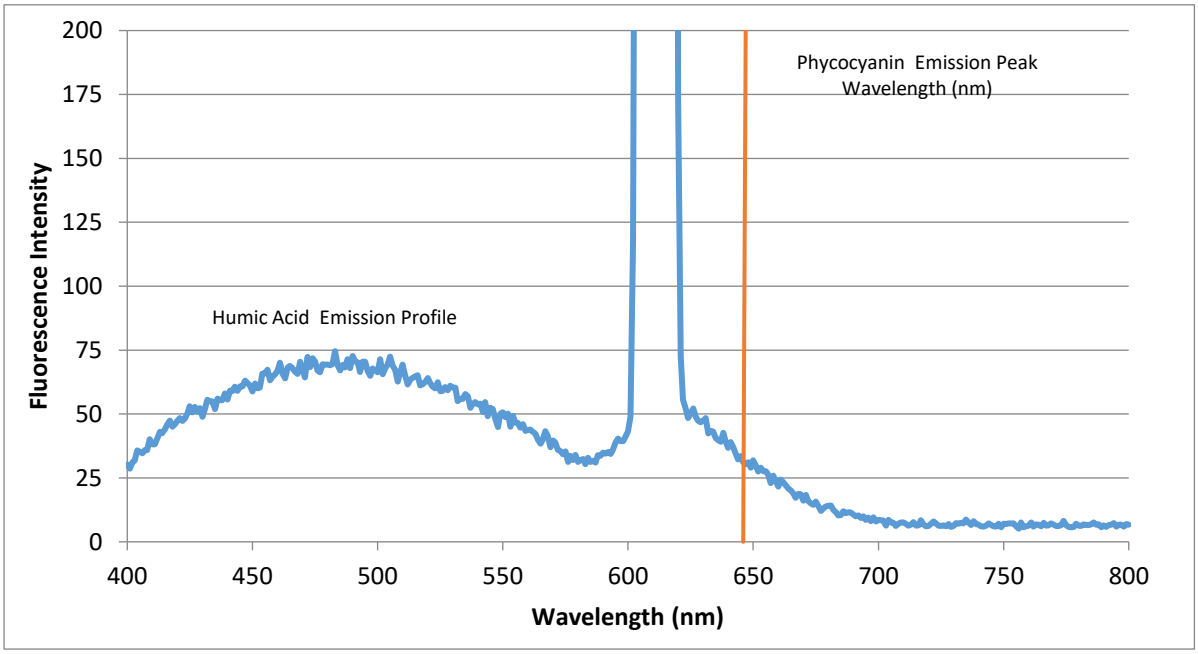


Figure 4.5b: Humic acid, 10 mg/L, emission profile between 400-800 nm when excited at 609 nm.

This emission of purified humic acid sodium salt dissolved into Milli-Q water shown in Figure 4.5a and 4.5b demonstrates similar characteristics to that presented by Bridgeman et al. (2011). Bridgeman et al., (2011) investigated the excitation and emission ranges of aromatic, humic-like and fulvic-like substances. Humic-like substances were determined to be excited within 250-400 nm with emission measured between 380-540 nm.

The EEM profile of humic acid demonstrated the potential interference with the broad emissions between 420-620 nm with the excitation 280-580 nm. Of most concern for the emission of the humic acid profile was the width of the emission range and the potential carryover of humic acid signal emission into the path of phycocyanin signal emission at 647 nm.

Table 4.3: Influence of 10mg/L of CDOC on each cyanobacteria monoculture at 609 nm excitation/647 nm emission.

Species	***Microcystis aeruginosa***	***Cylindrosoermopsis raciborskii***	***Anabaena circinalis***
LOD in Deionised Water (cells/mL)	*22,864 ± 3,464*	*49,394 ± 7,484*	*43,274 ± 6557*
LOD in 10 mg/L of CDOC (cells/mL)	*108,200 ± 12,200*	*180,333 ± 20,333*	*135,300 ± 15,250*

With influence of 10 mg/L of CDOC, the limits of detection for all species of cyanobacteria increased to approximately 80,000-130,000 cells/mL for direct measurement by the fluorescence spectrometer used. These cell detection limits were determined to give a clear and undisputed signal that there was a presence of cyanobacteria and could be quantified.

Of the three compounds used to mimic natural organic matter, only humic acid demonstrated no interfering emission within the protein region when excited at 291 nm. There was a slight negative gradient proportional to the increasing humic acid concentration. This was determined to be due to

the additional colour from the increasing humic acid concentration; the negative gradient of emission intensity from increasing humic acid decreases the light able to pass through the cuvette compared to purified water. Whilst some spectrofluorometers and probes are able to adjust for the influence of organic matter as previous explored in Chapter 2, this research was designed to measure pigment and the influence of CDOM but not adjust for any influence of CDOM.

Table 4.4: Humic acid fluorescence emission at 337 and 647 nm

Dissolved Humic Acid conc. (mg/L)	**Fluorescence Intensity Units (FIU)**	
	Excitation wavelength = 291 nm, Emission wavelength = 337 nm	Excitation wavelength = 609 nm, Emission wavelength = 647 nm
10	-	5.41 ± 0.61
12.5	-1.16 ± 0.46	7.11 ± 0.61
15	-	9.66 ± 0.61
22.5	-	15.77 ± 1.01
30	-3.14 ± 0.46	20.91 ± 1.01
50	-4.79 ± 0.46	26.93 ± 1.95
75	-5.67 ± 0.46	32.88 ± 1.95
100	-	38.89 ± 1.95

When excited at 609 nm, humic acid demonstrated emission at 647 nm of up to 38.89 ± 1.95 FIU for concentrations up to 100 mg humic acid/L. For CDOC concentrations between 0 – 30 mg/L the concentration curve was linear. For concentrations between 15-100 mg/L, the best expression for the concentration curve was logarithmic (R^2 = 0.998). Whilst, any concentration above 15 mg/L of humic substances is potentially unlikely in any environmental water, there was a potential interference of humic acid emission with the board emission peak that could interfere with the 647 nm emission peak. This emission from humic acid was most likely due to aromatic compounds in humic acid fluorescing.

4.3.1.2 Interference from alginic acid

As the substitute for polysaccharides, alginic acid was excited at 291 nm and 609 nm to determine the level of interference produced when attempting to measure cyanobacteria. Alginic acid was measured through a five point calibration with a R^2 value of 0.991, demonstrated in Table 4.5.

Table 4.5: Alginic acid fluorescence signal emission at 337 and 647 nm

Dissolved Alginic Acid conc. (mg/L)	**Fluorescence Intensity Units (FIU)**	
	Excitation wavelength = 291 nm, Emission wavelength = 337 nm	Excitation wavelength = 609 nm, Emission wavelength = 647 nm
3	0.34 ± 0.20	-
10	4.88 ± 0.20	-0.05 ± 0.14
15	10.46 ±0.20	0.29 ± 0.14
30	20.90 ± 0.20	0.05 ± 0.14
50	42.95 ± 0.20	0.01 ± 0.14
100	-	0.06 ± 0.14

The lack of emission from alginic acid when excited at 609 nm with emission at 647 nm was a positive indication that alginic acid-like substance would not have an influence on phycocyanin detection. The abundance of polysaccharides in water systems from allochthonous and autochthonous organic matters is ubiquitous and is able to be measured, not with a single spectral scan but with multiple scans by employing extractions and purification techniques (Elliott et al., 2006, Henderson et al., 2008b, Her et al., 2004). The addition of fluorescence intensities from spectral interferences may diminish or suppress the detection at trace cell concentration of cyanobacteria; the addition of even 10 mg/L of alginic acid, a high polysaccharide concentration for natural waters, only alters the fluorescence emission intensity units by 5 FIU and requires 30 mg/L of alginic acid to obtain an emission intensity above 20 FIU as demonstrated in the right side column of Table 4.5.

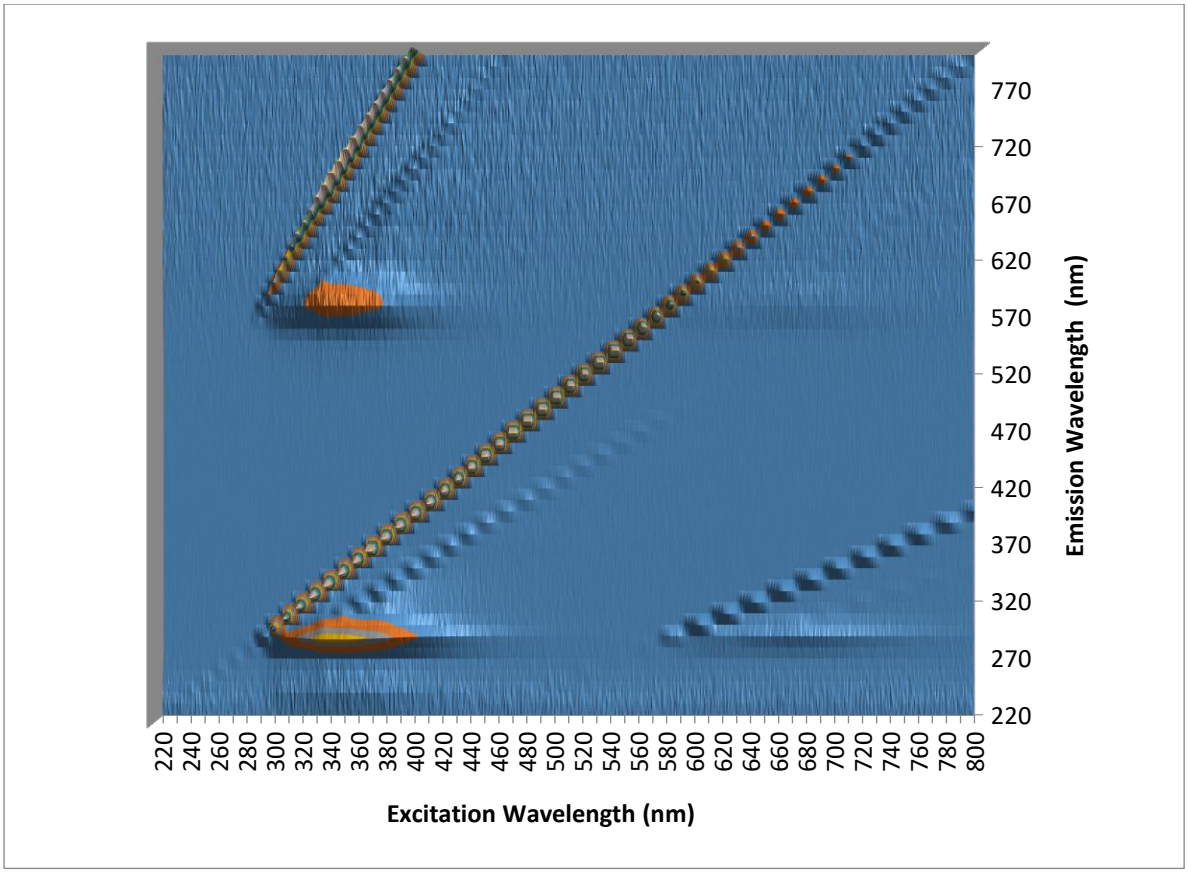


Figure 4.6: Fluorescence Excitation/Emission Matrix of Alginic Acid

This baseline emission of alginic acid from 609 nm excitation, providing all polysaccharides behave like alginic acid, means that any presence of polysaccharides in surface waters can be spectrally excluded from detection and thus from interfering with the detection of cyanobacterial pigment of phycocyanin.

4.3.1.3 Interference from bovine serum albumin

Bovine serum albumin (BSA) was used to simulate the presence of proteins in water. The concentration of the standard curve for protein that BSA emits at 337 nm is significantly high given the target cell concentration of cyanobacteria, 5,000-50,000 cells/mL. Despite the excitation range for protein-like substances lying between 250-280 nm, BSA was detectable from 3 mg BSA/L with linear growth of the emission intensity up to 50 mg BSA/L before concentrations were off scale and no longer measureable. Hence, excitation at 291 nm with emission at 337 nm was the region

for measuring proteins. As BSA standards were dissolved into Milli-Q water with no other interference from dissolved organic matter, any increase in emission fluorescence intensity was due to the presence of BSA.

Table 4.6: Bovine Serum Albumin fluorescence emission at 337 and 647 nm

Dissolved Bovine Serum Albumin conc. (mg/L)	**Fluorescence Intensity Units (FIU)**	
	Excitation wavelength = 291 nm, Emission wavelength = 337 nm	Excitation wavelength = 609 nm, Emission wavelength = 647 nm
3	6.215	0.372
10	71.090	2.054
15	-	3.870
30	575.476	5.339
50	884.847	7.662

Bovine serum albumin had significant spectral interference in the detection of cyanobacteria pigments by proteins when emission was measured at 337 nm from 291 nm excitation. In Table 4.6, the emission from BSA at 337 nm is highly problematic when emission from cyanobacteria only exceeded emission intensity above 350 FIU at cell concentrations of x10^8 cells/mL; this was a limitation of the fluorescence instrument utilised for this study. Hence, if excitation wavelength of 291 nm was utilised to detect cyanobacteria in the presence of proteins in the sample water, the excitation of proteins in the sample water will more likely overwhelm any emission from cyanobacteria.

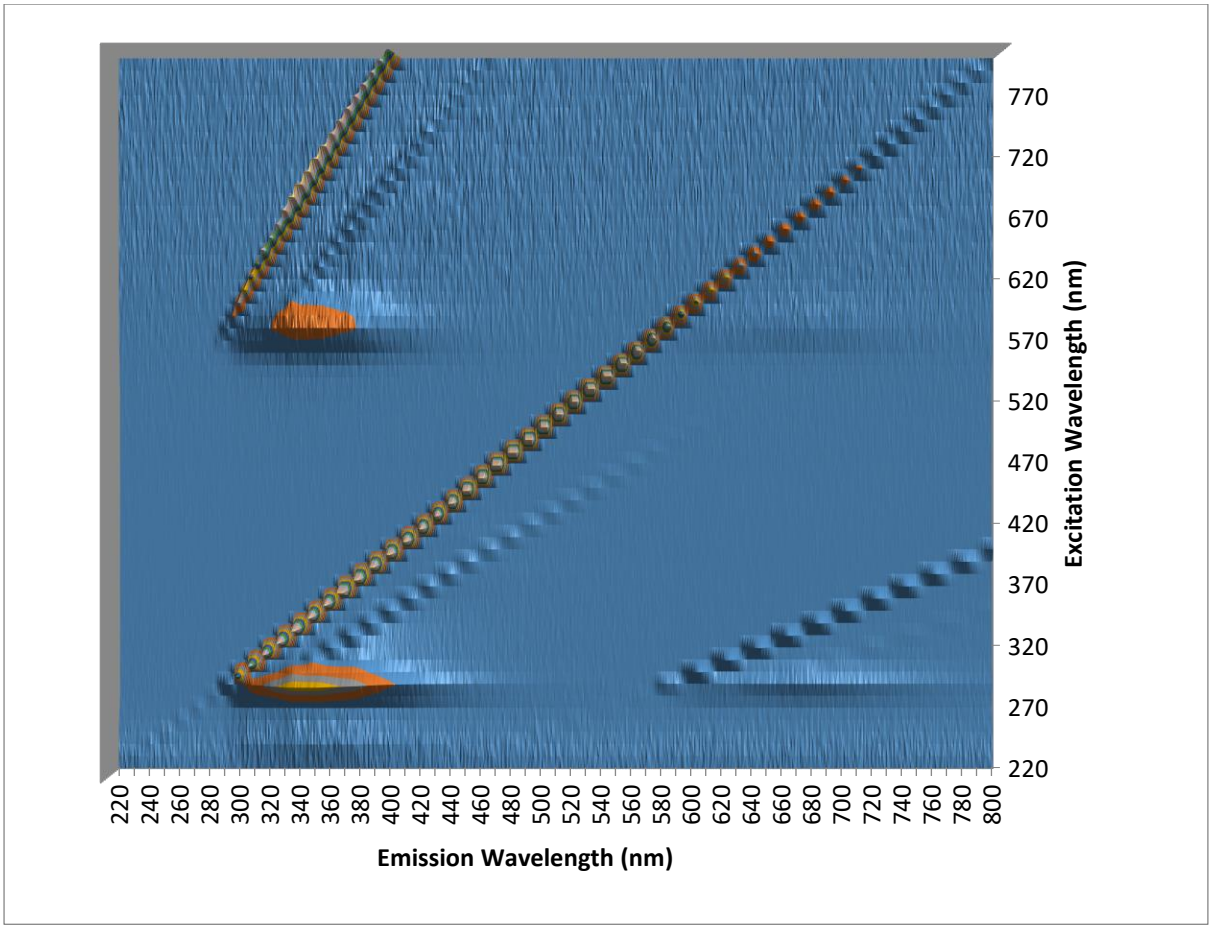


Figure 4.7: Fluorescence Excitation/Emission Matrix of 10 mg/L of BSA.

The linearity of BSA fluorescence versus concentration (mg/L) was $R^2 = 0.986$ when measured in the protein region, 291 nm excitation and 337 nm emission. The excitation and measurement of the pigment region for phycocyanin was 609 nm excitation and 647 nm emission. The concentrations of BSA used to determine the interference measured at this region were excessive. Natural water chemistry can be random but generally protein levels will not exceed concentrations of 5 mg/L (Filella, 2009, Frank, 1972). When measuring phycocyanin in the region 291 nm excitation and 337 nm emission, the presence of protein can interfere and produce inaccurate results for phycocyanin. It is unlikely for BSA to be zero-point or baseline corrected when exciting samples at 291 nm, as proteins are unable to be excited within any visible light region of the colour spectrum unlike CDOC like humic and fulvic acids.

From 609 nm excitation, the problem of high emission from BSA was markedly decreased and never reached an emission intensity over 23.81 ± 1.00 at concentrations of 200mg BSA/L, with a linear curve of R2= 0.989. The excitation of BSA at 609 nm demonstrates lower fluorescence emission compared to 291 nm. This was beneficial as high concentrations of BSA did not cause significant emission interference, and the low concentrations of proteins in natural waters means that any interference from proteins is likely to be small.

It was determined that any material associated with CDOC, like humic/fulvic acid, across the wavelength pigment region would potentially interfere with the pigment analysis of algal and cyanobacteria cultures. The application of applying zero-point correction and Gelbstoff measurements by current commercial devices is useful as long as the measurements are accurate to maintain a reliable correction and the water conditions remain constant, which if not could be influential to cause overestimations or underestimations. However, the mentioned applications only adjust the measurement for CODC rather than removing the CDOC influence or isolating the analytes.

4.3.2 Interference from green algae species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*

Various pigments from algae, diatoms and cyanobacteria can be present in the visible light spectrum when in high enough cell concentration to be detected. Whilst chlorophyll-a is present through all these species to undertake photosynthesis, the accessory pigments vary between different species as previously discussed in Chapter 2. The spectrum of live green algal cells, *Chlamydomonas reinhardtii*, in Figure 4.6 demonstrates the excitation wavelengths of chlorophyll-a with a fixed wavelength of 680 nm.

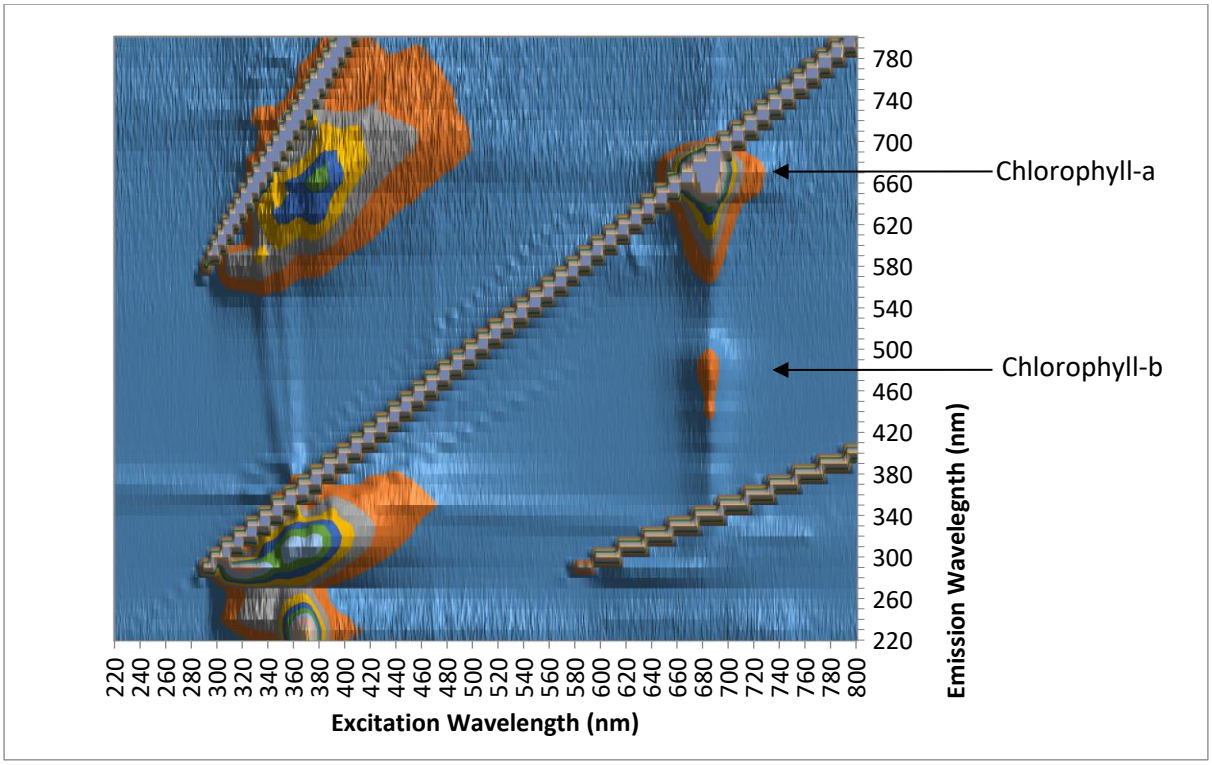


Figure 4.8: Fluorescence EEM of green algal cells, *Chlamydomonas reinhardtii,* at cell concentrations $1.8x10^7$ ± 2588 cells/mL.

One observation that was clear between the EEM of cyanobacterial cells and green algal cells was the presence of chlorophyll-b in *Chlamydomonas reinhardtii* cells, noticed in Figure 4.8, and the absence of this compound in *Cylindrospermopsis raciborskii* cells, noticed in Figure 4.4. The fluorescence region of chlorophyll-b for *Chlamydomonas reinhardtii* was 420-520 nm when excited at 660-700 nm.

In a similar manner to the fluorescence EEM of cyanobacteria cells, *C. raciborskii* in Figure 4.4 has peaks from excitation between the regions of 360-480 nm where the emission is close to the top and centre excitation lines of the EEM graph. The emission from the algal organic matter at this excitation region occur at three emission regions; 220-270 nm, 270-380 nm and 550-780 nm for the excitation region of 360-480 nm.

From this fluorescence EEM data of phycocyanin from Figure 4.3, *C. raciborskii* from Figure 4.4 and *C. reinhardtii* from Figure 4.6, spectral interferences from chlorophyll-a from green algae cells or the presence of chlorophyll-a from other photosynthetic plant matter from surface runoff was observed. This matter from surface runoff is further known as autochthonous and allochthonous organic matter (Henderson et al., 2008a, Her et al., 2004, Lee et al., 2006), previously discussed in Chapter 2.

An excitation profile was measured from 220-800 nm for the optimum excitation wavelength. This was achieved by assigning a fixed emission wavelength of 680 nm and varying the excitation wavelength to identify the optimum excitation wavelength.

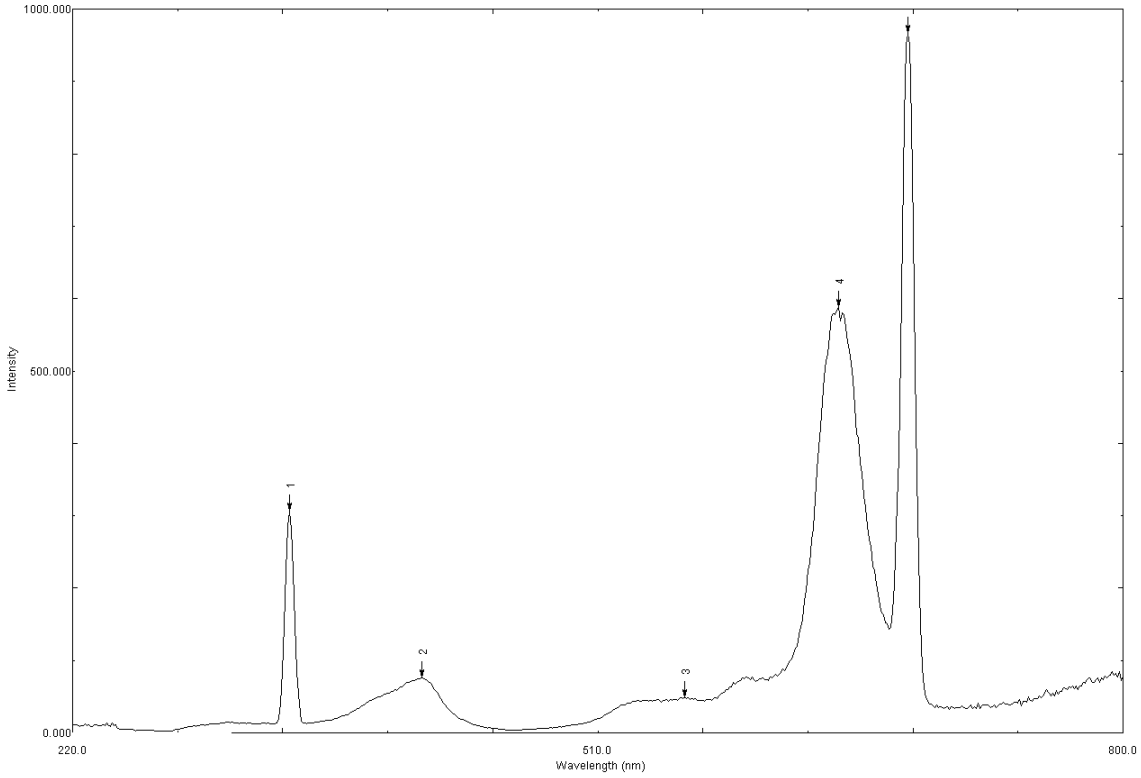


Figure 4.9: Excitation wavelength profile scan from 220-800 nm with a fixed emission point of 680 nm.

Excluding the high emission chlorophyll-a peak at 680 nm, there was a natural excitation

wavelength of 647 nm, peak 4, present in Figure 4.7. This wavelength is the excitation wavelength of phycocyanin with emission fixed at 680 nm, indicated by peak 4 of Figure 4.9.

Due to phycocyanin being an accessory protein, the excitation peaks for chlorophyll-a with a fixed emission point of 680 nm in Figure 4.9 demonstrates an excitation peak for chlorophyll-a at the same wavelength of phycocyanin's emission peak. Hence, the ability of phycocyanin to absorb photons of light and pass onto chlorophyll-a can be demonstrated through this observation. However, the problem associated with this observation is the spectral interference from chlorophyll-a when trying to detect pigment phycocyanin, which is specific to cyanobacteria. This interference can be observed in Figure 4.10 through the excitation of mono-culture cyanobacteria species of *M. aeruginosa*, *C. raciborskii* and *A. circinalis*, and mono-culture green algae, *Chlamydomonas reinhardtii*.

Baseline corrections, much like NOM zero-point correction or other measurements, may be employed to counter the spectral interference from chlorophyll-a from other sources. However, this leads to the same potential error in phycocyanin measurement of under or over estimations of cyanobacteria concentration if the concentration of green algae changes or the relationship used in the correction is not reliable as previously stated for CDOC interference.

As chlorophyll-a emits at wavelengths close to those for phycocyanin, attempting to apply any measurement correction would also impact the emitted phycocyanin measurement as the emission of phycocyanin overlap with chlorophyll-a as demonstrated in Figure 10a and b.

Figure 4.10a presents the overlay spectra of cyanobacteria species, *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*, and green algae, *Chlamydomonas reinhardtii*, excited at 610 nm with emission measured between 620 to 900 nm.

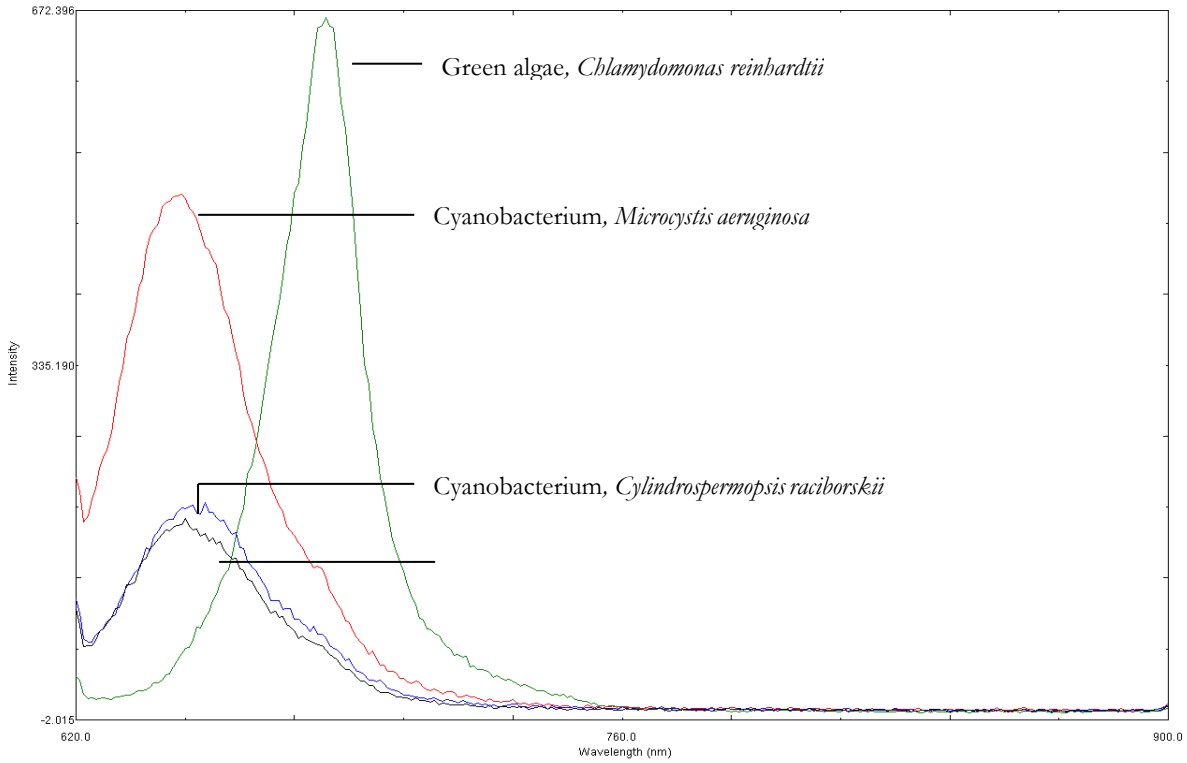


Figure 4.10a: Overlaying emission of 620 nm to 900 nm of all three cyanobacteria species, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii* and *Anabaena circinalis*, and green algae, *Chlamydomonas reinhardtii*, with excitation 609 nm.

From the overlay of four emission spectra, the separation of chlorophyll-a and phycocyanin was observed. Despite the separation of different pigments, the intensity of emission from chlorophyll-a and green algae, *C. reinhardtii*, at 682 nm overlays the emission measured at 647 nm for phycocyanin, the optimum wavelength to measure the blue pigment found in cyanobacteria. When the same emission spectra was observed between 620 to 750 nm as observed in Figure 4.10b, the peak from green algae, *C. reinhardtii*, was assigned as the emission of chlorophyll-a at 686 nm. As expected, there was no emission peak at 647 nm from green algae, which is due to the absence of phycocyanin. The width of the emission peak was of concern.

The width of emission from chlorophyll-a does and can cause some low level interference in the emission of phycocyanin at 647 nm. This interference could occur if the ratio of green algae species

is greater than the low level concentration of cyanobacteria attempting to be detected. Whilst this is based on the fluorescence spectrometer used in this study, it was an observation made by the vertical line of phycocyanin to the baseline in Figure 4.10b.

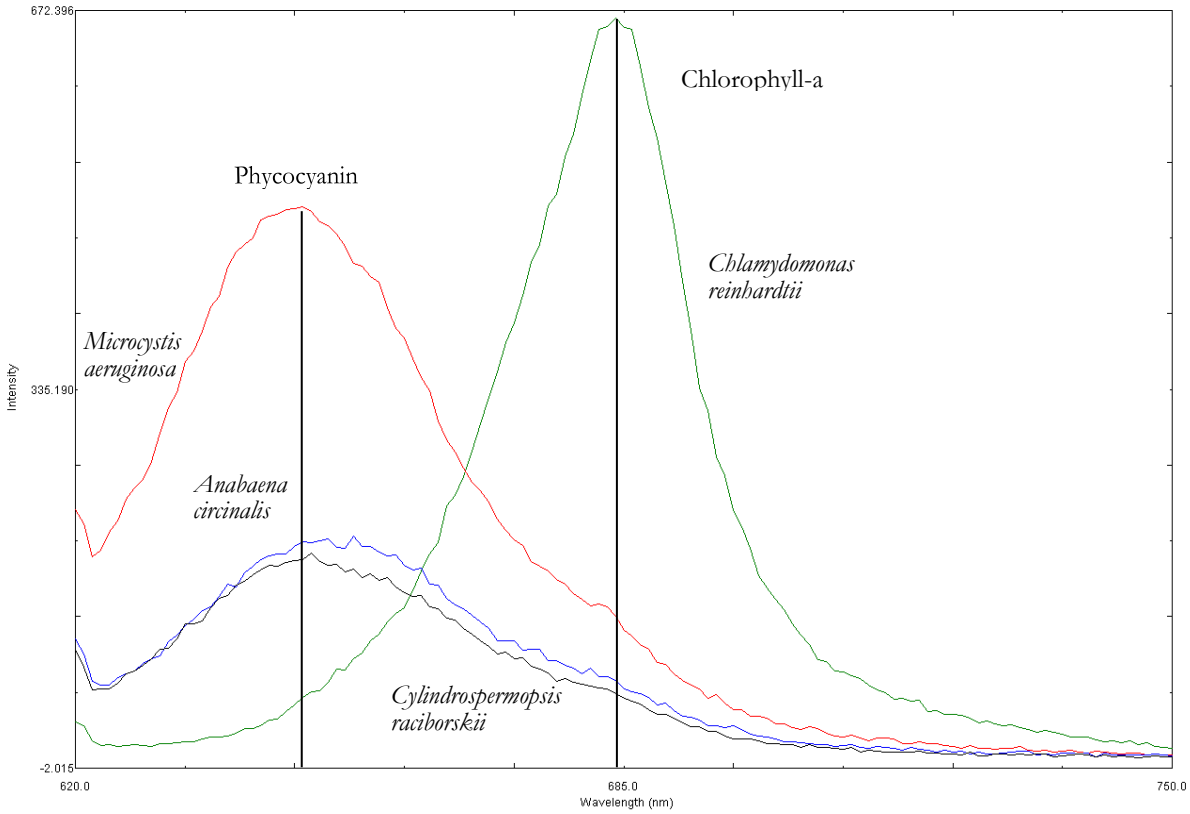


Figure 4.10b: Overlay of emission spectra between 620nm to 750 nm from cyanobacterial species, *Microcystis aeruginosa*, *Anabaena circinalis*, *Cylindrospermopsis raciborskii*, and green algae, *Chlamydomonas reinhardtii*, with excitation at 609 nm.

All three cyanobacteria species present in Figure 4.10b present peak tailing well through into the emission peak for chlorophyll-a. This was supported by Table 4.4 demonstrating the emission ratios between phycocyanin and chlorophyll-a within mono cultures. Taking a closer look at the emission spectra from cyanobacteria, *C. raciborskii* (blue line) and *A. circinalis* (black line), there was a slight secondary peak to the right which comes from the emission of phycocyanin at 647 nm at the same emission wavelength as that of chlorophyll-a. Hence, the ratio of chlorophyll-a to phycocyanin that was present can be measured by means of fluorescence emission.

From this observation, excitation for all species of cyanobacteria demonstrated linear responses up to 700 FIU (fluorescence intensity units) with cell concentrations of >1x10^7 cells/mL whilst green algae, *C. reinhardtii*, did not present much interference at 647 nm at similar cell concentrations.

Whilst the fluorescence intensity of green algae, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*, did not demonstrate a high influence on the emission of 647 nm, the potential for green algae spectral interference remains. Hypothetically, if any green algae species was the dominant culture in high concentrations, 100,000 to 10,000,000 cells/mL, the presence of chlorophyll-a detected could potentially hide the presence of low concentrations, 50,000 to 250,000 cells/mL of cyanobacteria, when measuring for phycocyanin at 647 nm.

Further, this observation is not limited to green algae, any species other than cyanobacteria containing chlorophyll-a when in high cell concentrations or in high biomass volumes, which will increase the presence of chlorophyll-a, has the potential to interfere with the detection of cyanobacteria.

4.4 Detection by Ultra-Violet/Visible Spectrophotometry

4.4.1 Measurement of cyanobacteria

There were differences in the physiology for each of the three cyanobacteria species analysed, but they are also some similarities. Each species of cyanobacteria, when in high concentrations exceeding >x10_7 cells/mL, demonstrated the presence of different light-harvesting pigments at various wavelengths.

Through the visible light region chlorophyll-a, chlorophyll-b and phycocyanin could be detected by UV/vis spectrophotometry at 680 nm, 440 nm and 620 nm, respectively (Nelson and Cox,

2005). Further, this research confirmed the absorbance of chlorophyll-a and phycocyanin, demonstrated in Figure 4.10 using the cyanobacteria species of this research, *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*.

The detection of cyanobacteria by UV/Vis spectrometry was possible if the pigments were in high enough concentration to be detected. The dominant absorbance peaks of chlorophyll-a and b, and phycocyanin dropped considerably during serial dilutions. The detection limit of *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii* and *Anabaena circinalis* were approximately 1,000,000 cells/mL. This was considerably higher than the detection limits of approximately 23,000 and 45,000 cells/mL with fluorescence excitation and emission of phycocyanin.

Figure 4.11 presences the absorbance profile of the three cyanobacteria species investigated. Absorbance was measured recorded between 300-900 nm using a 1 cm^2 quartz cuvette. Whilst 1cm^2 quartz curvettes were used and not 0.1cm^2, this was to maintain consistency by using the same volumes and concentrations as were used for fluorescence analysis.

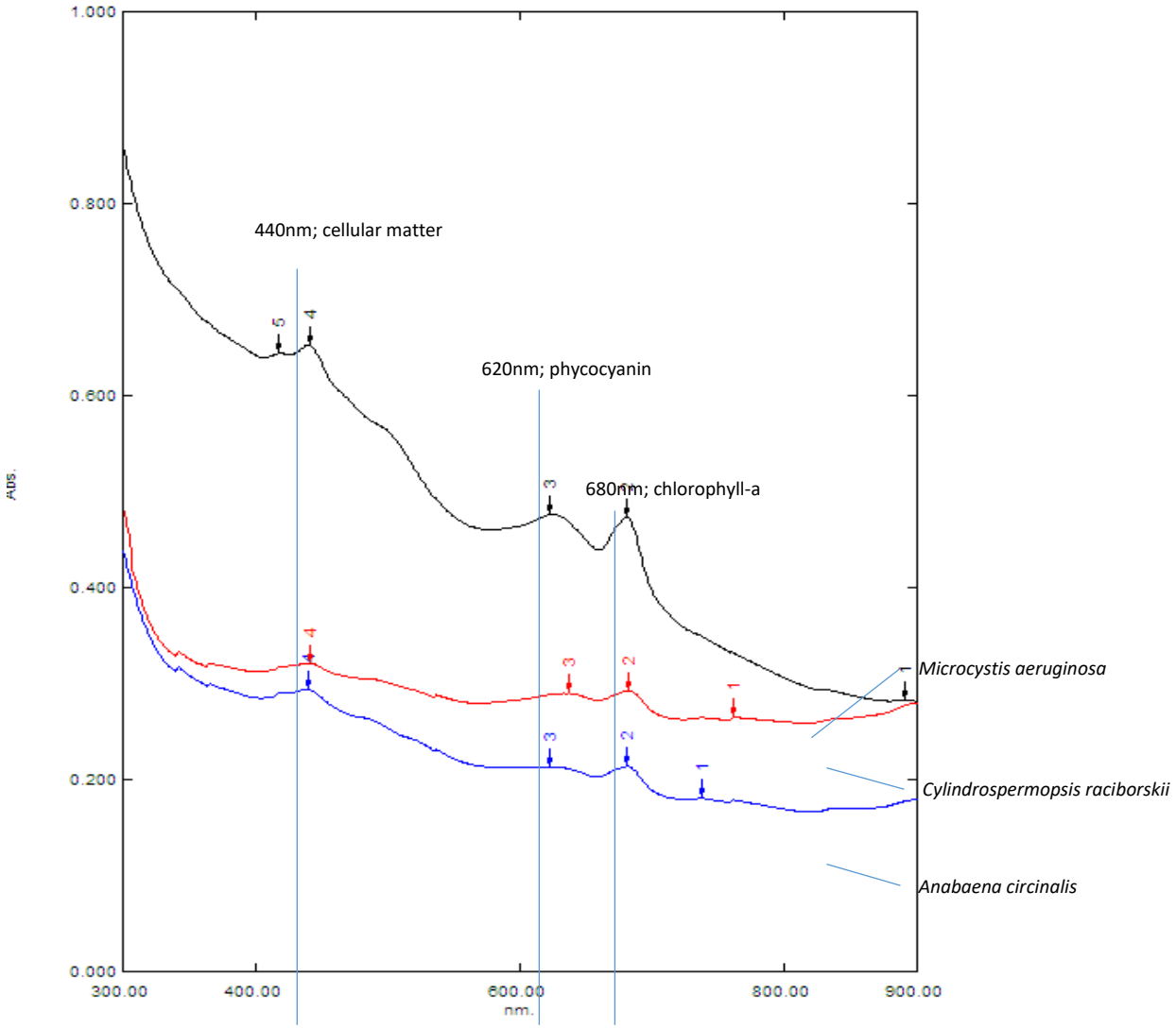


Figure: 4.11: UV-Visible spectrometry profiles of cyanobacteria species.

In Figure 4.11, there are three peaks labelled with the approximate wavelength absorbance peaked; 620 nm was phycocyanin, 680 nm was chlorophyll-a and 440 nm was labelled as cellular matter from the cyanobacteria cell. Overlooking the whole profile, there is a gradient increase from 800 nm towards 400 nm. Before 400 nm, the absorbance rapidly increased towards 300 nm and beyond. This rapid incline beyond 300 nm is due to intercellular matter. With the absorbance profiles presented, concentration curves could be estimated for each species of cyanobacteria. The concentration curves are present in the following figures 4.12 to 4.14.

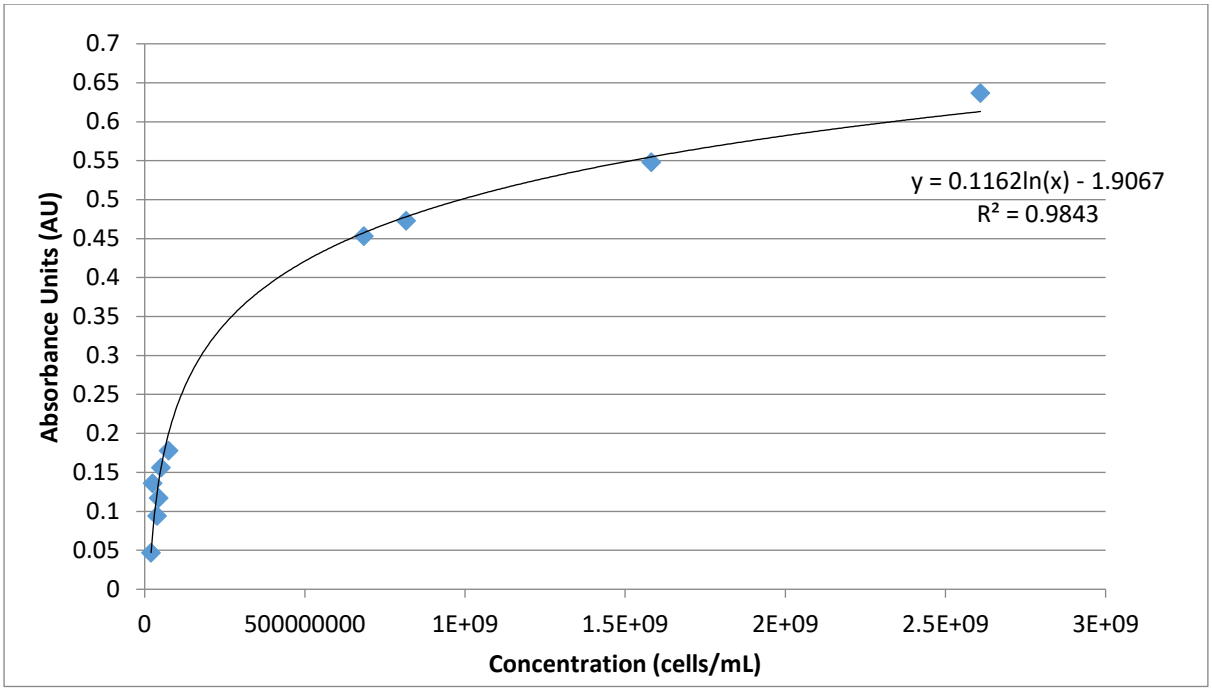


Figure 4.12a: Standard curve of *Microcystis aeruginosa*, optical density measurements of chlorophyll-a at 680 nm (y axis) against the observed cell concentration (x axis).

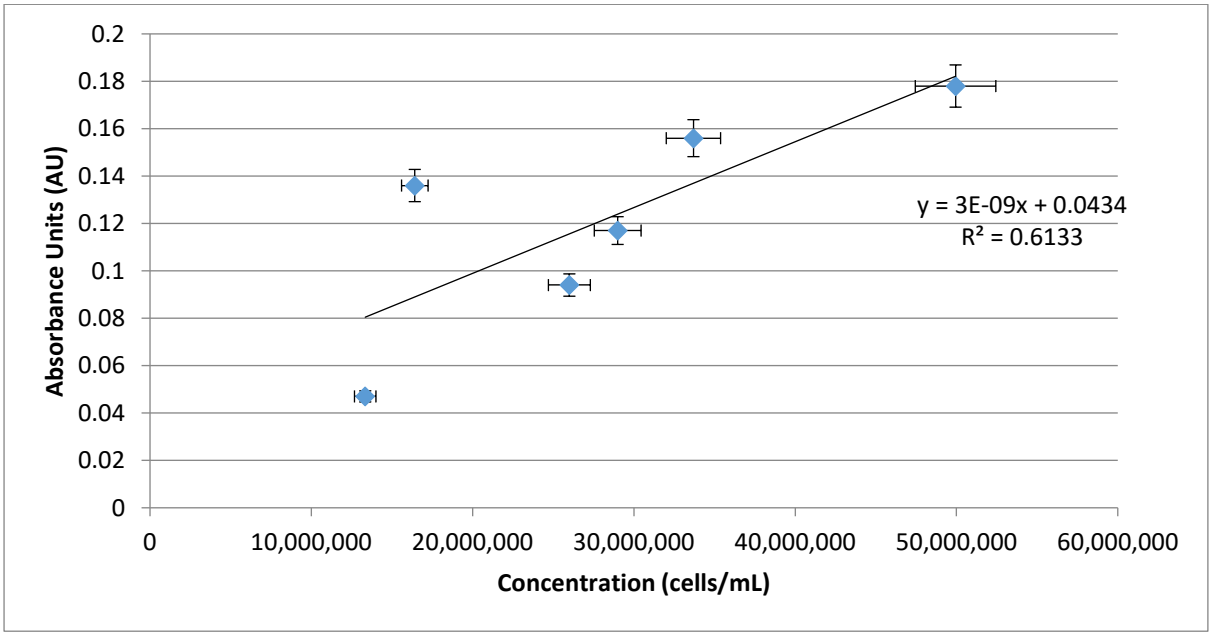


Figure 4.12b: Standard curve of *Microcystis aeruginosa*, optical density measurements of chlorophyll-a at 680 nm (y axis) against the observed cell concentration (x axis).

By considering only data from the lower cell concentration, linear range of the curve in Figure 4.12,

a R^2=0.613 can be obtained. However, even for this lower concentration ranged, the cell concentration of *Microcystis aeruginosa* was still within the tens of millions of cells/mL, well above detection limits of interest. Another observed limitation of the detection technique was the absorbance units versus the cell concentration in both Figure 4.12a and b. There was a significant difference in linear responses and cell concentration ranges between fluorescence and UV/Vis spectrometry. These characteristics were not isolated to one species of cyanobacteria as demonstrated for *Anabaena circinalis* in Figure 4.13, and for *Cylindrospermopsis raciborskii* in Figure 4.14.

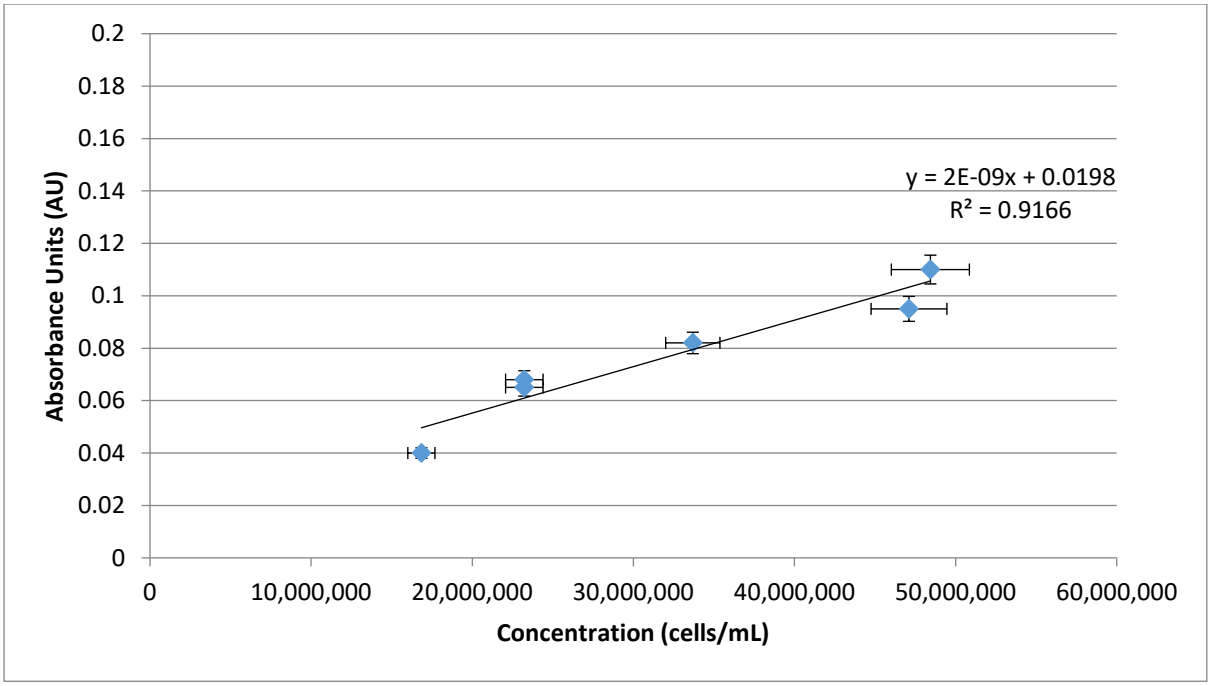


Figure 4.13: Standard curve of *Anabaena circinalis*, optical density measurements of chlorophyll-a at 680 nm (y axis) against the observed cell concentration (x axis).

Figure 4.13 demonstrates the linearity of cell concentration of *Anabaena circinalis* versus the optical density measurements using UV/Vis spectrometry. Even diluting the sample concentration to within a linear range for UV/Vis spectrometry would still be less sensitive compared to fluorescence spectrometry.

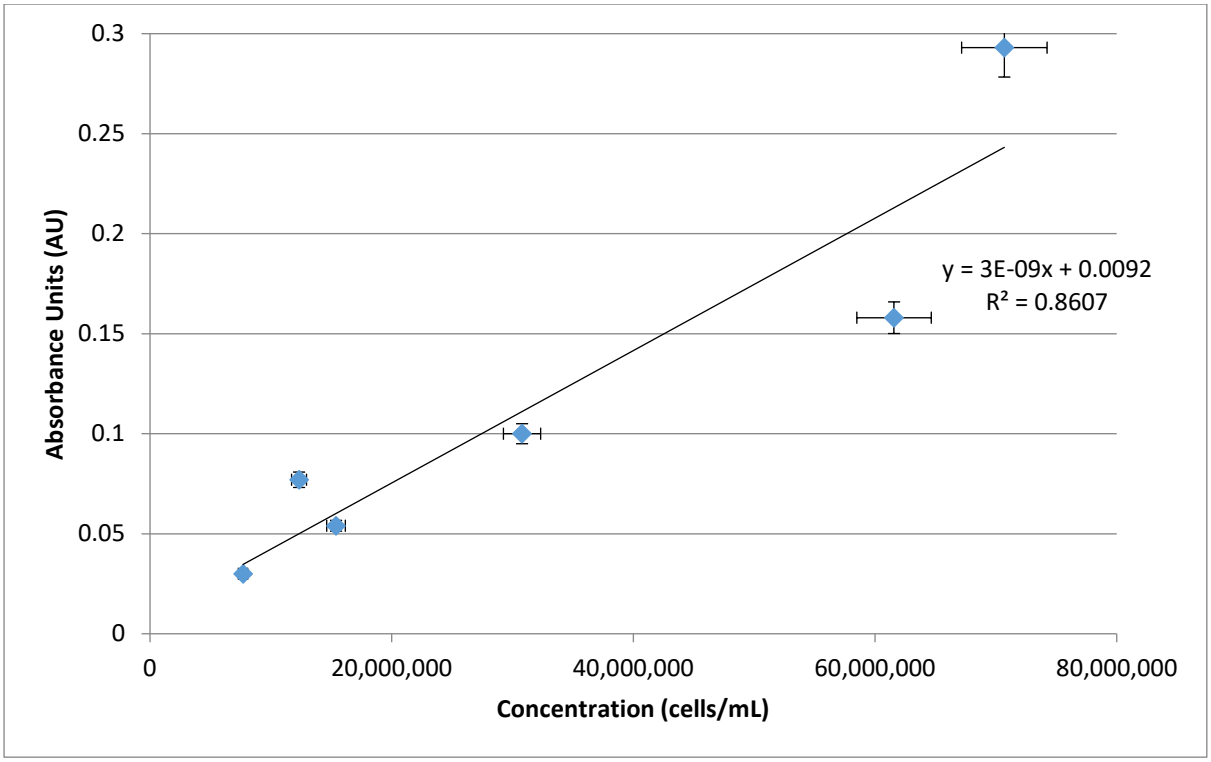


Figure 4.14: Standard curve of *Cylindrospermopsis raciborskii*, optical density measurements of chlorophyll-a at 680 nm (y axis) against the observed cell concentration (x axis).

Cyanobacteria cultures demonstrated some signs of saturation above $1x10^9$ cells/mL when the linear region turned into the logarithmic curve as demonstrated in Figure 4.12a. Within the linear region, ultra-violet/visible light absorbance compared to fluorescence was less linear with *Anabaena circinalis* at R^2 = 0.917, *Microcystis aeruginosa* with an R^2 value of 0.613 and *Cylindrospermopsis raciborskii* had an R^2 value of 0.861.

Anabaena circinalis and *Cylindrospermopsis raciborskii* were calculated to have a minimum detectable concentration limit of 6,250,000 cells/mL with an absorbance of 0.005 Absorbance Units (AU). *Microcystis aeruginosa* was detectable at approximately 1,670,000 cells/mL with 0.005 AU. Despite the use of UV/Vis absorbance to map the growth of each of the cyanobacterium species, this procedure is unable to detect cyanobacteria at the recommended low concentrations specified by the DSE (2008) and NHMRC (2011).

Data for cyanobacteria cell concentration versus absorbance by UV Visible spectrometry was stopped after the inintial assessment and data evaluation due to the limit of detection for each cyanobacterium was in excess of $>x10^7$ cells/mL. This limit of detection for cell concentration was not suitable as a sensitive detection technique without significant sample concentration. The technique was deemded not suitable for quanitication of cyanobacteria below cell concentrations of $1x10^7$ cells/mL.

4.4.2 Interference from natural organic matter

4.4.2.1 Interference from humic acid

Humic substances, including humic acid, are measured at 254 nm (Bolto et al., 2002b, Fan et al., 2008, Fearing et al., 2004, Humber et al., 2007, Myllykangas et al., 2002, Rodriguez and Nunez, 2011). Due to the colour addition from humic acid in water, there was a strong emission from humic acid which spans most of the visible light spectrum.

The concern, again, was the potential absorbance of humic acid over the whole visible light spectrum when attempting to detect various pigments such as absorbance from chlorophyll-a and phycocyanin.

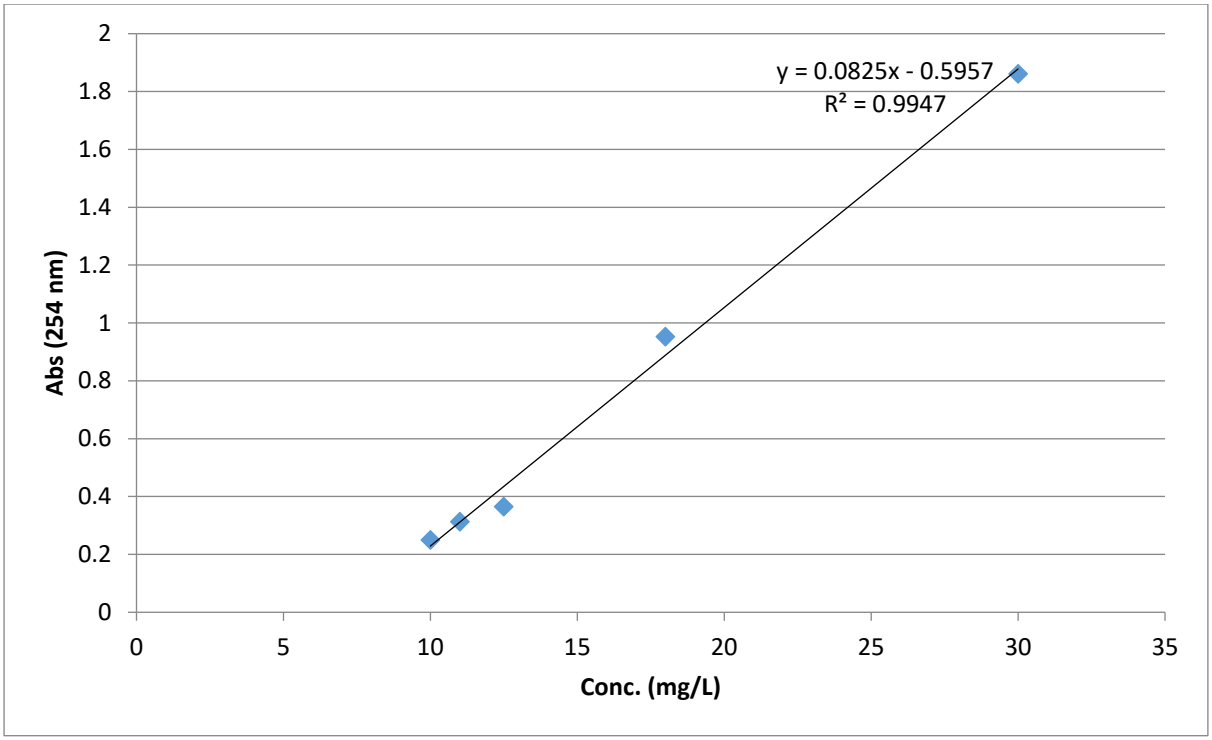


Figure 4.15: Humic Acid linearity curve at 254nm

The absorbance intensity of humic acid was optimal at 254 nm but did have spectral intensities ranging from 420-600 nm (Beckett, 1990, Rodriguez and Nunez, 2011, Sharma et al., 2011).

4.4.3 Interference from green algae species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*

Interferences from green algae using UV/visible spectroscopy were not as significant as when using fluorescence primarily due to the sensitivity, slightly increased separation between the absorption peaks and width of the peaks which relate of this detection technique. The lower sensitivity allowed for increased separation of the signal peaks between chlorophyll-a and phycocyanin. Exploring the absorbance peaks, phycocyanin was detected at 618 nm with chlorophyll-a measured at 680 nm for absorbance which gives greater separation between the chlorophyll-a and phycocyanin peaks. Further, the absorbance peaks are not as wide which did not overlap as was observed for fluorescence peak emissions which were observed in Figure 4.10b. However the lack of sensitivity

for measuring low level cell concentrations for this technique is not as suitable as fluorescence spectrometry despite any benefit for peak resolution

4.5 Conclusions

Detection of cyanobacteria pigment phycocyanin was highly sensitive to fluorescence spectrometry with a linear calibration and detection to ng/L concentrations of the pigment. Optimum wavelengths for detection of phycocyanin were 609 nm for excitation with 647 nm for emission. Detection limits for cell cyanobacteria concentrations in ideal purified water were approximately 60,000 cell/mL for this specific fluorescence spectrometer.

This limit of detection for cell concentration was not suitable as a sensitive detection technique without significant sample concentration. Technique was deemded not suitable for quanitication or qualication of cyanobacteria below cell concentrations of $1x10^7$ cells/mL.

Despite being unable to reach the recommended standards for the DSE (Department of Environment and Primary Industries, 2008)(2008) and NHMRC (National Health and Medical Research Council, 2011) (National Health and Medical Research Council, 2008), fluorescence spectrometry set the benchmark for improving detection of phycocyanin and cyanobacteria cells in this study. Detection of cyanobacteria at excitation of 291 nm and emission at 337 nm were not as reliable or reproducible with NOM interference from other proteins when excited at this wavelength region.

To apply zero-point corrections for measuring cyanobacteria concentration in the protein-region would require identifying the specific proteins present. This would require protein characterisation in an on-line instrument with the ability to separate phycocyanin from other proteins making such an approach complex.

Compensation for natural water composition variations using fluorescence spectrometery can apply zero-point correction on CDOC when the pigment emissions occur at higher wavelengths to the interfering analyst being subtracted. Chlorophyll-a was measured with 609 nm excitation and 680-686 nm emission. The presence of chlorophyll-a in cyanobacteria did not interfere with phycocyanin detection due to the difference in the ratio and signal emission capability of the pigments. However, other sources containing chlorophyll-a would interfere with phycocyanin emission. The interference of chlorophyll-a on phycocyanin occurs by the fronting from chlorophyll-a from the point of excitation at 609 nm to the peak of emission at approximately 680nm.

Proteins and polysaccharides did pose some interference with the detection of phycocyanin when measurement was taken within the protein region, excitation at 291 nm with 337 nm emission. However this could be overcome by exciting phycocyanin at 610 nm and taking emission at 647 nm, the visible light region. Nevertheless, coloured dissolved organic matter (CDOM) such as humic acid and humic/fulvic substances pose some concerns when attempting to measure phycocyanin within the visible light region. Whilst CDOM was observed to have higher emission intensity within the 400-500 nm region, the emission band of CDOM was broad and can tail into the emission wavelengths of phycocyanin and chlorophyll-a. Whilst CDOM can be potentially corrected for, this research was to establish a detection baseline with and without interference and attempt to achieve cyanobacteria detection without applying any corrections to achieve a lower detection limit.

In order to improve detection of cyanobacteria without interference from CDOC or chlorophyll-a, the capture or retention of intact cells and allowing for CDOC and/or chlorophyll-a to be removed or separated from cyanobacteria/algal cells would increase accuracy and potential sensitivity

without applying Gelbstoff measurements or applying zero-point correction. However, this requires continuous measurements and reliable corrections and calculations in place in order to avoid over or under estimations of cyanobacteria cell concentrations. This chapter has reviewed and demonstrated detection characteristics of phycocyanin using fluorescence and absorbance spectroscopy, which has established a foundation for research described in the following chapters.

Chapter Five

Capture and pre-concentration of cyanobacteria and algae by porous membranes

5.1 Introduction

Cyanobacteria physiology and morphology have been the focus of years of research, which has provided in depth detail into cell size, cell structure and shape, and other additional characteristics such as filaments. This information can be applied to the application of filtration, which provides physical separation between cell cultures and surface waters including DOM. Pre-concentration of intact cells from a large volume of water to a smaller volume allowed for increased detectability of pigments, as pigment concentrations increase as cell concentrations increase via retention on the filter. Further, physical separation of intact cells from DOM including CDOC, can remove interfering spectral signals and provide a cleaner signal from the pigments. Organic compounds such as humic and fulvic substances, proteins, and other natural organic matter (NOM) (Borisover et al., 2011, Filella, 2009, Henderson et al., 2008b, Her et al., 2004, Mergen et al., 2008, Rodriguez and Nunez, 2011, Sharma et al., 2011, Henderson et al., 2008c, Frank, 1972) and/or algal organic matter (AOM) from algal cells (Henderson et al., 2008b, Her et al., 2004)) can interfere with the detection of pigments.

Microfiltration has been used for the filtration and collection of algae, bacteria and other micro-organisms whilst ultrafiltration has been used for humic and fulvic compound collection in water purification (Fane et al., 2011, Watanabe and Kimura, 2011).

This chapter was conducted with a simple concept in mind: a water source would be pumped through a small pore size filter, ideally 0.45 μm in pore size, which would filter and collect cyanobacteria cells and elute dissolved organic matter (Bottino et al., 2001, Fane et al., 2011, Rossi et al., 2008, Watanabe and Kimura, 2011). Direct filtration of cyanobacteria provides physical

separation of cyanobacteria cells from humic substances that cause detection interferences and issues with the use of resins as discussed later in Chapter 7. However, a possible complication in purposely capturing intact cyanobacteria is fouling of the filter/membrane used. In this chapter, various membranes and filters were investigated to determine the most suitable membrane that would allow for minimal bleed through of cyanobacteria cultures whilst demonstrating minimal membrane fouling. Membrane survivability within the pigment extraction process involving ultra-sonication was also considered, as sonication is proposed for release of c-phycocyanin from cyanobacteria cells after the NOM has been cleared from the cells.

Operational procedures for analysis involving membrane investigations were outlined in Chapter 3 in section 3.4.3. There was no interaction, membrane degradation or observed effect of the growth media on membrane performance during these investigations.

5.2 Membrane types

Membrane types investigated consistent of flat disc membranes of various materials and tubular membranes. The application of a 0.45 μm membrane filter to separate NOM from algae is supported by the definition of dissolved organic matter in water systems and the cell size of algae and cyanobacterial species. As a definition, dissolved organic matter is defined to be organic matter able to pass through a 0.45 μm filter. Although, *Microcystis aeruginosa* is one of the smallest cell species found on the planet, it was not able to pass through any membrane of 0.45 μm pore size (Mergen et al., 2008, Filella, 2009, Sharma et al., 2011, Watanabe and Kimura, 2011).

Cell concentrations were determined by fluorescence spectrometry intensities using a linear relationship between fluorescence intensity and cell concentration as determined in Chapter 4. The bleed through the membrane was then calculated using equation 5.1;

Equation 5.1: Bleed through percentage

= (cell concentration in filtered water/cell concentration in initial solutions)*100

Cell retention for membrane efficiency was determined as 100 percent minus the bleed through percentage from 5.1.

Equation 5.2: Cell retention percentage

= 100 – Bleed through percentage

Equation 5.3: Backwashing cell recovery percentage

$$= \left[\frac{Cell\ Concentration\ in\ Backwashing\ solution\ (\frac{cells}{mL})}{Cell\ Concentration\ in\ inital\ test\ solution\ (\frac{cells}{mL})} \ x\ \frac{Volume\ of\ inital\ test\ solution\ (mL)}{Volume\ of\ Backwashing\ solution\ (mL)} \right] * 100$$

Calculations from Equation 5.1 – 5.3 were applied across all membrane types and only applied for experiments involving membrane filtration.

5.2.1 Whatman GF/F Glass Fibre

Whatman GF/F glass fibre disc membrane filters with nominal pore size of 0.7 µm as specified by the manufacture (47 mm in diameter), are preferred for concentrating phytoplankton from natural waters (Bidigare et al., 2005). The Whatman GF/F glass filters were effective in the capture of phytoplankton cultures of cyanobacteria and green algae from synthetic laboratory waters due to their small pore size and glass fibre membranes being low fouling for algae (Rossi et al., 2008).

Glass fibre membranes were set up as described in section 3.4.3 and graphically demonstrated in Figure 3.3 in Chapter 3.

In the setup for analysing the efficiency of disc filtration membranes, the glass fibre membrane was placed over the membrane manifold on a conical flask. Parafilm was wrapped around the edges of the membrane manifold and edges of the membrane to seal the membrane to the membrane manifold, which aided filtration when placed under vacuum.

Microcystis aeruginosa was the only species investigated for Whatman GF/F glass fibre filters due to the observations made during the resin backwashing and sonication cycle described in this Chapter.

The glass fibre membranes were able to retain an average of 99.25 ± 0.27 % of *Microcystis aeruginosa* cells with virgin membranes applied only for single use (n = 7). Test solutions contained 100,000 cells/mL of *Microcystis aeruginosa* in Milli-Q water without any additive such as BSA, humic acid or other species. Every 25 mL, an aliquot was taken for measurement by fluorescence spectrophotometry (excitation at 609 nm, emission at 637 nm) with a total of 400 mL filtered. The bleed through pattern observed during the analysis of glass fibre membranes in demonstrated by Figure 5.1.

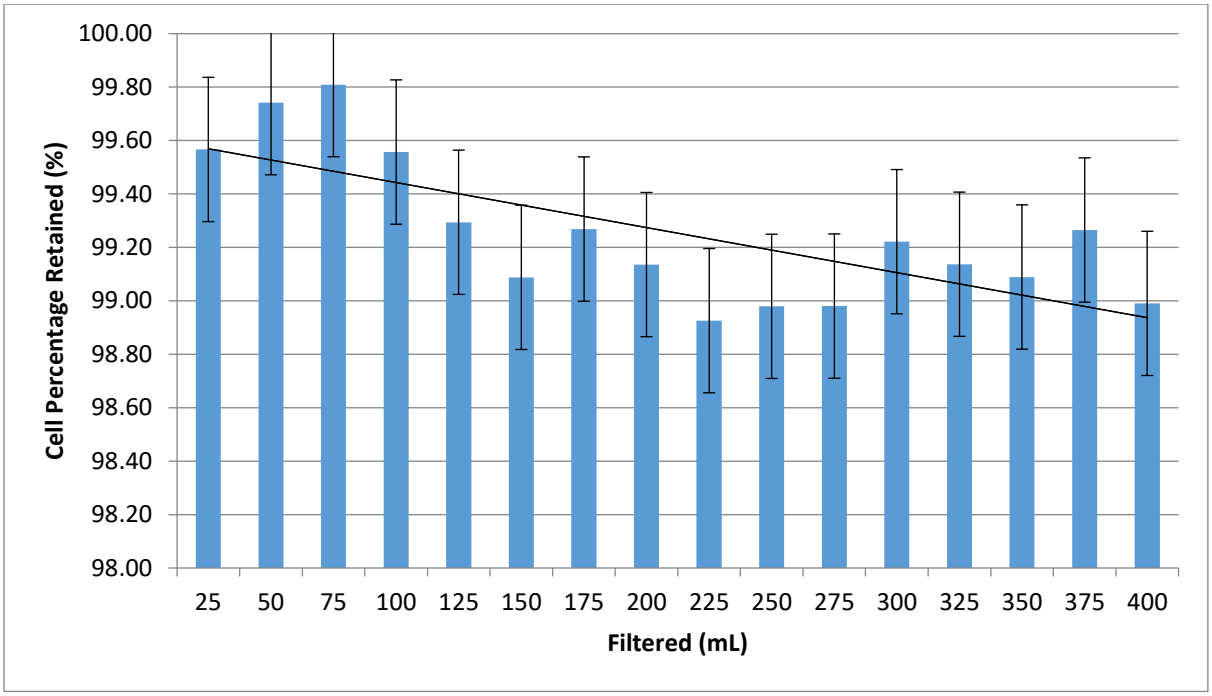


Figure 5.1: Percentage of *Microcystis aeruginosa* cells retained on GF disc membranes measured in 25 mL aliquots over a total of 400 mL filtration volume (n = 7).

The membranes were not able to be regenerated and re-used. Backwashing the membrane and/or sonication of the membranes to disrupt the retained cyanobacteria cells in order to liberate the cells for filter re-use was not successful. During sonication the membrane fibres were disrupted, which destroyed the membrane and rendered the membrane unusable. The membrane decayed and fragmented during the sonication cycle and additional filtration was required to remove the membrane fragments from the solution in order to analyse pigment concentrations. Phycocyanin was liberated from the cyanobacteria cells during sonication but at the cost of a glass fibre membrane each time.

Filtration of up to 400 ml of all test solutions, containing cyanobacteria and green algae monoculture, and CDOC or combinations, were conducted to determine the maximum cell culture able to be loaded onto the membrane before membrane fouling became apparent.

In one investigation, cell cultures from 500,000 to 1,000,000 cells/mL of the monoculture *Anabaena circinalis* were filtered with an average cell capture of 97.0 ± 2.7%. After the first 100 mL of filtration, the membrane generally had minimal bleed through of approximately 0.3 ± 0.1 %. From 200 mL to 400 mL, the minimum bleed through percentage increased to 0.9 ± 0.1 %.

Despite the shift of percentage bleed through, the glass fibre membrane was effective in the capture of cyanobacteria and green algal cultures before the implement of extraction procedures.

5.2.2 5 µm Metal Membrane

Cyanobacterium, *Microcystis aeruginosa*, was the smallest species used for filtration investigations. The average cell size is recorded as being between 2-5 µm in diameter (Batzing, 2002, Thain and Hickman, 2000, Warren et al., 2002). Capture of *Microcystis aeruginosa* cells proved to be difficult using 5 µm metal membrane filters. It was considered that the 5 µm metal disc membrane would not be as effective as other 0.45 µm disc membrane counterparts. The metal disc membranes were 47 mm in diameter with membrane thickness of approximately 0.2 mm.

Two species of cyanobacteria were investigated; *Anabaena circinalis* with an average cell size of 20 µm and *Microcystis aeruginosa* of an average cell size of 2-5 µm. Three aliquots of 400 mL of each monoculture of cyanobacteria were used in continuous filtration studies. In applications with continuous filtration utilising a 5 µm metal membrane, the initial feed of *Anabaena* had concentrations equal to 255,000 cells/mL and 140,000 cells/mL for *Microcystis aeruginosa*.

Table 5.1 presents the results for the filtration of *Microcystis aeruginosa* and *Anabaena circinalis* cultures through 5 µm metal membranes.

Table 5.1: Effectiveness of cell retention by 5 µm metal membranes

Continue Filtration Cycles	***Retention of Microcystis aeruginosa cells (%) (2-5 µm cell size)***	***Retention of Anabaena circinalis cells (%) (20 µm cell size)***
1st	5.2	40.7
2nd	19.7	41.9
3rd	17.7	39.5

In Table 5.1, *Microcystis aeruginosa* cell cultures were utilised to determine the potential bleed through 5µm membranes. The bleed through the membrane was 85.8 ± 9.0 %. This increase in cell retention for *Microcystis aeruginosa* was a result of cells being retained in the pores of the membrane after the first filtration, which resulted in increased cell retention for the second and third filtrations. The first filtration on the virgin membrane retained cyanobacteria cells in the membrane pores that led to reduced pore size and subsequently increased cell retention on the 5.0 µm metal membrane.

Trials of backwashing membranes retaining *Anabaena circinalis* identified that only 39.2 % of cells retained on the membrane were removed. The remaining 1.5 ± 0.8 % of cells unaccounted for was determined to arise from experimental error associated with fluorescence measurements.

Anabaena circinalis cultures were retained more easily on the 5 µm metal membrane compared to *Microcystis aeruginosa* due the obvious size cell difference between the two species. Low cell retention made this membrane not suitable for use.

5.2.3 1 µm Metal Membrane

The membrane was 47mm in diameter with 2 mm thickness with a nominal pore size of 1 µm. The cell concentration used for filtration of *Microcystis aeruginosa* was 140,000 cells/mL.

The *Microcystis* cells retained on the 1 µm metal membrane filter was calculated to be 64.7 %. This was calculated from the 35.3 % bleed through of *Microcystis aeruginosa* cells through the membrane. Applying Equation 5.3, of the 64.7 % cells retained on the membrane, 62.8 % of cells were backwashed from the membrane.

Figure 5.2 illustrates the filtration of *Microcystis* cultures through this metal membrane. The slight greenish tint discolouration in the centre of the membrane represents ~ 65% of cell capture on the membrane.

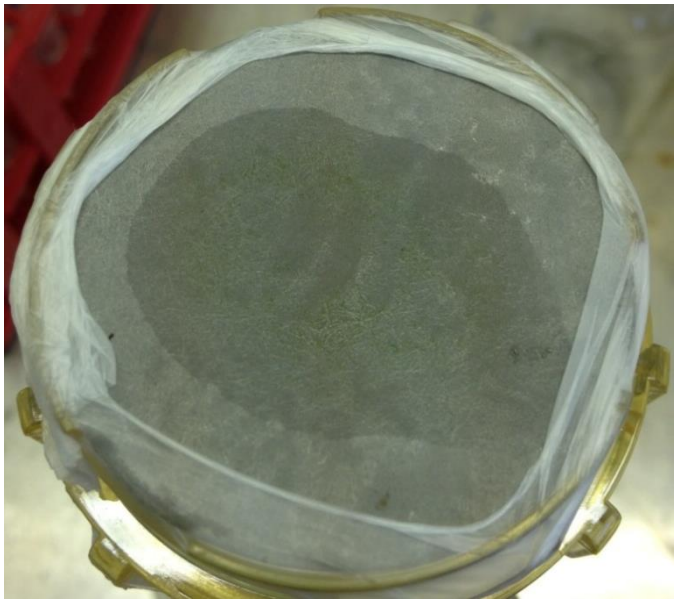


Figure 5.2: Cell capture of *Microcystis aeruginosa* culture on a 1 µm metal membrane filter.

Despite the lack of cell capture, cell disruption by sonication of captured cell cultures was not effective through the membrane housing in a sonication bath. As presented in Chapter 3, Figure 3.4, the membrane was enclosed in the membrane housing. This allowed for any sonication to be absorbed by the housing and not disrupt any cells at all. Whilst it would be effective to backwash the membrane and/or expose the membranes to a sonication probe, the membrane bleed through was too large for further consideration of this membrane. The 1 µm metal membranes had similar physical characteristics to the 5 µm membrane but demonstrated better cell retention.

5.2.4 0.45 µm Tubular Metal Membrane

Tubular metal membranes with a nominal 0.45 µm pore diameter (supplier data) were investigated for their filtration properties with filtration of water occurring perpendicular to the direction of flow. As both ends of the tubular membrane were open, one end of the membrane was sealed and the other was the inlet for flow. This allowed for perpendicular flow through the cylinder side which was the 0.45µm membrane surface. This design has the potential for dual flow inlets/outlets unlike a disk membrane that has only one flow direction possible at a time outlined in Figure 3.5 and Section 3.8.2 in Chapter 3. Further the metal membrane was more suitable for withstanding sonication and more aggressive cell disruption techniques or the use of various solvents than polymer and glass fibre membranes.

On average, approximately 225 mL of water was filtered through the membrane. However, the small tubular membrane was able to filter up to 375 ± 25 mL with 1,000,000 ± 200,000 cells/mL of *Microcystis aeruginosa* before filtration began to show signs of reduced filtration speed. Cell concentrations of 150,000 cells/mL of *Microcystis aeruginosa* were used for flux investigation. The flux results for the tubular membrane were considerably lower than the flux for the disc membranes due to the increase in surface area. At 1.0 mL/min flow rate, the flux was calculated to be 16.7 L/m^2/h and 167 L/m^2/h at 10 mL/min. The average filtration time for filtration of 10 mL of cell culture was 54.8 ± 3.68 seconds. Hence, the average flux was 182.9 L/m^2/h with a range between 171.3 to 196.0 L/m^2/h.

The tubular membrane was placed in a suitable bag, submerged in Milli-Q water, washed and sealed before being sonicated in a sonication bath. This was used because of difficulties sonicating the tubular membranes effectively in a similar manner to the other disc membranes. An 80% methanol wash was performed after every 4th backwash to completely remove any remaining cells

retained on the membrane. The small cell diameter of cyanobacteria, *Microcystis aeruginosa*, yielded 4.6 ± 2.9 % bleed through the tubular membrane.

The membranes were able to retain 95.4 ± 2.9 % of *Microcystis aeruginosa* from suspension. Backwashing of the tubular membranes resulted in 89.4 ± 5.7 % of the total *Microcystis aeruginosa* cell concentration in suspension being recovered from the membranes. Table 5.2 shows results for repeat investigations into cell retention and back washing of the membrane.

Table 5.2: Cell concentrations of *Microcystis* cultures washed from the tubular membrane

	Investigation 1	Investigation 2	Investigation 3	Investigation 4
Initial Cell Concentration in 400 mL	1,140,000	1,140,000	1,140,000	1,140,000
Series of Backwash	Backwashing Cell Recovery (%)			
1st Backwash in 10 mL	824.3	557.9	407.8	539.9
2nd Backwash in 10 mL	312.6	172.7	159.1	15.2
3rd Backwash in 10 mL	43.0	62.6	24.9	19.6
4th Backwash in 10 mL	11.3	18.3	14.8	4.0

Table 5.2 shows concentrations of *Microcystis aeruginosa* cells washed from the tubular membranes over four 10 mL backwashes. The high backwash percentage recoveries from the first and second backwash cycle were a result of the release of high cell concentrations retained on the membranes into 10 mL aliquots. The initial concentrations of each investigation were between 616,000-1,170,000 cells/mL which was significant evidence that *Microcystis aeruginosa* cells were retained in the tubular membrane.

Aliquot solutions containing 250,000 cells with 10 mg/L dissolved humic acid were also filtered through tubular membranes for evaluation. There was no significant variation with cell retention

with the addition of dissolved humic acid. Cell retention was 94.6 ± 2.7 % (n = 7). Filtration of humic acid demonstrated 95% removal with the remaining 5% remaining on the membrane until a wash through membrane removed the residual humic acid.

5.2.5 0.45 µm Nylon membrane

Nylon membranes were similar in shape and size to the glass fibre membranes; 47mm in diameter, 0.45 µm pore size and flat disc.

Initial concentrations of cyanobacteria suspensions used for the nylon membrane investigations were 660,000 ± 20,000 cells/mL. Cultures of *Microcystis aeruginosa* had a bleed through percentage of 2.0 ± 1.3 %. The bleed through of the cultures demonstrates the efficiency of the nylon membrane to retain 98.0 ± 1.3 % of *Microcystis aeruginosa* (n= 7).

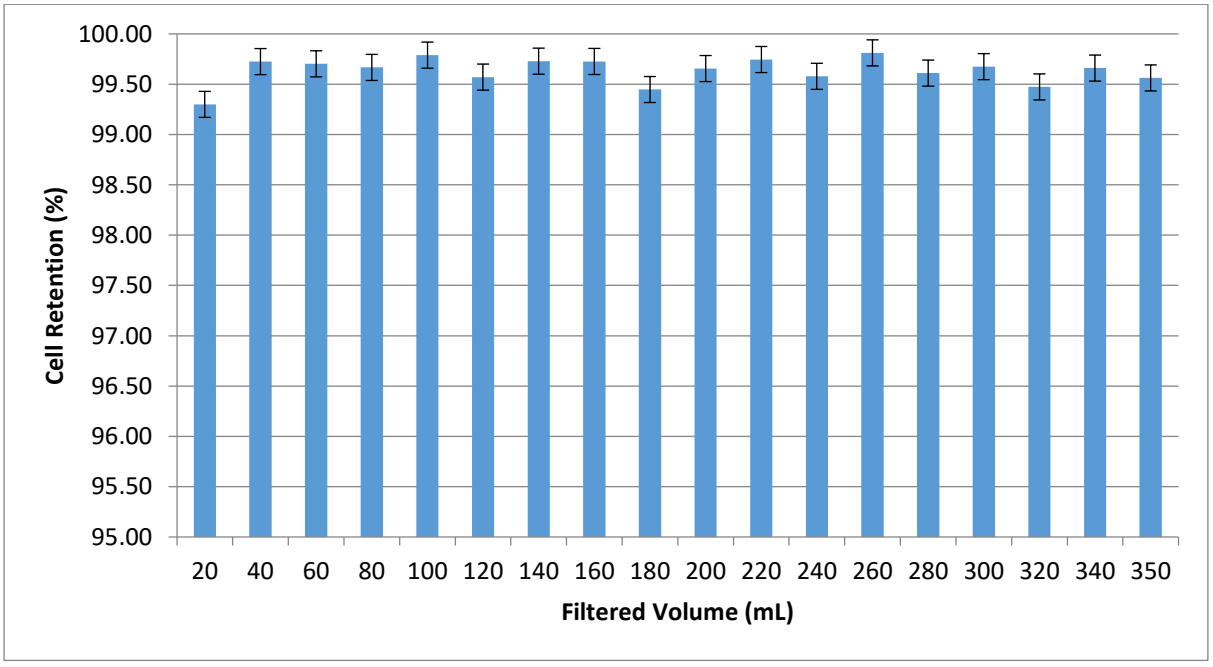


Figure 5.3: Cell retention percentage of *Anabaena circinalis* cells culture filtered through nylon membrane

From Figure 5.3, cultures of *Anabeana circinalis* with an approximate concentration of 393,500 ± 135 cells/mL, were diluted to 1 L with deionised water. This reduced the concentration to approximately 559 ± 135 cells/mL, which was well below the limit of detection and not measurable by fluorescence spectrometry. After the litre was filtered through the 0.45 µm nylon filter under vacuum, it was calculated the cultures retained on the membrane were concentrated to 3,200,260 ± 135 cells/mL after the bleed through percentage was considered.

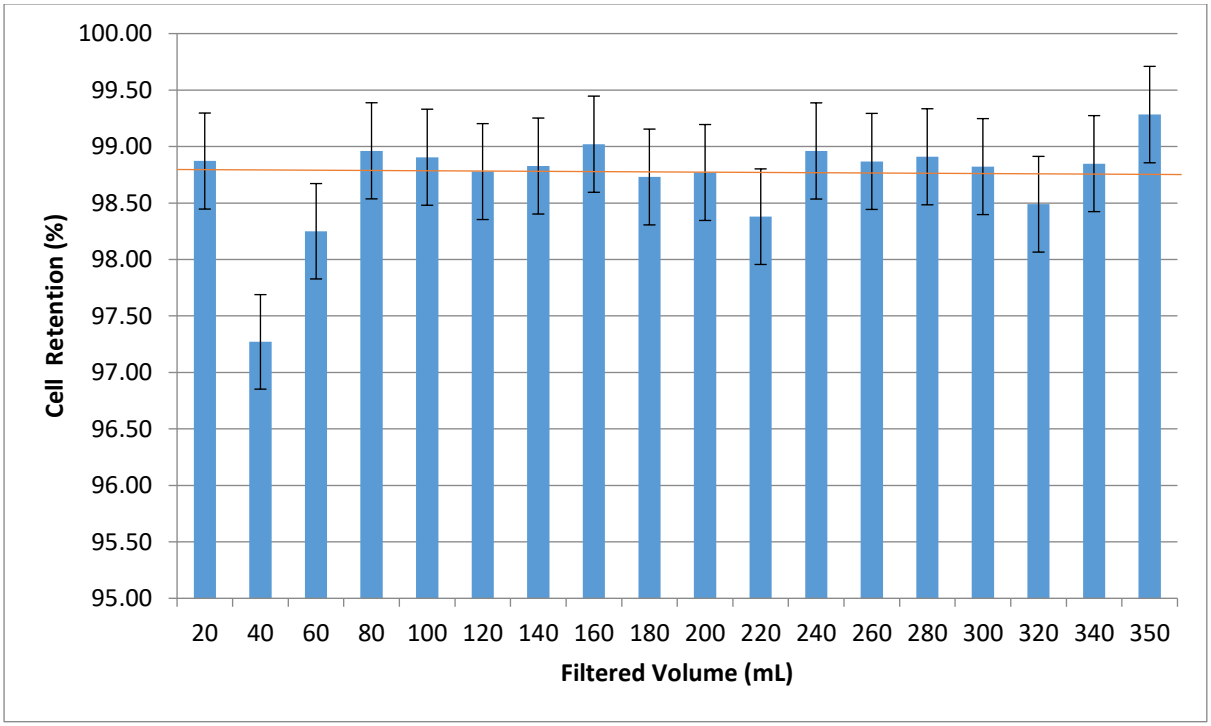


Figure 5.4: Cell retention percentage of *Microcystis aeruginosa* cells culture filtered through nylon membrane

Average cell concentrations for *Microcystis* cultures used were 450,000 ± 75,000 cells/mL with bleed through of 1.3 ± 0.4%. Cultures of *Microcystis aeruginosa* demonstrated more percentage bleed through than *Anabaena circinalis* during filtration investigations utilising nylon membranes. This was the result of different size cells between the two species of cyanobacteria. Cultures of *Anabeana circinalis* demonstrated less than 1 % cell bleed through compared to *Microcystis aeruginosa* cell cultures where the bleed through was generally <2 % as shown in Figure 5.4.

Backwashing of the nylon membranes over five consecutive backwashes cycles resulted in a decrease of cell recovery from the nylon membranes. The cell cultures were backwashed with 10mL of Milli Q water over five wash cycles. The first backwash concentration was measured to be 8,900,000 cells/mL for the first 10 mL backwash, and for the second backwash the concentration was 2,300,000 cells/mL. The third and fourth backwash cycles resulted in backwash waters containing approximately 500,000 cells/mL and reduced to 300,000 cells/mL by the 5th cycle.

Flux investigations on nylon membranes used cell concentrations of 150,000 cells/mL of *Microcystis aeruginosa*. At 1.0 mL/min flow rate, the flux was calculated to be 34.6 L/m^2/h. The average filtration time of 10 mL was 89.3 ± 8.6 seconds. The average flux was calculated to be 232.5 L/m^2/h with a range between 212.2 to 257.2 L/m^2/h.

Nylon membranes were demonstrated to be more durable than glass fibre membranes but less durable then metal membranes when undergoing repeated sonication. The durability of a thin nylon filter was not as suitable for pressure or any filtration of cell cultures in a liquid medium under vacuum. This was easily rectified by placing the nylon filter over the top of a larger pore size metal membrane filter. This allowed collection of cyanobacteria cells whilst supporting the nylon membrane from the effects of vacuum pressure, and enabled filtration of the liquid medium. The effect of vacuum pressure and instrumental set up is demonstrated in Figure 5.5.

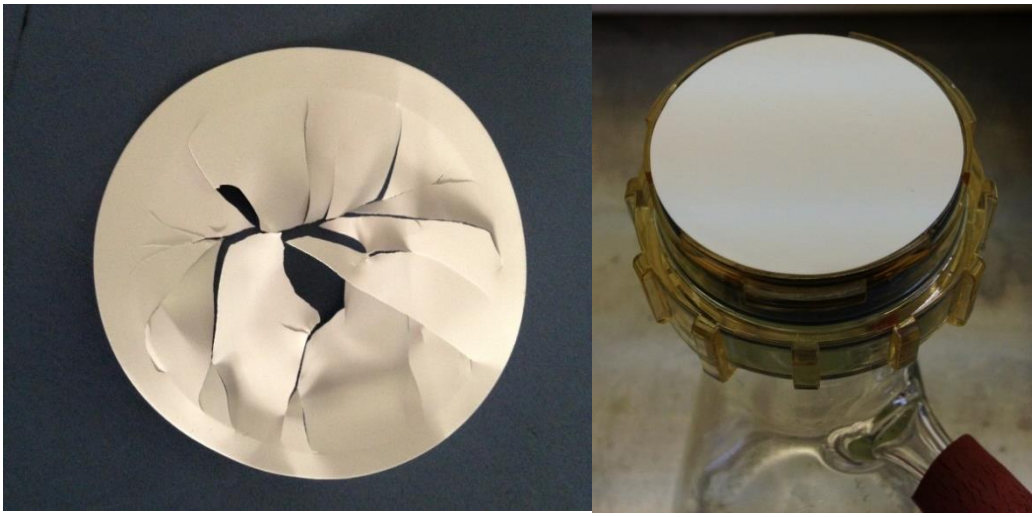


Figure 5.5: Nylon filter broken under vacuum pressure (left), a nylon filter supported on a porous metal membrane was able to withstand vacuum pressures (right).

Treatment with sonication and the use of methanol to aid cell lyse and removal from the membrane increased the pace of the membrane degradation. Despite this observed decrease in membrane performance, the nylon membrane performed better than glass fibre membranes.

Filtration under pressure was not suitable for nylon membranes without a support. The experimental set up for the Nylon membranes is described in Section 3.3.1 of Chapter 3.

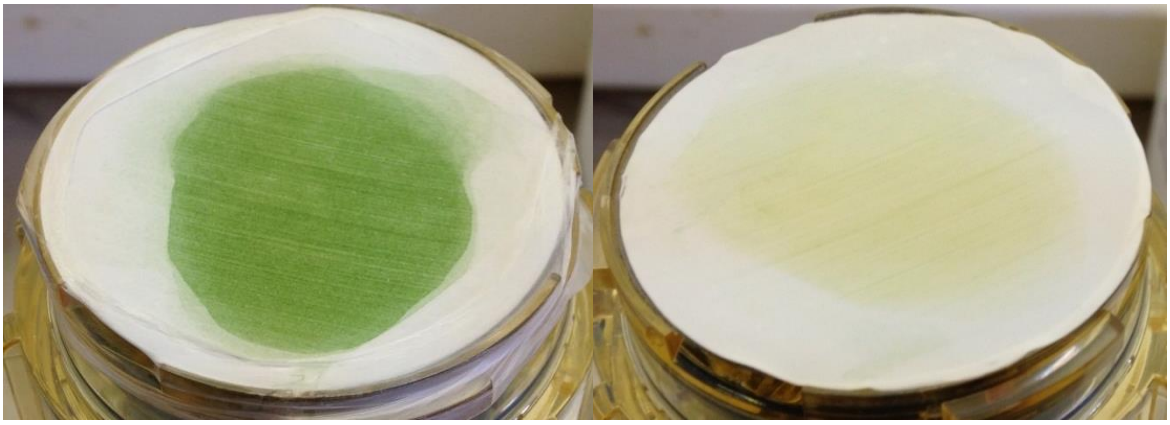


Figure 5.6: Discolouration of nylon membrane after filtration of cyanobacteria and membrane washing.

Figure 5.6 presents the retention of cyanobacteria cells on the nylon to the left and the discolouration of lyse cell cultures that could not be removed from the membrane after backwashing the membrane on the right.

5.2.6 0.45 µm Ceramic membranes

The ceramic membranes were constructed with a ceramic support which was cream-brown in colour, with the 0.45µm active filter layer on top of the support (Li et al., 2011). All three cultures of cyanobacteria, *Microcystis aeruginosa*, *Cylindrospermopsis raciborskii* and *Anabaena circinalis*, were used for ceramic membrane tests. As these species were the smallest and largest cell cultures of this study, these species were first investigated to test the retention efficiency of the membrane with varying cyanobacteria size.

Cultures of *Anabaena circinalis* filtered through virgin ceramic membranes were conducted using initial stock concentrations of 635,000 cells/mL; aliquots of 200 mL filtered for seven measurements.

Initial filtrations using virgin filters demonstrated a slight increase in bleed through with each sample of permeate measured for cyanobacteria concentration. Measurements for bleed through and cell retention were taken every 10 ml; measured under fluorescence 609 nm excitation and 647 nm emission.

The bleed through increased from 0.81% for the first permeate sample after filtration of every 20 ml, to 3.32% percentage bleed through for the permeate sample after 200 mL total permeate. Despite the slight increase of bleed through from the first permeate measurement, the membrane was able to concentrate *Anabaena circinalis* from 635,000 to 1,736,000 cells/mL.

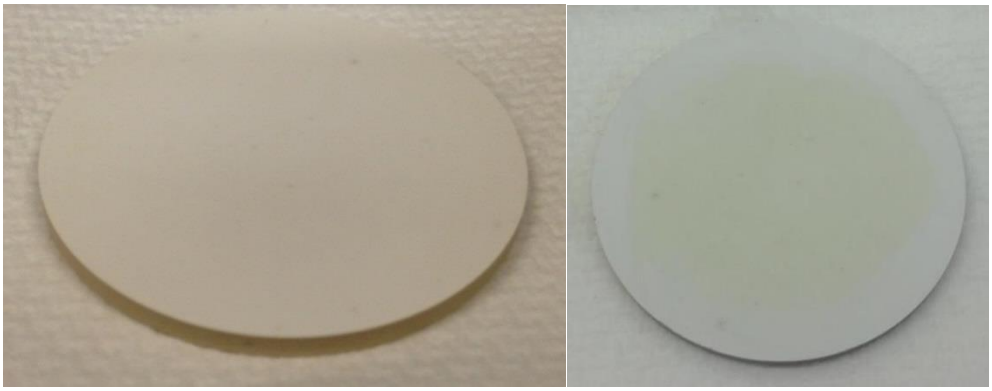


Figure 5.7: Virgin 0.45µm ceramic membrane (left), and the same membrane after the filtration of *Anabaena circinalis* and membrane backwashing (right).

Figure 5.7 presents the virgin membrane, (left photograph) and the membrane after use and backwashing (right photograph). The photograph of the membrane after use was discoloured from the excretions and disrupted cyanobacteria cells.

Investigations into the effectiveness of *Microcystis aeruginosa* filtration through ceramic membranes began with initial concentrations of 1,200,000 ± 10,000 cells/mL. The filtration of *Microcystis aeruginosa* cultures by ceramic membranes also demonstrated effective collection of intact cells with minimal bleed through of 0.5 ± 0.1 % (total permeate filtered = 400 ml).

Backwashing of the ceramic membranes were conducted after 140 mL of stock water was filtered, and a single backwash of 10 mL was used. The concentration of backwashed cyanobacteria increased by a factor of approximately 9 compared to the stock suspension, with cell concentrations increasing from 1,200,000 cells/mL to 11,000,000 cells/mL. Further, 65.5% of *Microcystis aeruginosa* cells were recovered from the membrane in a single membrane wash.

Despite the advantages of using the 0.45 µm ceramic membrane, there were some design complications that made the use of this type of membrane impractical. The resistance of the membrane significantly increased the filtration time compared to the nylon membrane. Filtration times were recorded to be 89.2 ± 8.6 seconds/10 mL. Another potential downfall of the ceramic membranes was the thickness of the membrane.

Continuous filtration involving *Cylindrospermopsis raciborskii* cultures began with initial cell concentrations of approximately 1,340,000 cells/mL. The bleed through with the first treatment of stock suspension was 0.5 ± 0.1 % and recovery upon backwashing was 56.7 ± 7.5 %. Cell Recovery was calculated Equation 5.3.

Phycocyanin extraction using bath sonication for algae retained on the membrane was 42.8%. After the first backwash, the membrane was loaded again with the same solution and concentration of *Cylindrospermopsis raciborskii* culture. The bleed through with the second treatment increased to 1.07 ± 0.01 % with cell disruption for c-phycocyanin extraction of 43.7 % and cell recovery following backwash of 55.7 %.

The effectiveness of this membrane to capture cyanobacteria and algal cells was similar to the nylon membranes, however, it had a lower filtration rate and required a specialised membrane housing.

5.3 Membrane Comparison

Comparisons between the different types of membranes were undertaken to evaluate the most effective membrane. Comparisons were drawn from all the membranes investigated through this chapter. Bleed through and total recovery percentages are compared in Table 5.3 and the observations made throughout the chapter led to conclusions based on practical operational characteristics.

Whatman GF/F disc membrane filters initially demonstrated effective filtration of cyanobacteria cell with a similar bleed through to the ceramic and nylon membranes also investigated. The average bleed through was 0.33 ± 0.11 % for 100 mL when filtered by virgin Whatman GF/F membranes. From 200 mL to 400 mL filtration volumes, the bleed through percentage increased to 0.89 ± 0.13 %. These results are similar to the other disc membranes. However, the degradation of the membrane during physical cell disruption made the membrane unsuitable for repeat filtrations.

Tubular metal membranes demonstrated greater cell retention using *Microcystis aeruginosa* cultures with 95.38 ± 2.93 %, than the other metal membranes, and being metal allowed for increased durability with the pigment extraction techniques. The tubular membranes demonstrated cell retentions similar to the nylon, glass filter and ceramic membranes. The advantage of being able to directly flow feed either on the surface or within the tube was a potential benefit for the tubular metal membranes, as was the ability to flush from two different directions of the tube. Filtration of humic acid demonstrated ~95% removal with the remaining 5% retained on the membrane until a further Milli Q water was washed through the membrane.

Virgin nylon membranes with a pore size of 0.45 µm, the cell bleed through decreased to 1.28 ± 0.42% making the membrane more effective with repeated use. With continuous use, nylon membranes presented with discolouration and signs of membrane degradation when exceeding more than 10 repeated cycles of filtration, sonication and backwashing. Treatment with sonication and chemicals increased the pace of the nylon membrane degradation, however, it performed better than glass fibre membranes. Filtration under pressure was not suitable for nylon membranes without a support.

Ceramic membranes demonstrated the most effective cell capture with an average membrane bled through of 0.51 ± 0.07 %. The average filtration time of 10 mL was 89.3 ± 8.6 seconds, which was

the longest filtration of all the membranes. Backwashing of the membrane demonstrated effective removal of cyanobacteria from the membrane with the same discolouration as observed for the nylon membranes. The longer filtration time was a result of the proportional thickness of the membrane compared to the other membranes investigated. Whilst this characteristic does not reduce the efficiency to retain cells, it does reduce the effectiveness of the membrane compared to other membranes investigated as analysis time is increased.

Table 5.3: Bleed through and backwashing percentages of cyanobacteria, *Microcystis aeruginosa* through various membranes

Membrane Type and Pore size (µM)	**Bleed Through Percentage (%)**	**Total Recovery Percentage (%)**
Glass Fibre 0.7 µm	0.9 ± 0.1	97.0 ± 2.7
Nylon 0.45 µm	2.0 ± 1.3	92.9 ± 2.6
Metal 5 µm	59.3 ± 1.2	96.2 ± 2.0
Metal 1 µm	35.3 ± 5.1	97.1 ± 2.9
Metal Tubular 0.45 µm	4.6 ± 2.9	98.3 ± 1.4
Ceramic 0.45 µm	0.5 ± 0.1	56.7 ± 7.5

From Table 5.3, metal disc membranes performed the worst for cell retention with high cell bleed through percentages compared to all other membranes investigated. Tubular metal membranes had better cell retention but not as much as nylon, ceramic and glass fibre membranes.

When investigating total cell recovery from the membranes, ceramic membranes demonstrated the worst recovery which could be directly related to membrane fouling. Despite the poor cell recovery from ceramic membranes, the other membranes investigated demonstrated similar total cell recoveries from the membranes between 92.9 to 98.3 %.

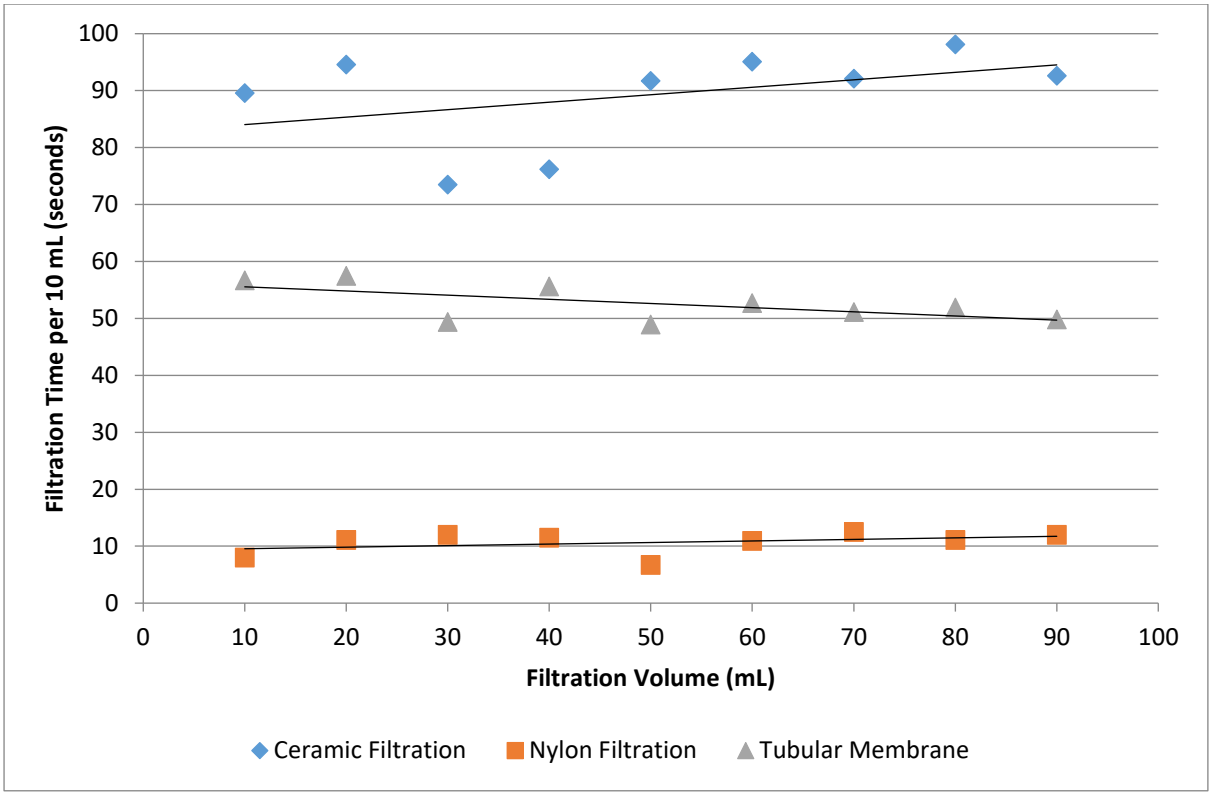


Figure 5.8: Comparison between filtration times through various membranes plotted against filtration volume.

Figure 5.8 compares filtration of 100 mL aliquots through ceramic, nylon and metal tubular membranes. All membranes were of 0.45 µm pore size. The 100 mL aliquots were sampled from a 1.5L stock solution containing 350,000 cells/mL of *Anabaena circinalis*. Timed measurements were recorded every 10 mL for the total volume of 100 mL. From this investigation, nylon membranes demonstrated the fastest filtration times. Whilst all filters were effective in retaining cyanobacteria cell cultures, rapid filtration was a benefit for the overall process and would allow a faster analysis.

5.4 Conclusion

Of all the characteristics and types of membranes analysed through this chapter, pore size was a critical factor which determined cell retention and cell bleed through. It was demonstrated that membranes with pore sizes of 0.45 µm were more effective at cell retention than membranes with

larger pores. The 0.45 µm membranes were the nylon, ceramic, glass fibre and tubular membranes. The metal flat disc membranes with 1 and 5 µm pore sizes failed to effectively retain cell concentrations.

Flat disc and tubular metal membranes and ceramic membranes proved to be more durable following cell disruption via sonication. Flat discs membranes required a membrane housing. The membrane housing absorbed sonication and did not allow for cell disruption unless the membrane were directly exposed to the effect of sonication. Tubular metal membranes were easily sonicated as they did not require a membrane housing and could be easily exposed to sonication. Operations with tubular metal membranes were more practical and functional whilst demonstrating similar filtration and backwash results to the disc membranes, and therefore it was used in subsequent tests.

Chapter Six

Suitable extraction methods and matrices for pigments from cyanobacteria and green algae

Following the investigation into retaining cell cultures by membrane techniques in Chapter 5, this chapter explored suitable extraction methods and different extraction matrices to liberate phycocyanin from cyanobacteria cultures. Primarily phycocyanin was the focus of the investigation due to specific links to cyanobacteria presence in water sources. Chlorophyll-a was also investigated to determine a specific extraction matrix as a secondary pigment found in cyanobacteria cultures and also for the potential interference it could have on liberated phycocyanin from cyanobacteria cultures. Cell disruption to liberate pigments from the cells required a suitable extraction technique and suitable extraction matrix to maintain pigment integrity. Initial investigation on both chlorophyll-a and phycocyanin from Chapter 4 demonstrated the detectability of the pigments associated with cyanobacteria cultures. This chapter focuses on the stability of phycocyanin and chlorophyll-a in various extraction media and using different methods of cell disruption, to determine optimum conditions for maintaining a high yield of phycocyanin. During the investigation into pigment extractions, the emission signal of both pigments, chlorophyll-a and phycocyanin, within a cyanobacteria cell were expressed as a ratio.

The emission ratios between phycocyanin to chlorophyll-a in the cyanobacteria investigated were 3.1 for *Microcystis aeruginosa* and 2.2 for *Anabaena circinalis* and 2.0 for *Cylindrospermopsis raciborskii* as demonstrated in detail in Table 4.2, Chapter 4. These ratios were calculated by the concentration of phycocyanin over chlorophyll-a. The selected wavelengths used to determine the ratio were excitation at 609 nm with phycocyanin measured at 647 nm and chlorophyll-a at 680 nm. *Microcystis aeruginosa*, the smallest culture, had the highest ratio. This means *Microcystis aeruginosa* has a higher proportion of phycocyanin to chlorophyll-a compared to *Cylindrospermopsis raciborskii* and *Anabaena circinalis*.

Table 6.1: Phycocyanin and chlorophyll-a concentration per million cells

Species	Phycocyanin (ng/1,000,000 cells)	Chlorophyll-a (mg/1,000,000 cells)
Cylindrospermopsis raciborskii	22.7 ± 0.03	1.13 ± 0.02
Anabaena circinalis	23.2 ± 0.08	1.10 ± 0.04
Microcystis aeruginosa	26.2 ± 0.04	0.84 ± 0.02

Concentrations of phycocyanin and chlorophyll-a within a million cells were determined by plotting the fluorescence emission intensity of each pigment in cell concentration of 1,000,000 cells/mL against the linear curves of pure standard of chlorophyll-a (Figure 3.8) and phycocyanin (Figure 3.9) from Chapter 3.

The uncertainty measurements were calculated from the standard deviation of cell concentrations in replicate measurements of phycocyanin and chlorophyll-a fluorescence responses. The variation for phycocyanin and chlorophyll-a concentrations versus emission were low as presented in Chapter 4, whilst cell concentrations demonstrated higher variance due to the method of cell concentration determination, also presented in the same chapter.

6.1 Cell Disruption

Cell disruption was implemented with the objective of liberating high yields of phycocyanin from cyanobacteria cell cultures. Cell disruption was investigated using techniques from literature to determine the optimum conditions for the cell disruption of cyanobacteria cells. The techniques utilised for cell disruption were sonication by bath and probe (Furuki et al., 2003, Rajasekhar et al., 2012a, Rajasekhar et al., 2012b, Wu et al., 2011, Wu et al., 2012b, Zhang et al., 2006a, Zhang et al., 2006b), and freezing/thawing cycles (Chaiklahan et al., 2012, Chaiklahan et al., 2011, Lello Zolla and Bianchetti, 2001, Lemasson et al., 1973, Moraes et al., 2011, Niu et al., 2007, Sarada et al., 1999, Benedetti et al., 2006, Silveira et al., 2008, Simis et al., 2007).

As an alternative method for phycocyanin extraction from cell cultures, a chemical method observed during liquid chromatography sample preparation was further investigated to determine the effectives of the process, as a viable method of phycocyanin extraction. This method is further discussed later in section 6.4.4.

To maintain membrane and tubing integrity during and following repeated sonication cycles, the sonication probe could not be too destructive but powerful enough to lyse cyanobacteria cells. The observed destruction of membranes under sonication disruption was evident with glass fibre membranes as stated in Chapter 5.

Extraction yields of the pigments, phycocyanin and chlorophyll-a, in solvent medium were measured by fluorescence spectrometry. Cell disruption yield percentage was determined by the yield of phycocyanin for cyanobacteria and chlorophyll-a for green algae. To determine the effect of cell disruption on pigment stability, the test solutions were measured before and after various treatments to identify any drop in measured pigment signal arising from degradation of the pigment. Disruption efficiency was determined by the measurement of intact cell concentrations determined by filtration through disposable 0.45 μm filters of initial solutions before treatment and after treatment.

6.1.1 Sonication Bath

Cell disruption by sonication bath demonstrated mixed results between the monocultures. Individual monocultures of cyanobacteria were exposed to ultra-sonic frequencies for 10, 20, 40 and 60 minutes. Table 6.3 shows the data collected from monocultures exposed to various sonication times. The high degree of extraction variability between sonication times for each

cyanobacteria culture, however, made this extraction procedure impractical for application when the output was so unpredictable.

Table 6.2: Percentage of phycocyanin liberated by various sonication times

	Phycocyanin extraction percentage (%)		
Culture	*Microcystis aeruginosa*	*Anabaena circinalis*	*Cylindrospermopsis raciborskii*
Sonication 10 minutes	3.7 ± 2.0	6.7 ± 3.5	17.8 ± 4.6
Sonication 20 minutes	5.5 ± 5.1	3.8 ± 1.3	9.1 ± 4.3
Sonication 40 minutes	18.0 ± 15.1	6.7 ±5.3	18.0 ± 10.8
Sonication 60 minutes	13.0 ± 0.3	30.0 ±28.8	-

Phycocyanin extraction by 40 minute sonication treatment seemed more effective for *Microcystis aeruginosa*, the smaller culture species of cyanobacteria. *Microcystis* cultures demonstrated an exponential yield as sonication time increased. Cell disruption with *Cylindrospermopsis* cultures were similar at 10 and 40 minute durations, however, the data was more consistent for 10 minutes sonication with 17.8 ± 4.6 % for phycocyanin yield. *Anabaena circinalis* demonstrated low yield cell disruption not exceeding 10 % for this sonication technique.

The difference in extraction of phycocyanin following cell disruption with a sonication bath compared to a sonication probe was significant. The sonication bath could generate ultra-sonic frequencies using 50 Watts of power in a 500 mL volume of water.

The sonication probe could generate up to 400 Watts of power. The probe was altered via output, amplitude of the frequency and the duration of the ultra-sonic wave from 0.1 second to 1.0 second exposure. To determine an effective comparison between exposure to a sonication bath and sonication probe, the output of a sonication probe was limited to the same output as the sonication bath.

6.1.2 Sonication Probes

Sonication probes were used for the disruption of cyanobacteria and green algae cells using a Branson Sonifier 450. The applied conditions of the sonication probe were as described in section 3.6.1.1.1 with an output of 60 Watts.

Cell disruption of microscopic monocultures was primarily conducted by a sonication probe with the head of the probe submerged vertically into a sample suspension containing the culture (Furuki et al., 2003, Rajasekhar et al., 2012a, Rajasekhar et al., 2012b, Wu et al., 2012a, Wu et al., 2011, Wu et al., 2012b, Zhang et al., 2006a, Zhang et al., 2006b). The sonication probe had interchangeable sonication heads. For this research, only the 3 mm sonication probe was utilised when submerged into the solution.

Cultures treated by sonication were both filtered and unfiltered to determine the effectiveness of the different sonication times. In order to measure the potential effect of phycocyanin emission during cell disruption, a comparison was made between treated unfiltered cultures exposed to ultrasound versus non treated cultures of the sample at the initial concentration.

Microcystis aeruginosa monocultures were treated at various cell concentrations between 75,000 and 2,400,000 cells/mL. In cell concentrations that were mixed, the ratio between unfiltered samples at an initial concentration versus filtered after exposure to the sonication probe at different times demonstrated an initial wide range of the data up to approximately 12 minutes. Most of the data points were between 80-100 % of phycocyanin extraction from the beginning of sonication treatment. As the exposure time increased, the data points converged within 85-100% of total phycocyanin extraction from *Microcystis aeruginosa* cells.

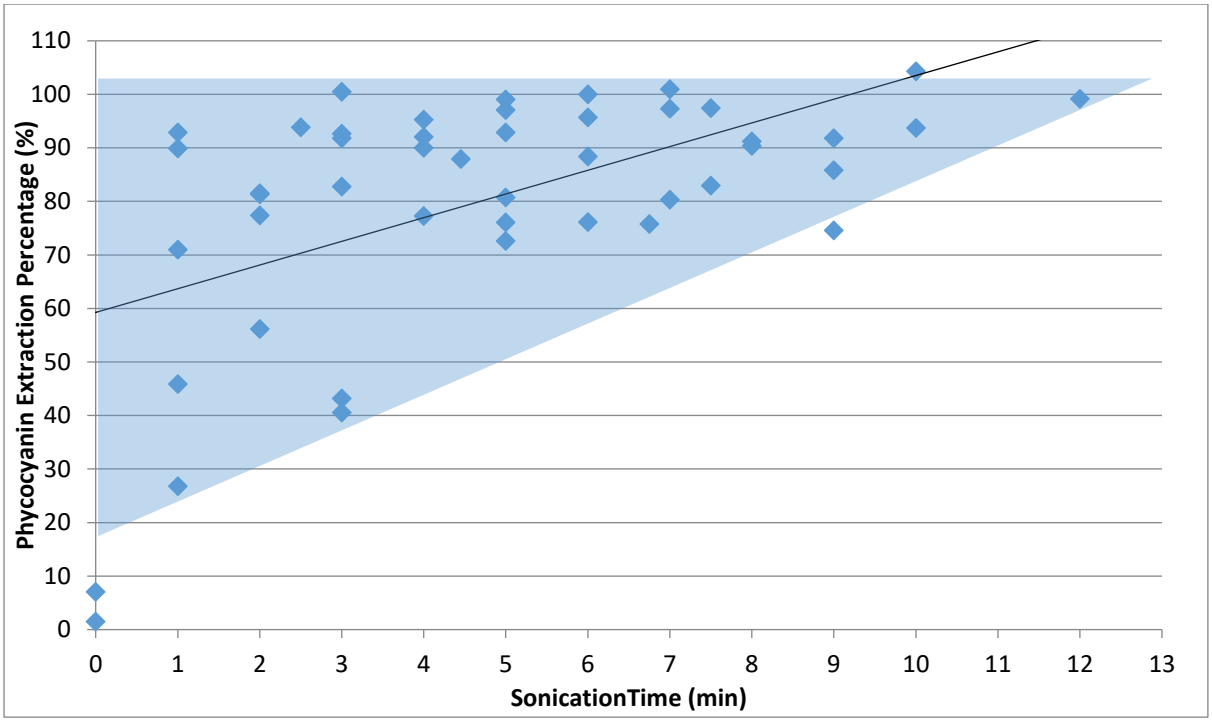


Figure 6.1: Percentage of phycocyanin extraction

Figure 6.1 demonstrates the wide variance of data points at the initial exposure to sonication from 0-12 minutes. Another factor that was observed was the variation in concentration when exposed to sonication. Higher concentrations of *Microcystis* cell cultures demonstrated lower extraction of phycocyanin in the earlier minutes of sonication treatment compared to lower cell concentrations or required longer sonication times for increased cell disruption. The opposite was also observed. Lower cell concentrations required less energy to disrupt the cells in the suspension. The blue highlighted area in the figure demonstrates the extracted phycocyanin from cyanobacteria with increasing sonication time.

Unlike *Microcystis aeruginosa*, the treatment of *Anabaena circinalis* cultures did not yield 100% phycocyanin extraction with increased sonication times as presented in Figure 6.2.

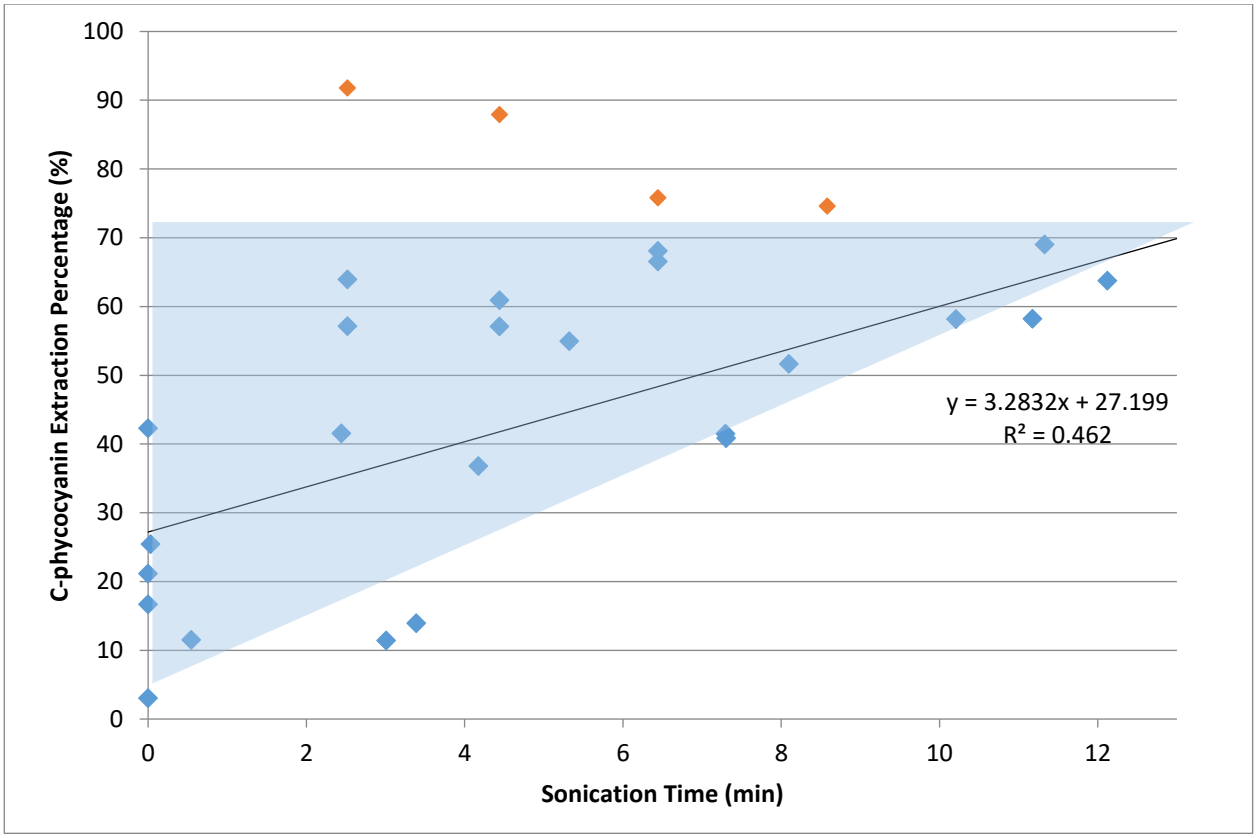


Figure 6.2: *Anabaena circinalis* presented with a different phycocyanin extraction

Figure 6.2 demonstrates the behaviour of phycocyanin extracted from the *Anabaena circinalis* cell cultures. Unlike the phycocyanin extraction behaviour of *Microcystis aeruginosa* of an increasing extraction yield over time, phycocyanin extraction from *Anabaena circinalis* demonstrated a wider variance of extraction from the first minutes which converged at 12 minutes to 65.2% recovery (n=7). One of the trials was regarded as an outlier due to the abrupt high phycocyanin recovery which decreased over sonication time; these data points are indicated by the red marks and were not taken into calculations. *Anabaena* cultures were larger in cell size compared to *Microcystis* which would not take as much potential energy to disrupt the cell. *Microcystis* was a smaller cell culture which could potentially retain cell integrity longer than larger celled cyanobacteria (Batzing, 2002, Thain and Hickman, 2000, Warren et al., 2002). Conditions were not changed between cell disruption of *Microcystis* and *Anabaena* cultures meaning any effect on phycocyanin intensity was related to the effect of sonication on that species.

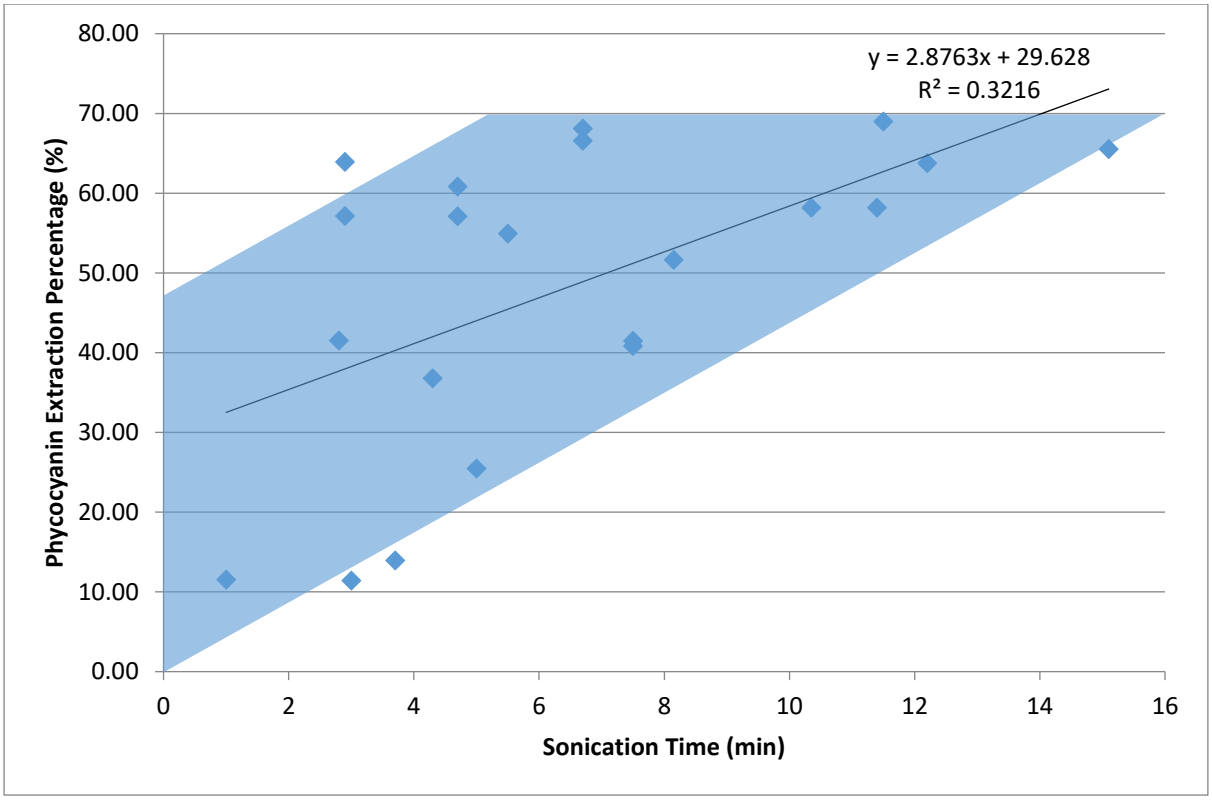


Figure 6.3: *Cylindrospermopsis raciborskii* presented with a different phycocyanin extraction

Liberation of phycocyanin from *Cylindrospermopsis raciborskii* demonstrated an extraction profile similar to *Anabaena circinalis*. Over 15 minutes a total phycocyanin extraction recovery percentage of 69.8 % was achieved.

Use of the sonication probe was limited to a total treatment time of 15 minutes. This included cooling periods every two minutes as the heat generated from the probe decreased the detectability of phycocyanin by heating the water temperature. This phenomenon was investigated further in section 6.2.2.

6.1.3 Freeze-Thaw cycling

Freeze thaw cycling is a successful technique demonstrated by numerous publish research articles (Benedetti et al., 2006, Chaiklahan et al., 2012, Chaiklahan et al., 2011, Lemasson et al., 1973, Moraes et al., 2011, Niu et al., 2007, Sarada et al., 1999, Simis et al., 2007, Zolla and Bianchetti,

2001). It is cost effective and a relatively easy process to implement. Of the three cyanobacteria monocultures investigated, only *Cylindrospermopsis* cultures demonstrated effective pigment extraction yields that would allow further use of this technique. However, *Microcystis* and *Anabaena* cultures demonstrated recoveries of only <40 % phycocyanin yields. Table 6.3 represents the data collected from the freeze-thaw technique.

Table 6.3: The percentage of cell disruption by freeze-thaw cycle

Culture	*Microcystis aeruginosa*	*Anabaena circinalis*	*Cylindrospermopsis raciborskii*	*Chlorella vulgaris*
Freeze –Thaw yield	37.3 ± 10.1	33.6 ± 5.0	73.3 ± 14.9	0.004*

* - Baseline data – instrument variation of the baseline

The cyanobacteria, *Cylindrospermopsis raciborskii*, demonstrated the most effective cell disruption of the cultures investigated with 73.3 ± 14.9 % of phycocyanin recovered. *Microcystis aeruginosa* and *Anabaena circinalis* demonstrated approximately 30% cell disruption which was relatively consistent with a 60-minute sonication treatment. Despite the baseline emission of extraction from *Chlorella* cultures, the technique was effective in cell disruption but no value could be confirmed. Due to the lack of stability of chlorophyll-a in deionised water, no percentage on cell disruption could be calculated for green algae by the freeze-thaw technique. Optimal extraction solvents will be discussed later in this chapter.

Despite the effectiveness of the freeze-thaw technique, the length of the technique to be effective and to take the technique from a laboratory method into field operations would be difficult. The main disadvantage to the freeze-thaw method was the time to achieve effective cell disruption for all species of cyanobacteria. One freeze-thaw cycle was 2 hours: 60 minutes in the freezer and 60 minutes on the bench at room temperature to thaw. The complete freeze-thaw technique required three cycles or approximately 6 hours.

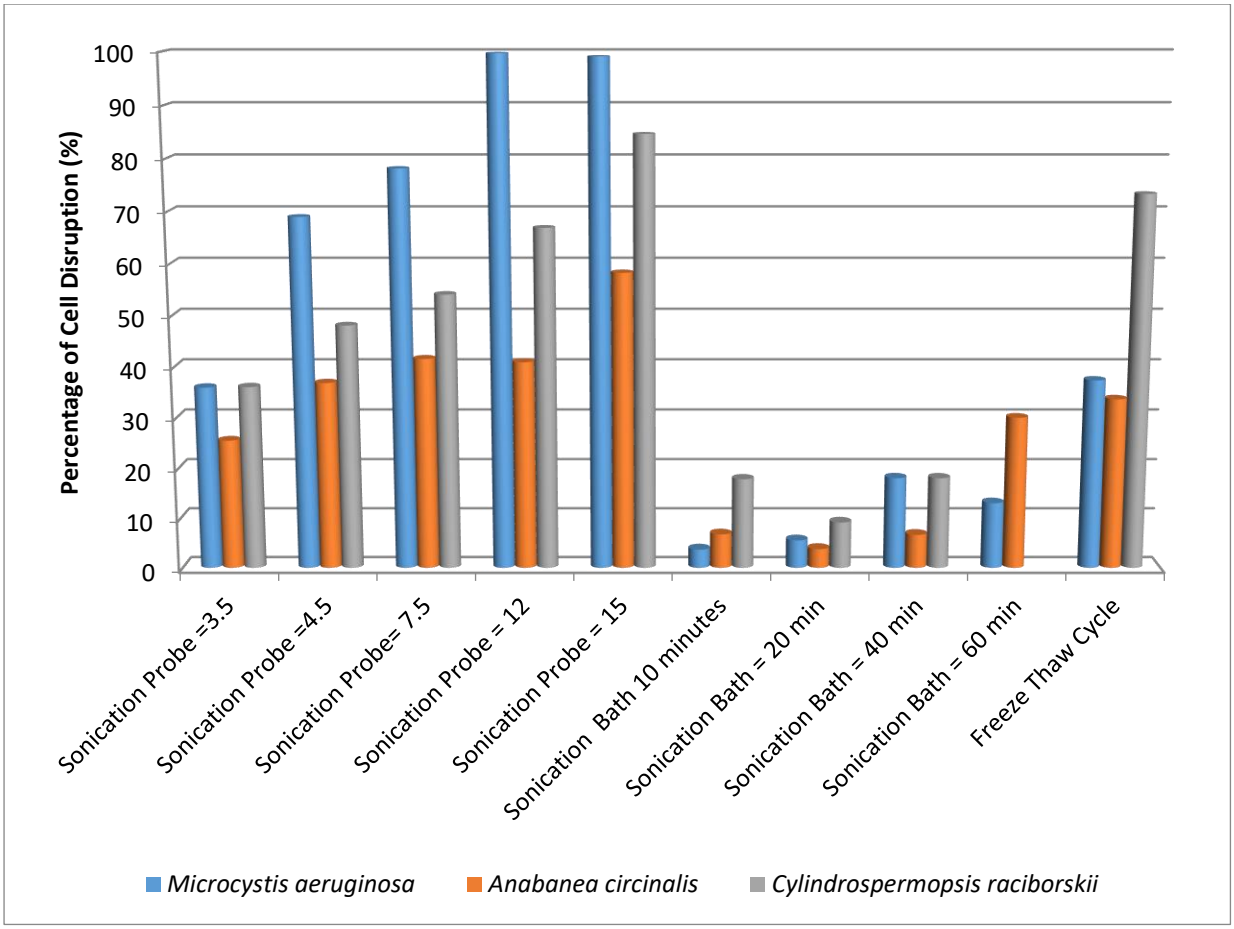


Figure 6.4: Cell disruption of *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii* using increased sonication times and completion of a full freezing-thawing cycle.

The trial of the sonication probe for cell disruption demonstrated the most cell disruption in the shortest treatment time as shown in Figure 6.4. The freeze-thaw technique would be suitable for cell disruption over use of the sonication bath. However, the length of treatment time required was not a suitable application for a rapid analysis technique.

6.2 Physical factors

Whilst there are alternative methods of chemical extraction such as acid extraction (Sarada et al., 1999), the extraction of phycocyanin into high concentrations of hydrochloric acid was not considered in this research. Section 6.3.1 demonstrates the stability of phycocyanin within acidic

and basic solutions, whilst the previous Chapter on detection demonstrated the unpredictability of phycocyanin measurement when attempting to calculate concentration within the protein region. The errors in attempting to measure phycocyanin within a protein region are due to the abundance of additional proteins from the cyanobacteria culture within a solution.

6.2.1 pH

Purified phycocyanin was placed into three concentrations of sodium phosphate buffer at various pH values that ranged from 2 to 10.3. The region of pH 6-7 demonstrated the most fluorescence intensity whilst anything outside this narrow range of pH had a negative effect on the emission of phycocyanin when excited at 609 nm (see Figure 6.5). Further there was no observed wavelength shift from phycocyanin during the pH investigation. This observation of pH effect on phycocyanin supports the work by Chaiklahan et al. (2012).

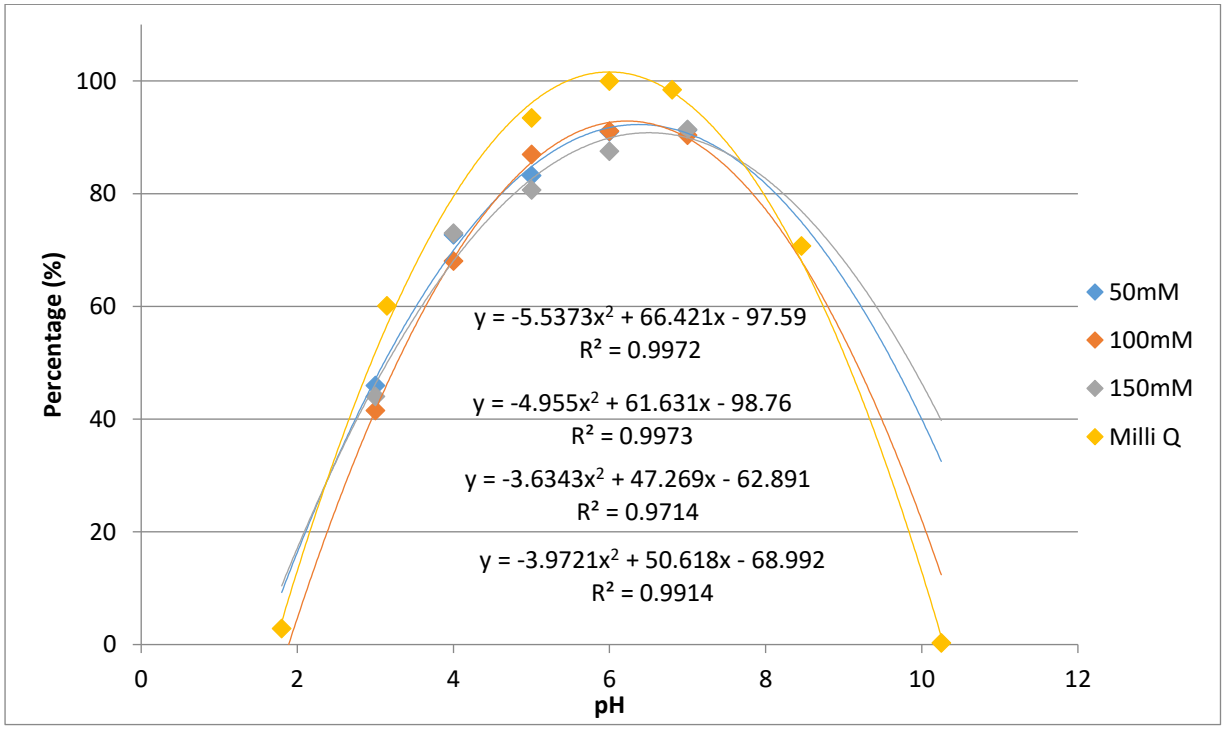


Figure 6.5: pH effects on phycocyanin emission intensity in various concentrations of buffer (excitation at 609 nm, emission at 647 nm).

There was little difference between deionised water and the various concentrations of sodium phosphate buffer when exposed to various pH values. Further, phycocyanin demonstrates no pKa, only an optimal pH to maintain relative emission. Phycocyanin dissolved into Milli Q water was used as a blank.

6.2.2 Temperature

The fluorescence intensities of pure 10 µg/L phycocyanin solutions in Milli Q were measured at temperatures ranging from 2 °C to 50 ºC. The temperature was varied by first placing the solution in a slurry ice bath at 2 ºC and slowly increasing the temperature to 50 ºC on a hot plate. Ambient light exposure was limited, with the samples stored in 50 mL amber test tubes during the experimentation. The temperature was recorded from a thermometer suspended in a vial similar to that containing the phycocyanin solution, so that the temperature of the solution and not the water bath was recorded. A second control was stored at 20 ºC and measured with each increase in temperature to record any potential drop in emission from pigment degradation in Milli Q water. Phycocyanin stability to temperature was determined by measurements at 609 nm excitation and 647 nm emission.

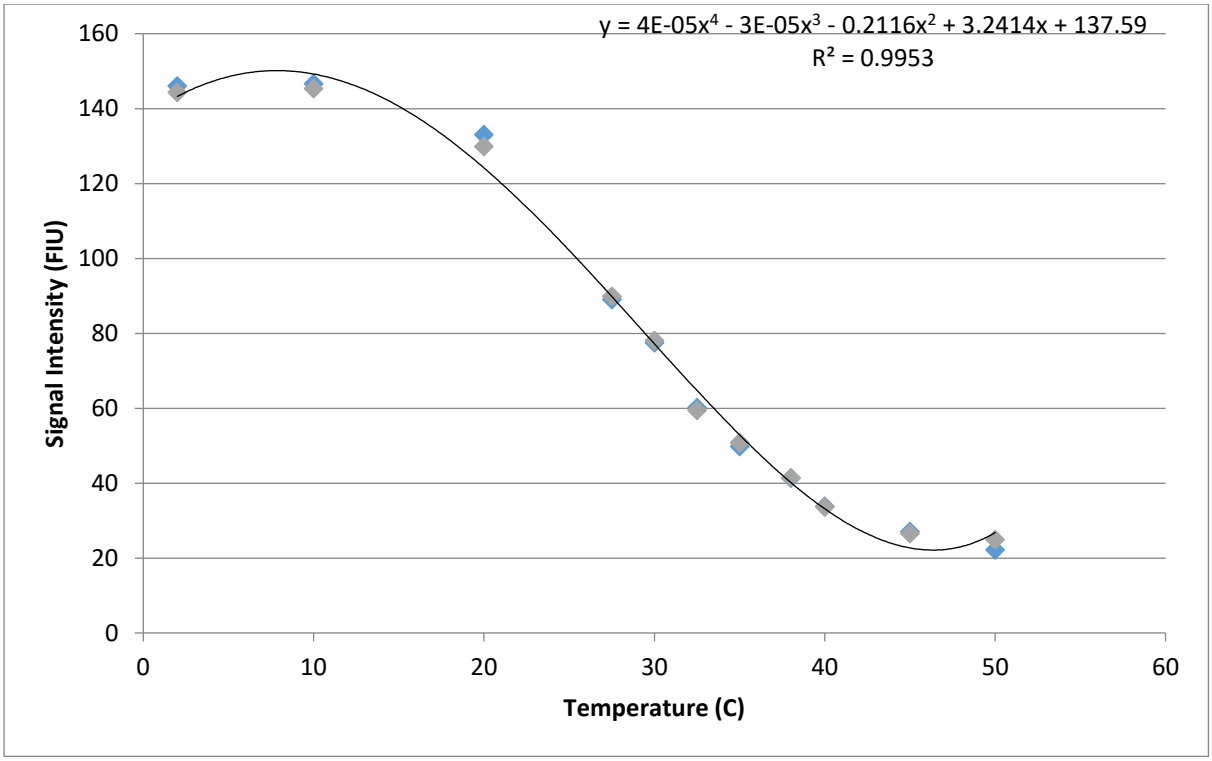


Figure 6.6: Temperature effects on phycocyanin (excitation 609 nm, emission 647 nm)

As the temperature was increased, measurements were taken with the emission response demonstrated in Figure 6.6. Phycocyanin fluorescence intensity began to decrease after 15 °C at a steady rate until about 40 °C, where phycocyanin emission began to stabilise. Optimal temperatures for measuring extracted or phycocyanin dissolved into Milli-Q water was ≤10 ºC. After 20 ºC, there was a significant drop in emission responses of phycocyanin.

6.3 Matrix effects on phycocyanin

Solution matrix effects on phycocyanin fluorescence emission were investigated with pure compounds obtained from Sigma Aldrich shown in Table 6.4. Table 6.4 demonstrates the decreasing signal emission organic solvents have on phycocyanin. An increase in solvent strength decreased the emission response.

Table 6.4: The effect of organic solvent (Methanol, Acetonitrile and Acetone) in Milli Q water on phycocyanin fluorescence (pure phycocyanin from Sigma Aldrich).

Solvent Strength (%)	Methanol	Acetonitrile	Acetone
0	100.00	100.00	100.00
10	85.74	8.49	77.98
20	68.93	1.77	37.44
30	42.13	0.93	22.19
40	20.43	0.45	6.59
50	4.47	0.42	2.26
60	5.46	0.63	1.78
70	5.88	0.14	1.04
80	4.87	0.23	0.63

6.4 Extraction matrices used on cyanobacteria and green algae cultures

In the extraction of pigments, phycocyanin and chlorophyll-a were investigated in different extraction matrices. This investigation covered the effect of the various extraction solutions on the pigments.

The parameters of the investigation covered pigment stability and extraction yields into the various solutions. These solutions were deionised water, potassium dihydrogen phosphate buffer used for the extraction of phycocyanin from cyanobacteria in previous research (Sarada et al., 1999, Hunter et al., 2010, Chaiklahan et al., 2011) and 90 % acetone to 10 % deionised water used in the extraction of chlorophyll-a by (Moberg et al., 2001, Parésys et al., 2005). In determining the optimum extraction solution for phycocyanin detection, these solutions extracted pigments from three cyanobacteria species, *Microcystis aeruginosa*, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*, and green algae species, *Chlamydomonas reinhardtii* and *Chlorella vulgaris*.

6.4.1 Extraction into Milli-Q

The wavelengths of interest for phycocyanin were excitation at 609 nm and emission at 647 nm emission and all statistical data was done using the software program SPSS PAWS Statistics 18.

6.4.1.1 Extraction of phycocyanin

The extraction of phycocyanin by ultra-sonication in Milli-Q water as the liquid medium, provided an increased intensity from the untreated control. This is shown in Figure 6.7 and measured by fluorescence excited at 609 nm with emission measured at 647 nm. This increase in the signal emission from phycocyanin after sonication was produced by the removal of the cell membrane. When the cell membrane was disrupted by sonication, the signal emission of phycocyanin increased for all species shown in Figure 6.1. Whilst all three cyanobacteria species demonstrated an increase in signal emission, *Anabaena circinalis* presented the highest signal increase, with *Microcystis aeruginosa* demonstrating the lowest signal increase. Cyanobacteria, *Microcystis*, had the smallest average cell size of the species investigated and gave the lowest increase in signal emission after extraction. The data was analysed by comparing the fluorescence intensity of the liquid suspension of the sample, Pm with the initial concentration of each cyanobacterial species measured before the samples were placed into the sonication bath, Po. The ratio of Pm/Po was always greater than 1.00 as shown in Figure 6.7.

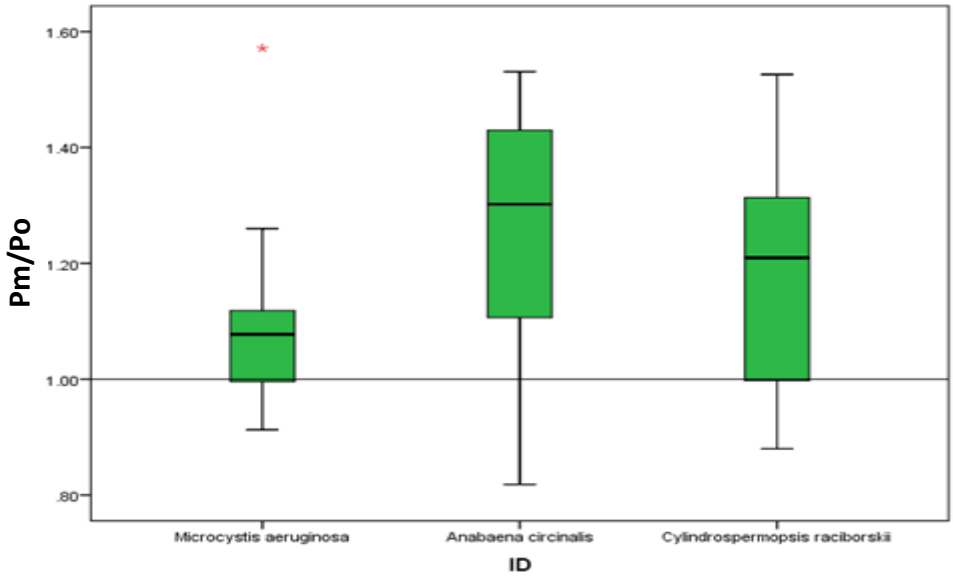


Figure 6.7: The effect of ultra-sonication on phycocyanin liberation from three cyanobacteria species using water as the liquid medium; fluorescence excitation at 609 nm, emission at 647 nm. Red star was an outlier within two standard deviations (n = 25).

With a 95% confidence interval of the mean, sonication of *Microcystis aeruginosa* cultures demonstrated an increase of phycocyanin emission between 3.0 to 12.4 % with a mean increase of 7.7 %. *Cylindrospermopsis raciborskii* demonstrated an average mean increase in phycocyanin emission of 21.0 % with a range of 12.6-25.9 %. *Anabaena circinalis* demonstrated an average mean increase in phycocyanin emission of 30.5 % with a range of 18.1 to 33.5 %.

Anabaena circinalis and *Cylindrospermopsis raciborskii* had a wider range of Pm/Po ratiosin relative to the intact cyanobacterial cell culture, illustrated in Figure 6.7. Whilst the ratios of phycocyanin emission were between 0.82 to 1.54, the box in the results for both species were displayed above the initial reading.

Whilst *Microcystis* cultures demonstrated an increase between Pm/Po, the increase was more consistent distribution of results compared to *Anabaena* and *Cylindrospermopsis*, however *Anabaena* and *Cylindrospermopsis* presented with a greater increase of Pm/Po.

6.4.1.2 Extraction of chlorophyll-a

Extraction of chlorophyll-a was investigated to determine if chlorophyll-a could be extracted along with phycocyanin from cyanobacteria. This would allow for potential spectral interference when measuring phycocyanin.

The extraction of chlorophyll-a into Milli Q water as a liquid medium was slightly less effective than phycocyanin (see Figure 6.8). The extractions were slightly higher than the initial readings with *Microcystis aeruginosa* that recorded an increase of 1.5% in detection with a 95% confidence interval for mean ratio between 0.99-1.08. *Anabaena circinalis* was observed having a 2.8% increase with a 95% confidence level for a mean ratio between 0.97 and 1.13. *Cylindrospermopsis*

raciborskii recorded an increase of 8.8% in the detection from the initial level with a 95% confidence range and the mean ratio between 1.03 and 1.16.

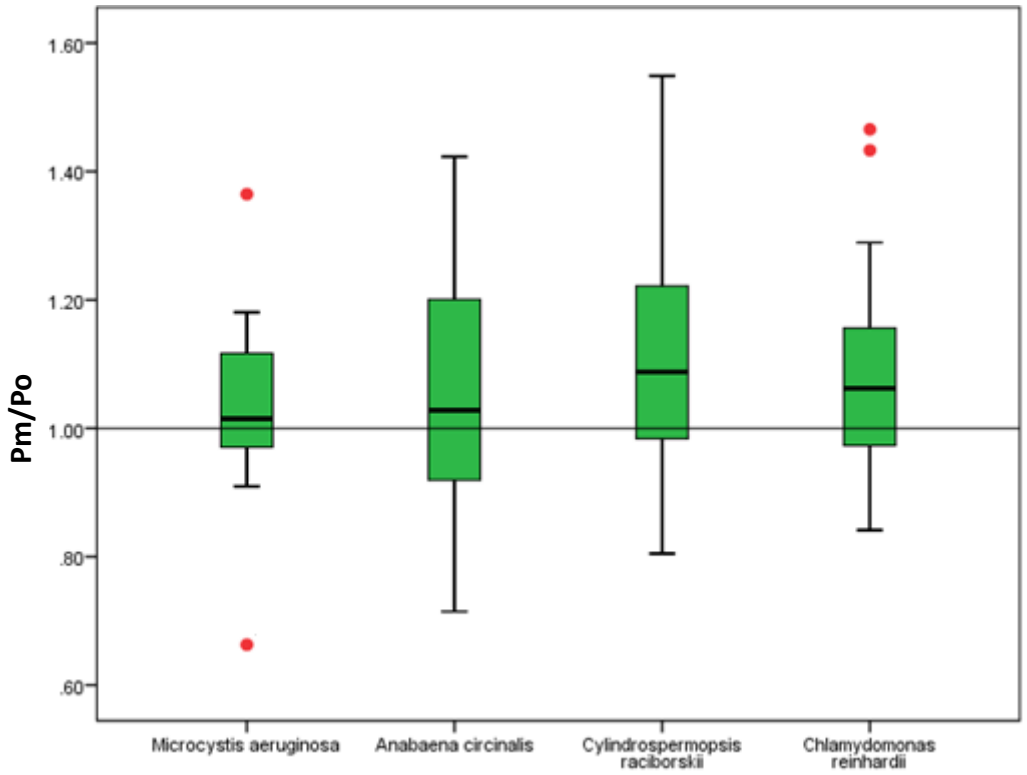


Figure 6.8: Statistical analysis of effect of ultra-sonication on chlorophyll-a detection from three cyanobacteria and one green algae species using Milli-Q water as the liquid medium (fluorescence excitation at 609 nm, emission at 680 nm). Red dots were outliers >1 standard deviation.

Green algae species, *Chlamydomonas reinhardtii*, showed an increase of 6.2% after the pigment was extracted from the cell with a 95% confidence range from 1.03 to 1.14 of the mean. However, unlike the data present in Figure 6.7 for phycocyanin, the extractions for chlorophyll-a into deionised water did not demonstrate any significant benefit or effect of extracting chlorophyll-a into deionised water. The mean increases in emissions for green algae, *Chlamydomonas reinhardtii*, and cyanobacteria, *Microcystis aeruginosa* and *Anabaena circinalis*, are slightly larger than their initial reading. The data is further plagued with a wider variance in the data range and

contains multiple outliners for *Microcystis aeruginosa* and *Chlamydomonas reinhardtii*. *Cylindrospermopsis raciborskii* had with a higher mean than the other species investigated but with a wide data range ratio between 0.8-1.57: no outliners. The wide data range was not considered ideal as the data was not reproducible for *Cylindrospermopsis raciborskii* extractions of chlorophyll-a into deionised water.

6.4.2 Extraction into Sodium Phosphate Buffer

Sodium Phosphate Buffer at pH 6.5 was investigated as an extraction medium for phycocyanin. The efficiency of the sodium phosphate buffer decreased phycocyanin signal emission compared to Milli-Q water extraction emission. This was presented in Table 6.5.

Table 6.5: Fluorescence signal emission comparison of sodium phosphate buffers versus Milli-Q

Sodium Phosphate Buffer Concentration (mM)	Fluorescence signal emission comparison of phycocyanin in Milli-Q (%)
0	101.3 ± 1.7
50	91.7 ± 0.47
100	92.1 ± 0.9
150	90.2 ± 0.6

The 50-150 mM concentrations of sodium phosphate buffer presented with similar consistent results. Whilst the approximate 10 % drop in signal emission was not significant in the measurement of phycocyanin, the extraction medium of Milli-Q water was more effective than using sodium phosphate buffer.

6.4.3 Extraction by Potassium Phosphate Buffer, pH 6.2

Initial observations for phycocyanin emission using potassium phosphate buffer as an extraction solvent demonstrated a peak shift from 647 nm to 619 ± 2 nm. This shift of the wavelength was related to potassium phosphate buffer interactions with phycocyanin. Potassium phosphate buffers were adjusted to a pH of 6.2 that mimicked the pH of phycocyanin in Milli-Q and sodium

phosphate buffer (50-150 mM) as per sections 6.4.1 and 6.4.2. With variation of pH removed as a factor that may affect emission of phycocyanin, the shift in wavelength was due to the matrix effect of the potassium phosphate buffer on phycocyanin. This is problematic as the excitation of phycocyanin was optimally conducted as 609 nm which also has a wide emission profile that does interfere with 620 nm as seen in Figure 4.2, Chapter 4. When the excitation wavelength was changed to 580 nm, which is the minimal excitation wavelength, there was an emission peak at 619 ± 2 nm for phycocyanin.

6.4.3.1 Extraction of phycocyanin

The extraction of phycocyanin from the cyanobacterial species by sonication into a 50 mM potassium phosphate buffer at pH 7.0 had mixed results. The statistical analysis of extracting phycocyanin from *Microcystis aeruginosa* into 50 mM of potassium phosphate buffer showed a slight decrease in the mean emission after sonication compared to before sonication, presented in Figure 6.9 below. This indicates that there is some interaction from the buffer solution that limits the emission of phycocyanin at 647 nm compared to sonication and suspension in Milli-Q water, presented in Figure 6.7.

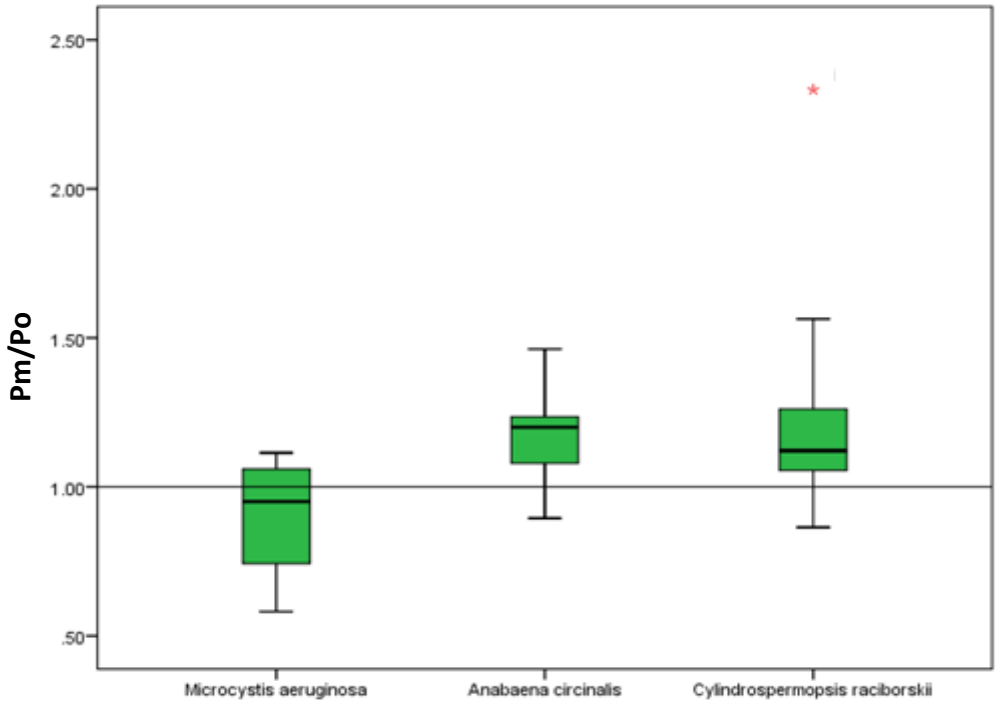


Figure 6.9: Statistical analysis of effect of ultra-sonication of three cyanobacteria and one green algae species using 50 mM potassium phosphate buffer as the liquid medium; fluorescence excitation at 609 nm with measurements recorded at 647 nm emission

From Figure 6.9, the reading of 1.0 on the y-axis is a reference line to indicate fluorescence intensities before pigment extraction. Whilst cyanobacterial species *Anabaena circinalis* and *Cylindrospermopsis raciborskii* present with a higher mean emission intensities after sonication compared to before sonication emission, this representation was deceiving. The matrix effect of potassium phosphate buffer on *Anabaena* and *Cylindrospermopsis* cell cultures instantly reduces the fluorescence intensity to a significantly low intensity even before sonication. This instant and significant reduction in fluorescence intensity is demonstrated in Figure 6.10.

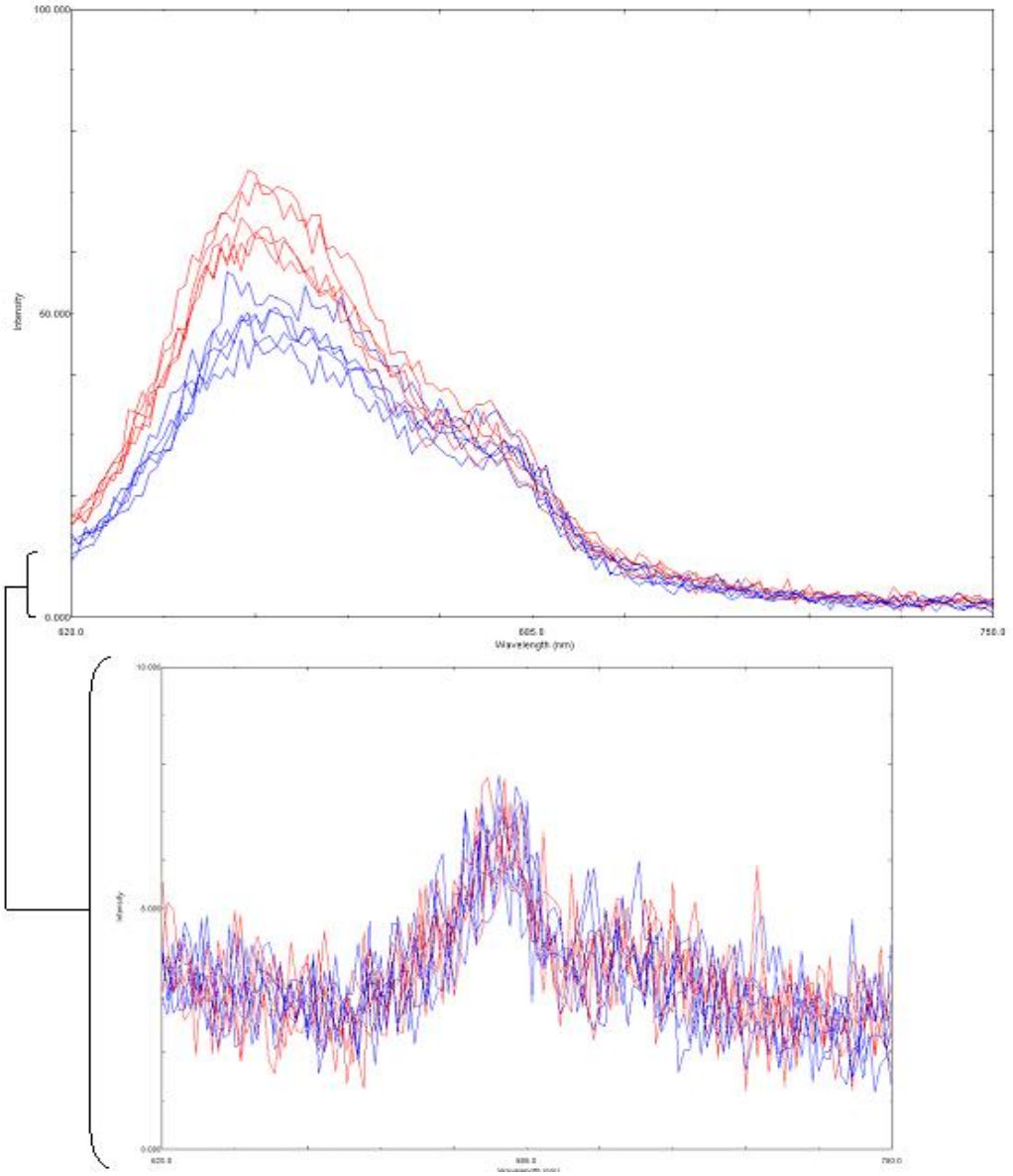


Figure 6.10: Emission effect of pigment extraction into a water medium (top graph) and potassium phosphate buffer solution (bottom graph), phycocyanin liberated from *Cylindrospermopsis raciborskii* (Ex 609 nm, Em 647 nm)

For these two cyanobacteria species, 50 mM potassium phosphate buffer suppressed the emission of phycocyanin unlike that of *Microcystis aeruginosa* which demonstrated no adverse effect from extraction into phosphate buffer. In Figure 6.10 the emission of phycocyanin was demonstrated with five individual cultures each of *Cylindrospermopsis raciborskii*, blue emission lines were before extraction and red emission lines were after extraction.

The top spectrum in Figure 6.10 represents the effect of using deionised water as the extraction medium. It is clear that the emission peaks of phycocyanin (approximately 647 nm) and chlorophyll-a (approximately 680 nm), were not affected, but an increase in signal emission occurred after the compounds were liberated upon cell disruption (red emission lines in the top spectrum). The bottom spectrum used phosphate buffer in the method of extraction with the same cell concentration of cyanobacteria cultures. However, the scale of the emission from phosphate buffer is one tenth the scale and emission of the extraction of phycocyanin into water. In the spectrum, the initial emission signal was suppressed almost to signal noise.

The interaction between the water soluble phycocyanin and potassium phosphate buffer led to the suppression in fluorescence signal emission for phycocyanin.

6.4.3.2 Extraction of chlorophyll-a

Extraction of chlorophyll-a was investigated for the potential interference that it could cause when liberated. Species investigated were cyanobacteria, *Microcystis aeruginosa*, *Anabaena circinalis*, *Cylindrospermopsis racborskii* and green algae, *Chlamydomonas reinhardtii*.

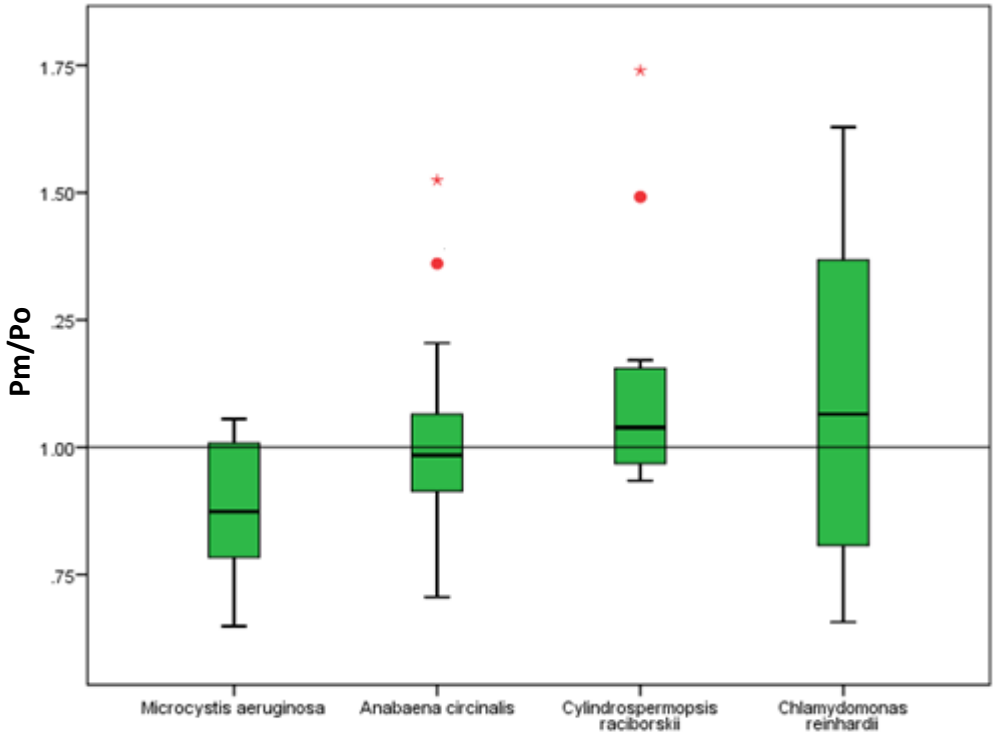


Figure 6.11: Ratio comparison of chlorophyll-a before and after extraction into buffer solution from green algae and cyanobacteria cultures.

The red dots in Figure 6.11 relate to outliers >2 standard deviation, the red star within two standard deviations. Individually, the emission from *Microcystis aeruginosa* was potentially limiting when cells were lysed. The initial emission ratio from chlorophyll-a from *M. aeruginosa* was generally lower than the initial emission intensity, which means the buffer in some capacity slightly suppresses the emission signal. The emission from *Anabaena circinalis* and *Cylindrospermopsis raciborskii* had similar proportional ratios between the initial fluorescence emissions before and after the cell cultures were lysed.

Whilst the data was presented as a ratio, the actual emission response of the cell concentrations for *A. circinalis* and *C. raciborskii* suppressed the chlorophyll-a emission as well. This is demonstrated in Figure 6.11. Initial cell cultures of the same concentration from *C. raciborskii* and *A. circinalis* were measured in deionised water and buffer solution with different results. The ratio from

chlorophyll-a before to after extraction from green algae, *Chlamydomonas reinhardtii*, demonstrated the most variance of all cell cultures analysed. In Figure 6.11, *C. reinhardtii* presents ratios from 0.69 to 1.58.

Extraction of chlorophyll-a via sonication into a 50mM phosphate buffer medium is not recommended. From all species investigated, there is no potential benefit as chlorophyll-a was suppressed when it was liberated from the cell into the buffer medium. The expression of chlorophyll-a from cyanobacteria and green algae demonstrated a wide range of emission. The emission of chlorophyll-a from cyanobacteria demonstrated no potential benefit of extraction into buffer solution compared to Milli-Q water or organic solvent.

6.4.4 Extraction into organic solvents

Chlorophyll-a extractions were conducted using a 90% methanol and 90% acetone matrices (Bidigare et al., 2005).

6.4.4.1 Extraction of chlorophyll-a from green algae, *Chlorella vulgaris* and *Chlamydomonas reinhardtii* cultures

Extraction of pigments from green algae culture *Chlorella vulgaris*, were tested at cell concentrations between 1,000,000 to 12,000,000 cells/mL. Despite the large number of cells, the limit of detection was approximately 44,000 ± 414 cells/mL with a fluorescence signal of ~0.7 FIU. Cell concentrations were determined by cell counting in a Neubauer cell chamber as per section 3.4.1 in Chapter 3. The various concentrations were then measured by Fluorescence spectrometry and plotted in a linear relationship. Taking the fluorescence emission signal response (FIU) one step further, pure chlorophyll-a was measured at various concentrations as per Figure 3.8 in Chapter 3. The fluorescence response of a known concentration of chlorophyll-a was converted into a cell

concentration by matching the fluorescence response between the two linear curves; as long as the matrix was the same for pure chlorophyll-a calibration as cell concentration linearity.

The detection was more suitable for recovery determination at respectable FIU of ~35 and ~300 FIU. The extraction for chlorophyll-a in 80 % methanol matrix yielded an average of 98.8 ± 0.2 between the various cell concentrations and 80 % acetone yielded extraction percentages of 97.9 ± 0.3.

Green algae, *Chylamydomonas reinhardtii*, yielded a few near outliers above 100 % which set the average yield of chlorophyll-a extraction to be approximately 103.4 %. These outliers were 108.6 and 110.8 which were concluded to be a manual dilution error. The removal of the outliers from the calculated yield average reset the yield to 98.1 ± 1.4 %.

6.4.4.2 Extraction of chlorophyll-a from cyanobacteria cultures

Extraction of chlorophyll-a from *Microcystis aeruginosa* by 80 % methanol matrix yielded an average of 97.7 ± 1.3 between the various cell concentrations of ~300,000 to ~ 1.2 million cells/m. Solutions of 90 % acetone yielded extraction percentages of 98.3 ± 1.0. There were no outliers or deviations from the extraction of chlorophyll-a.

Chlorophyll-a from cyanobacteria cultures were measured at 670 nm due to the slight shift in wavelength emission due to the effect of 80 % acetone solutions. Whilst, green algae cultures demonstrated increased yields with the use of acetone as demonstrated with *Chlamydomonas reinhardtii* in Figure 6.12, the extraction was not as effective for the cyanobacteria cultures. There were numerous outliers but with the means and bulk of the extracted values close to the initial value before extraction further demonstrated in Figure 6.12 as well.

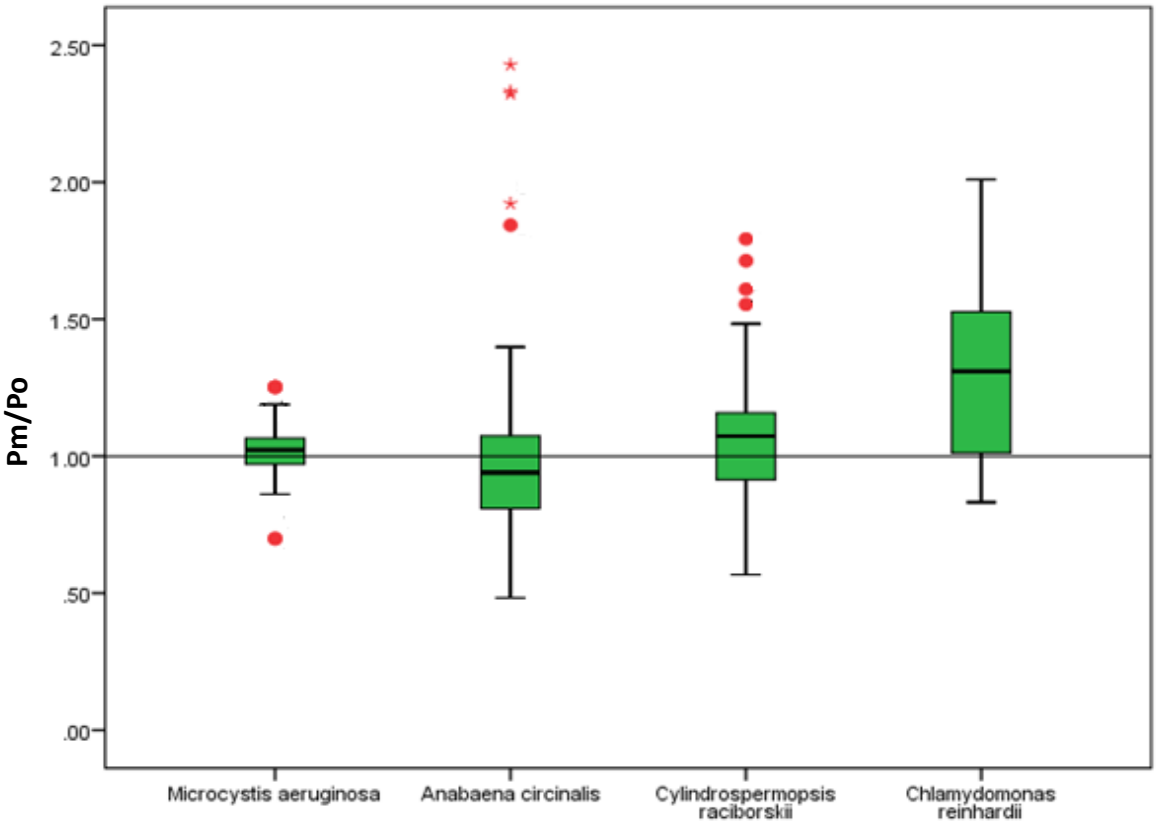


Figure 6.12: Extraction yields of chlorophyll-a from cyanobacteria cultures and green algae, *Chlamydomonas reinhardtii*.

Figure 6.12 demonstrated the various extraction ratios of chlorophyll-a into an 80% acetone solution from the species listed. This data demonstrates the acetone solution was less suitable for the extraction of chlorophyll-a in the cyanobacterial species with a larger increase in the number of outliers. Also, the average means of chlorophyll-a demonstrated a similar emission ratio between chlorophyll-a intact in the cell or extracted, and was within 100 ± 3 % recovery of chlorophyll-a for all three cyanobacteria cultures. The effect of the acetone solution on green algae species, *Chlamydomonas reinhardtii*, presented a different outcome to that the chlorophyll-a extractions from cyanobacteria. There is a wider range of ratio between 0.80-2.00 compared to 1.00 as the measurement of each solution before sonication. The difference chlorophyll-a extraction results between cyanobacteria and green algae was theorised to be the cellular wall green algae have compared to cyanobacteria's cellular membrane (Batzing, 2002, Thain and Hickman, 2000).

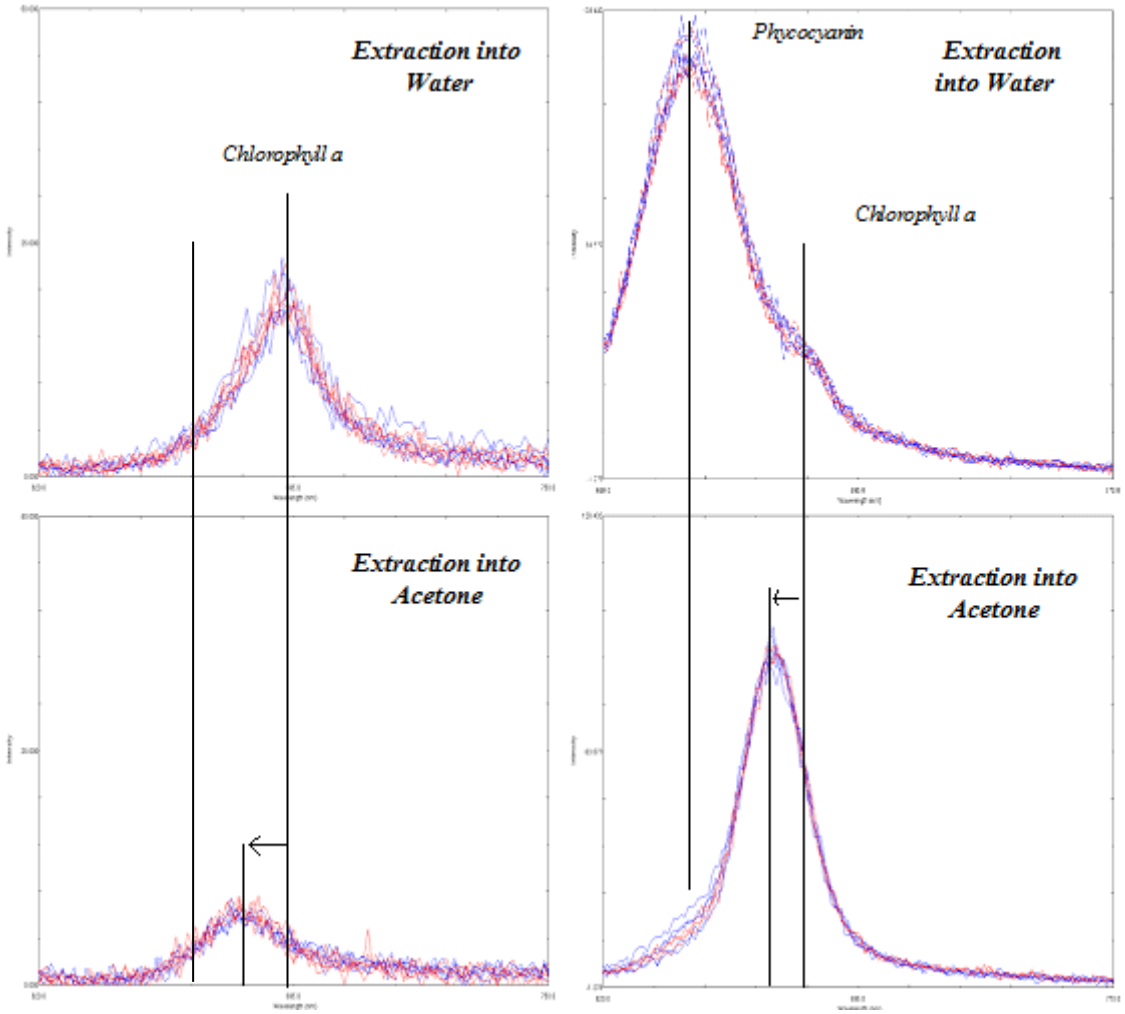
6.4.4.3 Extraction of phycocyanin from cyanobacteria cultures

Whilst the extraction of chlorophyll-a is well documented and has a standard method, the use of organic solvents in high concentration suppresses the emission of phycocyanin. Table 6.6 demonstrates the suppression of phycocyanin in 80 % acetone compared to deionised water. This was measured using the excitation of phycocyanin at 609 nm with emission at 647 nm. A pure standard of phycocyanin was used for this observation.

Table 6.6: Fluorescence suppression of phycocyanin in 80 % acetone versus deionised water when excited at 609 nm and 647 nm

Phycocyanin Concentration (ng/L)	**DI Water**	**80% Acetone**
11.7	93.771 ± 0.032	0.084 ± 0.029
1.13	9.052 ± 0.032	0.057 ± 0.029
0.21	1.711 ± 0.032	0.037 ± 0.029
0.10	0.82 ± 0.032	0.066 ± 0.029

To further demonstrate the effect of organic solvents on intact living cyanobacteria cells, the signal suppression of phycocyanin and chlorophyll-a were observed on the disruption of cyanobacteria culture *Microcystis aeruginosa* and green algae, *Chlamydomonas reinhardtii*. The fluorescence spectral emission between 620-750 nm was observed following excitation at 609 nm, demonstrated in Figure 6.13



Chlamydomonas reinhardii *Microcystis aeruginosa*

Figure 6.13: Excitation at 609 nm with emission between 620-750 nm of green algae, *C. reinhardtii*, and cyanobacteria, *M. aeruginosa*, with extraction of pigments into deionised water and 80% acetone.

The emission of phycocyanin extracted into acetone demonstrated total suppression when extracted from *Microcystis aeruginosa* whilst there were hypochromic shifts for chlorophyll-a emissions from approximately 680 nm in water to 670 nm due to the solvent use. Chlorophyll-a from *Chlamydomonas reinhardtii* demonstrated the same shift of chlorophyll-a when exposed to organic solvents.

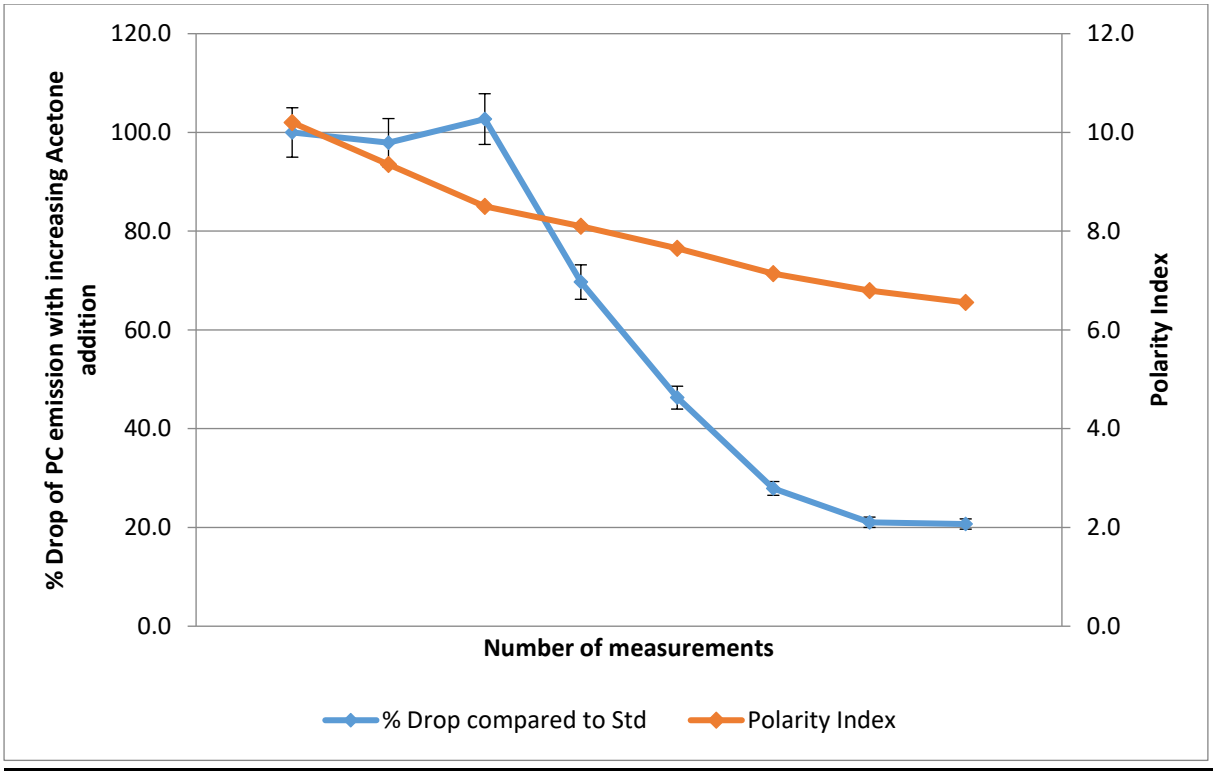


Figure 6.14: The expression of phycocyanin and potential percentage decrease of phycocyanin expression with a decreasing polarity index from the addition of acetone.

With increasing concentration of acetone in the extraction solution, there was a drop in the polarity in the solution (see Figure 6.14). This drop in the polarity index had the effect of solidifying phycocyanin, as phycocyanin is insoluble in solvents. The rate of solidification is dependent on the polarity index. Higher polarity index solutions such as 20 % acetonitrile will take longer to solidify phycocyanin compared to 80 % acetone. The active functional groups on phycocyanin are more stable in polar solutions compared to non-polar solutions (Adlnasab et al., 2010, Garrido and Zapata, 2006). When phycocyanin is exposed to a highly non- polar solvent and in order to maintain its polar functionally, phycocyanin binds with itself in a weak ionic form that is highly unstable and easily re-dissolved into the solvent with gentle mixing.

Figure 6.15: Precipitation of pure phycocyanin in 20c% acetonitrile (left tube) and 20% acetone (right tube).

Precipitation of phycocyanin from high concentration solutions could be visually observed as shown in Figure 6.15 (100mg/L of phycocyanin).

Extraction of phycocyanin from cyanobacteria in organic solvent followed by sonication was more effective than treatment by sonication bath with Milli-Q water as the extraction medium. Results showed that for phycocyanin the extraction yield was 78 ± 5 % and chlorophyll-a yielded approximately 97 ± 3% with cell cultures above 1,000,000 cells/mL. The additional benefit of such an extraction method was the capability to extract chlorophyll-a initially in high yield whilst physically separating phycocyanin from dissolved organic matter. The effect of precipitation and dissolving phycocyanin led to a slight loss of 22 ± 5 % of the fluorescence emission after extraction

from the cell cultures. The loss is associated with the potential residue of undissolved phycocyanin on the filter paper that was not dissolved by the wash of Milli-Q water. Despite the loss of phycocyanin signal emission, this loss could not be confirmed by any other additional means, as the mass of phycocyanin was at the nanogram level.

Figure 6.16 shows the capture of phycocyanin from cyanobacteria cultures, *Anabaena circinalis* and *Cylindrospermopsis raciborskii,* after cell disruption into 80 % acetonitrile medium.

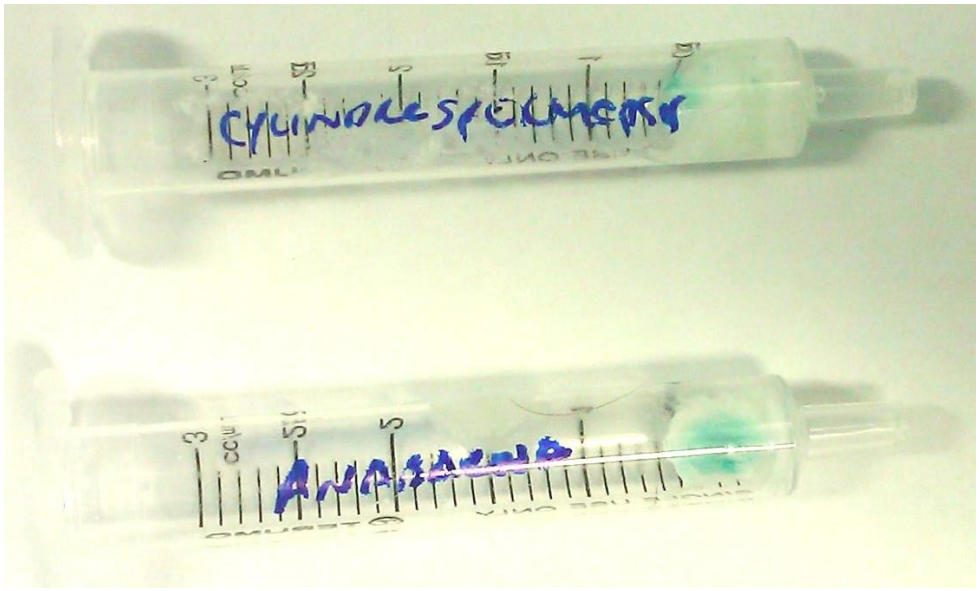


Figure 6.16: Chemical extraction and capture on Whatman GF/G filter paper of phycocyanin from cyanobacteria cultures, *Anabaena circinalis* and *Cylindrospermopsis raciborskii*.

6.5 Discussion and Conclusions

It was discovered through this investigation that the submerged sonication probe was more effective than the sonication bath due to the action of cavitation in direct contact with the culture. The sonication probe acted directly on the suspension containing the algae/cyanobacteria while the sound waves from the sonication bath were mediated by the filter holder through which they were required to travel before impacting the culture suspension. The mediation of the filter holder dampened the effectiveness of the sonication bath.

The medium used for cell disruption of cultures was deionised water, as it was suitable and phycocyanin was stable over short durations of approximately 4 hours. Whilst there was a drop/loss of emission signal with the disruption of the cell; chlorophyll-a was not determined to be extraction into a water medium. Further, in freeze-thaw cycles, any organic medium could not be frozen readily due to the lower freezing point of the organic solvents investigated.

The extraction of pigments, chlorophyll-a and phycocyanin, from cyanobacteria proved to be a complex issue. With phycocyanin stable in Milli-Q water and 150 mM sodium phosphate within 5.9-6.5 pH ranges at temperatures below 20ºC and chlorophyll-a stable in 80 % organic solvents (mainly methanol or acetone), the most suitable solution was water.

Cell disruption by sonication probe was time efficient and highly effective to allow rapid cell lyse and extraction of phycocyanin. The sonication probe was flexible allowing for changes in sonication time and treatment intensities to optimise conditions compared to freeze thaw and chemical extractions. Cell disruption by sonication bath was not practical due to the plastic and housing absorbing sonication waves and inhibiting cell disruption.

Disruption by sonication probe was achieved within 13 minutes with high yields of 68 % for *Anabaena* cultures and 100 % for *Microcystis* cultures. The proximity of the cells being within the same medium and distance to the cells proved satisfactory. Hence, the most effective method for this experiment was the use of a sonication probe that gave an effective cell disruption within 13 minutes. Considering phycocyanin was the main pigment focused on in these extractions, the stability of phycocyanin in Milli Q or buffer solution also benefited from the use of a sonication probe. This was further demonstrated with treatment by sonication bath, in which most cultures could not achieve cell disruption above 30-40 % extraction of phycocyanin.

Freeze-thaw cycles could be effective in the extraction of phycocyanin with increased cycles beyond 3. However, the procedure was lengthy and extraction efficiency between cyanobacteria species inconsistent, with *Cylindrospermopsis raciborskii* cultures yielding 75% pigment extraction whilst the *Anabaena* and *Microcystis* gave much lower extractions. Organic solvents were able to extract precipitated phycocyanin out of solution for capture on 0.7 µm glass fibre filters, however the emission signal loss was 22 % of the original signal. Further, this method is only suitable for larger concentrations of cyanobacteria species that will allow for larger quantities of precipitated phycocyanin to be captured on the membrane filters. The loss of fluorescence signal would also hinder the detection of cyanobacteria cultures at lower cell concentrations.

Therefore, further investigations focused on the use of sonication, namely sonication probes, due to the speed cell disruption can occur. Freeze/Thaw cycles, whilst effective require time for the technique to take effect. Sonication probe can be implemented more easily from an engineering perspective to target cyanobacteria compared to the implementation of a sonication bath or Freeze/Thaw system, which would require custom built specific designs and implementation.

Chapter Seven

Isolation and purification of phycocyanin from interference from source waters by chromatographic means

This chapter explores the use of different types of chromatographic resins to isolate and purify phycocyanin, primarily, and chlorophyll-a. The spectral interferences to low concentration phycocyanin detection were coloured dissolved organic matter (CDOM) and chlorophyll-a, and these interferences were discussed earlier in Chapter 4. The use of various size exclusion and anionic resins were to investigate any potential properties that could be used to capture and potentially pre-concentrate both pigments for improving detection of cell concentrations lower than 2,000 cyanobacterial cells/mL. The use of anionic exchange resins has been utilised for the separation, isolation and purification of proteins for decades (Jandera and Churáček, 1985, Minkova et al., 2003, Patel et al., 2005, Patil et al., 2006).

The use of resins to purify phycocyanin has been recorded within the food industry. The purity of phycocyanin was measured by the ratio of UV/Vis absorbance at 620 nm divided by the absorbance at 280 nm, with a ratio >4 indicating a high purity (Patel et al., 2005, Patil et al., 2006). Absorbance at 620 nm is indicative of phycocyanin concentration while protein absorbance is measured at 280 nm. Therefore, the higher the ratio indicates that phycocyanin has been successfully separated from other proteins and/or amino acids that only absorb light at 280 nm. Phycocyanin was of most interest in this investigation as the pigment is closely related to cyanobacteria, unlike chlorophyll-a. The abundance of chlorophyll-a in any photosynthetic system has the capacity to produce a false positive for cyanobacterial detection. Hence, the focus was to isolate and pre-concentrate only phycocyanin with a specific resin and ensure the resin was able to provide reproducible results and would not foul.

The isolation of phycocyanin was conducted by using and observing the interaction between the various resins employed and the model compounds such as purified phycocyanin, humic acid in sodium salt form, bovine serum albumin, alginic acid and chlorophyll-a in both an aqueous and organic matrix. Following the evaluation of model compounds, live algal and cyanobacterial cultures with naturally occurring CDOM and synthetic organic carbon, that were mixtures of humic acid and BSA, were investigated.

7.1 Size Exclusion Resin

Due to the various sizes of matter present in open water systems, the use of size exclusion would allow the smaller molecular weight fractions such as chlorophyll-a and phycocyanin to be potentially separated from CDOM. This separation may be efficient for removing the spectral interferences of humic substances from spectral detection of the pigments. The larger, highly aromatic fractions of humic acid allow for increased interaction with the size exclusion gel resin compared to the smaller pigment compounds of phycocyanin and chlorophyll-a (Filella, 2009, Lee et al., 2006, Mergen et al., 2008). The advantage of pigment separation utilising size exclusion resin is that the difference in ionic interaction, chlorophyll-a being low ionic compared to phycocyanin, which could potentially decreases any fouling effect of the resin.

7.1.1 LH-20 Sephadex

LH-20 Sephadex size exchange resin was one type of size exclusion resin investigated. The resin contains a gel type interconnecting structure of deltan which is also referred to as Sephadex. The specifications of the resin were 25-100 μL bed volume per g dry gel in either 4 mL of water, methanol or chloroform solutions. The potential benefit of investigating this size exclusion resin was the capacity of the resin to be used the resin for separation of chlorophyll-a in high methanol solutions or phycocyanin in water based solutions from CDOC.

The elution of pure phycocyanin, purchased from Sigma-Aldrich, was investigated with concentrations of 14 µg of phycocyanin/L. As per the general procedure in Chapter 3, section 3.5, flow rates were approximately 2 mL/min maintained by a peristatic pump. Investigations using this resin were conducted by measuring pure compounds individually and later as mixed samples or synthetic laboratory waters. Synthetic laboratory waters were a mixture of approximately 5 mg/L of BSA, 10 mg/L of dissolved coloured organic matter (produced from dissolved humic acid) and 14 µg/L of phycocyanin.

LH-20 Sephadex resin was investigated by loading single aliquots of 14 µg/L of phycocyanin and measuring the elution of phycocyanin with 18 mL of Milli-Q water; measurements were taken every 1.5 mL with a 1.5 mL cuvette. This investigation revealed there was a gradual elution between 3-8 mL elution volumes of phycocyanin using Milli-Q water and achieved 73.3 ± 1.3 % of phycocyanin recovery.

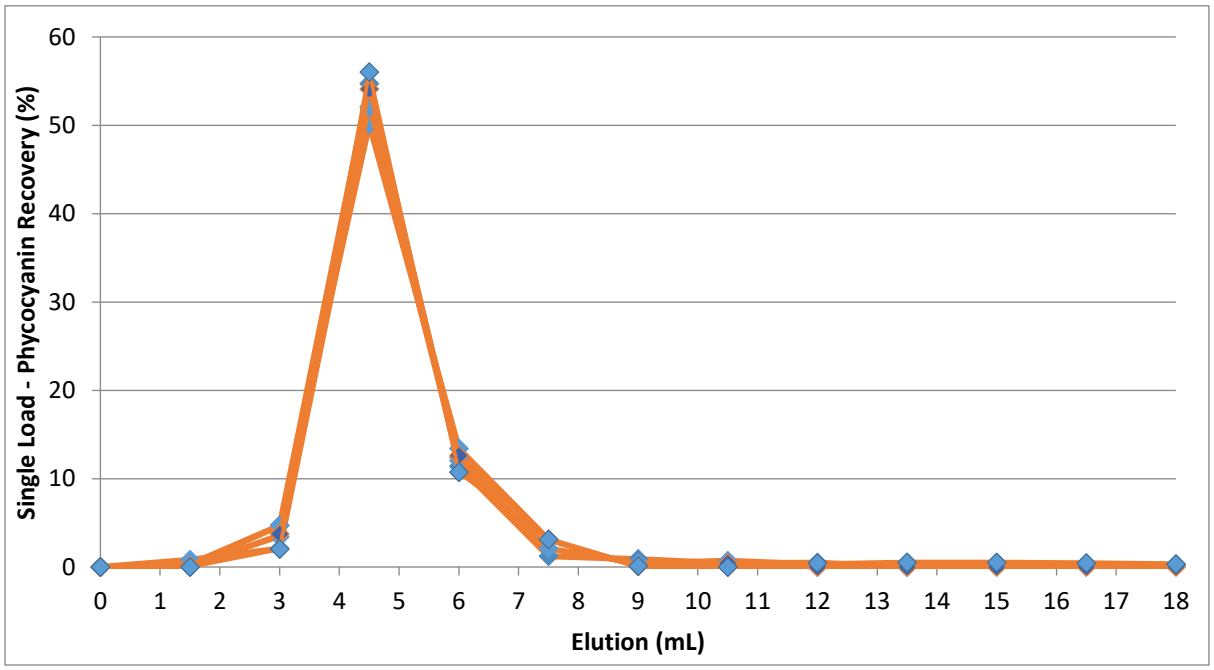


Figure 7.1: Single Load Elution Patterns of phycocyanin from LH-20 Sephadex resin (n=7)

In Figure 7.1, there was a mass elution after 3 mL which peaked at a calculated elution volume of 5 mL (range between 2.8 to 7.9 mL).

With only 73.3 ± 1.3 % of phycocyanin recovered from the resin column, the remaining 26.7 ± 1.3 % was not eluted and permanently retained on the column. Despite this percentage retained on the column, there was no observed fouling of the resin. However, the column did not exceed the point of saturation. Flushing of the resin column with 100 mL of Milli-Q water did not reveal any fouling of the resin when using purified phycocyanin, as no phycocyanin was detected in the eluant.

Continuous serial elution of phycocyanin was measured at every 4 mL. Elution of phycocyanin demonstrated the first 4 mL eluded was approximately 50.4 %. The elution quickly increased to 91.7, 97.0 and 100.5 % eluted from the resin column over the remaining 3 measures. The continuous elution cycles demonstrated no retention of phycocyanin on the column and also no separation from any components during continuous elution cycles.

7.1.2 Sephadex G25

The particle size of Sephadex G25 from the manufacture's specifications was specified as being between 10-40 microns with water retention of approximately 2.1 to 2.7 mL/g of resin. This neutral gel resin was only slightly effective in the isolation of phycocyanin, molecular weight of 689, from humic acid, with a higher and diverse molecular weight.

This was obtained by colorimetric observation; however, the elution flowrate was approximately 0.3-0.5 mL/min for a total of 6 mL. The elution of both compounds was observed in the first 6 mL. Figure 7.2 depicts the theoretical chemical structure of Sephadex G25 from Sigma Aldrich.

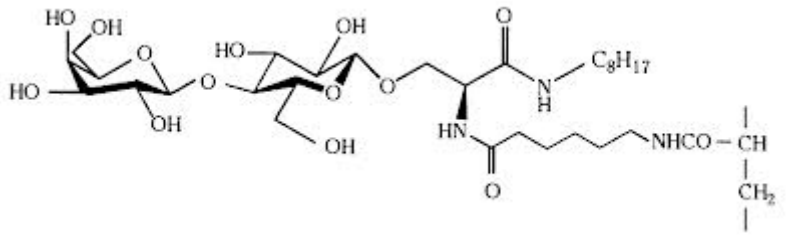


Figure 7.2: Chemical structure of size exclusion Sephadex G25 gel resin

Investigations with this resin did not demonstrate any separation between chlorophyll-a, phycocyanin and CDOC, namely dissolved humic acid. Despite the lack of separation between chlorophyll-a and phycocyanin, there was a slight separation time between the pigments and CDOM, dissolved humic acid.

With a 3 mL loading of a synthetic sample onto the resin, containing compounds humic acid, phycocyanin and chlorophyll-a; the pigment molecules chlorophyll-a and phycocyanin were eluted first while the humic substances had a slightly longer retention time. This column elution study demonstrated repeatable elution order and elution times.

Milli Q water was used after the initial loading of sample to elute the different standards through the gel resin. This procedure could not be done with continuous flow, but rather only as a sample load and elute method due to the presence and potential interference from humic substances. Further, this procedure did not separate chlorophyll-a from phycocyanin. Due to the similar and small molecular weights of chlorophyll-a and phycocyanin, the separation could not be measured. Using another size-exclusion gel resin with a smaller pore size this may to subject to resin fouling or blockage from humic acid, larger molecular weight proteins, polysaccharides and/or any other macro-molecules. With the use of a smaller pore gel size exclusion resin, the elution time will also greatly increase.

Humic acid was eluted from the column and yielded approximately 7-8 mg with continuous loading of 10 mg/L concentrations of humic acid dissolved in deionised water. This was determined by visual colorimetric analysis and fluorescence responses in excitation and emission. Due to the smaller particle size, the elution flow rate under gravity was decreased from 1.0 mL/min to 0.5±0.1 mL/min for chlorophyll-a and phycocyanin solutions. When operating with humic acid solutions, the elution flowrate decreased to approximately 0.2 ± 0.1 mL/min.

7.1.3 Sephadex G75

Sephadex G75 size exclusion gel resin had larger neutral particle sizes between 40-120 microns as specified by the supplier, Sigma Aldrich, with a bed volume of 12-15 mL of water per gram of resin. The larger pore size of this resin did not separate the pure compounds and the elution times were short because the flowrate was approximately 1.5 mL/min. Hence, the resin demonstrated little separation of the compounds that interfered with the pigments of interest, namely chlorophyll-a and phycocyanin. The loading of humic acid onto Sephadex G75 resin gave faster elution than the G25 size exclusion resin; however, the elution time of humic acid was close to the elution of the pigments. There was little data collected on this resin due to the lack of separation of the pigments, chlorophyll-a and phycocyanin, from the interfering colour of humic acid.

7.1.4 Conclusions of size exclusion resins

Sephadex G25 was the most suitable size exclusion resin for separation of CDOM substances and pigments, phycocyanin and chlorophyll-a. Increasing the length of the resin column for Sephadex G25 could increase separation and elution time between the elution of pigments from humic acid. There was potential to further investigate with size exclusion resin and attempt to utilise one that would limit potential fouling without sacrificing filtration time. By increasing pore size of the resin with an increased length of resin column, effective separation may have been achieved. However, a resin was not identified in this study to provide adequate separation between chlorophyll-a and

phycocyanin. The co-elution of chlorophyll-a and phycocyanin is problematic. The process of loading a column with a mixed solution of chlorophyll-a and phycocyanin did not separate out phycocyanin from chlorophyll-a. Due to phycocyanin being directly related to cyanobacteria and no other algae unlike chlorophyll-a which is common in any organism that can undertake photosynthesis, chlorophyll-a was declared to be an interference in the measurement of phycocyanin.

A noticeable disadvantage with the use of chromatographic resin was it was only able to be utilised in single aliquot loading. Chromatographic resins may allow for separation of compounds but only from a batch sample. Also, there was no benefit for separation of pigments in applying chromatographic resins in this application.

7.2 Activated Silica Resin

The activated silica resin size was 1.0 mm and formed a loose filter cake after settling. The size of the resin beads allowed Milli-Q water to elute through the column. Phycocyanin was dosed in low concentration between 10-20 μg/L and 0.5-2.0 mg/L. After the addition of low concentration phycocyanin solutions onto the resin, phycocyanin could not be observed spectrofluorometrically or visually by colour. Phycocyanin was not observed to elute through the column during or after resin regeneration. Higher concentration solutions of phycocyanin were visually observed to be loaded onto the resin. After regeneration with 1.0M NaCl solution, the blue-pigment of phycocyanin remained loaded onto the resin and phycocyanin was not observed in the eluted solutions as measured by fluorescence spectrometry.

Figure 7.3 demonstrates the lack of phycocyanin being eluted from the column by extraction solution, washed from the column with Milli-Q water and high retention of phycocyanin on the

resin column. These low percentages were consistent and demonstrated the irreversible retention of phycocyanin on the silica resin column.

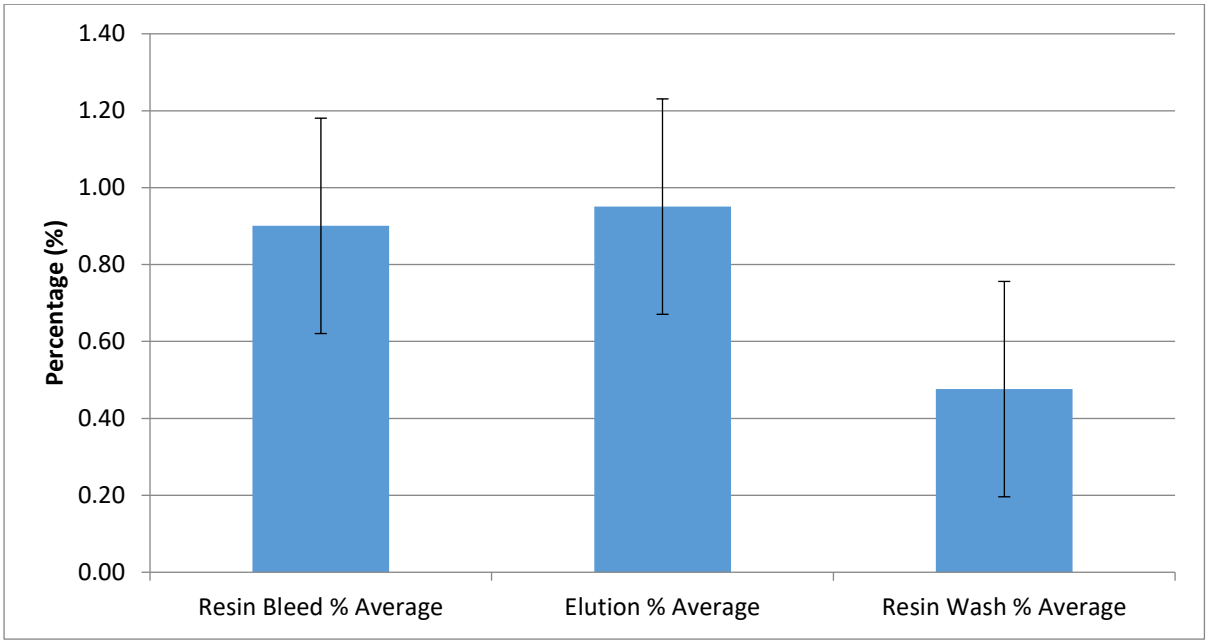


Figure 7.3: Percentage elution yield of phycocyanin from silica resin in a 10 mL syringe column using Milli-Q water as a eluent (n = 5).

The irreversible capture of phycocyanin on the silica resin led to a slight blue discolouration of silica resin at the top of the resin column, which demonstrated the irreversible loading onto the silica column after the attempted elution of phycocyanin with various increasing concentrations of NaCl solutions up to 4M. Elution of phycocyanin was not achieved after sample loading.

7.3 Anionic Exchange Resins

Investigations into anionic exchange resin interaction with pure compounds and extracted pigments were useful for the separation of negatively charged compounds from water systems and were supported by literature. The anion exchanged resins investigated in this chapter were; DEAE-Sephadex A25, QAE Sephadex A25, Amberlite IRA-416 and Amberlite IR-93. The selected resins include both strong and weak base interactions. Equilibrium between phycocyanin and the resins

was demonstrated by the quaternary resin, Q+, and the competing binding between the analyte or phycocyanin, A-, and regeneration ion, C-, which was chloride in this case.

Equation 7.1 $K = Q^+C^- + A^- \leftrightharpoons Q^+A^- + C^-$ (Jandera and Churáček, 1985)

The simple expression of an anion exchange model in Equation 7.1 shows that the sorption capacity, K, is the result of an equilibrium between binding of counter ions, C, and the competing ion, A for the quaternary ammonium ion.

7.3.1 DEAE-Sephadex A25

DEAE Sephadex A25 has a Dextran backbone, shown in Figure 7.4 (Jandera and Churáček, 1985) with the additional functional group of diethylaminoethyl for anionic interaction (see Figure 7.5). This functional group on the dextran/sephadex backbone ionically bonds to exchange the retained phycocyanin with low concentrations of sodium chloride solution, approximately 0.25 M as stated in previous literature (Chaiklahan et al., 2011, Liao et al., 2011, Patel et al., 2005, Patil et al., 2006, Silveira et al., 2008).

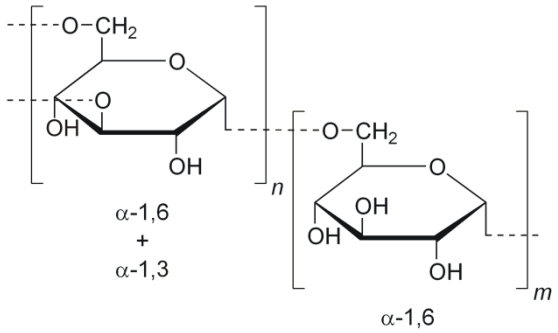


Figure 7.4: The reported Dextran or Sephadex backbone (Jandera and Churáček, 1985)

The nitrogen atom is contained in a positively charged, quaternary amine group of which there are two on each monomer along the dextran backbone. The resin is commonly used in chloride form,

with chloride ions ionically bound to the positive charge on the quaternary amines and participating in ion exchange. Cross linkages from the addition of water between the dextran backbone structures forms a gel. Figure 7.5 illustrates the reported chemical structure of DEAE-Sephadex. This allows for the elution of anion compounds using concentrated chloride ions, i.e. sodium chloride solutions.

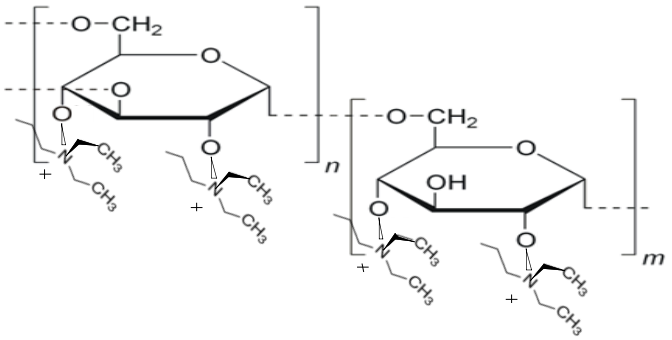


Figure 7.5: Reported chemical structure of DEAE-Sephadex A25 anionic resin (Liao et al., 2011, Tchernov et al., 1999)

DEAE-Sephadex A25 has been used for the purification of phycocyanin from cyanobacterial species, *Spirulina*, and for pharmacologically products (Jandera and Churáček, 1985, Liao et al., 2011, Moraes and Kalil, 2009, Patel et al., 2005, Patil et al., 2006, Sarada et al., 1999, Silveira et al., 2008). Diethylaminoethtyl (DEAE) Sephadex A25, an anionic resin, exchanges chloride ions for negatively charged ions which can be eluted off the gel resin with different concentrations of sodium chloride solutions. The charge density of the DEAE Sephadex A25 resin is 3.5 ± 0.5 meq/g and it has a resin size of 40-120 microns.

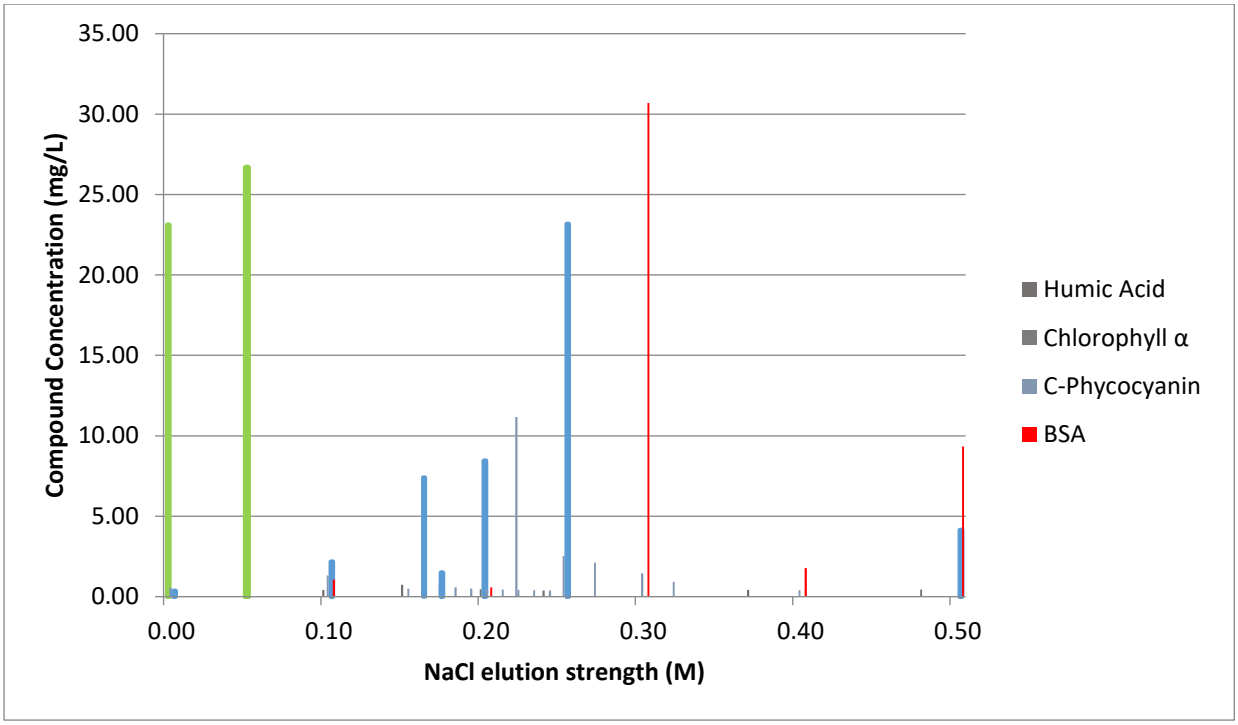


Figure 7.6: Compounds eluted using increasing NaCl strength concentrations in a DEAE-Sephadex A25 resin column in a water matrix at 1.0 mL/min.

Figure 7.6 shows the separation as various pure compounds, prepared in Milli Q, though DEAE-Sephadex column. During the experimentation, a 4 mL of sample was loaded onto the resin column and allowed to filter through. Then 4 mL aliquots of elution solutions (various concentrations of sodium chloride) wereused to elute the adsorbed compounds from the resin using sodium chloride solutions ranging from 0.0 M to 0.5 M with step increases of 0.05 M.

The elution of these pure compounds was measured by fluorescence spectrometry at specific optimal wavelengths for each compound as determined in Chapter 4; phycocyanin was excited at 609 nm with emission measured at 647 nm, chlorophyll-a was excited at 609 nm with emission measured at 680 nm, BSA was excited at 291 nm and measured at 337 nm, humic acid was excited at 475 nm with emission measured at 530 nm.

Data was collected and combined from individual pure standards and mixtures of compounds through pure resin columns. The green lines represent chlorophyll-a, blue lines represent phycocyanin and red lines represent bovine serum albumin. Humic acid is represented by brown lines but they are within baseline limits and hence are not clearly visible in Figure 7.6. This diagram shows the lack of humic acid elution from the resin. Humic acid was irreversibly retained on the resin column and permanently fouled the resin which is further discussed in section 7.4.1.2. The isolation of phycocyanin can be achieved by adsorption followed by elution with 0.16-0.27 M sodium chloride solution through the column. Using gradient elution, the optimum range for phycocyanin elution from DEAE-Sephadex A25 resin was determined to be 0.22 ± 0.03 M. Hence, the isolation and detection of phycocyanin is promising, however, the presence of natural organic matter in the form of humic acid and humic substances may still pose a challenge due to fouling of the resin.

7.3.1.1 Spike recovery investigations involving phycocyanin

Whilst past research has demonstrated phycocyanin purification on resin columns (Benedetti et al., 2006, Liao et al., 2011, Patel et al., 2005, Patil et al., 2006, Sarada et al., 1999, Silveira et al., 2008), the recovery of phycocyanin was investigated under various individual concentrations of sodium chloride elution. As investigated in this section, 0.16-0.27 M sodium chloride was able to effectively elute phycocyanin from the DEAE-Sephadex A25 resin employing gradient elution. There are no reports, however, in the literature demonstrating the yield of phycocyanin from the resins, or the result of a single elution with various concentrations of sodium chloride or the effect elution concentrations has on phycocyanin recovery.

7.3.1.1.1 Effect of single elution concentration

For elution using single concentrations, the recovery of eluted phycocyanin from DEAE-Sephadex A25 resin was investigated using sodium chloride concentrations of 0.2, 0.3, 0.5 and 1.0 M. These

solutions were based on the gradient elution results for phycocyanin. Solutions at 0.2 and 0.3 M yielded the highest recovery of phycocyanin whilst 0.5 and 1.0 M were higher concentration ranges in which phycocyanin should be eluted with less volume. With a stock solution concentration of 20.3 μg of phycocyanin/L, the bleed through percentage was 5.4 ± 3.8 % with a single filtration/load of phycocyanin. However, continuous loading of phycocyanin led to increased bleed through with each additional sample load. The second loading increased the bleed though to 7.1 % and the third loading increased bleed through to 11.1 %. This was a result of increased concentration of phycocyanin on the resin column with an increased concentration breaking though the resin column during the loading of phycocyanin. This was determined over four individual resin columns of approximately 0.7 ± 0.07 grams dry virgin resin, washed with Milli-Q water, phycocyanin loaded and regenerated between runs.

The elution of phycocyanin from the resin was investigated with 0.2 M sodium chloride. The elution with the first 4 mL presented with an average recovery of 22.6 ± 5.6 % whilst the following elution of 4 mL demonstrated an additional 20.1 ± 4.5 % removal of phycocyanin loaded onto the resin (n = 12).

In an attempt to increase yield recovery, the sodium chloride concentration was increased to 0.3 M. The elution with the first 4 mL yielded an average elution of 20.0 ± 11.1 % whilst the following elution of 4 mL demonstrated 17.0 ± 5.1 % removal of phycocyanin loaded onto the resin. The elution of phycocyanin from the resin was also investigated with 0.5 M sodium chloride. In this case elution with the first 4 mL gave an average elution of 24.8 ± 6.2 % whilst the following elution of 4 mL demonstrated 20.1 ± 3.7 % elution of phycocyanin.

The concentration of the elution solution was further increased, and two elution aliquots of 4 mL sodium chloride solutions of 0.5 M were investigated. Each aliquot achieved a 20-25 % total yield with the first elution and combined yield recovery of 40-45 % with the second elution. By investigating elution concentrations of sodium chloride solutions of 0.2, 0.3 and 0.5 M individually, it was determined the recoveries of phycocyanin eluted from the column were comparable and between 20.1 to 24.8 % with the first elution and 17.0 to 20.1 % with the second elution. The elution profile of phycocyanin was then investigated further with increased elution volumes in the following section.

The elution of phycocyanin with 1.0 M sodium chloride solutions demonstrated a different behaviour profile with a decrease in the recovery of phycocyanin. The recovery was 11.3 % for the first elution followed by 7.4 and 7.1 % phycocyanin recovery for subsequent elutions.

This was approximately half the recorded recovery for 0.2, 0.3 and 0.5 M sodium chloride solutions. The higher concentration of sodium chloride salt interfered with the emission characteristics of phycocyanin at 647 nm. Phycocyanin was eluted from the resin column and did not salt out. Higher ionic interaction with concentrated sodium chloride solutions did decrease phycocyanin emission intensity in the visible light region. However, phycocyanin was eluted from the column as determined from measurements in the protein region; measured by fluorescence with 291 nm excitation and 337 nm emission. Sodium chloride elution solutions at any concentration resulted in a slight wavelength shift from 647 to 637 nm presented in Figure 7.7.

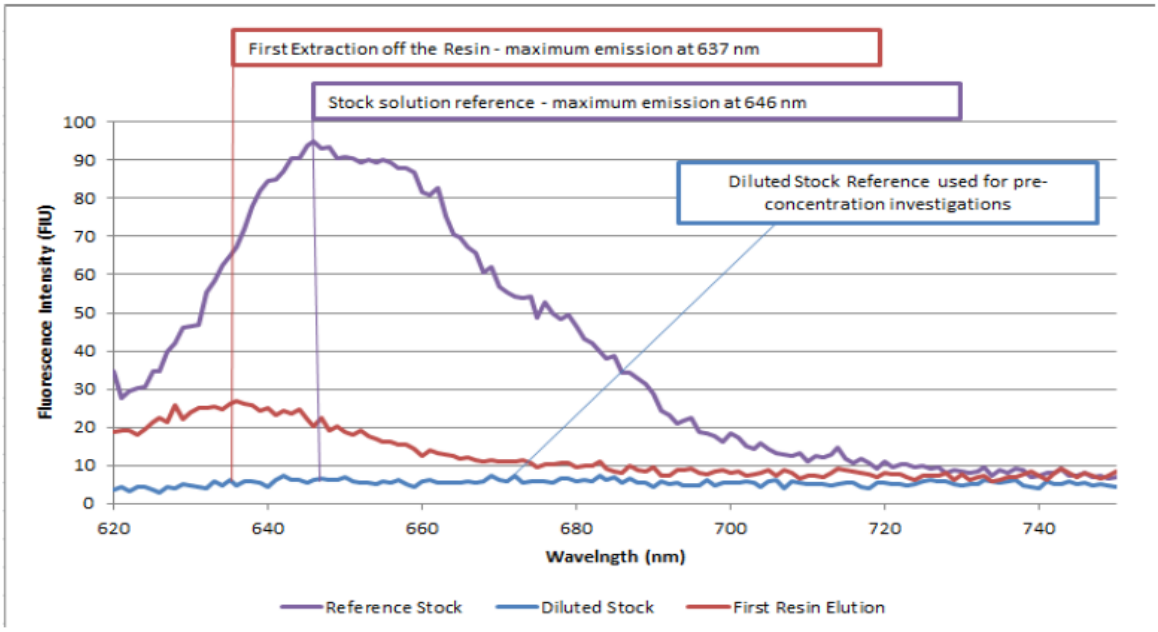


Figure 7.7: Wavelength shift from phycocyanin in Milli-Q (purple line and blue lines) and elution of phycocyanin in 0.3M sodium chloride solution.

In Figure 7.7, the purple line represents the phycocyanin stock solution used for pre-concentration investigations. From this stock solution, concentrations were diluted which did not present a clear fluorescence emission signal, represented by the blue line. The red line represents the elution of phycocyanin off DEAE-Sephadex A25 resin using 0.3 M as sodium chloride elution solutions. Between the stock solution and the elution with phycocyanin using 0.3 M sodium chloride, there was a wavelength shift in the emission response of phycocyanin.

7.3.1.1.2 Resin effect involving phycocyanin

The efficiency of phycocyanin elution from DEAE-Sephadex A25 resin is shown in Figure 7.8. The elution of phycocyanin was measured between every 4 mL of eluent using 0.3 M sodium chloride. The percentage recovery demonstrated that a curved expression could be fitted to the data with a R^2 value of 0.967 and that it seemed to plateau at 80 %, accumulated recovery yield.

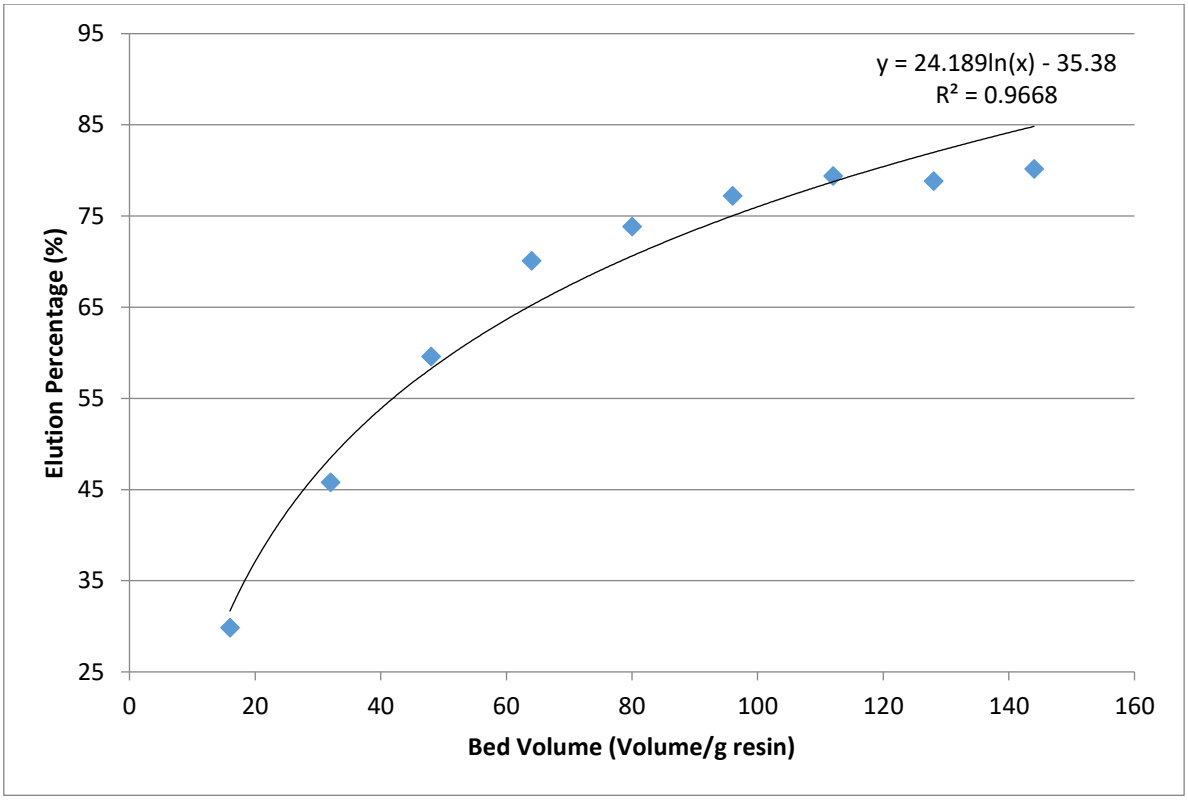


Figure 7.8: Elution profile of purified phycocyanin from DEAE-Sephadex resin with 0.3 M sodium chloride

The expression of the elution from DEAE-Sephadex could not allow for the full elution of phycocyanin. Even when the elution solution concentration was increased from 0.3 M to 0.5 M sodium chloride, the effect of percentage elution between each 4 mL demonstrated a similar elution percentage and a plateau at 80 % recovery. By increasing the concentration of sodium chloride in the elution solution, there was a decrease in the response signal for phycocyanin. This loss in fluorescence signal was believed to be due to interactions with the ionic strength of sodium chloride that led to structural changes in phycocyanin. By decreasing concentration of sodium chloride ions in solution, this was able to reverse some of the effects of phycocyanin signal suppression or any changes to signal emission. In using 0.3M sodium chloride solutions, phycocyanin recovery did not achieve more than 80% with the remaining 20% was unaccounted for or immeasurable due to the effect of increased sodium chloride strength demonstrated in Table 7.1.

Table 7.1: Phycocyanin elution percentage profile with standard derivation percentages using with 0.3M sodium chloride solution

Elution Volume (mL)	4	8	12	16	20	24	28	32	36
Phycocyanin eluted (%)	29.9	45.8	59.6	70.1	73.9	77.2	79.4	78.8	80.2
Standard Derivation of elution (%)	11.1	9.2	6.5	4.0	5.0	5.8	6.1	3.8	3.1

Table 7.1 together with the graphical representation of Figure 7.7 demonstrated the elution profile of phycocyanin through DEAE-Sephadex A25 resin. As shown graphically, the table also indicates a maximum phycocyanin recovery of approximately 80 %.

7.3.1.1.3 Effect of loading with various resin volumes

DEAE-Sephadex A25 resin volumes within the column were investigated to determine if the elution of phycocyanin was effected by the resin volume. It should be noted, smaller resin columns were utilised to maintain higher potential yield recoveries compared to larger resin columns that may have been able to filter greater volumes of solution at a given time. Small resin columns utilised 5 or 10 mL pipette tips compared to larger potential resin columns of approximately 50-100 mL resin. The design of the narrowing tip allowed glass wool to effectively stop loss of resin during loading or elution. Full resin columns utilised 5 mL of virgin hydrated DEAE-Sephadex A25 with glass wool at the tip of the small column. Half resin columns were half the resin used in the full columns.

Half resin and full resin columns demonstrated similar percentage of bleed through with similar variation of 6.0% ± 4.3% and 5.4% ± 3.8 %, respectively. In comparing half and full resin columns it was not only the phycocyanin bleed through that was consistent, but also the elution characteristics that were similar between both full and half resin columns. To ensure the elution behaviour was consistent, three full resin columns were investigated for their elution profiles up to

16 mL of eluent before approximately half the resin was removed from the columns. After the columns were regenerated and rinsed, as per resin pre-treatment procedures stated in section 3.4, elution profiles of the half resin columns were undertaken. The use of the same resin, only a different volume would limit any potential bias from variation in the resin.

Table 7.2: Phycocyanin elution percentage profile between full and half resin columns using with 0.3M sodium chloride solution

Elution Volume (mL)	4	8	12	16
Accumulated phycocyanin yields eluted from Full Resin Column (%)	29.9 ± 11.1	45.8 ± 9.2	59.6 ± 6.5	70.1 ± 4.0
Accumulated phycocyanin yields eluted from Half Resin Column (%)	35.1 ± 12.9	48.0 ± 11.3	52.0 ± 11.6	68.6 ± 1.3

Table 7.2 demonstrates the elution characteristics of phycocyanin for accumulated recovery yields up to 16 mL using 0.3 M sodium chloride eluent.

7.3.1.2 Chlorophyll-a isolation

Due to the non-polar properties and small molar size of chlorophyll-a, the pigment was eluted through the anionic gel resin column with water washing. This was graphically demonstrated in Figure 7.6, which presents the elution of various compounds using an increasing sodium chloride solution concentration. This is both of interest in the isolation and analysis of chlorophyll-a from other compounds as it was successfully separated from phycocyanin. With the use of this resin, it was possible to measure isolated chlorophyll-a concentrations.

7.3.1.3 Natural Organic Matter

The introduction of pure compounds, albumin bovine serum and humic acid sodium salt, was as follows. Albumin bovine serum as a substitute for additional proteins in laboratory mixed bench samples demonstrated elution with 0.30-0.50M sodium chloride. Whilst this is a potential interference from other proteins, the spectral interference from albumin bovine serum occurs within

the range of 280-350 nm for protein excitation and emission. There was no measured interference when phycocyanin was eluted from DEAE-Sephadex resin by 0.25M and measured by fluorescence. Despite the co-elution of albumin bovine serum, the emission of phycocyanin occurs at 647 nm which has no spectral interferences with albumin bovine serum or other proteins.

Dissolved humic acid was loaded onto a DEAE-Sephadex A25 resin column with 4 mL of 0.3 mg/L aliquots until the resin was saturated with humic acid. There was no measured bleed of dissolved humic acid during the loading of humic acid onto the column. The resin demonstrates that up to 5.8 ± 0.2 mg of humic acid was retained on the 5mL resin column. From this point of saturation, the resin column was regenerated using 1 M to 4 M NaCl. In addition to 4 M NaCl, a 4 M NaCl solution with 1 M NaOH was used in an attempt to regenerate the resin without any promising result.

Whilst DEAE Sephadex A25 was able to release phycocyanin by controlled elution from the column (Liao et al., 2011, Niu et al., 2007, Patel et al., 2005, Patil et al., 2006, Silveira et al., 2008), the introduction of humic substances from environmental samples introduces irreversible resin fouling. Due to the highly negatively charged complexes of humic substances as well as their hydrophobic sections, the humic acids were unable to be released by NaCl exchange and irreversibly fouled the resin.

7.3.2 QAE Sephadex

Anionic exchange resin QAE Sephadex (Quaternary Anionic Exchange Sephadex resin) was slightly less anionic that DEAE-Sephadex resin with a charge capacity of 3.0 ± 0.5 meq/g compared to the 3.5 ± 0.5 meq/g for DEAE-Sephadex. The anion exchanger had the same particle size of 40-120 µm, as DEAE-Sepahdex A25. Due to the weak anionic bonding associated phycocyanin ion exchange, the slight difference in resin was investigated for any potential benefit or improvement.

Resin column preparation consisted of 2.9901 grams of QAE Sephadex resin into 25mL Milli-Q with 0.5mL MilliQ wash through during settlement.

The elution of phycocyanin from a QAE Sephadex resin column demonstrated an unexpected result. The elution of phycocyanin from a weak quaternary anion exchange resin demonstrated lower elution percentages through series elution. The loading of phycocyanin on the resin demonstrated a relativity low bleed through of 5.01 ± 0.64 % which was comparable and relative to the loading of phycocyanin on the DEAE-Sephadex A25 resin column.

When phycocyanin was loaded onto the QAE Sephadex resin column, there was 5.28 ± 0.68 % bleed through of the concentration of phycocyanin loaded onto the resin column. The first elution aliquot, 4 mL of 0.3 M of sodium chloride, yielded 12.96 ± 1.89 %. The second 4 mL elution aliquot yielded a further 8.77 ± 2.97 %.

When the strength of sodium chloride was increased from 0.3 M to 1.0 M, the yield of recovery decreased to 9.29 ± 0.59 % elution with the first 4 mL aliquot. The following 4 mL of elution decreased to a yield percentage of 7.22 ± 1.49 %.

The interaction with the quaternary amine functional group of the resin decreased the recovery of phycocyanin when eluted from the resin, as phycocyanin was unable to be released from the resin. In comparison, DEAE-Sephadex A25 resin from section 7.4.1 contained diethylaminoethyl functional groups that provided improved desorption of the phycocyanin from the resin.

7.3.3 Amberlite IRA 93

An alternative to eliminate the effect of irreversible fouling by humic acid on a resin when phycocyanin was able to be eluted effectively, was to adopt a resin capable of capturing and eluting

humic acid. Amberlite IRA 93 is a weak base ionic exchange bead resin used for water processes (Bolto et al., 2002).

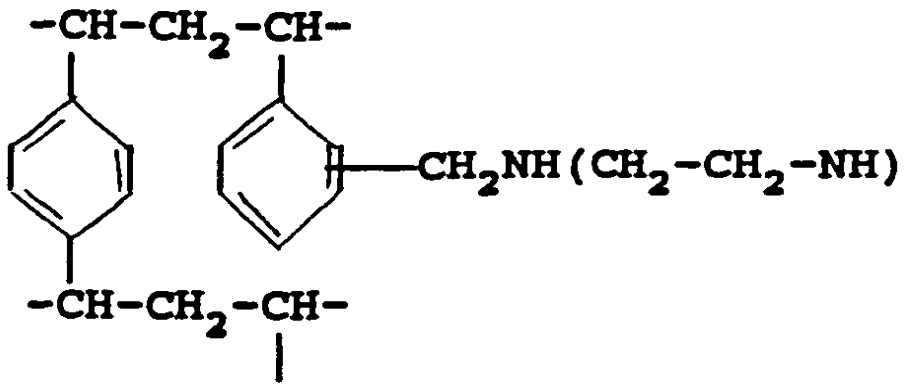


Figure 7.9: Amberlite IRA 93 resin structure

Amberlite IRA 93 is a macro-porous resin utilising a styrene structure as presented in Figure 7.9. The charge capacity of the resin is 0.9 meq/mL with a 58% water content at pH 7 and 3 % active sites charged at pH 7 (Bolto et al., 2002a). The use of a weak base, anionic exchange resin failed to capture chlorophyll-a as predicted. The lack of adsorption of chlorophyll-a onto Amberlite IRA 93 resin demonstrated the same behaviour and the same benefit for separation of phycocyanin from chlorophyll-a as did QAE Sephadex resin.

Table 7.3: Data Summary of resin performance related to pigment on Amberlite IRA93 resin

Pigment	**Phycocyanin**	**Chlorophyll-a**
Concentration of NaCl to elude from resin column (M)	ND	0 M – Eluted with Milli-Q
Bleed through percentage (%)	ND	98 ± 1 %

ND = Not determined.

Whilst there was separation between chlorophyll-a and phycocyanin upon loading onto the resin column, phycocyanin was not observed to elude through any bleed through percentage or elusion with any range between 0-4 M sodium chloride solutions. No elution of phycocyanin was recorded in the visible or region regions.

7.3.3.1 Natural Organic Matter

The stronger anionic capacity of IRA93 was more effective in the resin's ability to be used and regenerated with 4 M NaCl. Concentrations of 30.92 mg/L of humic acid were loaded onto a 5 mL IRA93 anionic exchange column. After each loading, the resin was regenerated with 4 M NaCl solutions.

During loading of humic acid onto the column, there was a bleed of humic acid through the column of approximately 4.62 mg/L ± 0.60, 95% STDEV with the exclusion of two outliers. The higher bleed through the column for these two runs could have contributed to the higher loading on run 2, which demonstrated a greater removal of humic acid. The percentage of bleed of humic acid being loaded onto the column was approximately 17.25%. Hence, it was calculated that approximately 82.75% of the humic acid was collected on the resin column during each loading of humic acid. However, the total calculated amount of humic acid loaded onto the column was 184.09 mg, with a total calculated bleed through of humic acid of 32.34 mg. When the column was regenerated, the sum of the total mass of humic acid in the eluant was 152.34 mg, the equivalent of 82.75% of humic acid loaded onto the column. Further, there was 0.59 mg/L addition from the calculations which could be explained by the additional matter coming off from the column during regeneration or spectral variation from reading the emission. The yield of humic acid from successive regenerations of the resin demonstrated a steady decrease during the seven loads of humic acid and regenerations. This is shown in Figure 7.10.

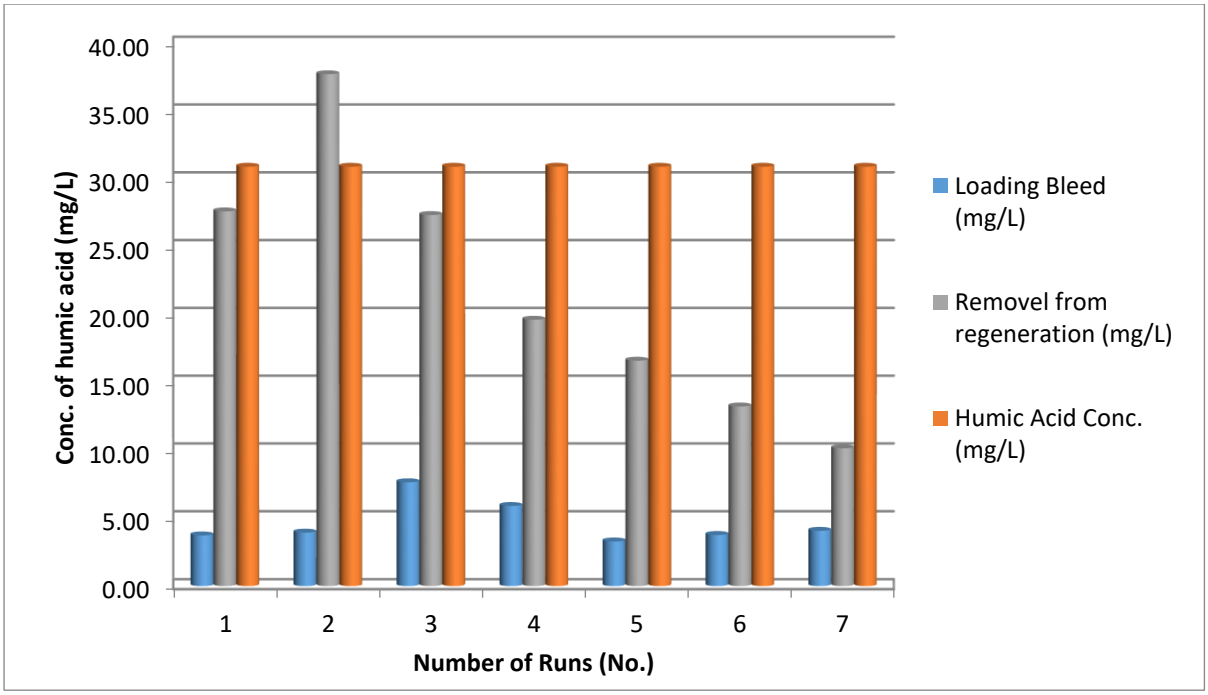


Figure 7.10: Investigations of humic acid with IRA93 when total concentration in solution is loaded onto a column (red), the bleed through the column (blue) and regeneration of the column to remove humic acid (green) is observed

Despite the decrease in humic acid being eluted from the column during resin regeneration, there was no observation of the resin failing to retain humic acid. The calculations demonstrate that all of the humic acid can be accounted for. The use of IRA93 anionic exchange resin is useful to limit the presence of humic acid in systems, however, it does not totally remove the issue of humic acid. Ideally, all of the humic acid present on the resin should be removed to not interfere with resin productivity. Further, the breakthrough of humic acid through the column was approximately 17.25 % which has the potential to interfere with phycocyanin detection.

7.3.3.2 Capture of phycocyanin

Phycocyanin was observed to be loaded onto an IRA93 anionic exchange resin column. The total amount of phycocyanin loaded onto the column was calculated to be 26.48 mg. The bleeding from phycocyanin through the column was 10.05%, which was approximately related to 89.95% of

phycocyanin that was loaded onto the resin column. However, the problem does not come from the bleeding of phycocyanin through the column nor the bleed of humic acid through the column, but rather from the regeneration of the resin and the removal of phycocyanin from the column.

In attempts to elute phycocyanin from the Amberlite IRA93 resin column, different NaCl molar concentrations between 0.1 M to 4.0 M were investigated. Despite experiments to utilise 0.3 M sodium chloride concentrations, which were optimal for eluting phycocyanin from DEAE-Sephadex A25, there was no observed elution of phycocyanin from the resin.

As phycocyanin has been observed to decrease or completely lose all fluorescence signal emissions with increasing sodium chloride elution concentrations, it may have been possible phycocyanin was eluted after its fluorescence capacity was completely diminished. However, if this was the case, there was no observed elution of non-fluorescent phycocyanin.

7.3.4 Amberlite IRA 416

The effect of humic acid on Amberlite IRA 416 anionic exchange resin (weak base resin) showed promising results for the capture and treatment of high humic acid waters. Amberlite IRA 416 demonstrated a potential to retain 76.35 % of humic acid on a 5mL resin bead volume. This allows for a bleed percentage of 23.65 % through the resin column. The results were similar to the Amberlite IR93 only with a higher potential for humic acid bleed through. The interaction with ethanolamine functional groups of the IRA 416 resin interfered with the elution of phycocyanin and it could not be eluted from the column making the resin not suitable for use.

Functional group of IRA 416 resin -[$CH_2N(CH_3)_2CH_2CH_2OH)^+OCH_3^-$]

7.4 Environmental Samples

The focus of environmental samples investigation was the capture of phycocyanin from a continuous flow process. Environmental samples were collected from Lake Fyans near Horsham, Victoria in March 2014. There was no presence of cyanobacteria, diatom or algal cultures or blooms. Waters were filtered through 0.45 μm and stored at 4 °C for 1 months or -18°C for 8 months. Turbidity was measured by a turbidity tube to be 4 ± 1 NTU, and CDOC was analysed by TOC analyser with measurements recorded between 6.6 to 7.8 mg/L of dissolved TOC.

For resin investigations on a bench scale analysis, sample stock solutions were 100 or 200 mL. For testing, 4 mL aliquots of these stock solutions were loaded on the resin columns for use. Due to the lack of cyanobacteria and algal cultures, natural waters were spiked with *Chlorella vulgaris* and *Chlamydomonas raciborskii* green algae cultures equivalent to 100,000 cells/L for each culture.

Cyanobacteria cultures were the variant of these investigations with waters spiked with a concentration rage between 0 to 150,000 cells/mL. To add to these environmental water sample investigations, the sample stock waters were spiked with pure phycocyanin, without cyanobacteria cultures, from 0 to 30 μg/L.

Cultures were only added to natural samples waters and were analysed within 10 hours to minimise cell concentration variation through cell death or growth. Stock sample waters spiked with phycocyanin were investigated and analysed within 4 hours. Size exclusion resins were useful in longer resin columns for increased practical separation but were not implemented as the resins were unable to be used in a continuous flow process. Anionic exchange resins were considered for these experiments, as they could be integrated into a continuous process and still achieve separation and potentially had the ability to concentration phycocyanin.

Of the resins investigated, DEAE-Sephadex A25 strong base, anionic exchange resin was the best resin to treat environmental and synthetic samples containing live cell cultures. This resin had the capacity to pre-concentrate, to isolate phycocyanin from chlorophyll-a, and had the highest phycocyanin recovery of approximately 80%. Environmental and synthetic samples containing cyanobacteria demonstrated similar elution profiles as demonstrated by purified compounds; namely phycocyanin, chlorophyll-a and humic acid.

Phycocyanin and chlorophyll-a extracted from cyanobacteria cells behaved the same way as purchased purified compounds. This was demonstrated through EEM fluorescence profile and elution behaviour through resin columns. Humic acid sodium salt demonstrated similar behaviour to humic substances when passed through DEAE-Sephadex A25 resin columns. Chlorophyll-a demonstrated no capture on the resin, humic acid substances from natural sources continuously fouled DEAE Sephadex A25 resin columns to which it was exposed and phycocyanin continued to demonstrate a logarithmic elution curve. This curve was same approximate to the observations of Table 7.1.

Live cultures of cyanobacteria exposed to cell disruption via sonication probe were then filtered through the resin column. However, this presented a new problem. When cyanobacteria cell were disrupted by the probe in order to liberate phycocyanin, the intercellular matter of the cells did lead to discolouration and membrane fouling when loaded on the DEAE-Sephadex A25 resin column. The effect of intercellular matter on DEAE Sephadex A25 resin was similar to the irreversible fouling effect of humic acid on the resin.

Even with pre-filtration to capture particulate matter above 0.45 µm, the dissolved organic matter of cells continued to foul the resin. By definition, dissolved organic carbon is classified as organic matter which can pass through 0.45µm filtration (Mergen et al., 2008, Sentana et al., 2009). The

combination of live cell cultures with humic acid substances did not affect the capture or elution of phycocyanin but simply fouled the active sites until the resin was completely and irreversibly saturated.

Chlorophyll-a passed through the column without adsorption. Phycocyanin was eluted from the column over a series of aliquot elutions up to a maximum yield of 80.1 % over 32 mL of regenerate solution. There was no variation of phycocyanin yield recovery from DEAE-Sephadex A25 with the presence or absence of humic acid or natural organic matter. The only effect humic acid or humic substances/natural organic matter had on phycocyanin yield and adsorption on DEAE-Sephadex A25 resin was when the resin was irreversible saturated and unable to retain any phycocyanin presented in Figure 7.11.

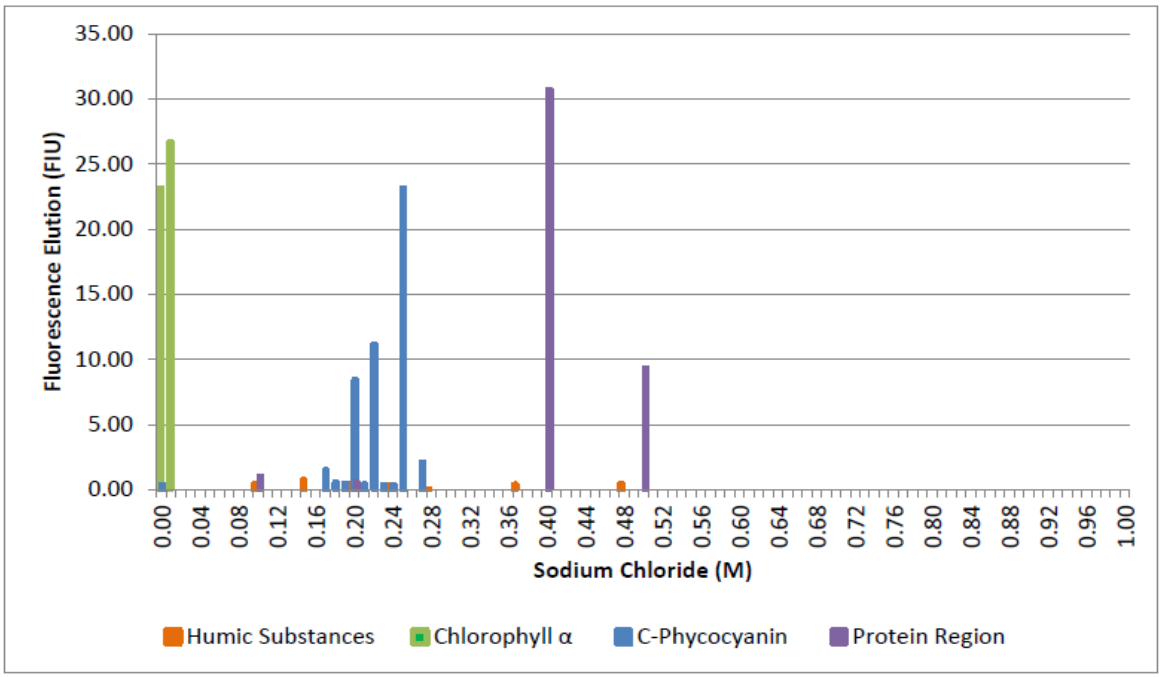


Figure 7.11: Elution order of pigments in environmental waters

Figure 7.12 demonstrates the insignificant effect of humic acid and intercellular organic matter on phycocyanin recovery during series elution.

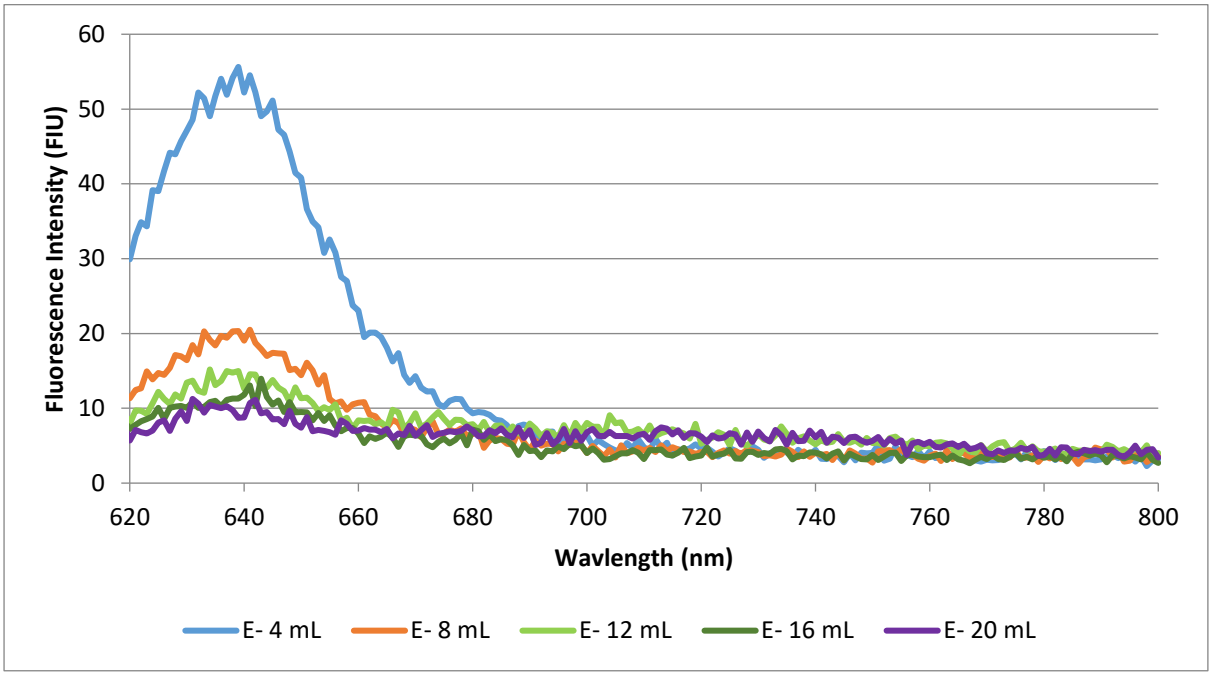


Figure 7.12: Series elution of loaded phycocyanin, previously liberated from cyanobacteria cell cultures, through a DEAE-Sephadex A25 resin column

As observed in Figure 7.12, there was a lack of humic acid emission between 500-650 nm as expected due to the irreversible loading of humic acid onto the resin; similar observations were made in section 7.4.1.3. Excitation was 609 nm with emission measured between 620-800 nm. It is also important to note, that chlorophyll-a was also not present in these serial elution due to the lack of retention on the DEAE-Sephadex A25 resin.

Whatever the solution, the performance of DEAE-Sephadex A25 resin demonstrated the same performance with environmental waters as with purified waters spiked with individual components.

7.5 Conclusions

The use of size exclusion resin demonstrated some benefit in the separation of phycocyanin and chlorophyll-a from humic acid substances and other coloured materials for fluorescence or

absorbance detection in the visible light region. The presence of proteins and polysaccharides do not pose any significant problem due to their lack of ability to fluoresce or absorb light within the measured regions for phycocyanin and chlorophyll-a. The downside of using size exclusion resins, however, is the lack of separation between chlorophyll-a and phycocyanin which are of similar approximate size; <900 Daltons. As discussed, decreasing particle size does increase the separation between the pigments and humic acid substances but does increase elution times, limits the recovery of the pigment concentrations if loaded in high concentration and eluted off with deionised water, and has greater potential to foul the resin over time. The use of larger particle size resins for size exclusion are able to combat the potential fouling property from humic acid substances but decreases the elution time of phycocyanin and chlorophyll-a within the interference range of humic acid substances.

Anionic exchange resins demonstrated the most promise with Sephadex A25 anionic exchange resin able to capture and elute off phycocyanin in small concentrations of sodium chloride; 0.2-0.5 ± 0.03 M. Yield investigations involving 0.2, 0.3 and 0.5 M sodium chloride solutions demonstrated similar percentage recovery of approximately 20-25% with the first 4 mL elution and 16-20% for the second 4 mL elution. Elution concentrations above 0.5 M sodium chloride interfered with the florescence emission of phycocyanin and the ability of phycocyanin to be measured within the visible light region. The introduction of humic acid into the solution fouls the Sephadex A25 resin by saturating the active sites. The saturation of humic acid on the resin could not be removed with the use of sodium chloride at high concentrations of 4 M or with alkali 4 M sodium chloride. QAE Sephadex anionic resin was not as effective as DEAE-Sephadex A25 for adsorption of phycocyanin with minimum breakthrough and efficiency of yield recovery.

Chapter Eight

Principles and model of the Isolated Pigment Analyser (IPA)

The Isolated Pigment Analyser (IPA) was designed to separate cyanobacteria cells and other algal species from natural organic matter, extract specific pigments related to cyanobacteria species for quantitative analysis and estimation of cyanobacteria cell concentrations. More specifically, the IPA was designed to eliminate the spectral interferences for phycocyanin analysis from chlorophyll-a and humic substances when employing fluorescence spectrometry as observed in Chapter 2.

During operation, the IPA prototype pre-concentrated cyanobacteria by filtration and semi-purified the sample by flushing with water to remove NOM, sonicated the captured cultures to liberate phycocyanin from the cell followed by concentration by adsorption and elution from ion exchange resin for detection by fluorescence spectrometry. The combination of individual components throughout this thesis led to the development of the Isolated Pigment Analyser. This chapter investigates the operational performance of a prototype as a whole and evaluates its performance. Due to the common nature of chlorophyll-a existing in other algal cultures and plant material, any measurement of chlorophyll-a could be incorrectly interpreted. Further, this chapter will discuss the concepts that led to the design of the isolated pigment analyser, the method of operation, and operational outcomes in laboratory for environmental waters.

8.1 Concepts and design of the Isolated Pigment Analyser (IPA)

The first concept associated with the Isolated Pigment Analyser (IPA) was to keep the prototype significantly smaller than the total volume processed. By filtering a large source of water into a smaller filtration point and then further capture of phycocyanin pigment onto an ionic exchange resin allowed for pre-concentration of the phycocyanin prior to fluorescence detection.

The optimum conditions of analysis for phycocyanin detection discussed in Chapter 6 were employed as an extraction medium. Due to the detectability of cyanobacteria monocultures above the DSE (2008) and NHMRC (2011) recommended safety guidelines determined in Chapter 4, it was necessary to pre-concentrate any potential phytoplankton cells from a large volume of water to smaller volume of water, .i.e. phycocyanin from 1L of sample of source water from a lake or reservoir to a10 mL of Milli-Q water. This would allow for a 100 fold concentration of phycocyanin by pre-concentration into purified water, rather than attempting to detect phycocyanin in the more dilute source water that contained interfering substances.

8.1.1 Initial design concept

Initial design concepts explored the use of flat disc membranes for the filtration of cyanobacteria and algae cultures. Whilst the filtration was successful using membranes with 0.45 um pore sizes such as the nylon, ceramic and even the glass fibre with 0.7 µm pore sizes, there was an unforeseen design issue that was not apparent until a prototype bench model was constructed.

Even though the use of flat disc membranes for filtration was reasonable, the combination of flat disc membrane filtration became impractical when implemented into the prototype.

Figure 8.1 demonstrates the design lay out of the prototype utilising flat disc membranes.

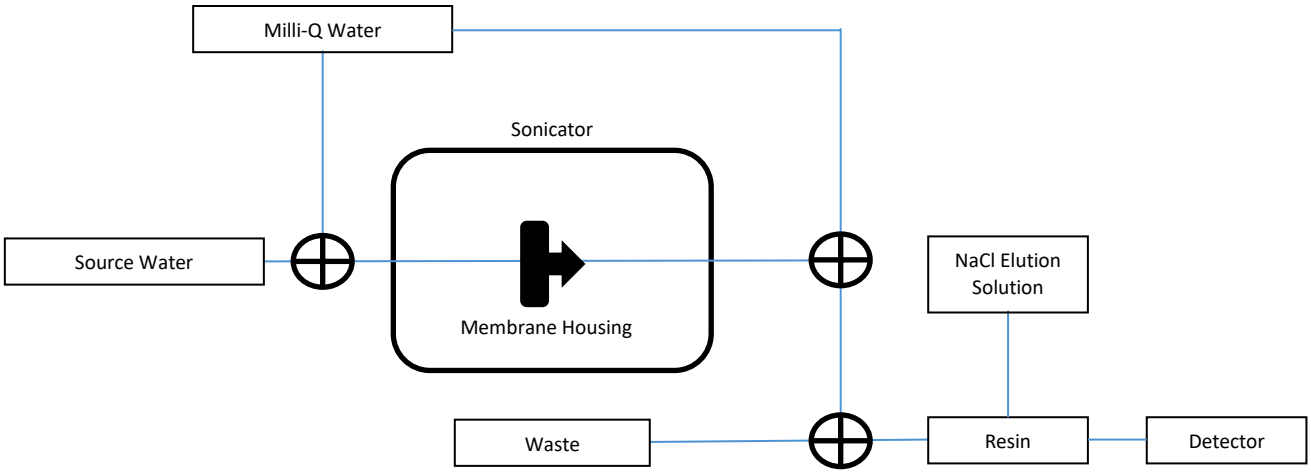


Figure 8.1: Prototype bench model of IPA using disc membrane and membrane housing in a sonication bath

The problems that this initial design faced were: the membrane housing could only support flat glass fibre, nylon or thin metal membranes and not the thicker ceramic membranes as discussed in Chapter 5, and the membrane housing did not allow for effective backwashing or effective sonication of the membrane. Alternatively, the membrane housing could be replaced by another membrane housing that may support a thicker ceramic membrane or increase the effectiveness of backwashing the membrane or sonication unit/system. However, this would be a custom part and would increase production cost of the prototype.

Sonication to disrupt cyanobacterial cells has been recorded in the literature by academics and industry (Furuki et al., 2003, Gupta and Sainis, 2010, Rajasekhar et al., 2012a, Rajasekhar et al., 2012b, Viskari and Colyer, 2003, Wu et al., 2012a, Zhang et al., 2006a). Reported in literature, sonication could be adapted into a field design rather than other low maintenance techniques for cell disruption and would be faster than some techniques (Lawrenz et al., 2011, Simis et al., 2007, Gupta and Sainis, 2010, Sarada et al., 1999).

Freezing and thawing techniques require cultures or samples to be frozen and thawed repeatedly thus inducing long cycles, before any effect of cell disruption becomes apparent. Cell disruption by the freeze/thaw and sonication techniques were discussed in Chapter 6, the time difference between effectiveness of cell disruption vs. length of cycle was significant. The freeze/thaw technique involved 3 complete cycles of 1 hour freezing and 1 hour to thawing at room temperature in amber centrifuge tubes, however, it took 6 hours in order to achieve 37.3% for *Microcystis aeruginosa*, 33.6% for *Anabaena circinalis* and 73.3% for *Cylindrospermopsis raciborskii* cultures to be disrupted. Alternatively, sonication via a sonication bath of identical concentration and cultures produced effective cell disruption of 18.0% for *Microcystis aeruginosa* and *Cylindrospermopsis raciborskii* after 40 minutes sonication, but after 60 minutes of sonication 30% of cells from *Anabaena circinalis* cultures were disrupted. This was for sonication of cultures that required the sound waves to propagate through a plastic container before disrupting the cells. Further, freeze/thaw cycles of a flat disc membrane and housing discussed here would be harder to control in a field experiment and lengthy compared to sonication.

A sonication bath was not ideal for use as the ultrasonic frequencies were heavily defused passing through the flat membrane housing. The use of tubular membranes demonstrated a potential benefit to combat this issue of ineffective sonication. The reformed design of the isolated pigment analyser was modified with the idea of allowing sonication directly on cell cultures by close proximity to the cultures. At the same time, this design would also eliminate any potential interference of the sonication by the housing or container. The main concern was the sonication of the filter and filter housing, which was required to be immobilised within the sonication bath. In this case, the ultrasonic waves did not penetrate effectively into the filter housing compared to the investigations on the different types of filters that did not use the filter housing for the instrument. This was theoretically supported by the backwash of the filter after sonication and elution of phycocyanin, and sonication of the filter without the filter housing. During the sonication phase for pigment

extraction, there was no additional removal of humic acid despite the build-up on the membranes. Even with the presence of undissolved humic acid on the membranes, the extraction of phycocyanin demonstrated no difference for the extraction of phycocyanin.

Sonication probes were initially overlooked as not being able to be implemented into a working design despite being highly effective throughout literature. As an alternative design, the second modification of the isolated pigment analyser was built around the sonication probe.

For operational efficiency, cultures were exposure to ultra-sonication at to 250 Watts for 1.5 minutes which was suitable to disrupt cell membranes. It was determined experimentally that close proximity of captured cell cultures to the sonication probe would determine the highest cell disruption given the setting for the sonication probe was optimum for effective cell disruption. Whilst backwashing a membrane, mainly flat disc membrane, to remove cell cultures could be achieved, there was a loss of cell cultures from cells retained on the membrane or dilution of the cell cultures in the backwash solution. The design of the prototype eliminated the need for an extra step of backwashing by using a membrane durable enough to withstand repeated sonication and not decrease filtration efficiency. Tubular membranes were in parallel which would allow for the dual function of filtration and easy sonication with the sonication probe inserted vertically through the centre of membrane housing and circled by the tubular membranes.

Figure 8.2 shows a schematic diagram of the IPA design layout.

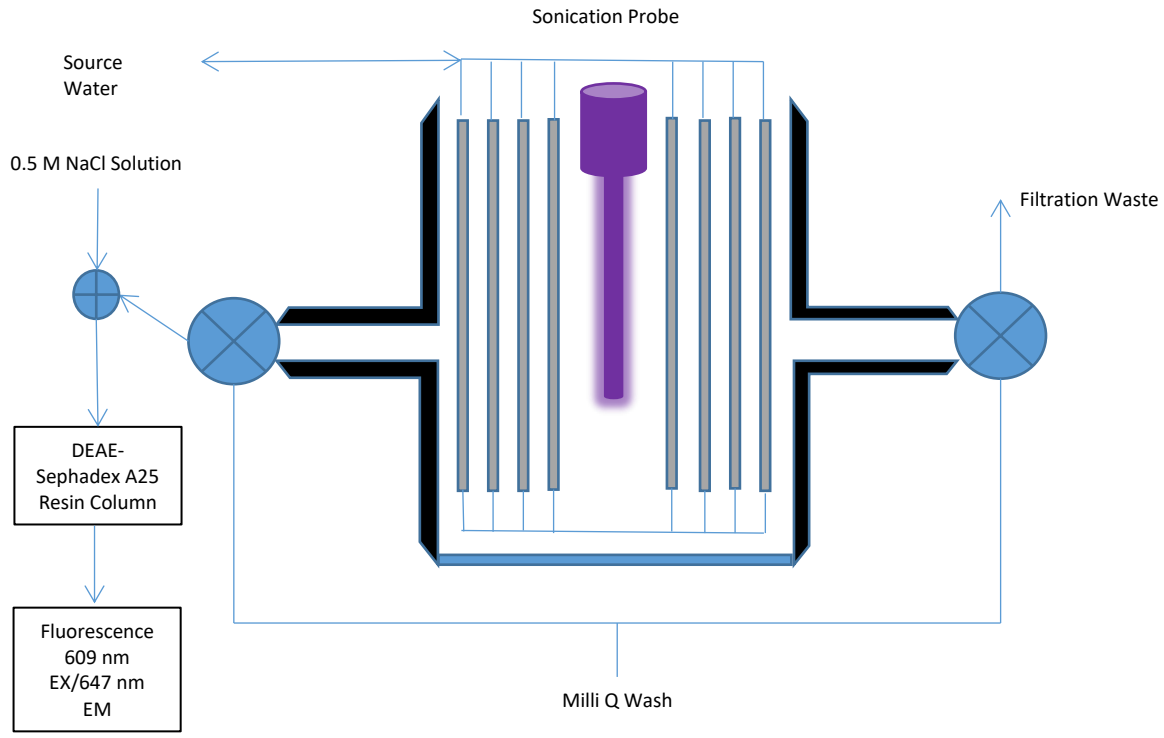


Figure 8.2: Isolated Pigment Analyser design flowchart

The design flow of IPA in Figure 8.2 demonstrates the practicality and simplified design concept compared to the design flowchart utilising flat disc membranes in Figure 8.1. The following subsections expand on the individual components that made up the operational prototype.

8.1.2 Filtration Component

The Isolated Pigment Analyser (IPA) was designed with the concept of isolating the pigments from any spectral interference by capturing intact cyanobacteria cells by filtration. The concept behind this stage was investigated throughout Chapter 5 in the determination of a suitable filtration membrane for easy use and re-use, with minimal potential fouling or any observed degradation of the membrane. Capturing intact cells by filtration provides two functions. First, by filtering a large volume of source water of approximately 5-10 L, the cyanobacteria cultures present were concentrated in 250 mL volumes in order to increase the concentration of cells/mL and hence the concentration of phycocyanin for increased detectability. Second, capturing intact cells by filtration

allows for natural organic matter and any potential spectral interference from dissolved species in the water to be separated from the cyanobacteria cultures.

The disadvantage of this approach was an expected gradual increase in membrane fouling with an increase in filtration times. In all cases of membrane fouling, the water chemistry was most important. With eight tubular membranes, the bench scale IPA could process 2 L of water containing cells concentrations <200,000 cells/mL in 20 mg/L of humic acid without any increase in filtration time or any other signs of membrane fouling. These concentrations are still well above detectable limit by fluorescence spectrometry. Naturally increased cell concentration in the starting solution before filtration led to more rapid membrane fouling.

On the other hand, solutions containing 50 mg/L of humic substances were filtered through the eight membranes with 43.2 ± 4.7 % of the dissolved humic substances filtered through the membranes. The remaining percentage of humic substances was eluted from the membrane through backwashing the membrane. The membranes were reversibly fouled and were able to be backwashed with 1Lof water to remove the NOM substances build up on the membranes pores. This fouling material could possibly be a NOM Filter cake or mixture of humic, protein and polysaccharide matter. This reversible fouling from this NOM Filter cake was due to natural organic matter and is a heterogeneous mixture containing various fractions of organic matters as demonstrated in research carried out by Fan et al (2008), shown in Figure 8.3.

Fan et al. (2008) and Lee et al. (2006) found that natural organic matter has fractions of approximately 50 % hydrophobic (HPO), approximately 25 % hydrophilic (HPI) and, approximately 25 % transphilic (TPI – containing both hydrophobic and hydrophilic properties). Whilst this figure was dependent on individual water chemistry from a specific source in a specific time, the fractions do exist and are common throughout global water sources. Thus, the results

indicate that natural organic matter contains components comprising both hydrophilic and hydrophobic properties. Further work conducted by Sentana et al. (2009) and, Costa and Pinho (2005) suggested that humic-like substances, much like humic and fulvic acids, account for approximately 50 % of dissolved organic carbon and are major constituents of natural organic matter in water sources.

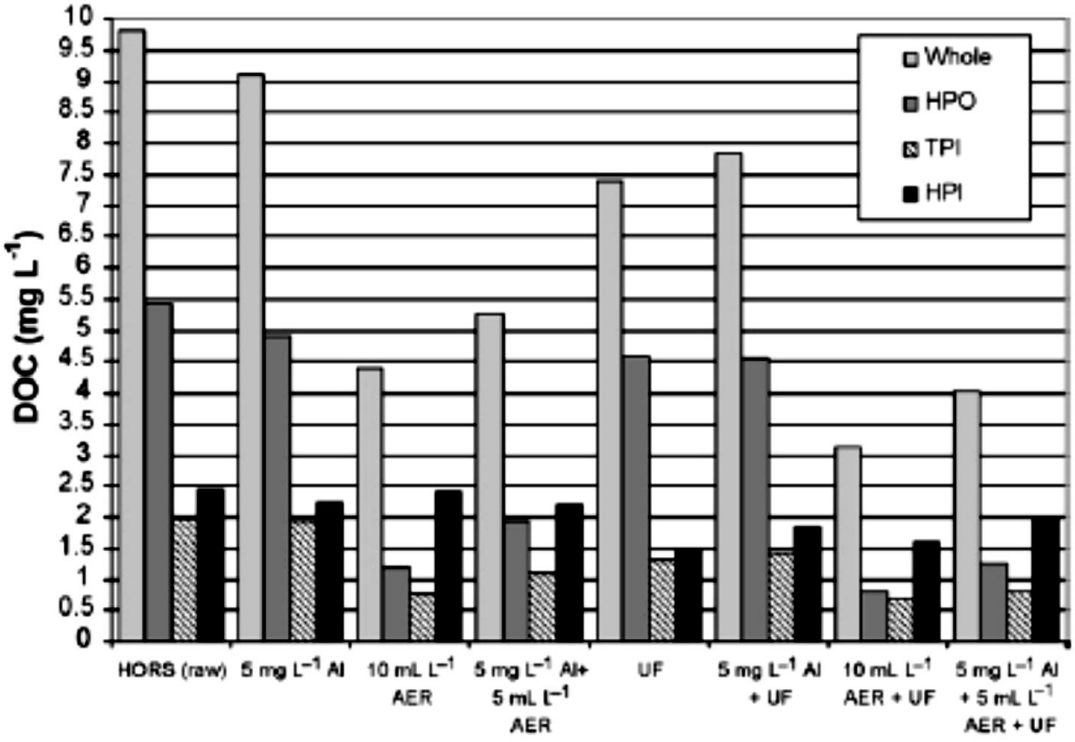


Figure 8.3: Dissolved organic fractions with various treatment applications on the same water source (Fan et al., 2008)

Figure 8.3 represents the results as DOC (mg/L) removal on the y-axis versus the experimental variables on the x-axis. Dissolved organic carbon on the y-axis is expressed as DOC, comprising hydrophobic (HPO), hydrophilic (HPI), and transphilic (TPI) fractions. It also demonstrated the use of single treatments as well as anion exchange resin (AER), ultra-filtration (UF) and coagulation using 5 mg/L Al^{+3} in combinations. Waste water used in Fan et al (2008) study was collected from a large municipal wastewater treatment plant in Melbourne, Australia. These

treatment waters did not contain any algae or cyanobacteria. Lee et al (2006) categorised the heterogeneous mixture on the basis of molecular weights, morphologies, and functional groups.

From this figure, the application of ultra-filtration (UF) and anion exchange resin (AER) demonstrated the highest removal of dissolved organic carbon.

Natural organic matter is derived from autochthonous and allochthonous matters. Work carried out by Stork et al. (2009), Lee et al. (2006), and Her et al. (2004) defined allochthonous matter as the natural inputs into water source by surface runoff. The latter is produced from the degradation of vegetables and animal matter. Autochthonous matter, in contrast, is derived from within the water body, such as algae, and is largely aliphatic with high concentrations of carboxylic acid functional groups.

Work by Her et al. (2004) demonstrated that algae species and algal organic matter are effectively removed by ultra-filtration membranes, however membrane fouling was significant. In addition to this finding, research conducted by Stork et al. (2009) have determined that extracellular organic matter secreted in the growth phase fouled the hydrophilic membrane more quickly. It had lower irreversible effect compared to fouling caused by the intracellular organic matter released in the death phase. The various phases of algal growth have been studied by Piorreck and Pohl et al. (1984). They determined the scale of metabolic production in a single growth phase of algal species, including green algae, *Chlorella vulgaris* and cyanobacterium, *Microcystis aeruginosa*.

A study by Henderson et al. (2008) also investigated the effect of the various algae including green algae, *Chlorella vulgaris*, and cyanobacterium, *Microcystis aeruginosa*, on the removal of natural organic matter from various water sources. The processes involved in this removal included coagulation and direct filtration processes, and these were found to be the most effective. Dissolved

organic matter such as humic substances were filtered through the membranes with minimal or no potential membrane fouling yielding concentrations <20 mg/L. Larger colloidal natural organic matter accumulated and fouled the membrane, reducing filtration efficiency. The removal of natural organic matter was required due to the irreversible effect of humic acid substances on DEAE-Sephadex A25 anionic exchange resin as described in Chapter 7.

Figure 8.4: Tubular membranes used for cyanobacteria filtration.

The image in Figure 8.4 shows the tubular membranes housed in the vertical section of the IPA. The tubular membranes remain vertical in the pipe housing to allow the sonication probe to slip between the tubular membranes. These were in close proximity to the captured cyanobacteria cells for improved cell disruption. The tubular membranes were interconnected together by rubber tubes to ensure filtration was maintained. This was to ensure any potential membrane fouling that

occurred, as a result of algal/cyanobacteria cell and/or macro-particle build up on the membranes, was overcome The top ends are interconnected, from the eight membranes in the prototype membrane housing unit, to one line for the sample to be pumped into the membranes. This division of a single water source to multiple membranes also dissipated water pressure away from cyanobacteria cultures that may cause early membrane disruption and phycocyanin loss. This was designed to filter the source water before cell disruption and to load phycocyanin onto DEAE-Sephadex A25 resin after cell disruption, as discussed in Chapter 3.8 in the operation procedure. The problematic influence that humic acid caused by permantely saturating the DEAE-Sephadex A25 resin was discussed at length in Chapter 7. From experimentation with humic acid and filtration through the membranes, it was determined that most of the humic acid was filtered using nylon, metal, ceramic or glass fibre most of the humic acid was not retained. Humic acid that remained could be easily washed out of the membrane with Milli-Q water. Hence, the design and procedure of operation for the IPA implements a method to capture intact cells for pre-concentration whilst removing the dissolved organic matter capable of fouling the anion exchange resin.

The design operations of the IPA for cyanobacteria filtration allows for cyanobacteria collection to be inside of the metal cylinder membranes. This is another advantage as not only can dissolved organic matter be filtered and removed to waste, but this allows for lyse cyanobacteria cells to be retained within the metal membranes whilst dissolved-phycocyanin can be liberated and collected for pre-concentration and analysis on the resin column. While, this procedure allows for an increase in potential membrane fouling with the initial loading of cyanobacteria on the membrane, the design allows for the retention of lysed cyanobacteria cells after sonication. This retention of matter only applies to matter >0.45 µm whereas any matter <0.45 µm is allowed to pass through to the resin column.

There was no operational difference in performance between the membranes investigated in Chapter 5 involving just the membrane, and the operation of the Isolated Pigment Analyser in Chapter 8.

8.1.3 Cell Disruption Component

Captured intact cells retained on the membrane surface would allow for natural organic matter and the potential for NOM interference on phycocyanin signal detection to be washed through the membrane. From this physical separation from one source of interference, namely humic substances, the brown discolouration of the water that is highly aromatic and anionic in nature, the implementation of a rapid and effective means of cells disruption was required.

From the literature research and investigations into the cell disruption by sonication by bath and probe (Furuki et al., 2003, Rajasekhar et al., 2012a, Rajasekhar et al., 2012b, Wu et al., 2011, Wu et al., 2012b, Zhang et al., 2006a, Zhang et al., 2006b), and by freezing/thawing cycles (Chaiklahan et al., 2012, Chaiklahan et al., 2011, Lello Zolla and Bianchetti, 2001, Lemasson et al., 1973, Moraes et al., 2011, Niu et al., 2007, Sarada et al., 1999, Benedetti et al., 2006, Silveira et al., 2008, Simis et al., 2007). Sonication probes were determined to be most effective way to liberate phycocyanin from intact cyanobacteria cultures. Sonication probes were used for the disruption of cyanobacteria and green algae cells. For this research, only the 3 mm sonication probe was utilised. This size head was employed because it was able to fit more easily within a vertical pipe containing captured cyanobacteria cultures.

8.1.4 Resin Component

The use of anionic resin demonstrated more potential benefit than size exclusion columns for two reasons. The design of the instrument allowed for separation to occur between humic substances along with other organic matter and intact cyanobacteria cells as discussed in Chapter 7. The role

of anionic exchange resins were for pre-concentration of phycocyanin rather than separation of components. Pre-concentration of phycocyanin on anionic resins, namely DEAE-Sephadex A25, did however allow for separation from intercellular organic matter, which began to collect on the resin.

This intercellular organic matter did foul the resin over time, and it was determined that the resin was able to be used and regenerated numerous times until saturation of the resin by the intercellular organic material. This saturation of the resin was dependent on the concentration of cultures and collection of intercellular matter on the resin with each run. The resin column was not backwashed to attempt to remove the cell debris for two reasons; the first was that attempting to backwash the column led to the formation of bubbles in the resin column and disrupted flow for the following analysis. The second, whilst some loss cell debris was potentially removed, there was still some fouling from intercellular matter. As a result, the resin column was determined to be a disposable consumable that would function for a number of phycocyanin analyses until saturation by organic matter.

Whilst the resin was continuously loaded with intercellular matter with each operation involving green algae or cyanobacteria, the resin was effective in its operation until the resin was saturated with dissolved organic matter. Approximately 80% of phycocyanin that was loaded onto DEAE-Sephadex A25 resin was eluted from the resin column, and the resin had the least interference with phycocyanin elution from a resin column. In other words, detection of phycocyanin was still achievable at 647 nm. Whilst phycocyanin demonstrated a logarithmic elution curve, this equation was simply implemented into the equation for cyanobacteria cell determination in the source water in the following section of this chapter.

8.1.5 Analysis

Analysis was conducted by fluorescence spectrometry with excitation at 609 nm and emission at 647 nm. The excitation and emission within these regions are directly related to expression of chlorophyll-a and phycocyanin cyanobacteria cell cultures. Chlorophyll-a was determined to be a potential source of interference from other organic sources capable of photosynthesis, such as green algae, plant matter, etc. This region of measurement also had the advantage of eliminating any potential interference from proteins and polysaccharides. Comparisons were drawn between stock solutions before treatment with the IPA and fluorescence measurements after treatment through the IPA. Cell concentrations for the stock solutions were measured by fluorescence at excitation 609 nm and 647 nm emission and compared to cell counting. Samples after IPA treatment were only measured by fluorescence spectrometry.

Due to the combination of different components, the elution of phycocyanin was multiplied by the resin effect with the elution volume directly related to this resin factor. The concentration of phycocyanin was then converted to an approximate cyanobacteria concentration. This was achieved by measuring and comparing literature values of phycocyanin and responses in fluorescence emission intensity to concentration. Experiments were conducted using the same linear response for intact live cyanobacteria cell cultures emissions by fluorescence. The following equation was determined and was implemented into IPA operations.

Equation 8.1:

$$\textit{Cell Concentration (cells/mL)} = \left(\frac{\textit{Conc.of Phycocyanin}}{\textit{Resin Factor}} \textit{x} \frac{\textit{Total filtered volume}}{\textit{Resin Elution Volume}}\right)$$

The determined cell concentration also incorporated the volume of source filtered to produce an estimated cell concentration within the source water. To precisely and accurately estimate the total concentration of cyanobacterial cells within a given solution, the individual components that made

up the IPA prototype were evaluated and Equation 8.1 accounted for their operation. The concentration of phycocyanin was determined by the response of the fluorescence signal from phycocyanin emission between 637-647 nm, multiplied by the resin factor and dilution factor. The resin factor was an elution percentage that was determined by the performance of DEAE-Sephadex A25 resin to elute phycocyanin with optimum regenerate solutions with the volume utilised as well. The resin factor for phycocyanin elution from DEAE-Sephadex A25 anionic resin was determined in Equation 8.2.

Equation 8.2: $Y= 2E\text{-}07x^4\text{-}4E\text{-}05x^3+0.0048x^2+1.3335x+9.6726$

The resin factor was calculated by elution of phycocyanin up to 40 mL. It was demonstrated that concentrations after 40 mL had limited potential variance. The maximum value of the resin factor was 0.80 ± 0.03, n = 119.

Table 8.1: Elution profile of phycocyanin from DEAE-Sephadex A25 resin by 0.3 M sodium chloride.

Elution Volume (mL)	4	8	12	16	20	24	**28**	**32**	**36**
Percentage Elution of phycocyanin (%)	29.86	45.80	59.59	70.09	73.86	77.21	**79.39**	**78.84**	**80.18**
Standard Deviation (± %)	11.05	9.19	6.55	3.99	4.96	5.77	6.06	3.85	3.10
Resin Bed Volumes (volume/g resin)	16	32	48	64	80	96	112	128	144

Table 8.1 demonstrates the elution profile of phycocyanin from DEAE-Sephadex A25 resin by 0.3 M sodium chloride regenerate solution. As highlighted in the table, volumes 28 to 36 mL gave similar elution concentrations of phycocyanin with no increase of phycocyanin detected above 40 mL elution volumes.

8.2 Operation of the Isolated Pigment Analyser

Controls of the IPA were determined by two variations of operations. The first was determined by the filtration of Milli-Q water not containing any cyanobacteria culture, green algae cultures or natural organic matter that could lead to any potential interference. Operations involving solutions containing green algae did not demonstrate any retention of chlorophyll-a on the resin as demonstrated in Chapter 7 and did not interfere with any measurement of phycocyanin.

The second variation involved IPA operations with green algae and/or natural organic matter. These operations were to determine the sensitivity of the IPA instrument in relation specifically to phycocyanin detection. Due to spectrum interference from chlorophyll-a and natural organic matter at the emission wavelength related to phycocyanin, the operation with green algae did not demonstrate any interference with chlorophyll-a or natural organic matter, because it was filtered and not eluted during measurement. Operations involving solutions containing nature organic matter or synthetic humic acid were either effectively filtered as discussed in Chapter 5 and later in section 8.3.2 and 8.3.3 of this chapter, or was irreversible retained on the resin column which did not interfere with the elution of phycocyanin. The operations with the IPA with either solution types did not result in any data illustrated in the following Figure 8.6 and Figure 8.7. The instrument could not be zeroed so the fluorescence emission was not zeroed during operations. A Milli-Q sample was taken and used as a blank for the estimate in calculations.

8.2.1 Investigation with cyanobacteria cultures

To determine the performance efficiency of the Isolated Pigment Analyser (IPA), measurements were taken of a stock cyanobacteria suspension of known concentration, and the calculated concentration from the IPA was based on a dilution factor versus the estimated cell concentration determined by the IPA using equation 8.1. Cell culture concentrations ranged from 2,000 to 350,000 cell/mL in 500-3000 mL volumes as treated by the IPA instrument.

Figure 8.5 demonstrates the linearity in response and deviation of the IPA prototype with a value of R^2 = 0.990 and a concentration range of 2,000-350,000 cells/mL (n =20). When the cell concentration was decreased, the limit of detection by fluorescence of cyanobacteria cultures was determined as given in Chapter 4.

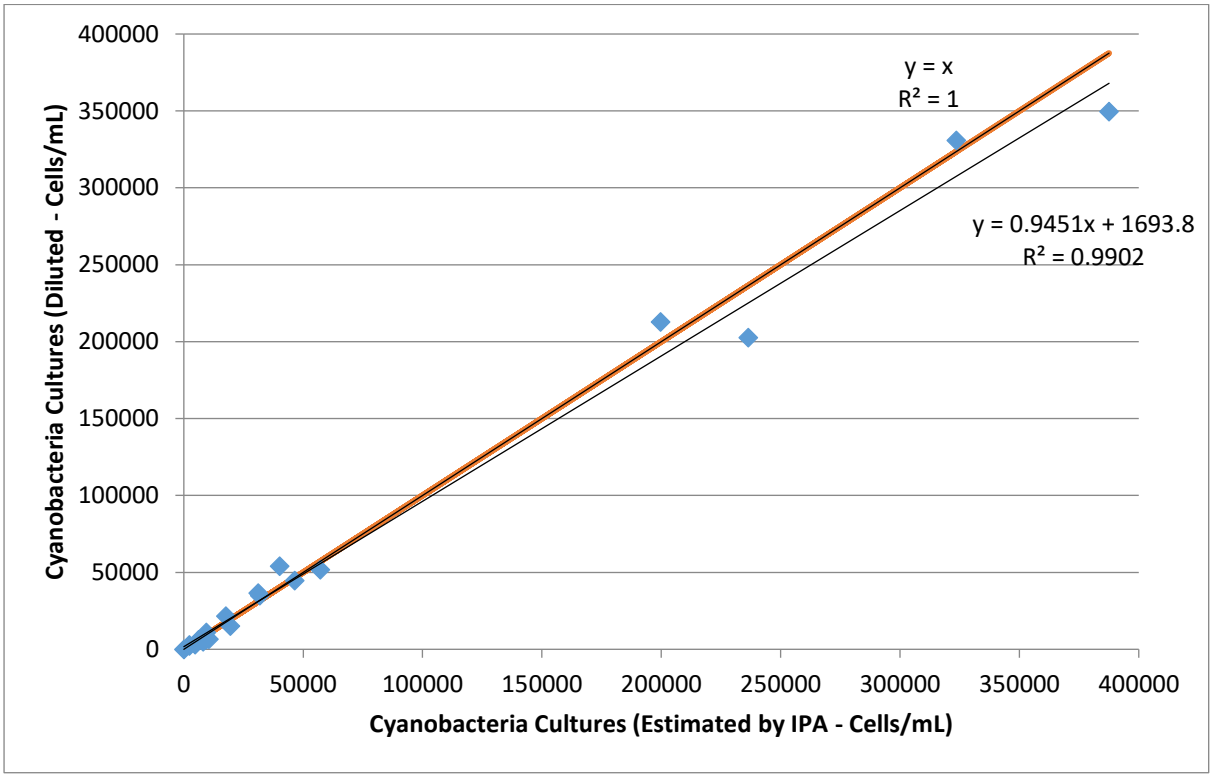


Figure 8.5: Actual diluted cyanobacteria cultures versus the estimated cell cultures determined by the IPA prototype.

The red line indicates actual diluted cell concentrations whilst the blue data points indicate the responses of the Isolated Pigment Analyser. When cell culture concentrations decreased to below 60,000 cells/mL, the linearity of the response decreased to a value of R2 = 0.941 for the concentration range of 2,000-56,000 cells/mL (n =15). This detection range between 2,000-56,000 was approximately 3-9 times lower than the detection limit for fluorescence without the use of pre-

concentrating the cyanobacteria cell cultures. Decreasing the working range between 2,000-20,000 cells/mL the prototype's linear response was $R^2 = 0.842$ (n = 11), plotted in Figure 8.6.

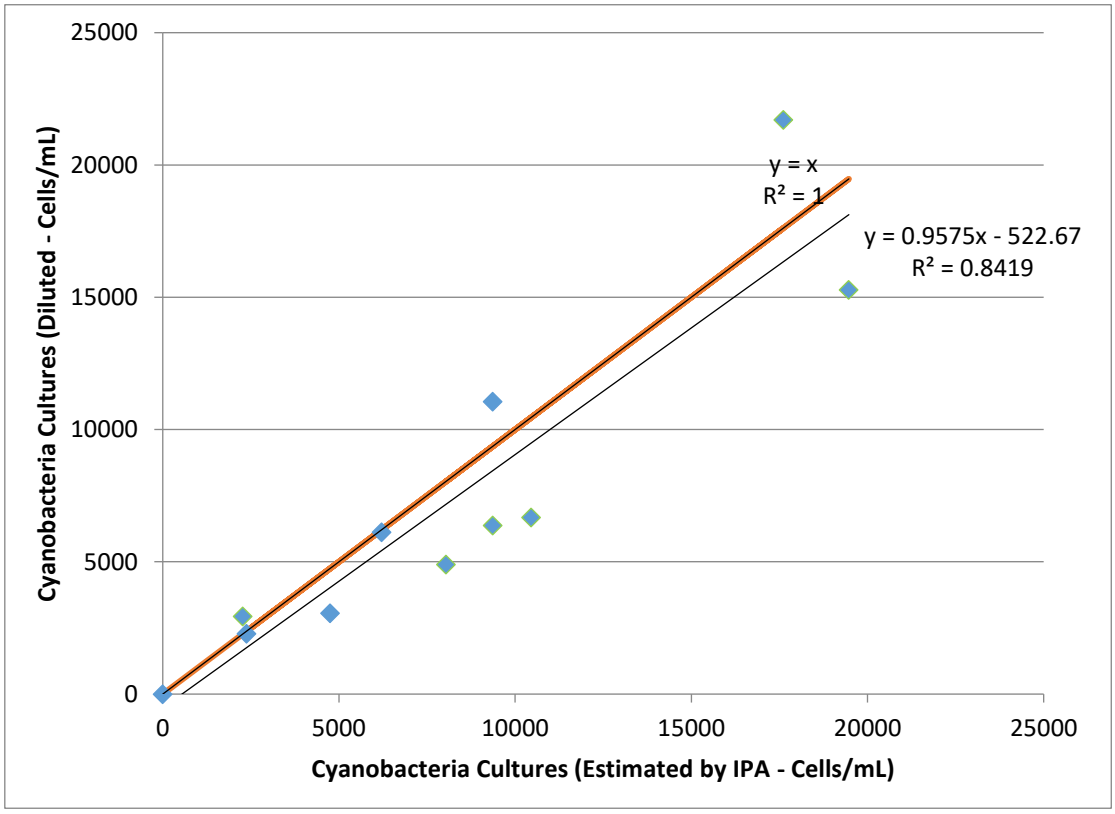


Figure 8.6: Diluted cyanobacteria cultures versus the estimated cell cultures determined by the IPA prototype of concentrations <20,000 cells/mL.

Data from experimentations conducted in Figure 8.5 and Figure 8.6 was limited to the specific experimentation of waters sources containing only cyanobacteria to demonstrate proof of concept.

By decreasing the concentration range from 2,000-350,000 to 2,000-20,000 cells/mL, the R^2 value decreased in accuracy. No outliers were generated and all data was utilised to produce an accurate analysis of the Isolated Pigment Analyser characteristics. Following from Figure 8.6, which demonstrated a linear response between the diluted cell concentrations and estimated cell

concentrations determined by the IPA instrument, Table 8.2 considers the individual variance between each data point.

Table 8.2: Variance of cell concentrations (cells/mL) between calculated diluted concentrations versus the concentration estimate determined by the IPA prototype

Data Variance (Diluted/Calculated)	Average %	Maxmum %	Minimum %	Data Points	Run within range (%)
Total Data Varance =	94.9	117.7	72.0	23	100.0
Within 40% deviation of data =	96.9	118.0	75.7	22	95.7
Within 30% deviation of data =	102.3	116.4	88.2	17	73.9
Within 20% deviation of data =	100.6	111.1	90.2	14	60.9
Within 10% deviation of data =	101.6	110.3	92.9	11	47.8

Table 8.2 shows the variance of estimated cell concentration determined by the IPA to the diluted cell concentrations, which assigned to 100 percent. The data was separated into five levels of variation between the diluted cell concentration and estimated cell concentration; total data, within 40% variance, within 30% variance, within 20% variance and within 10% variance. The variance of total data was wide, ± 22.9 % of cell accuracy between estimated and diluted cell culture concentrations (n = 23).

8.2.2 Investigations with natural organic matter

Investigations with the Isolated Pigment Analyser (IPA) involving natural organic matter were evaluated using synthetic CDOC solutions and filtered environmental waters. Filtered environmental waters were treated by filtering the water through a 0.7 µm glass fibre membrane. The initial filtration was to ensure algae and cyanobacteria cultures were captured from the water whilst dissolved organic matter was retained in solution.

Operation with the IPA demonstrated it was possible to filter dissolved organic matter through the metal membranes as previously investigated in Chapter 5. Dissolved organic matter was pumped

through the IPA with 96.6 % elution during culture loading onto the membranes. With the following treatment of a Milli-Q water wash, the remaining dissolved organic matter was removed from the membrane columns.

During operation of the IPA, the membranes filters demonstrated decreases in performance, filtration time, with the continuous retention of cyanobacterial cells and forms of NOM. On the completion of loading cyanobacteria onto the membranes, the membrane wash was performed to remove additional NOM from the membranes. Filtration with Milli-Q water showed there was a potential fouling of the membranes from residue CDOC. After thoroughly washing of the membranes, there was a notable decrease of NOM concentrations between each 50 mL measurement. The decrease of humic acid after 50 mL dropped to 56.0 % of the initial humic acid concentration, 46.5 % after 100 mL which decreased to 24.9 % by 200 mL. By the completion of the washing stage of the procedure, NOM concentrations of 10.6 % were still recorded by fluorescence spectrometry in the filtrate solutions through the membranes.

Despite the potential presence of humic acid substances or dissolved organic matter within the IPA system when sonication for pigment extraction takes place, this will ultimately not affect the determination of phycocyanin. If organic matter did manage to remain in the system until phycocyanin was loaded onto DEAE-Sephadex A25 resin, the matter would be irreversible retained on the column whilst allowing for the collection and elution of phycocyanin as long as the resin column does not reach saturation. However, this will require the DEAE-Sephadex resin to be changed for future operations before the resin reaches saturation and phycocyanin can no longer be retained for pre-concentration on the resin column.

8.2.3 Investigation with mixed cultures of cyanobacteria, algae and NOM

Mixed culture 1,000,000 cells of cyanobacteria and algae, were placed into 500 mL water with 5 NTU from the addition of humic acid (n = 3). *Anabaena circinalis* was the cyanobacteria culture utilised for these investigations, and *Chlorella vulgaris* was the green algae species used; in an approximate 25/75 percent ratio mix of cell cultures.

One of the outliers recorded was attributed to the loss of phycocyanin. After the collection of cyanobacteria cells on the membrane, there was a noticed detection of phycocyanin in the filtered water of 10.4 %. This indicated the 10.4 % was the result of cyanobacteria cells being disrupted during the filtration process or lysed in the sample water prior to filtration. For this result, this loss of phycocyanin during filtration led to a decreased in the predicted cell concentration of cyanobacteria at the end at the completion of the IPA process. Operation of the instrument determined there was no leaking from any connections or the filter housing that could account for an increase of 10.4 % emission from extracted phycocyanin, in 5 mL from 500 mL of processed water.

Table 8.3: IPA investigations on mixed cultures with NOM interference

Species	Initial Cell Concentration (cells/mL)	IPA's estimation of Cell Concentration (cells/mL)
Anabaena circinalis	236,443 ± 16,787	202,693 ± 7,094
Chlorella vulgaris	754,231 ± 10,512	*ND

*ND = not determined

Cell concentrations for *Chlorella vulgaris* could not be estimated by the IPA as it is unable to measure chlorophyll-a concentration. Sonication of the mixed culture faced a number of challenges that could account for the decreased extraction of pigments. The addition of more cyanobacterial cultures, green algae cultures or natural organic matter led to more potential membrane fouling and decreased filtrate flow rates over time.

Even with the presence of NOM and mixed cultures in high concentration during operations, the operations of the IPA slightly stressed the filtration stage to increase potential fouling of the membranes and decreased flow rate. Even though there was a the decrease in flow rate, the Isolated Pigment Analyser (IPA) was able to perform to the very end of the process and backwashing of the membranes decreased membrane fouling, thus allowing for continued operations.

8.2.4 Controls

As demonstrated in DEAE-Sephadex A25 resin investigations in Chapter 7, chlorophyll-a was not retained like phycocyanin. Operations involving water containing green algae did not demonstrate any retention of chlorophyll-a on the resin and did not interfere with any measurement of phycocyanin.

As control samples, the IPA was operated with green algae and/or natural organic matter without the presence of cyanobacteria in the sample solutions. These operations were to determine the sensitivity of the IPA instrument in relation specifically to phycocyanin detection. Due to spectrum interference from chlorophyll-a and natural organic matter at the emission wavelength related to phycocyanin, the operation with green algae did not demonstrate any interference with chlorophyll-a. Natural organic matter did not interfere because it was passed through membrane filters if dissolved, or it was retained on the filters if larger than 0.5 μm. If the organic matter passed through the membranes, the Milli-Q water wash after the filtration stage would flush the IPA system. If the natural organic matter was retained on the membrane filters, the sonication stage disrupted the cells to liberate phycocyanin and let it pass the membranes. The undissolved matter would then be backwashed off the membrane filters.

Operations involving solutions containing natural organic matter or synthetic humic acid solutions were effectively filtered as discussed in Chapter 5, section 8.2.3 and 8.2.5.3 of this chapter. If

organic matter, either intercellular or from the water source was irreversible retained on the resin column, it did not interfere with the elution of phycocyanin.

Negative controls were implemented to determine if any false positives arose with IPA operations. The negative controls consisted of purified water with green algae *Chlorella vulgaris* and *Chlamydomonas reinhardtii* with cell concentrations between 100,000-500,000 cells/mL, with or without the presence of 10 mg/L of humic acid. During calibration and the analysis of control samples, natural organic matter did not demonstrate any fluorescence signal increase during the measurement of phycocyanin. These measurements were incorporated into the investigations with cyanobacteria, as 0 cell/mL concentrations or control blanks.

It was concluded the IPA does not generate any false positives under the conditions tested and will only generate a positive result with the presence of cyanobacteria in sampled waters.

8.2.5 Limitations of the Isolated Pigment Analyser (IPA)

The investigations in this chapter led to the potential limitations of the isolated pigment analyser. These limitations of the Isolated Pigment Analyser are limited to the sonication probe, mainly height and treatment times, the on-going interference of humic acid substances and other organic matter, and the limitation with detecting cyanobacteria and phycocyanin.

8.2.5.1 Sonication probe

The sonication probe was not built into the instrument prototype. This poses a problem for operations with this prototype which could be overcome in a second generation design that has the sonication probe or bath built into the instrument at a fitted point. Due to the prototype being fitted for sonication treatment then removed to continue operations with the pumps, measurements were

taken to determine how deep the probe was submerged through the top of the membrane housing: it was determined the height of the probe tip was approximately vertically against the membranes.

The height of the prototype from the support stand was 285 mm with the height of the sonication probe from the base being 158 mm. The height of the membrane housing unit was 152 mm but the heights of the membranes were between 99-110 mm with an average of 105 mm long. The probe was submerged between 30-45 mm from the base of the membrane. Height of the probe against the membranes was important, not only for reproducing results but also optimising sonication with maximum cell disruption and for total phycocyanin recovery from cyanobacteria cells. Further, sonication time was another factor for the potential failing of the prototype.

8.2.5.2 Detection capacity and limitations

Fluorescence detection was proven to be the most effective way to detect phycocyanin in cyanobacteria cells. In order to concentrate cyanobacteria cells, the IPA was required to filter a large volume of source water. Due to the required detection concentrations of phycocyanin by fluorescence spectrometry, the concentration of cyanobacteria cultures captured is required to be equal to a detectable level of cyanobacteria cultures present by fluorescence spectrometry with excitation at 609 nm and emission measured between 635-650 nm or ideally emission at 647 nm. By increasing the number of membranes, this should allow for a greater volume to be filtered within the same time frame and potential capture and concentration the cyanobacteria cultures on the membranes.

8.2.5.3 Natural organic matter

Whilst the IPA was able to separate natural organic matter interferences and cyanobacteria from the source water, dissolved organic matter was effectively handled but organic matter >0.45μm particle size would greatly increase membrane fouling. The capture and pre-concentration of

phycocyanin on DEAE-Sephadex A25 resin was demonstrated to be nearly identical to resin investigations conducted in Chapter 5 of this research. Organic matter in IPA operations and during Chapter 5 demonstrated similar characteristic behaviour with both irreversibly fouling the DEAE-Sephadex resin. Whilst the IPA when operated efficiently is able to remove dissolved organic matter before it fouls the resin, there is another source of organic matter that does foul the resin.

Cell disruption of the cyanobacteria cells release intercellular organic matter also referred to as algal organic material (AOM) (Her et al., 2004, Villacorte et al., 2015). Algal-derived organic material (AOM) can be comprised of above 50% of biopolymers and refractory organic compounds (Villacorte et al., 2015). The intercellular matter is disruptive as it may irreversibly foul the resin. However, the concentrations of AOM likely to reach the resin still allows for multiple IPA operations before the resin needs to be changed. This fouling of the resin by intercellular matter is directly related to concentration of cell cultures captured on the membranes. Further, this intercellular matter is also dependent on the species of culture captured and treated by sonication. Each species will vary in intercellular matter present within a cell, which also partially depends on the growth phase in which the cell is found. The growth phase is likely to contain greater intercellular matter for increased colony/culture growth compared to a lag or death phase, before cells lyse which increases release of excreted intercellular organic matter (Her et al., 2004, Villacorte et al., 2015).

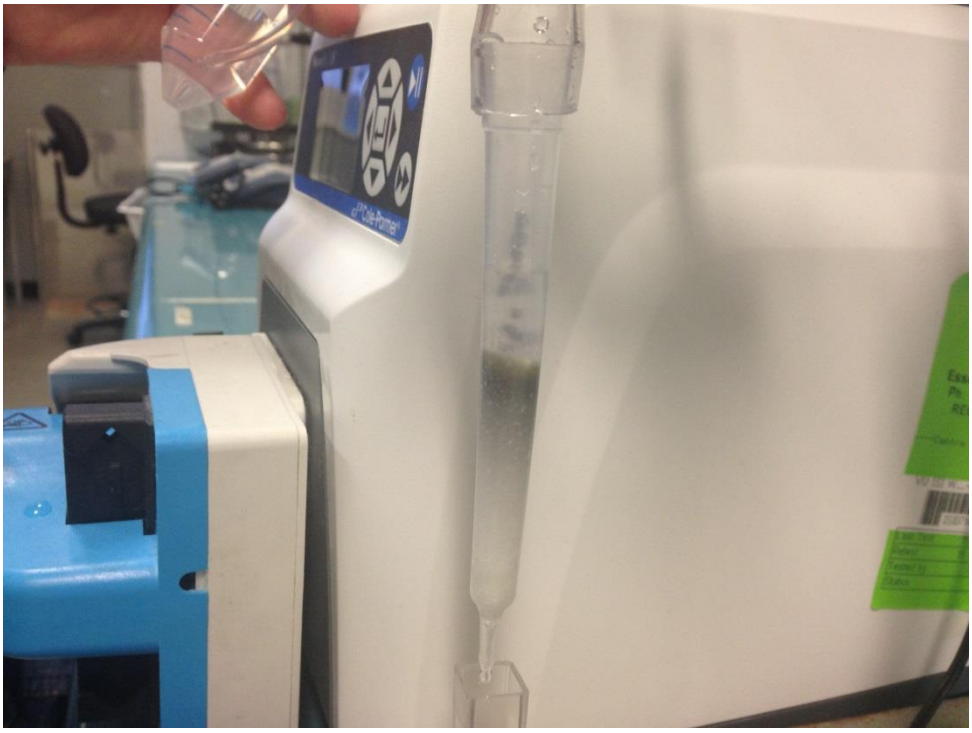


Figure 8.7: Intercellular matter build up on DEAE-Sephadex A25 resin

Figure 8.7 presents the image of the DEAE-Sephadex A25 resin column with discolouration from intercellular matter build up at the top of the resin column. Matter <0.45 μm will be able to pass through the membrane and continually discolour and irreversibly foul the resin column to the point that the resin column requires replacement.

8.3 Consumables

For operation of the IPA in field locations, the design requires low consumables to maintain operation and function for an extended period of time.

The main consumable of the Isolated Pigment Analyser is the resin column. As per Sigma-Aldrich, DEAE-Sephadex A25 anionic exchange resin in chloride form is commercially available between 10, 50, 100 and 500 gram containers. From Sigma-Aldrich, a 50 gram container of DEAE-Sephadex A25 resin costs $488. The resin column used by the prototype requires approximately 0.5 grams and can be used continuously until saturation by organic matter. This was either from

intercellular organic matter or from humic substances, in which a resin column can be exchanged between operations or during the filtration and cell capture stage of the operation. Other consumables consist of rubber tubing readily available in a hardware store which can degrade over time. After 25 cycles with the Isolated Pigment Analyser, the rubber tubing that holds the membranes together did not needed replacement. The resin column was a 5 mL pipette tip with glass wool located at the bottom of the tip. The viscous gel resin was retained by the glass wool and allowed for filtration under gravity or under pressure.

8.4 Conclusions

The Isolated Pigment Analyser was shown to be a promising prototype for the detection of cyanobacteria cultures or more specifically any aquatic culture/strain containing phycocyanin.

From these two factors, the specificity of the IPA to detect only phycocyanin was demonstrated; the specific pigment related to cyanobacteria cultures. Results from the controls also indicate any detection of phycocyanin is from cyanobacteria within the source water, thus the IPA was operated within protocol. This indicates the IPA designed to detect specific pigments related to cyanobacteria is accurate and it potentially demonstrates an estimation of cyanobacteria concentration relative to the actual concentration within a solution.

Treatment with cyanobacteria cultures had linearity curves of R^2=0.990 between 2,000-350,000 cells/mL and a linearity of R2=0.842 with a decreased range between 2,000-20,000 cells/mL. The data standard deviation between total data associated with the Isolated Pigment Analyser was decreased from ± 22.14 (n=20) whilst limiting the standard deviation to no more than < ± 10% that equaled ± 9.13 (n=10).

The operations of the IPA were effective but limited to specific conditions. These conditions include; the limitation by fluorescence detection which is related to the filtration volume required, the interference of natural organic matter and required automation of the sonication treatment. In order to combat these potential limitations, future work is required on the improved development of the IPA and its operation.

Chapter Nine

Conclusion and Future Research

9.1 Conclusions

A working prototype for the direct measurement of cyanobacteria in a water source was developed. This prototype was named the Isolated Pigment Analyser (IPA). It was designed to estimate cyanobacteria cell concentrations by means of the specific pigment, phycocyanin, within a water source.

Fluorescence spectrophotometry was utilised for its sensitivity in the detection of cyanobacteria, using chlorophyll-a and phycocyanin. Spectrometric studies were employed to produce a working benchmark to lower the limit of detection for cyanobacteria cells compared to fluorescence: this was achieved by removing the substantial interference from dissolved organic matter. The capture and concentration of cyanobacteria cultures by direct sample filtration through the membrane was introduced and investigated by allowing the accumulation of cyanobacteria culture on the membrane. This accumulation of culture on the membrane served two functions; the first to remove dissolved organic matter interferences from the measurement of phycocyanin and second, to concentrate cyanobacteria cells within a specific volume to improve phycocyanin detectability.

Cell disruption was the next stage of the investigation. It was an important step as chlorophyll-a and phycocyanin pigments are retained within cyanobacteria cells. To liberate and isolate phycocyanin for measurement, these pigments were separated. The membrane(s) used for the IPA were required to be durable. After concentration of cyanobacteria occurred, the cultures underwent cell disruption, with sonication determined to be the most rapid and effective method investigated. A wash flushed the dissolved pigment(s) through the membrane and onto the resin column. The

undissolved material along with cellular matter that did not pass through the membranes was backwashed so the membrane could be reused.

The ion exchange resin was a secondary concentration step for phycocyanin and the stage for phycocyanin isolation. Any chlorophyll-a able to be washed through into the resin column was not retained and immediately washed through. Phycocyanin was retained and could be eluted in a controlled manner with a sodium chloride solution. Any presence of natural organic matter, trace levels from the water source or cellular matter, were irreversible and permanently retained on the resin. The resin was able to retain and elute off phycocyanin in a controlled matter until resin saturation occurred.

9.2 Environmental Application

For environmental applications the Isolated Pigment Analyser (IPA) is required to be implemented and field tested in a real world application with real world water based interferences and cyanobacteria. This requires integration of the IPA into a water station, calibration with phycocyanin and/or cyanobacteria cell cultures, operation and analysis. Automation of the IPA would allow for rural regions to operate, analyse and collect data remotely, unless human intervention was required, such as for routine or non-routine maintenance.

Whilst the IPA was initially designed to operate in freshwater applications, the capacity for operation may be expanded to marine operations as well. In theory, the IPA will operate under the same outlined protocol. The saline concentration in the marine water would be expected to be washed from the cell after collection on the membrane to dissolved organic matter interferences. This would allow the cyanobacteria culture to be taken from a salt water solution to a freshwater solution. If cell disruption occurred as a result of osmosis, the direct next stage of the IPA process is to disrupt the cell to liberate phycocyanin. The process would then continue to collect

phycocyanin on the resin column for the second concentration stage followed by controlled elution of phycocyanin and measurement.

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Appendix A

A list of chromatographic, anionic and cationic resin utilised in this research investigation along with their specifications can be located within Table 1 Appendix A.

Resin Type	Resin	Characteristics
Size Exclusion	LH-20 Sephadex	25-100 μ bed volume per g dry gel
Size Exclusion	Sephadex G25	10-40 microns with water retention approximately 2.1 to 2.7 mL/g of resin
Size Exclusion	Sephadex G75	40-120 microns
Anionic	Activated Alumina	0.1mm and 1.0 mm resin size
Anionic	Activated Silica	0.1mm and 1.0 mm resin size
Anionic	DEAE-Sephadex A25	3.5 ± 0.5 meq/g with a particle size of 40-120 microns
Anionic	QAE Sephadex	3.0 ± 0.5 meq/g compared to the 3.5 ± 0.5 meq/g. particle size of 40-120 μm
Anionic	Amberlite IRA93	Weak base, 0.49 mm, capacity 1.2 meq/mL:
Anionic	Amberlite IRA416	1.4meq/mL by wetted bed volume, 40-47 % moisture, particle size of 20-25 μm