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Application of Cytotoxicity Assays for Detection of Potentially Harmful Bioactive Compounds Produced by Freshwater Bloom-Forming Algae

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APPLICATION OF CYTOTOXICITY ASSAYS FOR DETECTION OF POTENTIALLY HARMFUL BIOACTIVE COMPOUNDS PRODUCED BY FRESHWATER BLOOM-FORMING ALGAE

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Abstract

Detection of harmful bioactive compounds produced by bloom-forming pelagic algae is important to assess the potential risks to communities. We applied two cell-based assays, an erythrocyte lysis assay (ELA) that assesses membrane integrity, and a RTgill-W1 cytotoxicity assay (RCA) that detects changes in cell metabolism, to evaluate the cytotoxic effects of: (1) individual toxins and noxious compounds; and (2) complex mixtures of compounds produced by cyanobacteria and chrysophyte isolates. ELA was insensitive to toxins and noxious compounds except at exceptionally high concentrations ($EC_{50} \geq 10^6$ nM). RCA was sensitive to noxious compounds only, at concentrations greater than reported environmental averages ($EC_{50} \geq 10^3$ nM). Cultured isolates produced bioactive compounds that had recognizable, dose dependent, toxic effects. Toxicity of these bioactive compounds depended on the taxa (cyanobacteria, not chrysophytes), growth stage (stationary phase more toxic than exponential phase), location (intracellular more toxic than extracellular), and iron status (iron-replete cells more toxic that iron-deplete cells).

Keywords

Assay, Bioactive, Cyanobacteria, Chrysophyte, Freshwater , Harmful Algal Bloom , Lake, Odour, Taste, Toxin,

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Chapter 1
General Introduction

1.1 Problem Statement

Freshwater algal blooms dominated by cyanobacteria and chrysophyte algae are a growing concern in affected communities (Skulberg et al., 1984; Carmichael, 2001; Winter et al, 2011). The risks posed to biota exposed to the compounds produced by these algae are somewhat understood; however, techniques designed to evaluate the biological and/or cytotoxic effects of these compounds as they occur in mixtures are generally lacking (Watson, 2004; Dayeh et al., 2005). This thesis aims to: (1) review the current state of knowledge in terms of toxic, noxious, and/or bioactive compound production by bloom forming freshwater algae; and (2) investigate the use of two cell-based assays designed to evaluate the cytotoxic effects of these compounds.

1.2 Scientific Justification

Freshwater cyanobacteria are prokaryotic microorganisms that are commonly found in water bodies around the world (Carmichael, 2008; Paerl et al., 2008; Fortin et al., 2010; Huber et al., 2012). The presence of cyanobacteria has been verified in freshwaters on every continent, and the ability to accumulate in high number (known as a bloom) has been a main reason for the widespread awareness of these microscopic bacteria (Carmichael, 2008; Heisler et al., 2008). Colloquially named blue-green algae, cyanobacteria are actually autotrophic bacteria that have pigments such as chlorophyll a, phycoerythrin, and phycocyanin which give it a blue-green appearance when observed under a microscope or on the surface of the open water during bloom events (Sivonen & Börner, 2008). Cyanobacteria exist in a variety of arrangements, including filamentous, colonial, and single cell orientations (Sinclair & Hall, 2008). The specific arrangement varies from species to species (Sinclair & Hall, 2008).

Some genera of cyanobacteria have the ability to produce toxins and noxious compounds which can pose risks to exposed biota (Pouria et al., 1998; Falconer, 1999). A number of hepatotoxins, neurotoxins, and other compounds have been isolated from

strains of cyanobacteria, and these findings have led Environment Canada to designate every algal bloom that is dominated by cyanobacteria a harmful algal bloom (HAB), indicating its potential to cause harm (Charlton et al., 2008; Sivonen & Börner, 2008). The production, mode of action, biological and/or ecological risks associated with each toxic and noxious compound are unique, and information regarding the antagonistic, synergistic, or additive effects of the compounds is generally lacking (Watson, 2004).

Chrysophyte algae are also common to freshwaters, and there has been an increase in reports of chrysophyte bloom events across a number of systems (Paterson et al., 2008; Winter et al., 2011). Chrysophytes are eukaryotic microorganisms known as 'golden-brown algae', and the pigment primarily responsible for their unusual colour is fucoxanthin (Nicholls, 1995). Chrysophytes are commonly found in colonies, but also exist as single cells (Nicholls, 1995). Although freshwater chrysophytes do not produce toxins, they do produce a variety of noxious compounds associated with poor taste and odor events that can impact the water quality at very low concentrations (Watson, 2003).

Many peer-reviewed articles have assessed individual compounds produced by cyanobacteria and chrysophyte algae in terms of their toxicological properties and production routes, but there is generally a lack of material that compiles this important information in one location (Nicholls, 1995; Sivonen & Jones, 1999). Similarly, many of the reviews that have been conducted focus on compounds produced by one type of algae, neglecting to acknowledge many of the analogous compounds produced by other types of algae (MacKintosh et al., 1990; Falconer, 1999). Further synthesis of the toxicological data available for all of the common compounds produced in freshwaters by cyanobacteria and chrysophyte algae is essential for drawing conclusions and identifying the compounds likely to be responsible for the most deleterious effects on the environment and exposed biota.

A unifying theme among the compounds produced by cyanobacteria and chrysophyte algae is the relationship that exists between physiology and compound production (Watson, 2003; Paterson et al., 2004; Kaplan et al, 2012). Aside from physical forces that are continuously acting on the cell, an important physiological control is nutrient limitation (Nicholls, 1995; Paterson et al, 2004; Kaplan et al., 2012). Based on this knowledge, it is not surprising that the link between cyanobacterial toxin production

and the micronutrient iron (Fe) is well established (Alexova et al., 2011; Kaplan et al., 2012). Fe limitation is a trigger of toxin production in some cyanobacteria, and although less well defined, the relationship between chrysophytes and Fe has also been examined (Paterson, 2004; Watson, 2004). Elucidating the link between Fe stress and toxic and/or noxious compound production is a major goal of this thesis and will aid in our understanding of how blooms respond to Fe limitation in open systems.

Another physiological control is growth stage. The literature suggests that cyanobacteria have the ability to produce toxins at the highest rate during exponential phase growth, likely due to the high rate of cellular processes (photosynthesis) occurring during this stage of growth (Alexova et al., 2011; Kaplan, et al, 2012). Identifying the timing of production is important in furthering our understanding of the hazards of bloom events as they occur in natural systems.

Furthermore, physiological controls on the storage and/or excretion of the toxins or noxious compounds are important. Compounds that are primarily stored in the cell are more likely to build up and be released in a large pulse during bloom senescence, while compounds that are excreted throughout the duration of their production may be found in the open water for great periods of time at relatively lower concentrations (Nicholls, 1995; Watson, 2004; Kaplan et al, 2012). Identifying the storage and/or excretion patterns of these compounds over the course of a cells' growth is an important factor to consider when assessing the impacts that freshwater algal blooms may present to exposed biota.

Identifying analytical methods suitable for the task of quantifying the biological impacts of complex mixtures can be challenging, and cell based assays offer a modern solution to the limitations of traditional sampling techniques (Fent, 2001; Graham et al., 2008; Lee et al., 2009). The erythrocyte lysis assay (ELA) and RTgill-W1 cytotoxicity assay (RCA) are two assays which have been applied to studies of the compounds produced by marine phytoplankton (Ling & Trick, 2010; Dorantes-Aranda et al., 2011). The adaptation of these assays for use in freshwater testing may prove valuable in improving understanding of the biological/cytotoxic effects of the compounds produced by cyanobacteria and chrysophyte algae. The ELA uses rabbit erythrocytes to evaluate the hemolytic capacity of the test substance and is used to evaluate how compounds

impact membrane integrity (Wang et al., 2007; Hemholz et al., 2010; Zhang et al., 2011). The RCA is used to evaluate the effects that tested substance have on cellular metabolism (Schirmer et al., 1998; Schirmer et al., 2001). Using these two techniques in tandem may prove advantageous in that it will allow us to gain further detail on the mode of action of the substances of interest.

1.3 Goals and Hypotheses

The first goal of this research is to critically assess the state of knowledge with regards to the toxic and noxious compounds produced by freshwater cyanobacteria and chrysophyte algae. Synthesizing the available information with regards to these compounds will aid in clarifying the importance of the relevant compounds, and understanding how the toxic mechanism may work in tandem. The conclusions drawn from this review will be useful in the analysis of the results observed from the experimental portion of the thesis.

The second goal of this research was to combine the use of the RCA and the ELA to determine their effectiveness in the detection of cytotoxins in freshwater samples, and allow us to gain insights into the production of toxic, noxious, and/or bioactive compounds under differing nutrient (high and low Fe) conditions. This was examined through: (1) the application of individual analytical standards of toxins and noxious compounds produced by freshwater algae; and (2) the analysis of complex mixtures of metabolites produced by freshwater algae isolates of cyanobacteria and chrysophyte algae (Figure 1.1).

The following hypotheses were tested:

H1: The application of individual analytical standards of toxins and/or noxious compounds produced by freshwater algae will result in concentration-dependent decreases in viability of cells in both the RCA (assesses damage to cell metabolism) and the ELA (assesses damage to cell membrane). I predict that concentrations of these compounds at or below their environmentally measured averages will result in significant decreases in cell viability in both assays.

H2: The application of complex mixtures of algal metabolites extracted from cultures of toxic and/or noxious compound-producing isolates of cyanobacteria and

chrysophyte algae will result in concentration-dependent decreases in viability of the RCA and ELA. I predict that: (1) due to the increased rate of cell growth and toxic and/or noxious compound production during exponential phase, exposure solutions derived from exponential growth phases will result in significant decreases in cell viability in both assays relative to those of the stationary phase; (2) due to the predominant intracellular storage of toxic and noxious compounds, exposure solutions derived from lysed cultures (intracellular + extracellular compounds) will result in significant decreases in cell viability in both assays relative to solutions derived from non-lysed samples (extracellular compounds only); and (3) due to toxin production stimulated by Fe limitation of toxin-producing cyanobacteria, exposure solutions extracted from low Fe (0.1 μM) treatments will result in significant decreases in cell viability in both assays relative to the high Fe (10 μM) treatments.

The application of cytotoxicity assays for detecting toxic, noxious, and/or bioactive compounds produced by cyanobacteria and chrysophyte algae will allow us to gain insights into the production, storage, and biological effects of these compounds as they occur in complex mixtures (Dayeh et al., 2005).

1.4 Thesis Organization

This thesis is divided into four parts. Following this general introduction, chapter 2 presents an in-depth critical review of toxic, noxious, and bioactive compounds produced by HAB-forming freshwater algae. This chapter deals with the various types of compounds produced, their chemical structures, the genera responsible for their production, their mode(s) of action, and includes a discussion of the need for improved detection techniques. Chapter 3 presents experimental work investigating the use of two cell-based assays (RCA and ELA) to evaluate the toxicity of compounds produced by HAB-forming freshwater algae. This chapter includes the evaluation of individual compounds, as well as complex mixture derived from algal cultures. Chapter 4 presents the general conclusions drawn from this thesis, and discusses the future research directions and management implications of this work.

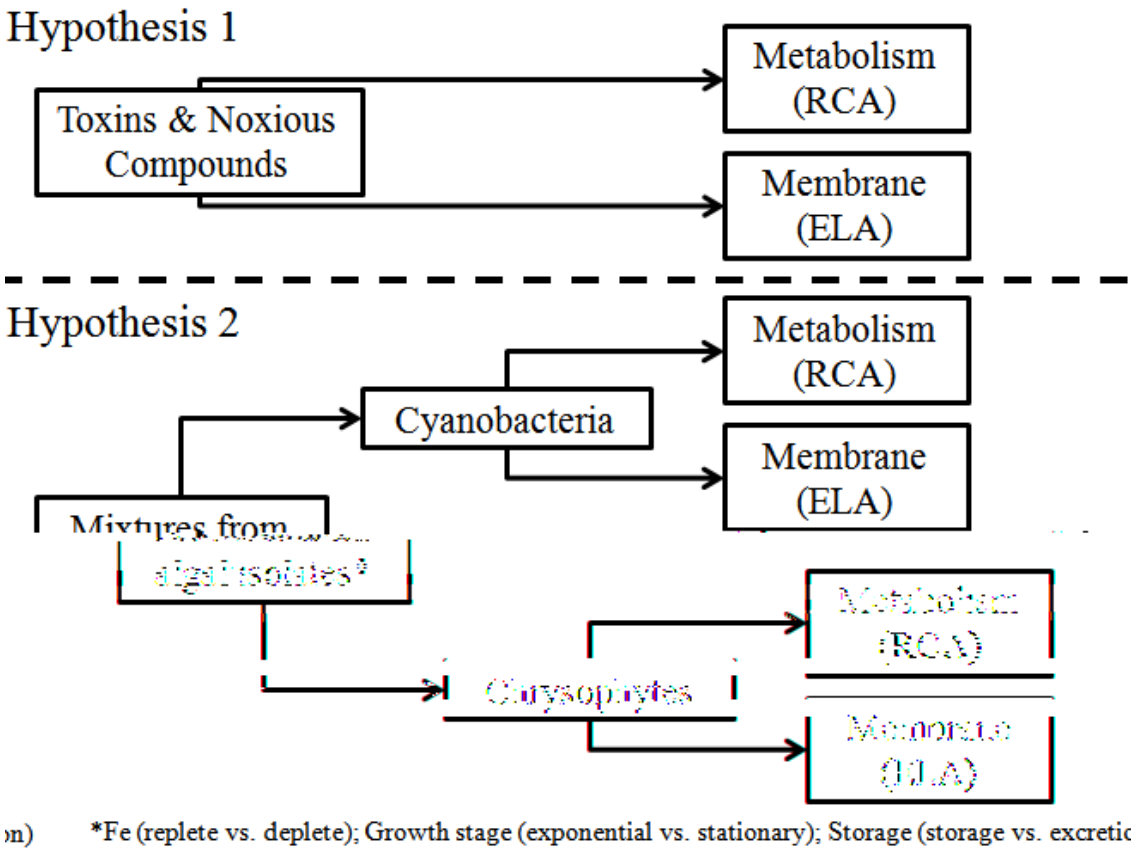

Figure 1.1. Flow chart of experimental design for laboratory testing of toxic and noxious compounds produced by cyanobacteria and chrysophyte algae.

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Chapter 2

Toxic, Noxious, and Bioactive Compounds Produced by Freshwater Harmful Algal Blooms

2.1 Introduction

Freshwater HABs are able to exist in a wide variety of climatic regions, and have been reported in systems across the globe (Sinclair & Hall, 2008; van Gremberghe et al., 2011). Cyanobacteria-dominated HABs (cHABs) have been reported on all continents, including remote parts of the world such as Alaska, Antarctica, Iceland and Hawai'i (Sinclair & Hall, 2008; van Gremberghe et al., 2011; Figure 2.1). This widespread distribution illustrates how these events affect water quality on a global scale and demonstrates the need to develop a concrete understanding of the risks associated with HABs.

Recent work has also addressed the increased incidence of chrysophyte bloom events, which are not known for their ability to produce toxic compounds (in freshwater genera), but rather their production of a wide variety of noxious compounds responsible for the fouling of surface waters and causing unpleasant taste and odour events (Watson, 2004; Winter et al., 2011). Interest in the areas of freshwater cHABs and chrysophyte blooms has risen in recent years (Figure 2.2). Figure 2.2 shows that the general awareness and concern over algal blooms dominated by cyanobacteria have by far been more covered by publications available to the general public (Google results) relative to chrysophyte blooms. This is likely a result of the toxic potential of freshwater cyanobacteria and their ability to effect animal health and cause of beach closures. While chrysophyte blooms are common, their lack of visibility (can occur below the surface of the water) may be a cause of underreporting or lack of awareness of these events in mainstream publication (Nicholls, 1995).

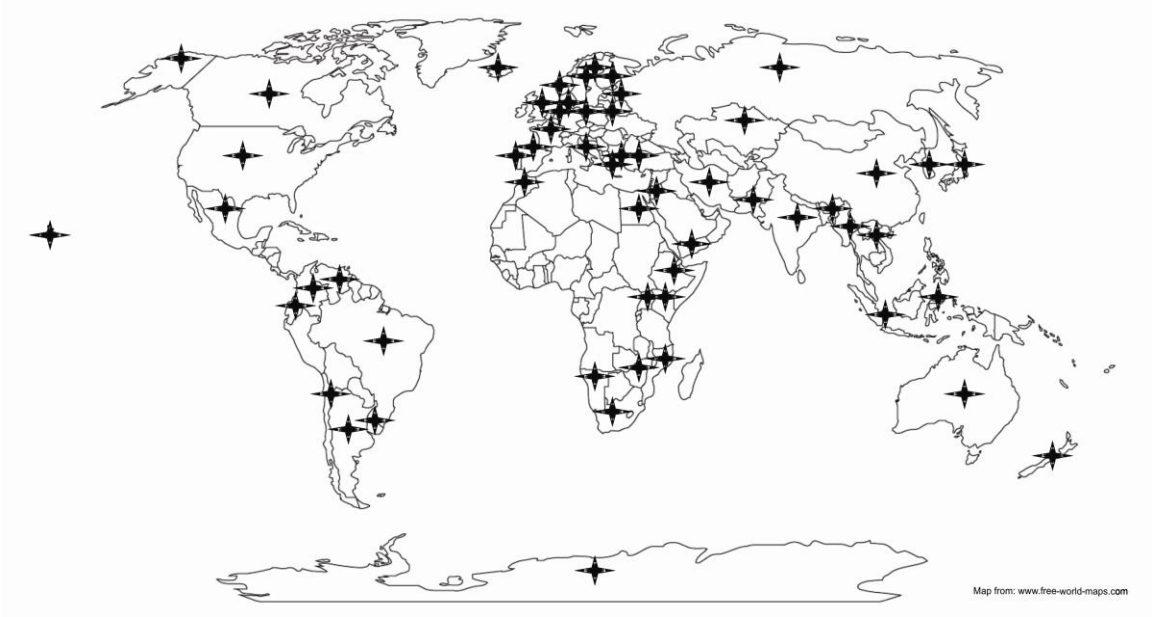

Figure 2.1. Worldwide distribution of cHAB events. Stars indicate countries (or states) with documented presence of one or more cHABs, and are not an indicator of cHAB density in a particular region. (Synthesized from Sinclair and Hall, 2008 and van Gremberghe et al., 2011).

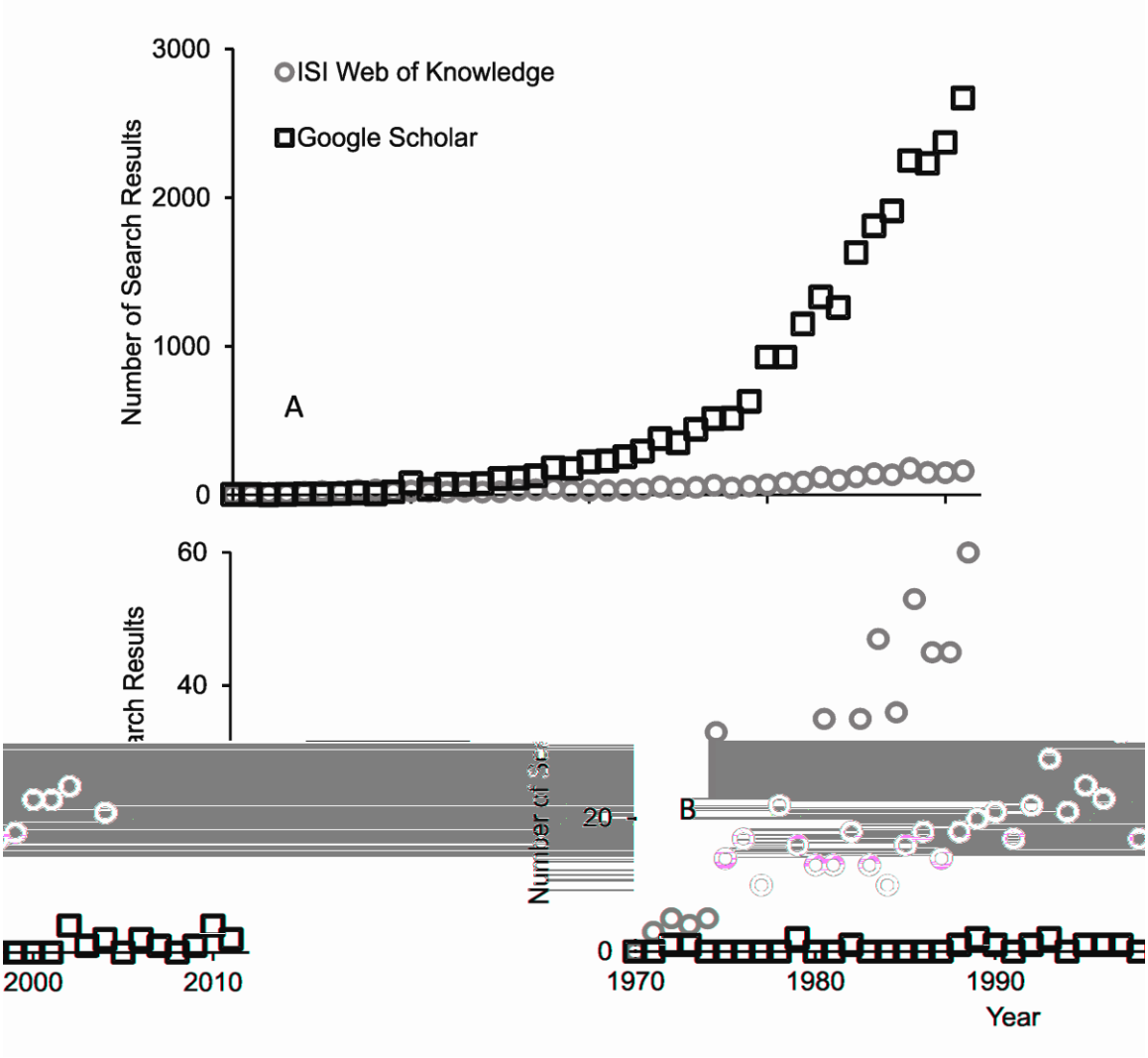

Figure 2.2. Number of search results per year from major databases: A) Search topics: cyanobacteria* AND bloom* AND freshwater*; B) Search topics: chrysophy* AND fresh* AND bloom*.

2.2 Freshwater Harmful Algal Blooms: Associated Compounds, Taxa, and Consequences

The compounds present in freshwater blooms can vary drastically from basin to basin (Sinclair & Hall, 2008). However there are a number of compounds, or groups of compounds, that are frequently present in bloom events dominated by cyanobacteria or chrysophyte algae (Watson, 2004; Sivonen & Börner, 2008) The genera responsible for the production of toxic compounds in freshwaters are exclusively cyanobacteria, while genera of both cyanobacteria and chrysophyte algae are capable of producing noxious compounds (Table 2.1). These compounds can have direct and/or indirect effects on surrounding biota, with a great deal of diversity in terms of the type of mechanism that these substances use to cause harm (Table 2.2).

2.3 Cyanotoxins

Cyanotoxins: microcystins and nodularins

Microcystins (MCs) are the toxins most commonly produced by cyanobacteria in freshwaters (Sivonen & Börner, 2008). These heptacyclic peptides have been implicated in the poisonings of a number of animals, and have the potential for endangering human health if ingested at high concentrations (Jochimsen et al., 1998; Carmichael, 2001). The primary method of cytotoxicity exhibited by MCs is the inhibition of type 1 and 2A protein phosphatases (Honkanen et al., 1990; Mackintosh et al., 1990). These enzymes act by dephosphorylating proteins that are essential to cellular processes such as differentiation, transport, cell cycle regulations, and metabolism (Barford, 1995).

The presence of hemorrhaging and tumor formation in the livers of exposed animals, suggests that MCs disrupts the primary function of this organ (glucose metabolism) (Cohen, 1989; Nishiwaki-Matsushima et al., 1992; Jochimsen et al., 1998; Carmichael, 2001; Pearson et al., 2010). Based on these observations, MCs are classified as hepatotoxins that act primarily on the liver, although alternative/secondary effects of MCs on an organism level have been identified (Gupta et al., 2003; Sedmak & Elersek, 2006; Sivonen & Börner, 2008; Amado & Monserrat, 2010).

Table 2.1. Cyanobacteria and chrysophyte taxa and their corresponding production of toxic and noxious compounds. +, – indicate the ability, or inability, respectively, of a genus to produce a compound (MC – Microcystin; NOD – Nodularin; ANTX – Anatoxin; STX – Saxitoxin; CYN – Cylindrospermopsin; BMAA – β-methylamino-L-amine; Synthesized from Watson, 2004; Sinclair and Hall, 2008; Sivonen and Börner, 2008; Pearson et al., 2010).

	Toxins					Noxious compounds					
Genera	MC	NOD	ANTX	STX	CYN	BMAA	Terpenoids	PUFAs	Sulfurous	Amines	Pyrazines
Cyanobacteria											
Microcystis	+	-	+	-	-	+	+	+	+	-	-
Anabaena	+	-	+	+	+	+	+	+	+	-	-
Aphanizomenon	-	-	+	+	+	+	+	+	-	-	-
Planktothrix	+	-	+	+	-	+	-	+	-	-	-
Cylindrospermopsis	-	-	-	+	+	+	-	+	-	-	-
Nodularia	-	+	-	-	-	-	-	+	-	-	-
Chrysophyte											
Dinobryon	-	-	-	-	-	-	-	+	-	+	+
Synura	-	-	-	-	-	-	-	+	-	+	+
Uroglena	-	-	-	-	-	-	-	+	-	+	+

Table 2.2. Direct and indirect effects of exposure to toxic, noxious, and/or bioactive compounds produced by bloom-forming freshwater algae (MC – Microcystin; NOD – Nodularin; ANTX – Anatoxin-a; ANTX-a (S) – Anatoxin-a (S); STX – Saxitoxin; CYN – Cylindrospermopsin; BMAA – β-methylamino-L-amine).

Class	Compound	Direct effects	Indirect effects	References
Toxic	MC	Inhibition of protein phosphatase 1, 2A; promotes tumor formation	Infochemicals; inhibit growth	Kaplan et al., 2012
	NOD	Inhibition of protein phosphatase 1, 2A; promotes tumor formation		Pearson et al., 2010
	ANTX-a	Depolarizing neuromuscular blocking agent (post synaptic); disrupts muscle function		Carmichael et al., 1975
	ANTX-a (S)	Inhibits the enzymatic breakdown of acetylcholine; disrupts muscle function		Matsunaga et al., 1989
	SAX	Alters the current of Na ion channels; associated with paralytic shellfish poisoning		Strichartz, 1981; Pearson, 2010
	CYN	Inhibition of protein synthesis		Fastner et al, 2003
	BMAA	Acts on NMDA & mGluR5 receptors	Oxidative stress response	Lobner et al., 2007; Liu et al., 2009
Noxious	Terpenoids	Potent T&O causing substances; earthy		Srinivasan & Sorial, 2011
	PUFAs	Potent T&O causing substances; fishy	Pheromones; defense mechanisms	Nicholls, 1995; Watson, 2004
	Sulfurous	Potent T&O causing substances; sulfurous	Cellular identification	Hofbauer & Juttner, 1988
	Amines, pyrazines	Potent T&O causing substances		Young et al., 1996; Watson et al., 2004
Bioactive	Cyanopeptolins	Some are serine protease inhibitors	Toxic effects towards crustaceans	Weckesser et al., 1996; Gademann et al., 2010
	Hexapeptides	Some are non-specific protease inhibitors		Sivonen & Börner, 2008
	Microviridins, microginins, aeruginosins	Some are non-specific protease inhibitors	Toxic effects towards zooplankton	Rohrlack et al., 2004

All MCs display the general structure *cyclo*(d-Ala-X-d-MeAsp-Z-Adda-d-Glu-MdhA) in which X and Z represent variable single amino acids. d-MeAsp is d-erythro-β-methyl-aspartic acid, MdhA is *N*-methyldehydroalanine and Adda is (2*S*,3*S*,8*S*,9*S*)-3-amino-9-methoxy-2,6,8-trimethyl-10-phenyldeca-4,6 dienoic acid (Botes et al., 1984; Rinehart et al., 1988). There have been at least 87 identified variants of MCs to this date and the advent of more sensitive analysis techniques and investigations into additional taxa capable of producing MCs will undoubtedly lead to an expansion of this list (del Campo et al., 2010).

The most cytotoxic variant has repeatedly proven to be microcystin-leucine-argenine (MC-LR), although evidence has suggested that MC variants that contain similarly hydrophobic amino acids (such as Phe, Try, or Ala) in the variable regions of the molecule will have similar cytotoxic effects (Stotts et al., 1993; Codd et al., 1999; Gupta et al., 2003). Conversely, MCs that contain amino acids that are more hydrophilic in their variable regions may be less harmful, as the interactions with the lipid bilayer of cells may be affected (Stotts et al., 1993; Gupta et al., 2003; Teixeira & Rosa, 2012).

Structurally, nodularins are also classified as cyclic peptides and are closely related to MCs. The general chemical structure is *cyclo* (D-MeAsp-L-argenine-Adda-D-glutamate-Mdhb, in which Mdhb is 2-(methylamino)-2-dehydrobutyric acid (Rinehart et al., 1988). The mechanism of toxicity by nodularins is reported to be very similar to that of MCs, although much less data is available on the subject (Yoshizawa et al., 1990).

A difference between nodularins and MCs in natural systems is their spatial distribution. Nodularins are more prevalent in brackish/estuarine waters, while MCs are commonly found in freshwaters (Sivonen & Börner, 2008). Both nodularins and MCs are susceptible to microbial and bacterial degradation, and the variability observed in this type of degradation likely relates to the spatial differentiation generally observed between the two (Imanishi et al., 2005; Edwards et al., 2008). Genera that produce MCs include *Microcystis, Anabaena, Planktothrix*, and *Oscillatoria;* whereas, nodularin production is predominantly limited to *Nodularia* (Kaplan et al., 2012).

Beyond the direct hepatotoxic properties of these cyclic peptides, various allelopathic effects have been identified. Specifically, the MCs and nodularins in aquatic environments negatively affect the growth of other algal species and microorganisms,

reduce the animal and algal community diversity, and may affect bioaccumulation in food chains (Inderjit and Dakshini, 1994; Aboal et al., 2001; El-Sheek et al., 2010; Pearson et al., 2010).

Cyanotoxins: cylindrospermopsins

Cylindrospermopsins (CYNs) are hepatotoxic cyclic alkaloids, first observed in the tropical freshwater cyanobacterium *Cylindrospermopsis raciborskii* in Australia (Hawkins et al., 1985). Subsequent work has demonstrated the presence of these compounds in North and South America, Asia, and Europe (Sivonen & Jones, 1999; Kinnear, 2010). CYN production has also been observed in a number of different cyanobacteria genera including *Anabaena, Aphanizomenon, Umezakia, Raphidiopsis,* and *Lyngbya* (Fastner et al., 2003; Bazin et al., 2010).

CYNs consist of a tricyclic guanidine moiety combined with hydroxymethyluracil with two structural variants: deoxycylindrospermopsin and 7-epicylindrospermopsin. Both structural variants were identified in Australian waters, although only the latter was found to be toxic (Hawkins et al., 1985; Sivonen & Börner, 2008). The major concern with CYNs has been in drinking water-related health problems in Australia, although these events have been relatively isolated (Hawkins et al., 1985; Kinnear, 2010). Despite sharing the cyanobacterial hepatotoxic status of MCs, CYNs do not share the same tumor promoting potential, and they act though the inhibition of protein synthesis (Chong et al., 2002). This inhibition produces toxic effects on organs such as the kidneys, spleen and heart (Falconer et al., 1999; Shaw et al., 2000; Chong et al., 2002; Sivonen & Börner, 2008). Geno-toxic effects have also been observed via the use of cell lines (Bazin et al., 2010).

Cyanotoxins: saxitoxins

Saxitoxins (STXs) are potent neurotoxins originally detected in marine systems as a by-product of some dinoflagellates. STXs can accumulate to high concentrations in shellfish, which has led to instances of human poisoning (Kao & Nishiyama, 1965; Kao, 1966; Anderson et al., 1990). These incidents prompted the development of a detailed chemical characterization of STXs. They are a type of carbamate alkaloid that contains

three rings and can be generally described as a 3,4,6-trialkyl tetrahydropurine (Schantz et al., 1975; Strichartz, 1981). Variants of this general structure include non-sulfated, singly sulfated, or doubly sulfated types (Sivonen & Börner, 2008). SAXs act on the nervous system by altering the current of sodium ion channels, although this inhibition is reversible (Strichartz, 1981). Although there has not been a confirmed instance of human poisoning from consumption of freshwater shellfish, the potential for STXs contamination alone is cause for concern (Sivonen & Jones, 1999). The locations of these reports have been widespread, with STX being identified in freshwaters in North and South America, Europe, Asia, and Oceania (Smith et al., 2011). The genera responsible for the production of STXs in freshwaters are variable, but all are cyanobacteria (Sivonen & Jones 1999; Sivonen & Börner, 2008). Specifically, *Anabaena, Aphanizomenon, Cylindrospermopsis, Planktothrix,* as well as benthic cyanobacteria such as *Lyngbya, Scytonema, and Raphidiopsis,* have been linked to the production of STXs in freshwaters (Sivonen & Jones, 1999; Smith et al., 2011). Relatively few attempts have been made to assess the allelopathic potential of STXs, and existing research has not identified a secondary role for STXs beyond sodium ion channel inhibition (Leflaive & Ten-Hage, 2007; Juhl et al., 2008).

Cyanotoxins: anatoxins

Anatoxin-a is also a neurotoxin that is classified as an alkaloid compound with the general chemical structure 2-acetyl-9-azabicyclo (4-2-1) non-2-ene with two variants (homoanatoxin-a, 4-hydroxyhomoanatoxin-a) (Sivonen & Börner, 2008). This compound acts as a post-synaptic depolarizing neuromuscular blocking agent and is associated with uncontrollable muscle contraction and respiratory paralysis when ingested (Carmichael et al., 1975; Hawkins et al., 2006). Evidence of animal deaths due to anatoxin-a exposure in natural systems, as well as the potent effects observed during laboratory testing, make this toxin especially concerning for those exposed to affected waters (Carmichael et al., 1975; Hawkins et al., 2006; Faassen, 2012). It is thought that anatoxin-a is the most common cyanobacterial neurotoxin on a global scale, with frequent occurrences recorded in North American and European freshwaters (Skulberg et al., 1992; Sivonen & Börner, 2008). Genera recorded as producers of anatoxin-a include

Anabaena, Aphanizomenon, Microcystis, Cylidrospermum, Lyngbya, Planktothrix, Oscillatoria, and *Phormidium* (Sinclair & Hall, 2008; Sivonen & Börner, 2008).

Anatoxin-a (S) shares a similar name to anatoxin-a, although the chemical structure, mode of toxicity, and occurrence in natural systems are all notably different. Anatoxin-a (S) is a phosphate ester of a cyclic N-hydroguanine in contrast to the alkaloid structure of anatoxin-a (Matsunaga et al., 1989). The neurotoxic mechanism of anatoxin-a (S) is that of an anticholinesterase; it inhibits the enzymatic breakdown of acetylcholine allowing it to build up and continue to act as a stimulant in the nervous system (Furchgott & Zawadzki, 1980; Sivonen & Jones, 1999). Despite these potent neurotoxic effects the natural occurrence of anatoxin-a (S) is relatively rare in freshwater systems (Sivonen & Börner, 2008).

Cyanotoxins: β-methylamino-L-amine

β-methylamino-L-amine (BMAA) is a non-protein amino acid that has potent neurotoxic potential if ingested at high concentrations (Sivonen & Börner, 2008). High levels of BMAA exposure, either through dietary or environmental vectors, have been linked to the development of Amyotrophic Lateral Sclerosis or Parkinsonism Dementia Complex and other neurodegenerative diseases (NTP, 2008; Pablo et al., 2009; Esterhuizen-Londt & Downing, 2011). BMAA promotes this type of neurological damage by acting on two key receptors (NMDA and mGluR5), and by inducing oxidative stress (Lobner et al., 2007; Liu et al., 2009). These types of neurological risks are heightened when considering the evidence of BMAA bioaccumulation in aquatic plant and animal life in tropical and temperate systems (Esterhuizen et al., 2011; Esterhuizen-Londt & Downing, 2011; Lurling et al., 2011; Mondo et al., 2012). The demonstrated ability of the many freshwater cyanobacteria – including isolates from the genera *Anabaena, Aphanizomenon, Microcystis, Cylindrospermopsins, Synechococcus, Planktothrix,* and *Nostoc* – to produce BMAA is also of biological and ecological significance given their presence in freshwaters around the globe (Cox et al., 2005).

In contrast to the above findings, some researchers have questioned whether the concern over BMAA exposure is warranted. Some neurological studies have not been able to make the link between BMAA exposure and its hypothesized storage in the

cerebral cortex, raising doubts about its ability to cause the aforementioned diseases (Snyder et al., 2009).

2.4 Noxious (Taste and Odour) Compounds

Noxious (taste and odour) compounds: terpenoids

Terpenoids are produced by a wide variety of freshwater algae and have intense odiferous properties (Watson, 2003). Notable terpenoids produced by freshwater cyanobacteria include geosmin (E-l, l0-dimethyl-E-9-decalol) and 2-methylisoborneol (MIB) (Izaguirre et al., 1982; Juttner et al., 1995; Watson, 2004). These two compounds are responsible for most of the reported taste and odour events in freshwaters, and they can be detected by the human nose at concentrations in the range of parts per trillion (Young et al., 1996). Because traditional forms of water treatment are ineffective in dealing with these compounds their presence can be especially noticeable, although they are not considered to be cytotoxic even at the concentrations observed in hyper-eutrophic systems (Blevins, 1980; Watson, 2003). The ability of these compounds to cause taste and odour events has led to investigation into the potential secondary effects of these compounds and the allelopathic interactions that may be a result of exposure to them. Much of this research has not offered analysis of chronic exposure to these compounds, and has been focused on the acute effects of these compounds via bioassays (Dionigi et al., 1993; Nakajima et al., 1996; Mochida, 2009; Srinivasin & Sorial, 2011).

Nor-carotenoids are a sub-set of terpenoids that occur in freshwaters and are also potent odour-causing compounds (Juttner, 1984, 1986). Their relatively limited occurrence in natural systems may help explain why these compounds collectively receive far less attention than the related geosmin and MIB (Watson, 2003). The most abundant nor-carotenoid in freshwaters is β-cyclocitral, a compound produced by cyanobacteria genera such as *Microcystis* and *Aphanizomenon* (Watson, 2003, 2004). Despite some evidence that nor-carotenoids such as β-cyclocitral have allelopathic effects on neighboring algae, the concentrations required for these effects are far above any observed maximum in natural systems (Ikawa, 2001).

Noxious (taste and odour) compounds: poly-unsaturated fatty acids

The presence of poly-unsaturated fatty acids (PUFAs) in freshwater algae has been repeatedly linked to the overall health of food webs and ecosystems (Ahlgren et al., 1992; Brett & Muller-Navarra, 1997). Despite these benefits, PUFAs are associated with the occurrence of taste and odour events both directly (by their presence in a system) and indirectly (by their role as precursor materials for various PUFA derivatives) (Watson, 2003, 2008). Counter to the common terpenoid compounds many PUFAs are not easily detectable by olfaction in contaminated waters but their presence is much more pronounced in terms of the potential biological, ecological, and allelopathic effects of these substances. PUFAs produced by both cyanobacteria and chrysophyte algae have can act as pheromones and defense mechanisms. Evidence for direct cytotoxic effects is generally lacking (Watson et al., 2001; Watson, 2003, 2004). PUFA derivatives such as alcohols, alkanes, esters, aldehydes, and ketones, are often found to be the root of taste and odour events. Nonadienals, heptadienals, octadienals, decadienals, and decatrienals are the common derivatives produced by freshwater chrysophyte algae, which generally emit an odour described as 'fishy' (Nicholls, 1995; Watson et al., 2001; Watson 2003). Chrysophyte genera such as *Synura, Dinobryon,* and *Uroglena* are most commonly associated with the production of these compounds, yet detailed work examining the secondary effects of these compounds has not been completed at this time (Nicholls, 1995; Watson, 2004).

Noxious (taste and odour) compounds: sulfurous compounds

Sulfurous compounds are a lesser-studied group of taste and odour causing metabolites most likely due to their relatively low abundance in natural systems compared to terpenoids and PUFAs (Watson, 2003). Common examples observed in freshwater systems include: methyl disulfide, dimethyl trisulfide, di-isopropyl disulfide, and di-isopropyl trisulfide, as well as compounds such as methanethiol, ethanethiol, propanethiol, and *t*-butythoil (Juttner, 1984; Hofbauer & Juttner, 1988, Watson, 2004). These compounds can be produced by freshwater cyanobacteria and green algae, although the biological role of the sulfur-containing compounds remains unclear.

Hofbauer and Juttner (1988) suggest that sulfur-containing compounds serve as chemical markers used by algae for identification purposes, but this hypothesis has not been further investigated.

Noxious (taste and odour) compounds: amines and pyrazines

Although relatively few amine- or pyrazine-based compounds have been characterized some of the compounds in these classes produce potent taste and odours (Young et al., 1996; Watson, 2004). Most notable are 2-isobutyl-3-methoxypyrazine and 2-isopropyl-3-methoxypyrazine, which give off highly potent earthy odours (Watson, 2004). These two groups are by far the least studied of the taste and odour causing compounds, and additional work needs to be done to improve the understanding of these compounds.

2.5 Bioactive Compounds

Bioactive compounds: cyanopeptolins

Cyanopeptolins are a sub-class of cyclic desipeptides (Gademann et al., 2010). The distinguishing feature of cyanopeptolins is that they are produced by freshwater cyanobacteria and contain a unique 3-amino-6-hydroxy-2-piperidone (Ahp) residue (Sivonen & Börner, 2008; Gademann et al., 2010). The cyanopeptolins are inclusive of a wide variety of compounds including micropeptins, microstylins, aeruginopeptins, and anabaenopeptilides. Cyanopeptolins are produced by the cyanobacteria genera *Anabaena*, *Microcystis*, *Nostoc*, and *Planktothrix*, and are generally recognized as serine or trypsin protease inhibitors (Weckesser et al., 1996; Harada et al., 2001; Grach-Pogrebinsky & Carmeli, 2008; Sivonen & Börner, 2008). Cyanopeptolins are also capable of having acute toxic effects on crustaceans although these effects are not produced by exposure to every compound of this group (Grach-Pogrebinsky & Carmeli, 2008; Gademann et al., 2010).

Bioactive compounds: hexapeptides

Similar to cyanopeptolins, but containing D-lysine and an uriedo linkage, hexapeptides are a sub-class of desipeptides produced by a range of cyanobacteria (Harada et al., 1995; Sivonen & Börner, 2008). Feritoic acids, anabaenopeptolins, and oscillamides all fall under this category (Williams et al., 1996). In freshwaters hexapeptides are produced by *Anabaena, Aphanizomenon, Microcystis, Nodularia*, and *Planktothrix*, and have the capacity to act as non-specific protease inhibitors, suggesting the ability to have similar biological impacts as cyanopeptolins or other protease inhibiting molecules (Sivonen & Börner, 2008).

Bioactive compounds: microviridins, microginins, and aeruginosins

The tricyclic desipeptide structure of microviridins and its production by *Nostoc sp.* are the major distinguishing factors from microginins and aeruginosins, which are both linear peptides (Sivonen & Börner, 2008). All three classes of compounds are produced by isolates of the cyanobacteria genera *Microcystis* and *Planktothrix*, although the toxicological properties of the three vary drastically (Fujii et al., 1997; Welker & von Dohren, 2006). Microviridins and aeruginosins are capable of causing cytotoxic responses, via protease inhibition, while the role of microginins has yet to be identified (Rohrlack et al., 2004; Sivonen & Börner, 2008). Microviridins exhibit toxic effects to zooplankton *in vitro*, and this result has served as an indicator of the toxic and/or bioactive potential of these compounds (Rohrlack et al., 2004).

2.6 Current Toxin Detection Methods

A wide selection of techniques and methodologies are employed to detect and quantify toxic, noxious, and bioactive compounds produced by freshwater algae. Common methods used for toxin analysis include: enzyme linked immunosorbent assays (ELISAs), protein phosphatase inhibition assays (PPIAs), 2-methoxy-1- methyl-3-phenylbuturic acid (MMPB), nuclear magnetic resonance (NMR), solid-phase micro-extraction-mass spectrometry (SPME-MS), high performance liquid chromatography (HPLC), thin layer chromatography (TLC), matrix assisted laser desorption/ionization-

time-of-flight-mass spectrometry (MALDI-TOF-MS), and liquid chromatography with electrospray ionization triple-quadrupole mass spectrometry (LC-MS) (Demirel & Sukatar, 2012). Despite these techniques and instruments the data available concerning the safe limits for acute and chronic exposure to individual compounds are generally lacking (Sivonen & Börner, 2008). The only available limit regarding exposure to the toxins produced by freshwater algae is set by the WHO for microcystin exposure. This guideline stipulates that total (intracellular + extracellular) MC-LR concentrations should not exceed 1.0 $\mu g\ l^{-1}$ in waters used for human consumption (WHO, 2003). This guideline does not include the other 86 variants of microcystin that have been identified and can be present in natural systems at concentrations higher than the LR variant (WHO, 2003; del Campo et al., 2010).

It is difficult to quantify the relationships among multiple compounds when they occur in complex mixtures. These effects can be additive, synergistic, or antagonistic, and establishing methods of analysis that elucidate the effects of these types of mixtures is the key to evaluating the true toxic and/or noxious potential of freshwater samples (Watson, 2003). Whole organism bioassays are useful but limited. The state of the art in whole-organism bioassay development for freshwater analysis is the 96-hour rainbow trout acute lethality test (Dayeh et al., 2003). While a good indicator of water quality for a wide range of tested substances, the inherent limitations of this assay include the inevitable (and difficult to assess) stress response of the organisms involved, the large volume of sample and the amount of time required to run the test, the sacrifice of organisms that perish during the test, and the cost of organism husbandry and storage for the period leading up to the trial (Dayeh et al., 2005). All of these factors have led some researchers to consider new, cost effective and reliable assays to avoid some of the pitfalls of the traditional whole-organism testing while maintaining the ability to evaluate samples that contain complex mixtures of toxic, noxious, and/or bioactive substances (Fent, 2001).

2.7 Alternative Toxin Detection Methods: Cell-Based Assays

Cell-based assays are the primary alternative to using of whole-organisms in freshwater testing and have been gaining popularity due to the ever increasing availability

of cell lines derived from a variety of organisms (Fent, 2001). Recent research has investigated the ability of animal cell lines to experience the cytotoxic effects of compounds produced by marine phytoplankton isolates, and this work has illustrated the potential for adapting this assay to analyze compounds produced by freshwater phytoplankton as well (Burkholder et al., 2005; Dorantes-Aranda et al., 2011). Specifically, the RTgill-W1 cell line cytotoxicity assay (RCA) has been used in conjunction with various *in vitro* and *in vivo* methods to assess a wide range of environmental pollutants and toxicants (Lee et al., 2009). Adapting the assay to investigate polycyclic aromatic hydrocarbons, industrial effluents, petrochemicals, jellyfish venom and many other compounds occurring in both freshwater and marine systems has made it a prominent candidate for use in freshwater research (Schirmer et al., 1998, 2001; Dayeh et al., 2005; Helmholz et al., 2010). The advantages of using the RTgill-W1 cell line rather than commercially available mammalian cell lines include the ability to conduct trials at room temperature (~20 °C), the tendency of fish cells lines to tolerate prolonged exposure, the availability of fluorescent dyes that yield multiple endpoints for a single trial, and its ability to serve as an appropriate substitute for the whole organisms used to test whole-water samples in 96-hour rainbow trout acute lethality tests (Dayeh et al., 2005).

Another cell-based assay of interest is the erythrocyte lysis assay (ELA). The ELA has also been used to examine the effects of a wide variety of compounds including the hemolytic activity of marine phytoplankton, bacteria, invertebrates, and higher plants (Eschbach et al., 2001; Ling & Trick, 2010). The ELA has been used in cyanobacteria bioactive compound analysis, highlighted by investigations into the hemolytic compounds produced by *Anabaena variabilis* (Wang et al., 2007; Zhang et al., 2011). The ELA determines the cytotoxicity/hemolytic activity of a sample through the evaluation of the integrity of the erythrocyte membrane by photometry, assessing the quantity of heme released from lysed erythrocytes following exposure (Eschbach et al., 2001).

The RCA and the ELA are prime examples of assays that could aid in the in the detection of toxic, noxious, and bioactive compounds in freshwaters.

2.8 Conclusions

The presence of toxic and noxious compounds in freshwater systems around the world is certainly cause for concern and is an issue worthy of additional research. The potent nature of the compounds produced by cyanobacteria and chrysophyte algae illustrate the need for developing a better means of understanding and predicting the effects of these compounds. The variety of compounds produced by freshwater cyanobacteria and chrysophyte algae makes this task extremely difficult if one relies on traditional methods of single compound identification. The majority of current methods neglect the potential additive, synergistic, or antagonistic effects that compounds may have in mixtures. The current state of toxin and noxious compound identification and quantification in freshwater systems may be greatly improved through the application of cell-based assays that can be used to fill in some of the gaps in modern assessment techniques.

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Chapter 3

Application of Cytotoxicity Assays for Detection of Potentially Harmful Bioactive Compounds Produced by Freshwater Bloom-Forming Algae

3.1 Introduction

Algal blooms are marked by significant increases in the population of pelagic algae resulting in the aesthetic, odorous, and/or biochemical fouling of surface waters (Reynolds & Walsby, 1975). Harmful algal blooms (HABs) are considered a sub-category of these events and are distinguished by the presence of particular taxa that have the ability to produce toxins, irritants, and/or noxious secondary metabolites (Carmichael, 1992, 2001; Watson, 2003; Watson et al., 2008). Freshwater HABs can have serious ecological, toxicological, and physiological effects on aquatic and terrestrial biota, and increased occurrence of these events over the past three decades is cause for concern (Skulberg et al., 1984; MacKintosh et al., 1990; Pouria et al., 1998; Falconer, 1999; Carmichael, 2001, 2008; Paerl et al., 2001; Sinclair & Hall, 2008; Fortin et al., 2010; Winter et al., 2011; Huber et al., 2012).

Blooms of high biomass are commonly attributed to excess nutrients entering the water body (Heisler et al., 2008; Paerl & Huisman, 2008). HABs are formed when these nutrients are preferentially consumed by harmful algal species that accumulate in biomass (Reynolds & Walsby, 1975; Paerl, 1988; Wetzel, 2001). Despite the multiple factors that promote HAB formation, accelerated eutrophication of surface waters by human inputs is the leading cause of HABs in freshwater systems (Schindler, 1987; Wetzel 2001; Smith, 2003; Christensen et al., 2006). Particularly of concern is the loading of macronutrients with phosphorus and nitrogen, which have been implicated as the key nutrients for bloom development (Schindler, 1977; Tett et al., 1985; Fisher et al., 1992; Guildford & Hecky, 2000).

Additional work has illustrated the importance of micronutrients in phytoplankton growth (Klausmeier et al., 2004; Molot et al., 2010; Fujii et al., 2011; Sorichetti et al., submitted). Specifically, Fe has been identified as a critical micronutrient for bloom

development and maintenance because it is required for essential cellular processes such as photosynthesis, pigment biosynthesis and (in some cyanobacteria) nitrogen fixation (Guikema & Sherman, 1983; Rueter & Ades, 1987; Paerl, 1988; Raven et al., 1999; Sterner et al., 2004). Fe has also been identified as a regulator of microcystin (potent cyanobacterial hepatotoxin) production (Kaplan et al., 2012). However, unlike most cellular functions the production of microcystin increases under conditions of Fe limitation (Utkilen & Gjølme, 1995; Alexova et al., 2011; Kaplan et al., 2012).

Cyanobacteria are the most common type of algae associated with freshwater HABs, and the most common genera are *Microcystis, Anabaena, Aphanizomenon,* and *Gloeotrichia,* all of which have the potential to produce toxins, noxious, and/or bioactive compounds (Skulberg et al., 1984; Carmichael, 2001; Paerl et al., 2001; Watson et al., 2008; Molot et al., 2010; Winter et al., 2011; Sorichetti et al., submitted). The incidence of blooms dominated by chrysophyte algae may also be on the rise (Paterson et al., 2004, 2008; Winter et al., 2011). Chrysophyte genera that are commonly observed dominating bloom events include *Dinobryon, Synura, Uroglena,* and *Mallomonas* (Nicholls, 1995; Watson, 2003; Paterson et al., 2004, 2008). While chrysophyte algae common to freshwaters do not produce toxins, they are infamous for their ability to produce a variety of noxious compounds that act as irritants or offensive taste and odour causing substances (Jüttner et al., 1986; Nicholls, 1995; Watson et al., 1999, 2008; Paterson et al., 2004; Winter et al., 2011). Collectively, the noxious compounds produced by chrysophyte algae are termed taste and odour compounds and are easily recognized by their ability to foul water bodies at even at very low concentrations (<10 nM) (Watson et al., 1999; Chorus, 2000; Watson, 2003).

This study targets the need for quick, reliable and cost effective tools to detect and evaluate the cytotoxicity of freshwater samples that may be contaminated with toxic, noxious, and/or bioactive phytoplankton metabolites. In 2008, Environment Canada released a report addressing the increase in HAB occurrence (Charlton et al., 2008). Three important research needs were outlined in this report, including: improving detection, characterization, and modeling of toxic and noxious algal metabolites produced during HABs (Charlton et al., 2008). The adaptation of existing cell-based assays to quantify the toxicity of whole-water samples is one way to improve detection, and may

offer a solution to the first step in the line of research needs. The main advantages of cell-based assays include: the low volume of sample required for analysis, the large number of samples that can be tested, the rapid exposure time, ease of sample preparation, and the avoidance of sacrificing whole organisms (Dayeh et al., 2005).

Recent research has investigated the ability of animal cell lines to detect the cytotoxic effects of compounds produced by marine phytoplankton isolates, and this work has illustrated the potential for the adaptation of these assays to analyze compounds produced by freshwater phytoplankton as well (Burkholder et al., 2005; Dorantes-Aranda et al., 2011). Specifically, the RTgill-W1 cell line cytotoxicity assay (RCA) has been used in conjunction with various *in vitro* and *in vivo* methods of analysis to assess a wide range of environmental pollutants and toxicants (Lee et al., 2009). The adaptation of the assay for investigations into polycyclic aromatic hydrocarbons, industrial effluents, petrochemicals, jellyfish venom and many other compounds occurring in both freshwater and marine systems has made it a prominent candidate for further use in freshwater research (Schirmer et al., 1998, 2001; Dayeh et al., 2005; Helmholz et al., 2010). The advantages of using the RTgill-W1 cell line rather than other commercially available mammalian cell lines include: the ability to conduct trials at room temperature (~20 °C), the tendency of fish cells lines to tolerate prolonged exposure to simple exposure medium, the availability of fluorescent dyes that can yield multiple endpoints for a single trial, and the ability to serve as an appropriate substitute for the whole organisms used to test whole-water samples in 96-hour rainbow trout acute lethality tests (Dayeh et al., 2005). For this study, the RCA was used with a resazurin-based dye, which served as an indicator of cellular metabolic activity.

Another cell-based assay of interest is the erythrocyte lysis assay (ELA). The ELA has also been used to examine the effects of a wide variety of compounds including the hemolytic activity of marine phytoplankton, bacteria, invertebrates, and higher plants (Eschbach et al., 2001; Ling & Trick, 2010). This work can be used in cyanobacteria bioactive compound analysis, highlighted by investigations into the hemolytic compounds produced by *Anabaena variabilis*, suggesting that the ELA may be suitable for further use in freshwater algae investigations (Wang et al., 2007; Zhang et al., 2011). The ELA determines the cytotoxicity/hemolytic activity of a sample by photometrically

assessing the quantity of heme released from lysed erythrocytes following exposure, which evaluates the integrity of the erythrocyte membrane (Eschbach et al., 2001).

The effectiveness of these two cytotoxicity assays were assessed through applying individual analytical standards of toxins and noxious compounds produced by freshwater algae, and analyzing of complex mixtures of metabolites produced by freshwater algae isolates – including both toxic and/or noxious compound producing isolates of cyanobacteria and chrysophyte algae. The following hypotheses were tested:

H1: The application of individual analytical standards of toxins and/or noxious compounds produced by freshwater algae will result in concentration dependent decreases in the viability of cells in both the RCA (assessing damage to cell metabolism) and the ELA (assessing damage to cell membrane). I predict that concentrations of these compounds at or below their environmentally measured averages will result in significant decreases in cell viability in both assays.

H2: The application of complex mixtures of algal metabolites extracted from cultures of toxic and/or noxious compound producing isolates of cyanobacteria and chrysophyte algae will result in concentration dependent decreases in viability as part of the RCA and ELA. I predict that (a) due to the increased rate of cell growth and toxic, noxious, and/or bioactive compound production during exponential phase, exposure solutions derived from exponential growth phases will result in significant decreases in cell viability in both assays relative to those of the stationary phase; (b) due to the predominant intracellular storage of toxic and noxious compounds, exposure to solutions derived from lysed cultures (intracellular + extracellular compounds) will result in significant decreases in cell viability in both assays relative to solutions derived from non-lysed samples (extracellular compounds only); and (c) due to toxin production stimulated by Fe limitation of toxin producing cyanobacteria, exposure solutions extracted from low Fe (0.1 μM) treatments will result in significant decreases in cell viability in both assays relative to the high Fe (10 μM) treatments.

The adaptation of cytotoxicity assays for detecting toxins, noxious, and/or bioactive compounds produced by cyanobacteria and chrysophyte algae will allow us to gain insights into the additive, synergistic and/or antagonistic biological effects that these

compounds may exhibit while produced in the complex mixtures by different isolates of cyanobacteria and chrysophyte algae (Dayeh et al., 2005).

3.2 Methods

RTgill-W1 cell line

The RTgill-W1, obtained from the American Type Culture Collection (ATCC CRL-2523), is a continuous cell line derived from gill explants of a healthy rainbow trout (*Onchorhunchus mykiss*) (Bols et al., 1994). The cell line culture was maintained in the dark at 20 °C in sterile, plug sealed, tissue culture treated (coating within the flask that promotes cell growth) flasks. Small flasks (culture area: 25 cm^2) were used for initial sub-culturing from cryopreserved samples, and large flasks (culture area: 75 cm^2) were used for the subsequent sub-cultures (353109/353110, BD Biosciences). This sub-culturing sequence ensured that an ample number of cells were available for use in multiple assays simultaneously. The cells were grown in 0.2 μm filter-sterilized L-15 Complete (Leibovitz's L-15 medium (MT10-045-CV Mediatech); 10% (v/v) fetal bovine serum (FBS, A15-204, PAA Laboratories); 2% (v/v) antibiotic/antimycotic solution (17-745E, Lonza)), which was renewed (old media removed and replaced with fresh media) twice weekly. Sub-culturing and/or cell cultivation was carried out once a confluent monolayer of cells was observed over the majority of growth surface via inverted microscopy (Axiovert 100 TV, Zeiss). Cell detachment was achieved by applying a phosphate buffered saline (PBS)-0.53 mM EDTA solution rinse (1 minute) followed by incubating (5 minute) with 0.25% Trypsin-2.21 mM EDTA in Hank's balanced salt solution (CA45000-664, VWR Canada). Cells were collected and concentrated by centrifugation at 200 × *g* for 5 minutes. The sub-culturing ratio was commonly 1:4, although this varied according to the degree of confluence observed prior to detachment. All solutions and equipment were sterilized via autoclave, 70% ethanol, or 0.2 μm filter prior to use, and handling occurred in a laminar flow hood with the appropriate aseptic techniques being employed to maintain the sterility of the culture (Dayeh et al., 2003, 2005).

Cryopreservation of RTgill-W1 cells

Cryopreservation of the RTgill-W1 cell line proved to be important to long-term cell storage, which allowed for a minimization of the use of consumables, the risk of microbial contamination, and the risk of genetic drift/morphological changes (Sigma-Aldrich, 2010). Cryopreservation also allowed me to conduct experiments using cells at a consistent passage number. Cells were cryopreserved by suspending cell solutions in L-15 Complete-5% (v/v) dimethylsulfoxide (DMSO) medium at a density of 10^6 cells mL^{-1}. Aliquots (1 mL) were pipetted into 2 mL polypropylene Cryule® vials (985746, Wheaton) which were immediately sealed. A -1 °C $minute^{-1}$ 'Mr. Frosty' freezing container (5100-0001, Thermo Fischer Scientific Inc.) filled with isopropyl alcohol was used to hold the vials, and was immediately placed into a -80 °C freezer. Cells remained viable in the freezer at least 1 year after the initial freezing event, as evidenced by successful culturing of cryopreserved cells (data not shown). Cryopreservation in liquid nitrogen was also successful when coupled with the use of an isopropyl alcohol bath to bring the temperature of the samples down to -80 °C prior to insertion into the liquid nitrogen dewar.

RTgill-W1 cell line contamination test

The absence of culture contamination by mycoplasma was confirmed through the use of a MycoAlert™ assay kit (LT07-118, Lonza). A small aliquot (1 mL) taken from the supernatant after the cell suspension was centrifuged at 200 × *g* for 5 minutes. The sample was combined with a buffer solution and pipetted into a 96-multiwell plate (MWP) in triplicate. Positive and negative control solutions were loaded into the plate, and all wells were analyzed by a fluorescence spectrophotometer (Cary Eclipse, Varian) with a multi-well plate attachment set to read chemi/bio-luminescence from 540 nm to 700 nm.

RCA experimental design

The RCAs were conducted in 96-MWPs. Cell concentrations were adjusted to density of 1.5 $x10^5$ cells mL^{-1} in L-15 Complete medium using a haemocytometer. Aliquots (200 µL) of the cell solution were transferred into the wells of a sterile, tissue

culture treated 96-MWP (353075, BD Biosciences). Not all wells of the 96-MWP were seeded with the cell suspension, as preliminary work suggested that the wells on the outer edge of the 96-MWP were not all suitable for use (data not shown). Seeded plates were immediately covered with a lid, wrapped with Parafilm M®, and incubated in the dark at 20 °C until a confluent monolayer was observed in each well via microscopy (2-3 days). The plates were moved into the laminar flow hood and inverted over a waste basin to drain the L-15 complete media overlying the monolayer at the bottom of each seeded well. Plates were kept upside down and placed onto a stack of paper towels to drain any remaining media. A rinse of PBS (200 µL) was then applied to all wells, followed by another inversion over the catch basin and drying on paper towel. Aliquots (200 µL) of the desired exposure solutions, controls, and blanks were loaded into the appropriate wells of the 96-MWP, and silicone sealing-mats (521-01-151, Axygen) were used to prevent interactions between wells. The plates were wrapped in Parafilm M® to limit evaporative loss, and inverted microscopy was used to ensure that no cells had become detached during the rinse and application steps. Exposure was carried out in the dark at 20 °C (Dayeh et al., 2003, 2005).

Measuring cell viability

PrestoBlue™ (A-13262, Life Technologies), a resazurin-based compound that uses the reducing environment of cells to measure metabolic activity, was used in place of AlamarBlue® to quantitatively assess cell viability (Dayeh et al., 2003; Dorantes-Aranda et al., 2011). Following removal of exposure solutions (via inversion, as described above) and a rinse with PBS (200 µL), aliquots of 5% (v/v) PrestoBlue™-L-15/ex solution (100 µL) were applied to all wells. The plate was wrapped in Parafilm M® and left to incubate in the dark at 20 °C for 2 hours. The plate was read by a fluorescence spectrophotometer (Cary Eclipse, Varian) with multi-well plate attachment set to read excitation/emission of 540/590 nm. Raw fluorescence (FU) was converted into cell viability (% of control) by using the formula outlined by Dayeh et al. (2003; Equation 3.1). To test for significant differences one-way ANOVA was used followed by Dunnett's test (α=0.05). When applicable, EC_{50} values were calculated for each compound (Alexander et al., 1998).

$$RCA\ Viability\ (\%) = \left(\frac{(\text{Exp. FU} - \text{Blank FU})}{\text{average}(\text{Neg. Control FU} - \text{Blank FU})}\right) \times 100\% \quad (3.1)$$

Erythrocyte Lysis Assay (ELA)

Rabbit blood in Alsever's solution was obtained from Inverness Medical Canada (QSRBA25) and stored in the dark at 4 °C. Cells were resuspended by hand at least twice weekly (they settle out of solution if left undisturbed), and erythrocyte density was determined through the use of a haemocytometer. Handling the blood at 4 °C rather than at room temperature aided in keeping the blood viable for longer (Inverness Medical, 2012). The decision to use rabbit erythrocytes in Alsever's solution was made on the basis of their commercial availability, ease of storage, and their history as a sensitive erythrocyte for detecting algal hemolysins (Kuroda et al., 2005; Wang et al., 2007; Ling & Trick, 2010; Zhang et al., 2011).

ELA experimental design

Erythrocytes were rinsed three times with ELA buffer in a 1.7 mL microcentrifuge tube (311-05-051, Axygen) by inversion and centrifugation at 2000 × *g* at 4 °C. Washed erythrocytes were diluted to a concentration of 10^7 cells mL^{-1} and used immediately (Eschbach et al., 2001; Ling & Trick, 2010). Equal parts erythrocyte solution (500 µL) and exposure solution (500 µL) were mixed in a 1.7 mL microcentrifuge tube and incubated at 20 °C for 4 hours. The data were converted from raw absorbance values into percent viability through the use of the formula similar to that proposed by Ling and Trick (2010; Equation 3.2). Significant differences were assessed using a one-way ANOVA followed by Dunnett's test (α=0.05).

$$ELA\ Viability\ (\%) = \left(1 - \frac{(\text{Exp. Abs 414 nm} - \text{Neg. Control Abs 414 nm})}{(\text{Pos. Control Abs 414 nm} - \text{Neg. Control Abs 414 nm})}\right) \times 100\% \quad (3.2)$$

Photometric profile of erythrocytes

A photometric profile of the erythrocytes was conducted to confirm that the optimal wavelength was being used to assess erythrocyte lysis. Whole erythrocytes in ELA buffer were prepared at a concentration of 5×10^6 cells mL^{-1}, while a solution of equal cell density was sonicated with a Virsonic 100 ultrasonic cell disrupter (SP Scientific) at a continuous power output of 10 for 20 seconds in an ice bath to provide the lysed sample. Absorbance scans of 350 nm to 700 nm were done with an integrated scanning DU 640 spectrophotometer (Beckman Coulter).

Erythrocyte fragility test

Erythrocyte fragility tests were performed weekly to evaluate the integrity of the erythrocytes over the course of their storage. Prior to each fragility test fresh stocks of saponin, originally isolated from tree bark from *Quillaja saponaria* (8047-15-2, Sigma) in ELA buffer were made to concentrations of 0-10, 15, 20 and 50 μg mL^{-1}. The solutions were mixed 1:1 with a 10^7 cells mL^{-1} erythrocyte solution in 1.7 mL micro-centrifuge tubes and incubated in the dark at 20 °C for 2 hours.

Toxins and noxious compounds tests

Thirteen compounds were selected for analysis by both cell-based assays (Table 3.1). Analytical standards of nodularin and 7 microcystin variants (LA, LF, LR, LW, LY, RR, and YR) (ALX-850-325-KI01, Alexis Biochemicals) were obtained and stored in the dark at -20 °C. Analytical grade solutions of (E,E)-2,4-decadienal (W313505, Sigma), (E,E)-2,4-heptadienal (W316407, Sigma), and β-cyclocitral (2,6,6-Trimethyl-1-cyclohexene-1-carboxaldehyde; W363928, Sigma), (±)-geosmin (G5908, Sigma) and 2-methylisoborneol (743364, Sigma) were obtained and stored in the dark at 4 °C. Exposure solutions consisted of single compounds dissolved in L-15/ex or ELA buffer solution, for use in the RCA or ELA, respectively (Eschbach et al., 2001; Dayeh et al., 2003).

Algal cultures

Nine phytoplankton isolates were obtained from the Canadian Phycological Culture Collection (CPCC) in Waterloo, ON, Canada (Table 3.2). Four chrysophyte isolates, *Dinobryon sp*. isolate 392, *Synura uvella* isolate 422, *Synura petersenii* isolate 495, and *Uroglena sp*. isolate 276 were selected to be used as representative isolates of phytoplankton species that cause the taste and odour bloom events (Nichols et al., 1995; Watson and Satchwill, 2003; Paterson et al., 2008). These cultures were isolated from small ponds in Maine, USA, and Newfoundland, Canada. Five cyanobacteria isolates, including *Anabaena flos-aquae* isolates 64 and 543, *Aphanizomenon schindlerii* isolate 631, and *Microcystis aeruginosa* isolates 124 and 299, were used as representative isolates of phytoplankton which produce freshwater HABs (Molot et al., 2010; Winter et al., 2011; Sorichetti et al., submitted). These isolates were obtained from lakes in Ontario, Saskatchewan, Manitoba, Ontario, and Alberta, Canada, respectively. Two isolates, *A. flos-aquae* 64 and *M. aeruginosa* 299, are confirmed microcystin producers. Maximum growth rates were calculated according to MacIntyre & Cullen (2005), by examining the exponential phase growth of each isolate. These data were used to calculate the optimum flow rate of the chemostat runs for each strain of cyanobacteria as described below.

Algal medium

Cyanobacteria isolates required BG-11 medium, which was prepared using ultrapure water (Barnstead Nanopure Infinity Ultrapure Water System), and adjusted to a pH of 7.5 (Andersen et al., 2005). BG-11 medium was then microwave-sterilized on 'High' (1000 W) power twice for 10 minutes prior to use, and stored at 4 °C (Andersen et al., 2005). BG-11 medium was warmed to room temperature (~20 °C) prior to addition to any culture. Chrysophyte isolates required WC growth medium, which was prepared using ultrapure water, pH adjusted to 7.8, microwave sterilized on 'High' power twice for 10 minutes prior to use, and stored at 4 °C (Andersen et al., 2005). Each isolate was grown in a low (0.1 μM) and high (10 μM) Fe medium that was prepared trace metal clean and stored in acid washed (soaked in 10% HCl for ≥ 24 hours) containers. Major nutrient additions were passed through a Chelex® 100 ion exchange resin (C7901,

Table 3.1. Chemical information and associated hazards of toxins and noxious compounds.

Compound	Chemical Formula	Molecular Weight	Associated Hazards
Microcystin-LA	$C_{46}H_{67}N_7O_{12}$	910.0	Hepatotoxic; skin, eye irritant
Microcystin-LF	$C_{52}H_{71}N_7O_{12}$	986.2	Hepatotoxic; skin, eye irritant
Microcystin-LR	$C_{49}H_{74}N_{10}O_{12}$	995.2	Hepatotoxic; skin, eye irritant
Microcystin-LW	$C_{54}H_{72}N_8O_{12}$	1025.2	Hepatotoxic; skin, eye irritant
Microcystin-LY	$C_{52}H_{71}N_7O_{13}$	1002.2	Hepatotoxic; skin, eye irritant
Microcystin-RR	$C_{49}H_{75}N_{13}O_{12}$	1038.2	Hepatotoxic; skin, eye irritant
Microcystin-YR	$C_{52}H_{72}N_{10}O_{13}$	1045.2	Hepatotoxic; skin, eye irritant
Nodularin	$C_{41}H_{60}N_8O_{10}$	825.0	Hepatotoxic; skin, eye irritant
(*E,E*)-2,4-Decadienal	$C_{10}H_{16}O$	152.2	Skin, eye irritant; fatty/citrus odour
(E,E)-2,4-Heptadienal	$C_7H_{10}O$	110.2	Skin, eye irritant; rancid fish odour
β-Cyclocitral	$C_{10}H_{16}O$	152.2	Skin, eye irritant; sweet tobacco odour
2-Methylisoborneol	$C_{11}H_{20}O$	168.3	Skin, eye irritant; musty odour
Geosmin	$C_{12}H_{22}O$	182.3	Skin, eye irritant; earthy odour

Table 3.2. Cyanobacteria and chrysophyte isolates obtained from the Canadian Phycological Culture Collection (CPCC) in Waterloo, ON.

Organism	Strain	Isolation location	Year of isolation
Cyanobacteria			
Anabaena flos-aquae	CPCC 64	Lake Ontario, ON, Canada	1987
Anabaena flos-aquae	CPCC 543	Burton Lake, SK, Canada	2001
Aphanizomenon schindlerii	CPCC 631	Experimental Lakes Area, ON, Canada	2005
Microcystis aeruginosa	CPCC 124	Heart Lake, ON, Canada	1987
Microcystis aeruginosa	CPCC 299	Pretzlaff Pond, AB, Canada	1990
Chrysophyceae			
Dinobryon sp.	CPCC 392	Sippewisset, MA, USA	1986
Synura petersenii	CPCC 495	Hwy 410, NL, Canada	1982
Synura uvella	CPCC 422	Arrowwood Lake, ND, USA	1995
Uroglena sp.	CPCC 276	Dickie Lake, ON, Canada	1991

Sigma) to remove excess trace metals prior to addition to the medium (Price et al. 1988/1989).

Measurement of algal growth

Optical density, calculated by measuring absorbance at 720 nm with a DU 640 spectrophotometer (Beckman Coulter), is an accurate measure of phytoplankton density (Held, 2011). Using optical density as a proxy for the biomass of samples allowed me to bypass performing cell counts on isolates which are filamentous and/or colonial, such as *A. flos-aquae* isolates 64 and 543, *Apha. schindlerii* isolate 631, *Syunra petersenii* isolate 495, and *Dinobryon sp*. isolate 392.

Cyanobacteria growth specifications

For exponential phase experiments, a continuous culture apparatus was used to maintain cultures at a constant growth rate with a fixed/known concentration of nutrients in the medium (Monod, 1949; Novick & Szilard, 1950). The chemostat apparatus was supported by a custom designed structure (Appendix A). Modified aquaria (25 cm × 50 cm × 30 cm) were placed ~15 cm from a side-mounted fluorescent light source. Aquaria were angled to reduce the incoming flux of light to 70 ± 10 μmol photons m^{-2} s^{-1} in each of the culture tubes. The tanks were filled near to the top with tap water, and wall-mounted heaters maintained a constant water temperature of 24 °C. Glass culture tubes (250 mL) were arranged haphazardly, suspended vertically in each tank, and capped with a modified rubber stopper to keep out ambient dust. An adjustable electric pump provided sterile media to the culture tubes, allowing the growth rate of each culture to be controlled via dilution (MacIntyre & Cullen, 2005). All parts of the chemostat apparatus were acid-washed, rinsed in ultrapure water, and ethanol-sterilized prior to inoculation.

For stationary phase experiments, all isolates were maintained in a batch culture. Each of the cyanobacteria isolates was grown in BG-11 medium at high (10 μM) and low (0.1 μM) Fe concentrations (prepared as described above) in 500 mL glass Erlenmeyer flasks. All flasks were acid-washed, rinsed in ultrapure water, and ethanol-sterilized prior to inoculation. All cyanobacteria cultures were grown at 24 °C under constant light conditions of 70 ± 10 μmol photons m^{-2} s^{-1}.

Following termination of experiments, cyanobacteria samples were stored in trace metal-cleaned bottles in the dark at -20 °C. These samples were kept in storage until immediately before application to both assays when they were thawed to room temperature. The cyanobacteria isolates proved to be resistant to lysis during freezing, which made this storage method ideal for the preservation of cells and bioactive compounds (data not shown) (Furtula et al., 2004; Kim et al., 2009).

Chrysophytes growth conditions

Chrysophyte cultures were grown under light conditions consisting of a 12 hour photoperiod and an irradiance of 20±5 μmol photons m^{-2} s^{-1}. Three of the chrysophyte isolates were grown at a temperature of 20 °C, while *Syunra petersenii* isolate 495 was maintained at 10 °C. Exposure of *Syunra petersenii* isolate 495 to higher temperatures (20 °C) resulted in degradation of the culture (data not shown). For exponential and stationary phase experiments, all isolates were maintained in batch culture in WC medium at high (10 μM) and low (0.1 μM) Fe concentrations (prepared as described above) in 500 mL glass Erlenmeyer flasks. All flasks were acid-washed, rinsed in ultrapure water, and ethanol-sterilized prior to inoculation. Chrysophyte samples were taken directly from the termination point of their growth period (either exponential or stationary) and processed for immediate application to both assays.

Testing algal metabolites

Algal samples were split into equal parts prior to assay application. Half of the sample was centrifuged (10,000 × *g* for 10 minutes for cyanobacteria, 1000 × *g* for 10 minutes for chrysophytes; higher speeds were required to pelletize physically smaller isolates of cyanobacteria like *M. aeruginosa* 124 and 299), and the other half was lysed using a Virsonic 100 ultrasonic cell disrupter (SP Scientific) at a continuous power output of 10 for 5 minutes per 10 mL of sample. During sonication, keeping the sample tube in an ice bath allowed the sample to stay cool and helped to avoid denaturing organics within the sample. Lysed samples were then centrifuged as described above. The resulting solutions were mixed with the appropriate buffer solution for each assay. A serial dilution of each exposure solution allowed for the assessment of the solutions over

a range of concentrations (0, 20, 40, 60, 80, and 100% concentration relative to the original culture density). This dilution series allowed for the assessment of the potency of each exposure solution at various cell densities. As discussed above, cell density was measured by determining the absorbance of each culture at 720 nm (optical density). The assays were conducted under the conditions described above.

3.3 Results

Cell line contamination test

The absence of culture contamination by mycoplasma is required for all of the cell line viability tests. It is critical that contaminating organisms are not in the bioassay. The prime contaminant is mycoplasma and cells from the cell line were surveyed prior to every bioassay experiment. The result of this test (repeated throughout the duration of the experiment) indicated that the tested cells were not contaminated with mycoplasma and were suitable for use in the assay (Figure 3.1).

Photometric profile of erythrocytes

The metric for the destruction of the erythrocytes is the release of heme from the cell. Three peaks can be seen in the scan of the both the lysed and whole erythrocytes: one at 414 nm, one at 540 nm, and one at 570 nm. Measured by the change in absorbance spectra, an alteration of the spectrum at 414 nm makes this the ideal wavelength to evaluate the proportion of lysed erythrocytes (Figure 3.2).

Erythrocyte fragility test

The results of these fragility tests indicated that the erythrocytes were suitable for use in the assay (Figure 3.3). By conducting this test on a weekly basis and confirming that the curve maintained its sharp sigmoid shape, we determined that the erythrocytes were able to maintain their integrity in 4 °C dark storage for 5 months (time series data not shown). Deviations from the sharp sigmoid curve to a more gently sloped curved indicate that erythrocytes are no longer suitable for use in the assay.

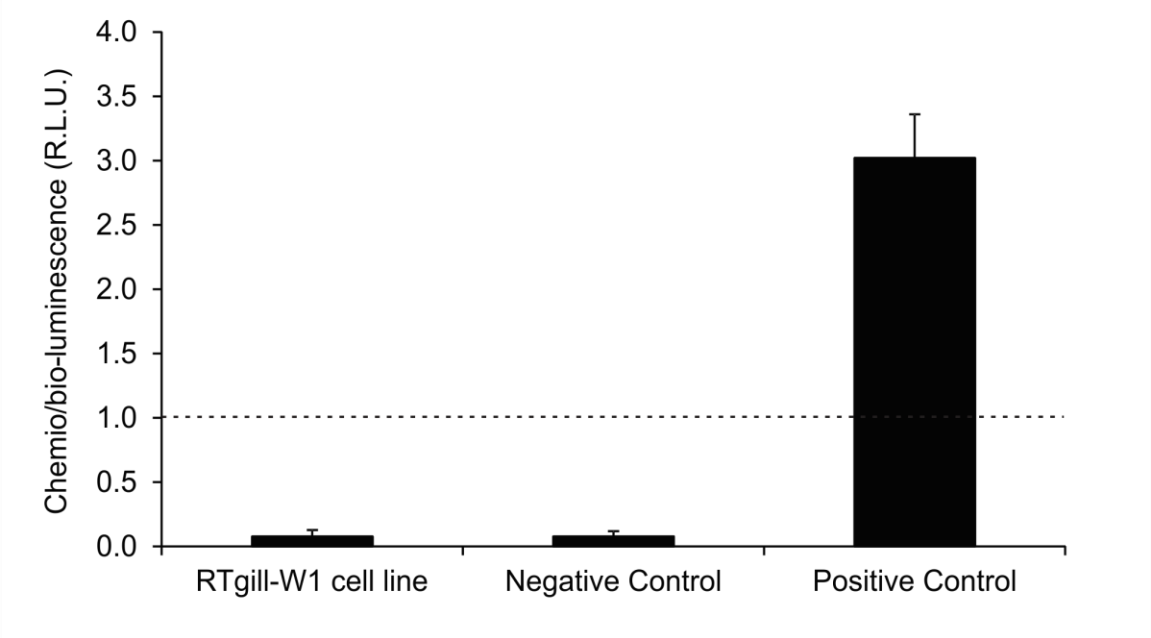

Figure 3.1. The mycoalert mycoplasma detection assay was used to detect chemi/bio-luminescence in relative light units (mean ± one standard deviation, n = 3) for the RCA. Corrected chemi/bioluminescence values >1.0 indicate mycoplasma contamination.

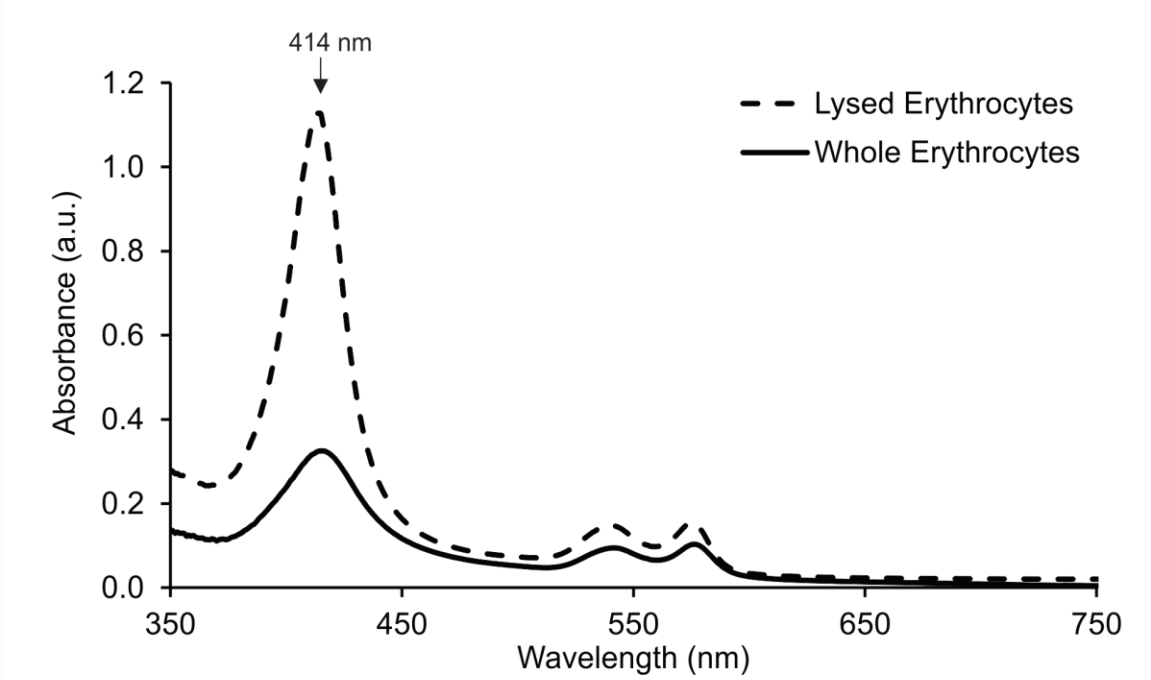

Figure 3.2. The absorbance spectra of lysed and whole erythrocytes concentrated to $5x10^{-6}$ cells mL^{-1} and read with a scanning spectrophotometer from wavelengths of 350-750 nm. Peak absorbance of lysed erythrocytes occurred at 414 nm.

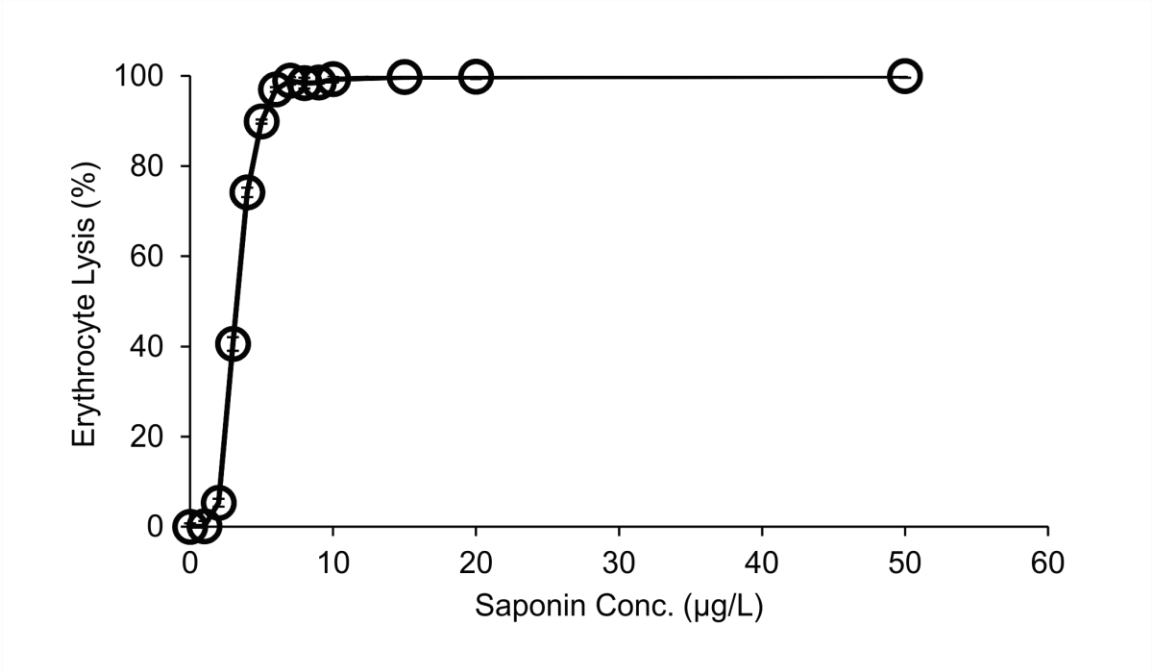

Figure 3.3. The fragility of cells during the ELA was determined by measuring percent erythrocyte lysis relative to saponin concentration (mean erythrocyte lysis ± one standard deviation, n = 3). Deviation from the sharp sigmoid curve indicates that erythrocytes are not suitable for use in the ELA.

ELA optimization

Establishing the optimal duration for erythrocyte incubation in the assay buffer solution was a key to determining the experimental setup used for further testing. Preliminary work showed differences in erythrocyte degradation rates under different incubation temperatures and the need for optimization was recognized. The percentage of erythrocyte degradation was evaluated at 4 °C and 20 °C every 2 hours to establish the optimum incubation period for ELA buffer solution at each temperature (Figure 3.4). Results indicated that an incubation time of 4 hours was appropriate for all conditions tested as this allowed for maximum exposure time with modest (<50%) erythrocyte degradation.

Several reports have shown that erythrocyte lysis is stimulated in conditions of high irradiance (≥100 μmol photons m^{-2} s^{-1}) (Kuroda et al., 2005; Ling & Trick, 2010). Preliminary testing investigated the effects of dark and light (100 μmol photons m^{-2} s^{-1}) conditions on erythrocyte lysis in ELA buffer solution (Figure 3.4). Light did not stimulate erythrocyte lysis in either the 4 °C or 20 °C treatment, indicating the ELA buffer solution was not reacting with the light to enhance lysis on its own (Figure 3.4). This suggested that the investigation into light and dark treatments for testing selected compounds in both ELA buffer was appropriate.

During preliminary work it was also observed that erythrocytes had a tendency to settle to the bottom of 1.7 mL micro-centrifuge tubes during the incubation period, potentially minimizing the interactions with hemolytic compounds and decreasing the sensitivity of the assay. Constant, yet gentle, shaking of the micro-centrifuge tubes was investigated to assess the effects of constant erythrocyte suspension. Shaking tests took place with ELA buffer solution, and occurred in the dark at 4 °C and 20 °C with the use of a shaker revolving at ~15 rpm (Gyratory Shaker Model-2, New Brunswick Scientific) (Figure 3.4). Shaking did not affect the rate of lysis of the erythrocytes and was not used in future experiments.

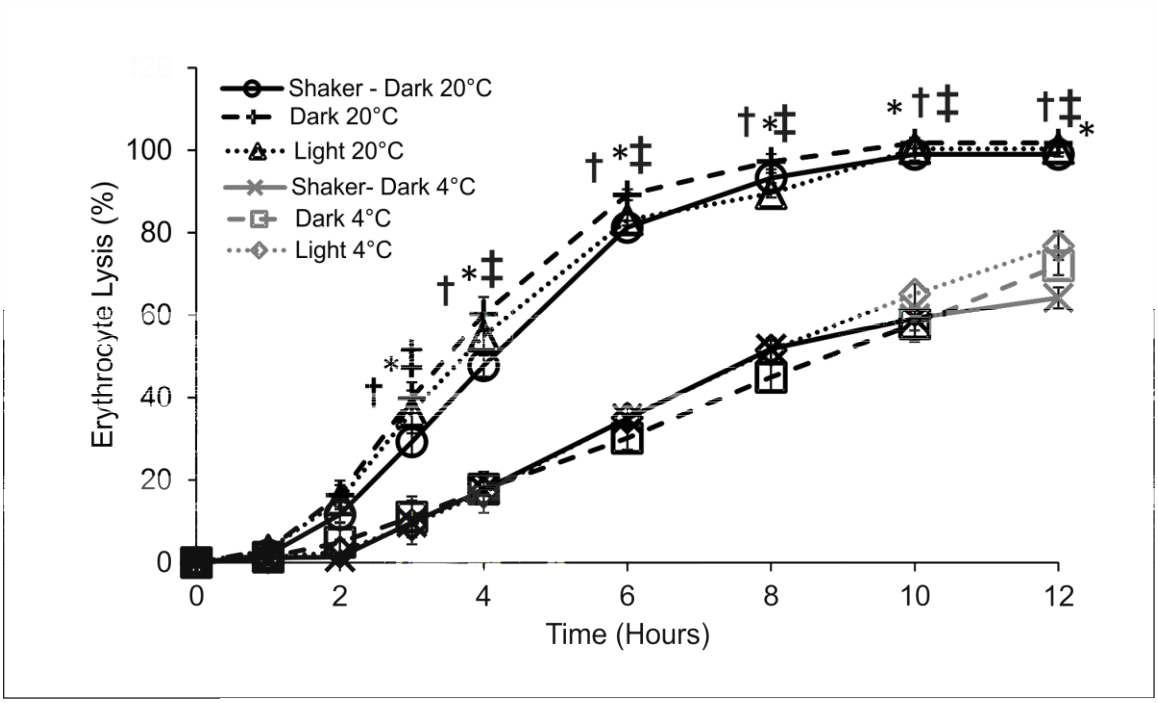

Figure 3.4. Rabbit erythrocytes incubated with ELA buffer to investigate the effect of light, temperature and shaking (mean erythrocyte viability ± one standard deviation, n = 3; *, ǂ , and ǂ indicate statistically significant differences relative to the control (4 °C treatments) for shaker, dark, and light treatments, respectively, $p<0.05$; students t-test).

Solvent selection for toxins and noxious compounds tests

While all attempts were made to dissolve the algal extract into the medium, the non-polar nature of some of the compounds required the use of an organic solvent. Compounds with low water solubility required an organic solvent to be used as a carrier. It was important to select a solvent that is not toxic to the cells of the assay, and the sensitivity needed to be evaluated prior to use. Four organic solvents - acetonitrile, DMSO, methanol, and ethanol - were investigated for use with the RCA over 24 hour incubations. Methanol was the least harmful of the solvents at higher concentrations, although DMSO and ethanol were suitable for use at lower concentrations (< 1%) (Figure 3.5). DMSO was used as for all subsequent experiments.

Cell growth rates

The dilution rate selected for each isolate grown in the continuous culture (chemostat) apparatus was equal the maximum growth rate of each isolate, μ_{max}, which was determined through analysis of the growth curves of each isolate in batch culture (Table 3.3). Two isolates of *M. aeruginosa* (124 and 299) had the highest maximum growth rates, while the lowest maximum growth rate was obtained for *A. flos-aquae* isolate 543.

BG-11 (algal medium) exposure to RCA and ELA

The RCA and ELA were evaluated for their ability to tolerate algal medium. Different ratios of algal medium: L-15/ex (1:0, 4:1, 3:2, 2:3, 1:4, 0:1) were applied to the RCA over a 24-hour exposure period. A 2:3 mix of BG-11: L-15/ex did not result in a loss of viability relative to the control (Figure 3.6). Based on this result, a conservative1: 1 ratio was used for all subsequent experiments. Similarly, the optimization of the exposure time of the ELA with algal medium (1:1) was accomplished by testing the percentage of erythrocyte degradation was evaluated at 4 °C and 20 °C every 2 hours to establish the optimum incubation period for ELA buffer solution at each temperature (Figure 3.7). Results indicated that an incubation time of 4 hours was appropriate for all conditions tested, as this allowed for maximum exposure time with middling (<60%) erythrocyte degradation.

Table 3.3. Maximum growth rates of cyanobacteria isolates determined by calculating the slope of the growth curve during exponential phase as described by MacIntyre & Cullen (2005).

Type	Organism	Strain	μ_{max} (divisions day^{-1})
Cyanobacteria	*Anabaena flos-aquae*	CPCC 64	0.23±0.03
	Anabaena flos-aquae	CPCC 543	0.17±0.02
	Aphanizomenon schindlerii	CPCC 631	0.21±0.02
	Microcystis aeruginosa	CPCC 124	0.28±0.03
	Microcystis aeruginosa	CPCC 299	0.26±0.03

Table 3.4. EC_{50} values for toxins, noxious compounds, and algal metabolites (after Alexander et al., 1999).

Compound	Mean Environmental Concentration (nM)	Maximum Environmental Concentration (nM)	EC_{50} Values (nM)	
			RCA	ELA
Microcystin-LA	$<10^0$	-	$>10^3$	$>10^3$
Microcystin-LF	$<10^0$	-	$>10^3$	$>10^3$
Microcystin-LR	$<10^0$	$<10^2$	$>10^3$	$>10^3$
Microcystin-LW	$<10^0$	-	$>10^3$	$>10^3$
Microcystin-LY	$<10^0$	-	$>10^3$	$>10^3$
Microcystin-RR	$<10^0$	-	$>10^3$	$>10^3$
Microcystin-YR	$<10^0$	-	$>10^3$	$>10^3$
Nodularin	$<10^0$	-	$>10^3$	$>10^3$
(*E*,*E*)-2,4-Decadienal	$<10^1$	$<10^2$	2×10^3	6×10^6
(E,E)-2,4-Heptadienal	$<10^1$	$<10^2$	5×10^3	5×10^7
β-Cyclocitral	$<10^1$	$<10^2$	3×10^6	10^7
Geosmin	$<10^{-1}$	$<10^1$	7×10^2	10^7
2-Methylisoborneol	$<10^0$	$<10^0$	6×10^3	10^7

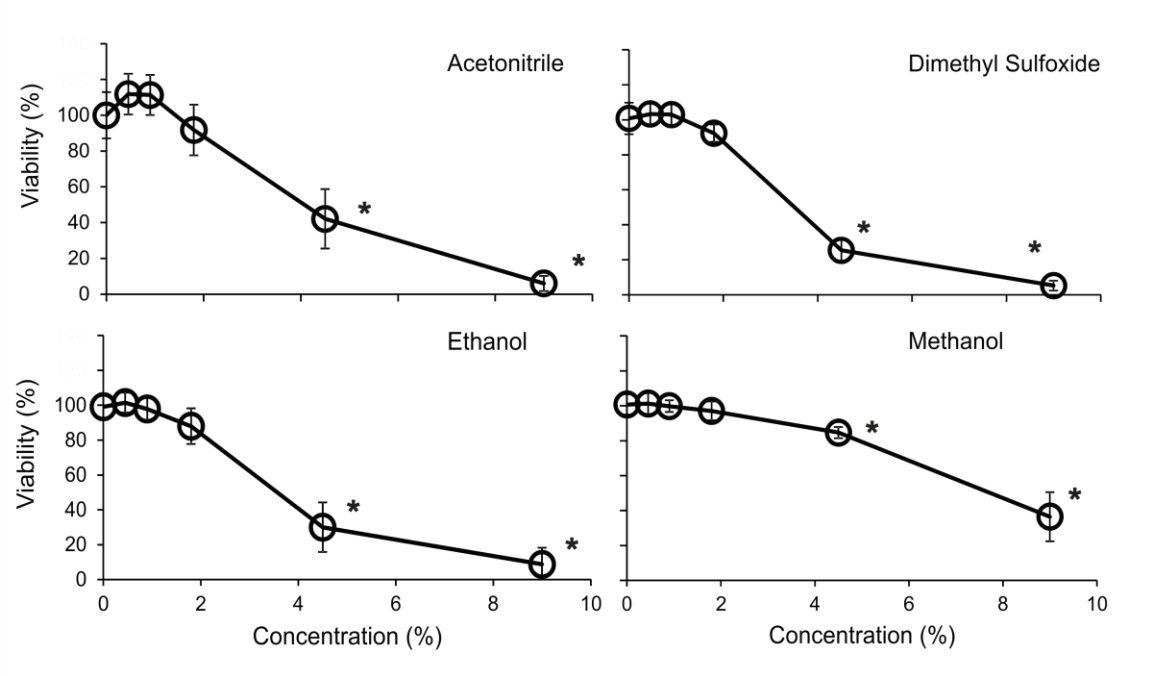

Figure 3.5. The effect of four organic solvents on the RCA for 24 hour incubations (mean cell viability ± one standard deviation, n = 10; * indicates significant differences relative to the control, $p<0.05$; Dunnett's test).

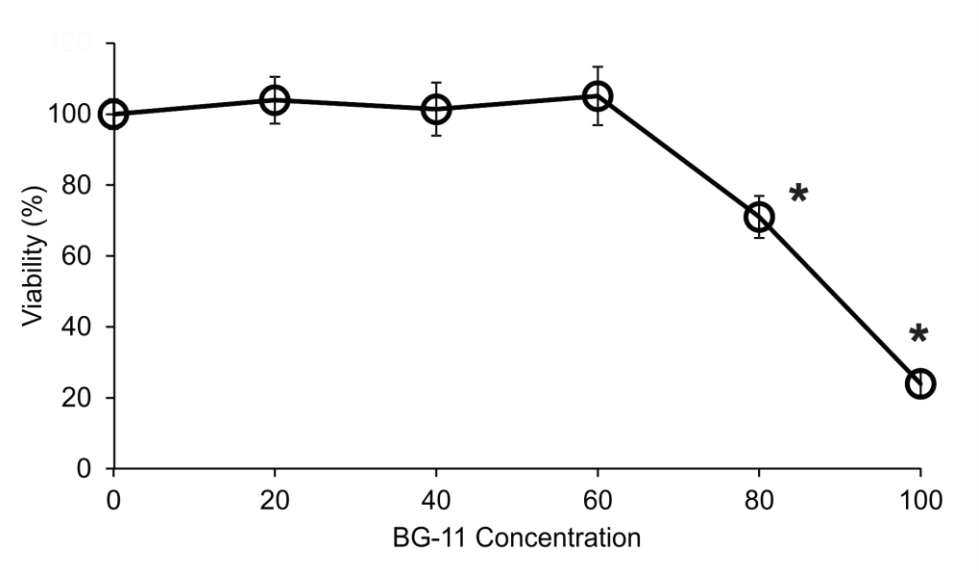

Figure 3.6. The effect of BG-11 medium on the RCA for 24 hour incubations (mean cell viability ± one standard deviation, n = 10; * indicates significant differences relative to the control, $p<0.05$; Dunnett's test).

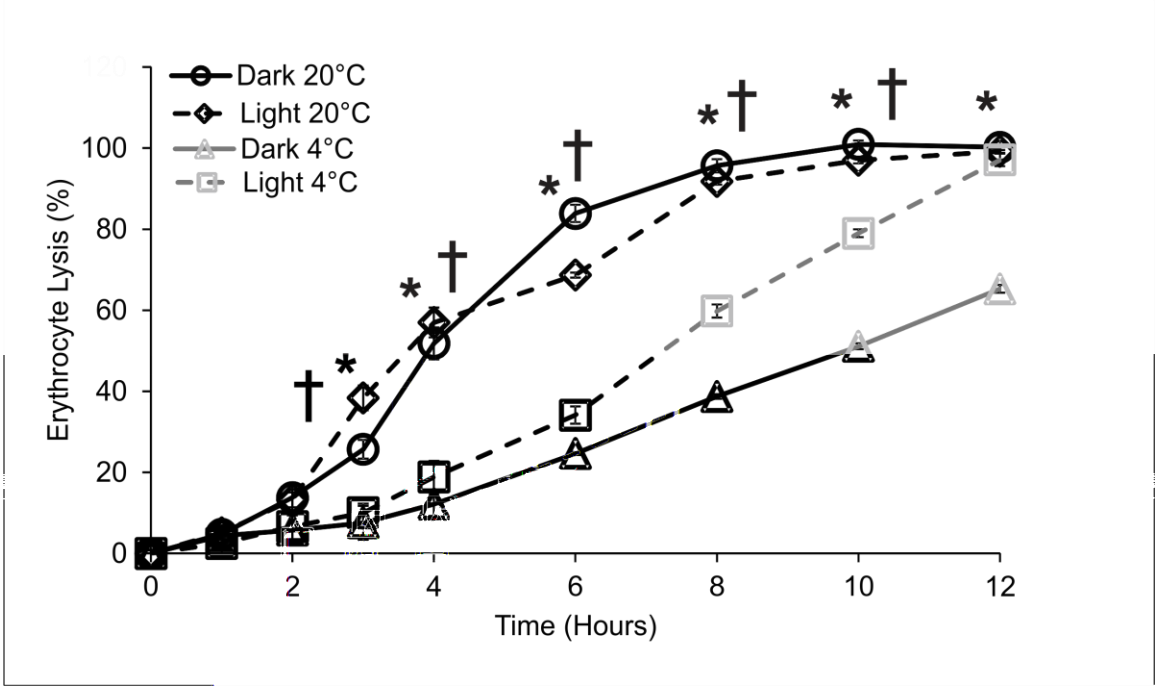

Figure 3.7. Rabbit erythrocytes incubated with BG-11 (algal medium) to investigate the effect of light, and temperature (mean erythrocyte viability ± one standard deviation, n = 3; *, and † indicate statistically significant differences relative to the control (4 °C treatments) for dark, and light treatments, respectively, $p<0.05$; students t-test) on the ELA.

RCA vs. toxins, noxious compounds and algal metabolites

The RCA was used to evaluate the effects of noxious and toxic compounds on cellular metabolism. The selected compounds were tested at concentrations well beyond their environmentally measured maximums. The RCA was insensitive to concentrations of individual microcystin variants and nodularin at or below environmental averages. In fact, the RCA did not show sensitivity at concentrations that are three orders of magnitude above these averages (Figure 3.8, Table 3.4). Losses of viability greater than 50 percent were not observed in any treatment, therefore the EC_{50} values for all microcystin variants and nodularin were beyond the tested range limit, and are generally many orders of magnitude higher than concentrations that would be measured in natural systems. Statistically significant declines in viability were observed in the MC-LR trial at the highest concentration tested (1000 nM), well beyond the maximum relevant environmental concentration.

The noxious taste and odour causing compounds tested with the RCA showed much greater sensitivity than the above noted toxins (Figure 3.9, Table 3.4). A notable reduction in viability was observed in the heptadienal trial, where a 50% reduction in viability was observed at concentrations between 10^2 and 10^3 nM following the 72-hour incubation (Figure 3.9). This suggests that heptadienal may be detectable individually near its environmental average concentration (Table 3.4). For the remainder of the compounds reductions in viability were observed at concentrations exceeding environmental averages (Table 3.4).

The RCA was also used to evaluate the effects of algal metabolites on cellular metabolism. Extracts from selected cultures were applied to the RCA at concentrations of 100, 80, 60, 40, and 20% so that the effects of the metabolites at different culture densities could be examined. These concentrations were expressed in terms of optical density at 720 nm. The tests of the RCA against the algal metabolites produced during exponential phase by cyanobacteria isolates showed no response, except for CPCC 64 (*A. flos-aquae)* which showed >80% decreases in viability in both lysed treatments (10 μM and 0.1 μM Fe), as well as the non-lysed 10 μM treatment (Figure 3.10). Conversely, algal metabolites harvested from stationary phase growth of the cyanobacteria isolates all showed decreases in viability, specifically the 10 μM Fe lysed treatment showed

reductions in cell viability > 90% across all isolates (Figure 3.11). CPCC 299 (*M. aeruginosa)* resulted in ~ 100% reductions in viability across all treatments during stationary phase tests (Figure 3.11). This result was not seen in any other isolate (Figure 3.11). Four broad trends were observed across all stationary phase tests of cyanobacteria isolates: 1) in treatments that resulted in significant reductions in viability at low concentrations, equal or greater reductions in viability were observed at higher concentrations (Figure 3.11); 2) 10 μM Fe treatments generally showed reductions in viability more frequently than the 0.1 μM Fe treatments; 3) exposure solutions composed of intracellular and extracellular material (lysed) generally resulted in greater reductions in viability than their counterparts containing only extracellular material (non-lysed); 4) optical densities of cultures that resulted in ≥ 50% reduction in cell viability ranged from ~0.1 to 0.6 a.u. (720 nm), with a large amount of variation among isolates and treatments.

The tests of the RCA against the algal metabolites produced during exponential and stationary phase by chrysophyte isolates generally showed no response (Figure 3.12, 3.13). Isolate CPCC 495 (*S. petersenii*) was the lone exception, showing >50% decreases in viability in both the 10 μM Fe lysed and 0.1 μM Fe lysed treatments of the stationary phase tests (Figure 3.12, 3.13).

ELA vs. toxins, noxious compounds and algal metabolites

The ELA was used to evaluate the effects of noxious and toxic compounds on membrane integrity. The selected compounds were tested at concentrations well beyond their environmentally measured maximums. Of the eight toxin analytical standards tested against the ELA, none produced a significant amount of erythrocyte lysis (Figure 3.14). This negative result is in spite of the application of toxins at concentrations that far exceed environmental averages by up to three orders of magnitude (1000 nM) (Table 3.4). Similarly to the toxins above, no significant amount of hemolysis was observed at environmentally relevant concentrations of noxious compounds tested (Figure 3.15). High percentage hemolysis was observed at extremely high concentrations (of these compounds, in most cases three to four orders of magnitude beyond an environmentally relevant concentration. Generally, this assay proved to be insensitive to all tested compounds at concentrations that are environmentally relevant.

The ELA was also used to evaluate the effects of algal metabolites on membrane integrity. Extracts from selected cultures were applied to the RCA at concentrations of 100, 80, 60, 40, and 20% so that the effects of the metabolites at different culture densities could be examined. These concentrations were expressed in terms of optical density at 720 nm. The ELA was generally not sensitive to cyanobacteria and chrysophyte metabolites (Figure 3.16, 3.17). Cyanobacteria isolates of *M. aeruginosa* 124 and 299 showed slight (10 to 20%) reductions in viability in samples from the lysed high (10 μM) Fe, stationary growth phase treatment (Figure 3.17). Likewise, samples from the lysed high (10 μM) and low (0.1 μM) Fe treatment of *A. flos-aquae* isolates 64 and 543 taken from stationary phase growth experiments also saw mild (20-40%) reductions in viability (Figure 3.17). Samples from exponential growth phase yielded an overall lack of response, the single exception being the moderate amount of hemolysis (~ 30%) observed in the lysed high (10 μM) Fe treatment of cyanobacterium isolate CPCC 543 (*A. flos-aquae*) (Figure 3.16). Chrysophyte metabolites showed no effect on ELA viability as a whole (Figure 3.18, 3.19). Generally, the ELA was relatively insensitive to the metabolites produced by cyanobacteria and chrysophyte algae irrespective of growth stage (exponential or stationary), growth conditions (10 or 0.1 μM Fe), or sample lysis (lysed or non-lysed) (Figure 3.18, Figure 3.19).

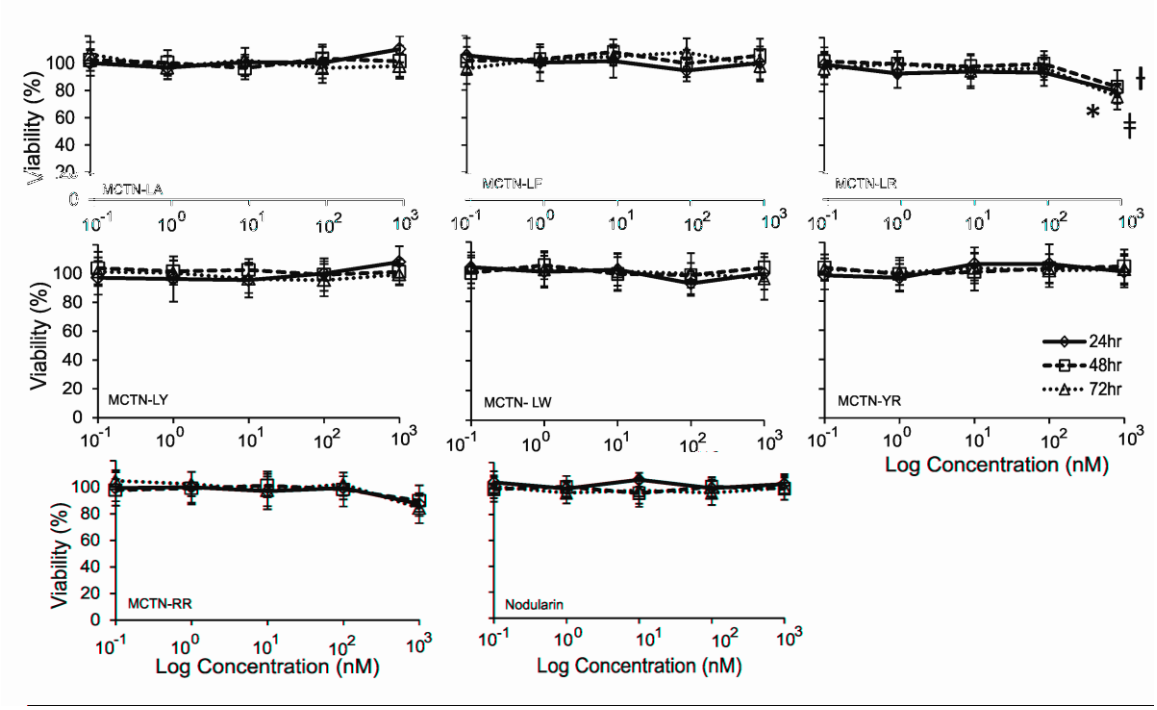

Figure 3.8. The effect of toxins on the RCA was assessed by incubating seven microcystin variants and nodularin for 24, 48, and 72 hours (mean viability ± one standard deviation, n = 10; *, ŧ, and ǂ indicate statistically significant differences relative to the control for 24, 48, and 72 hour treatments, respectively, $p<0.05$; Dunnett's test).

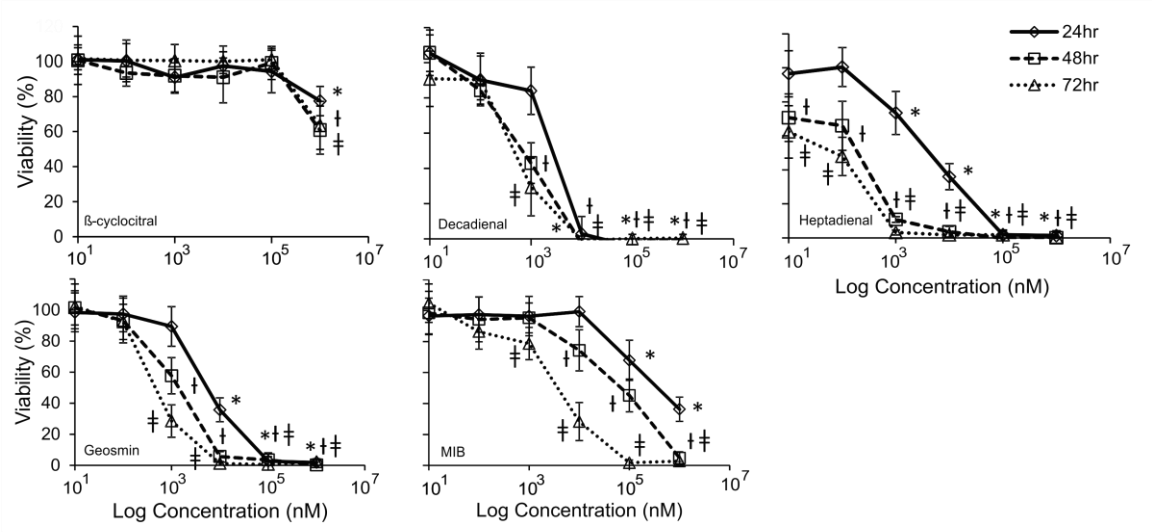

Figure 3.9. The effect of noxious compounds on the RCA was assessed by incubating five compounds for 24, 48, and 72 hours (mean viability ± one standard deviation, n = 10; *, ŧ , and ǂ indicate statistically significant differences relative to the control for 24, 48, and 72 hour treatments, respectively, $p<0.05$; Dunnett's test).

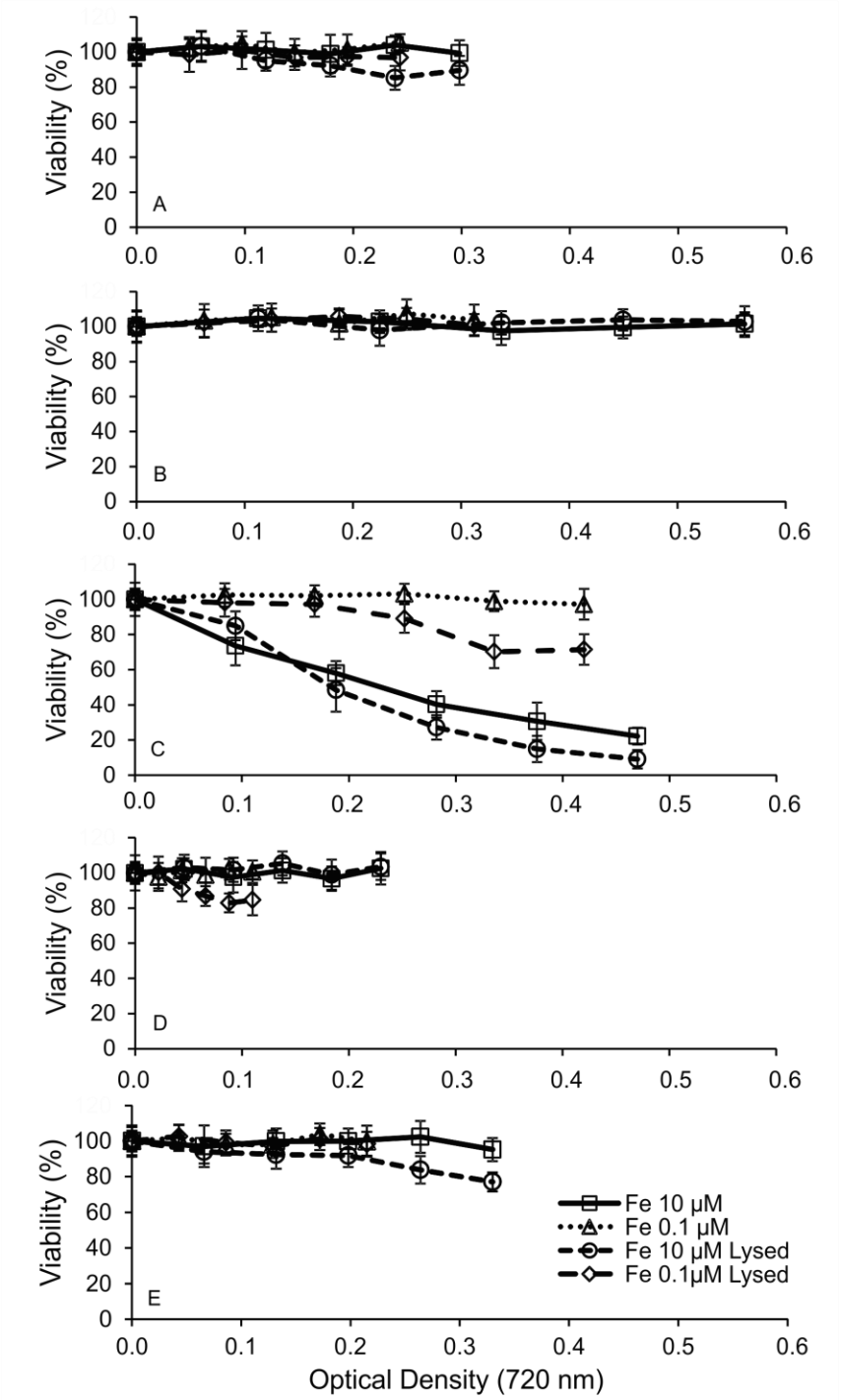

Figure 3.10. The effect of cyanobacterial metabolites produced in exponential phase was assessed with the RCA. Extracts from five isolates ((a) CPCC 124, *M. aeruginosa*, (b) CPC 299, *M. aeruginosa*, (c) CPCC 64, *A. flos-aquae*, (d) CPCC 543, *A. flos-aquae*, (e) CPCC 631, *Apha. Schindlerii)* and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

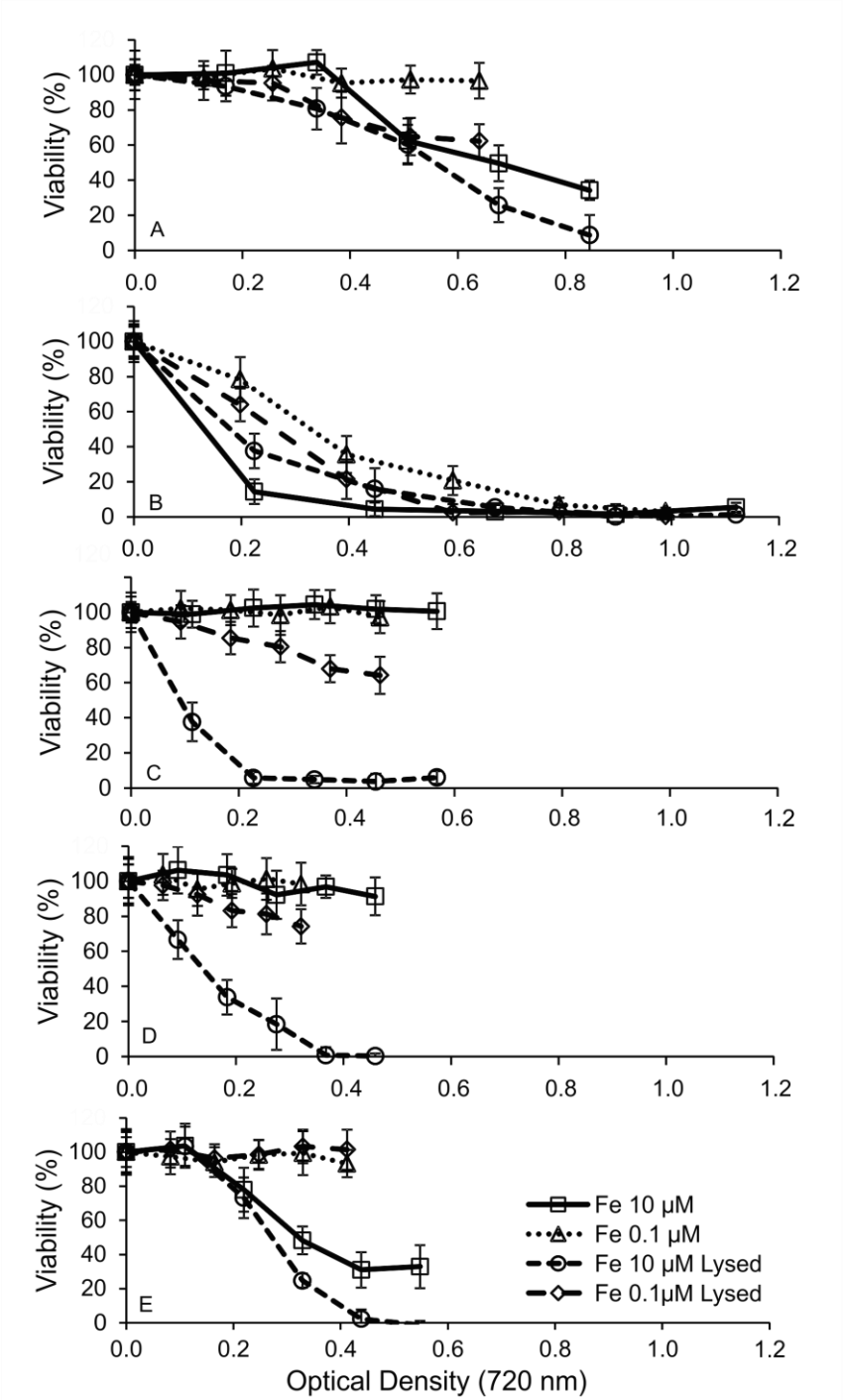

Figure 3.11. The effect of cyanobacterial metabolites produced in stationary phase was assessed with the RCA. Extracts from five isolates ((a) CPCC 124, *M. aeruginosa*, (b) CPC 299, *M. aeruginosa*, (c) CPCC 64, *A. flos-aquae*, (d) CPCC 543, *A. flos-aquae*, (e) CPCC 631, *Apha. Schindlerii)* and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

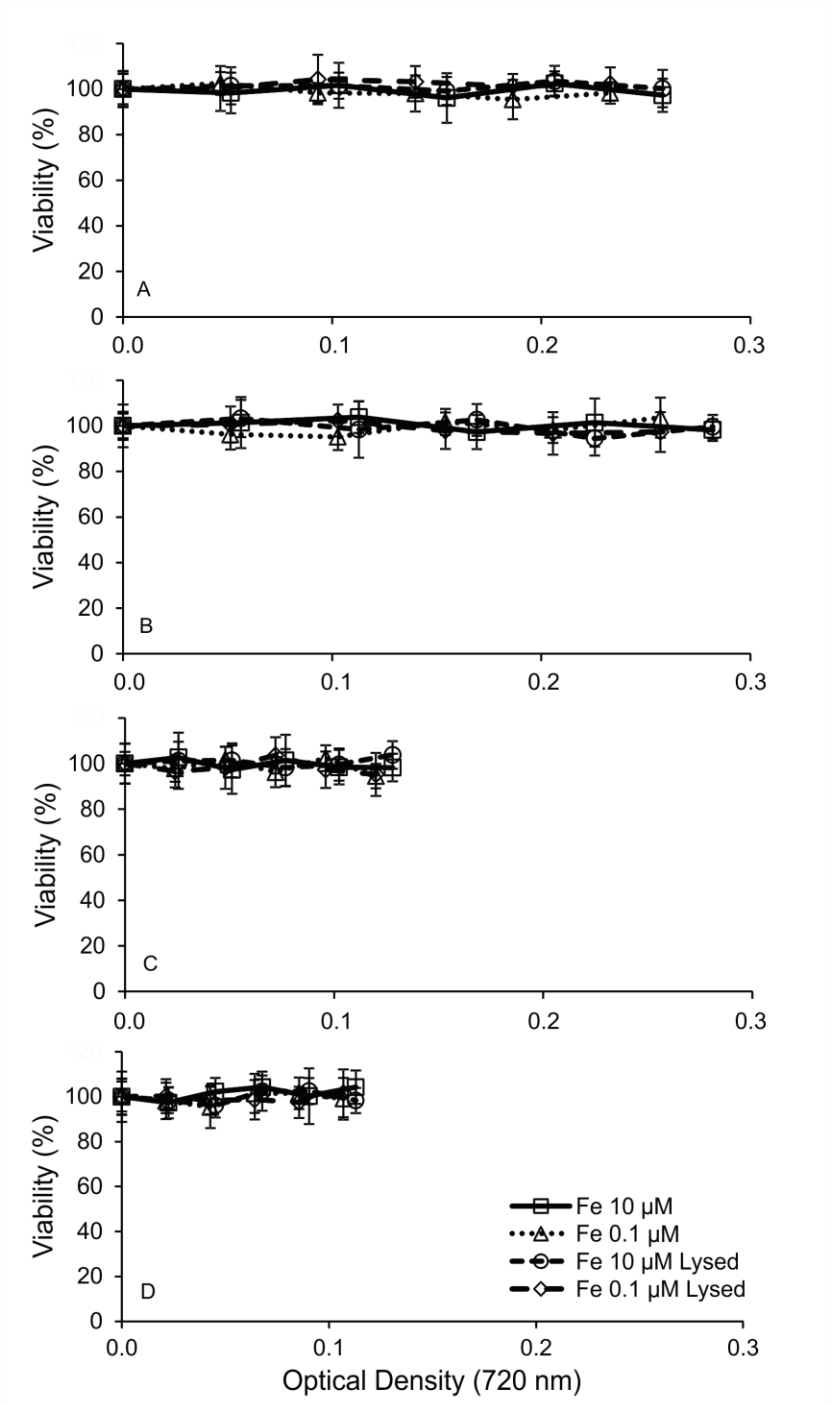

Figure 3.12. The effect of chrysophyte metabolites produced in exponential phase was assessed with the RCA. Extracts from four isolates ((a) CPCC 495, *Synura petersenii*, (b) CPCC 422 *Synura uvella*, (c) CPCC 392, *Dinobryon sp.* (d) CPCC 276, *Uroglena sp.*) and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

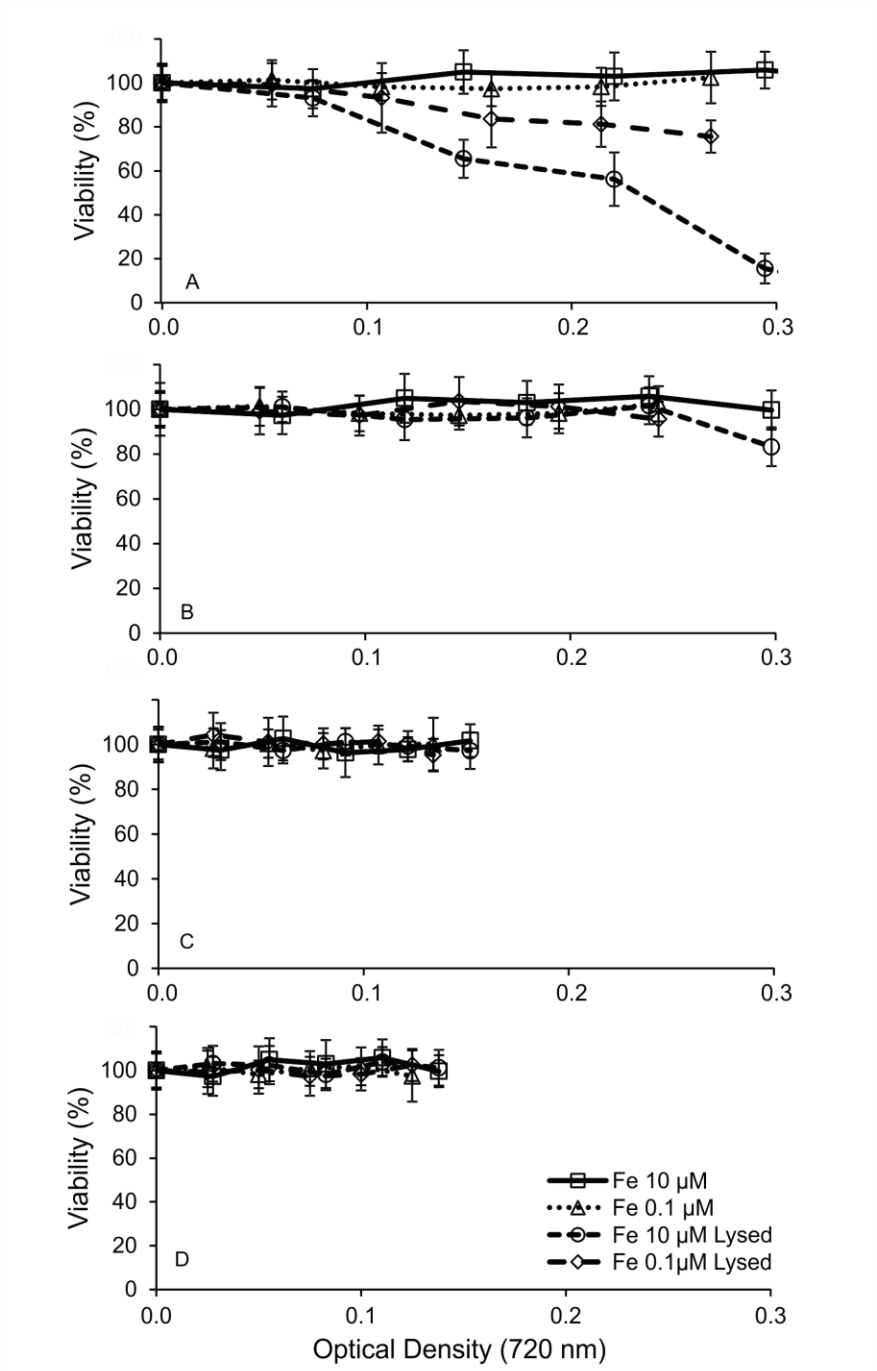

Figure 3.13. The effect of chrysophyte metabolites produced in stationary phase was assessed with the RCA. Extracts from four isolates ((a) CPCC 495, *Synura petersenii*, (b) CPCC 422 *Synura uvella*, (c) CPCC 392, *Dinobryon sp.* (d) CPCC 276, *Uroglena sp*.) and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

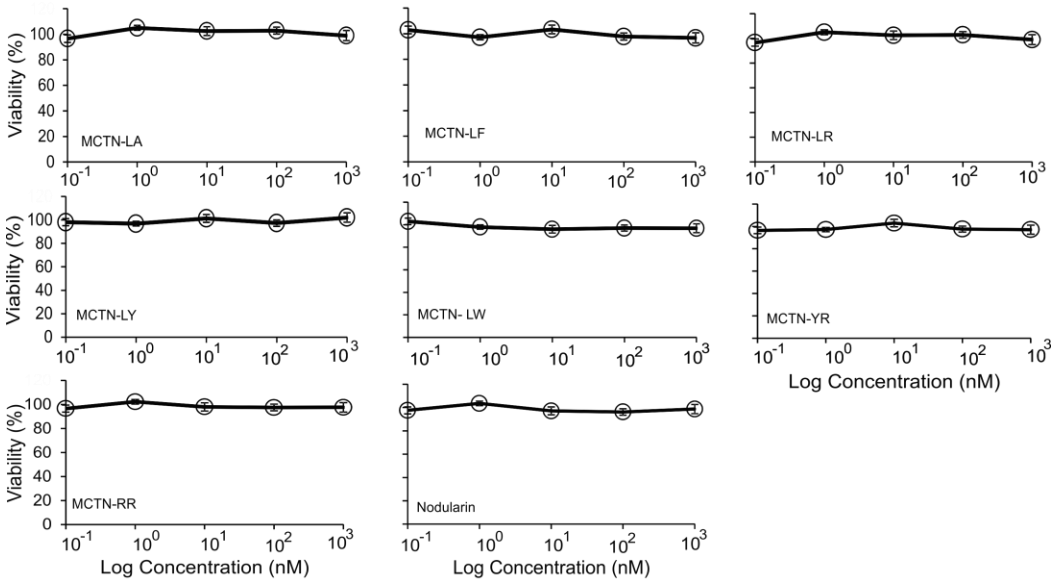

Figure 3.14. The effect of seven microcystin variants and nodularin on the ELA in 4 hour light incubations (dark trials not shown, mean viability ± standard error of the mean, n = 3; * indicates statistically significant differences relative to the control, $p<0.05$; Dunnett's test).

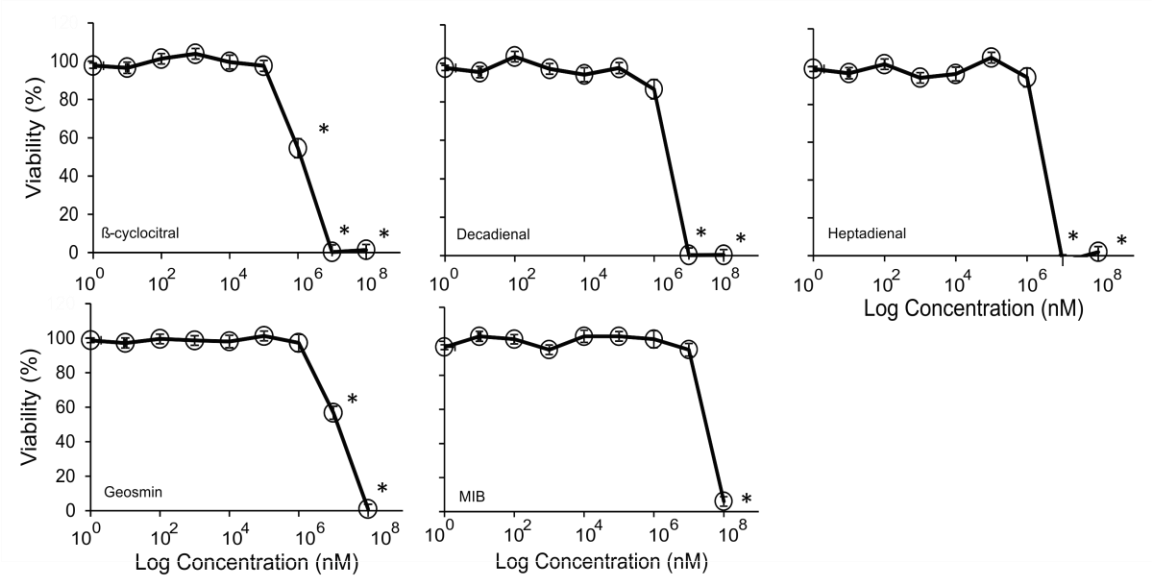

Figure 3.15. The effect of noxious compounds on the ELA in 4 hour light incubations (dark trials not shown, mean viability ± standard error of the mean, n = 3; * indicates statistically significant differences relative to the control, $p<0.05$; Dunnett's test).

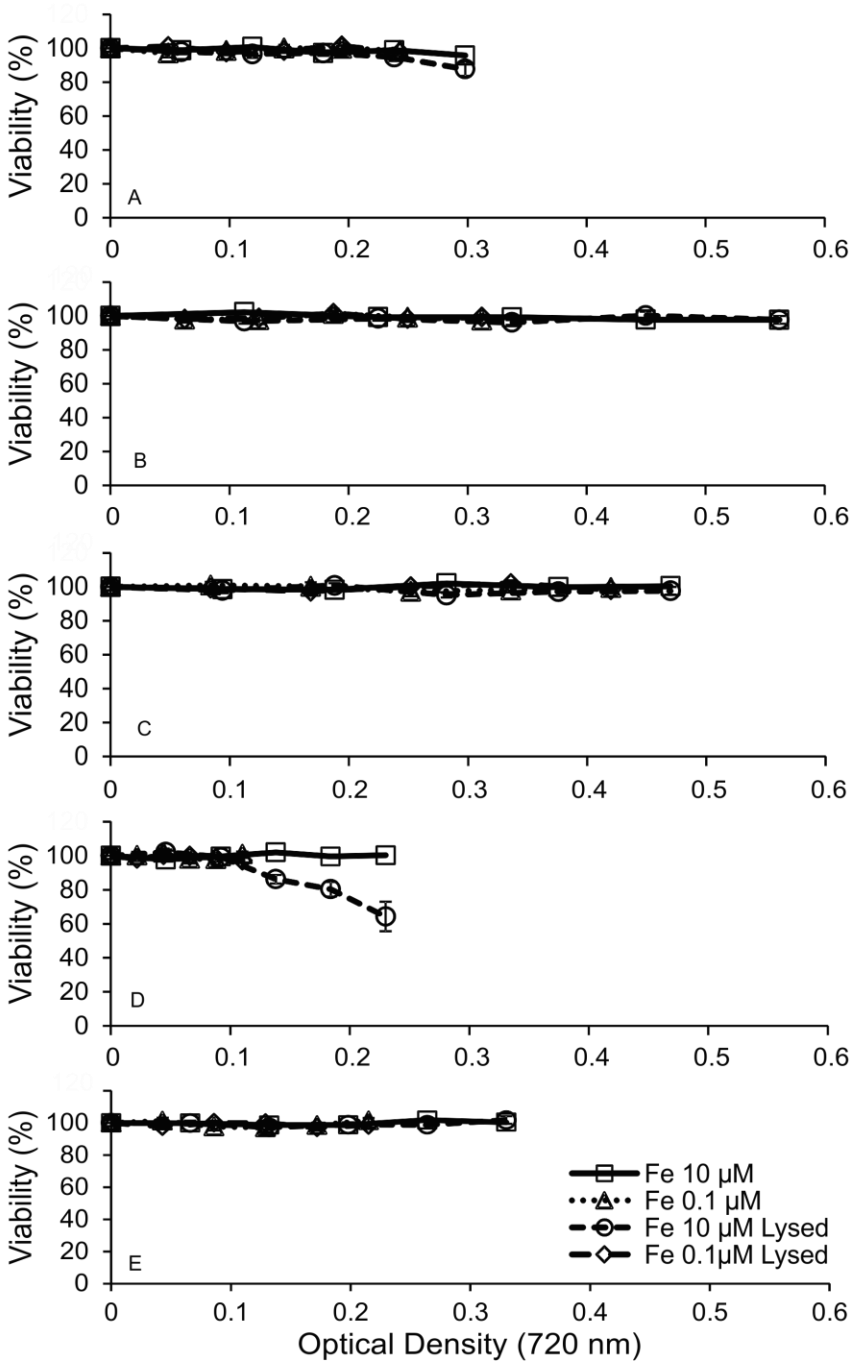

Figure 3.16. The effect of cyanobacterial metabolites produced in exponential phase was assessed with the ELA. Extracts from five isolates ((a) CPCC 124, *M. aeruginosa*, (b) CPC 299, *M. aeruginosa*, (c) CPCC 64, *A. flos-aquae*, (d) CPCC 543, *A. flos-aquae*, (e) CPCC 631, *Apha. Schindlerii)* and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

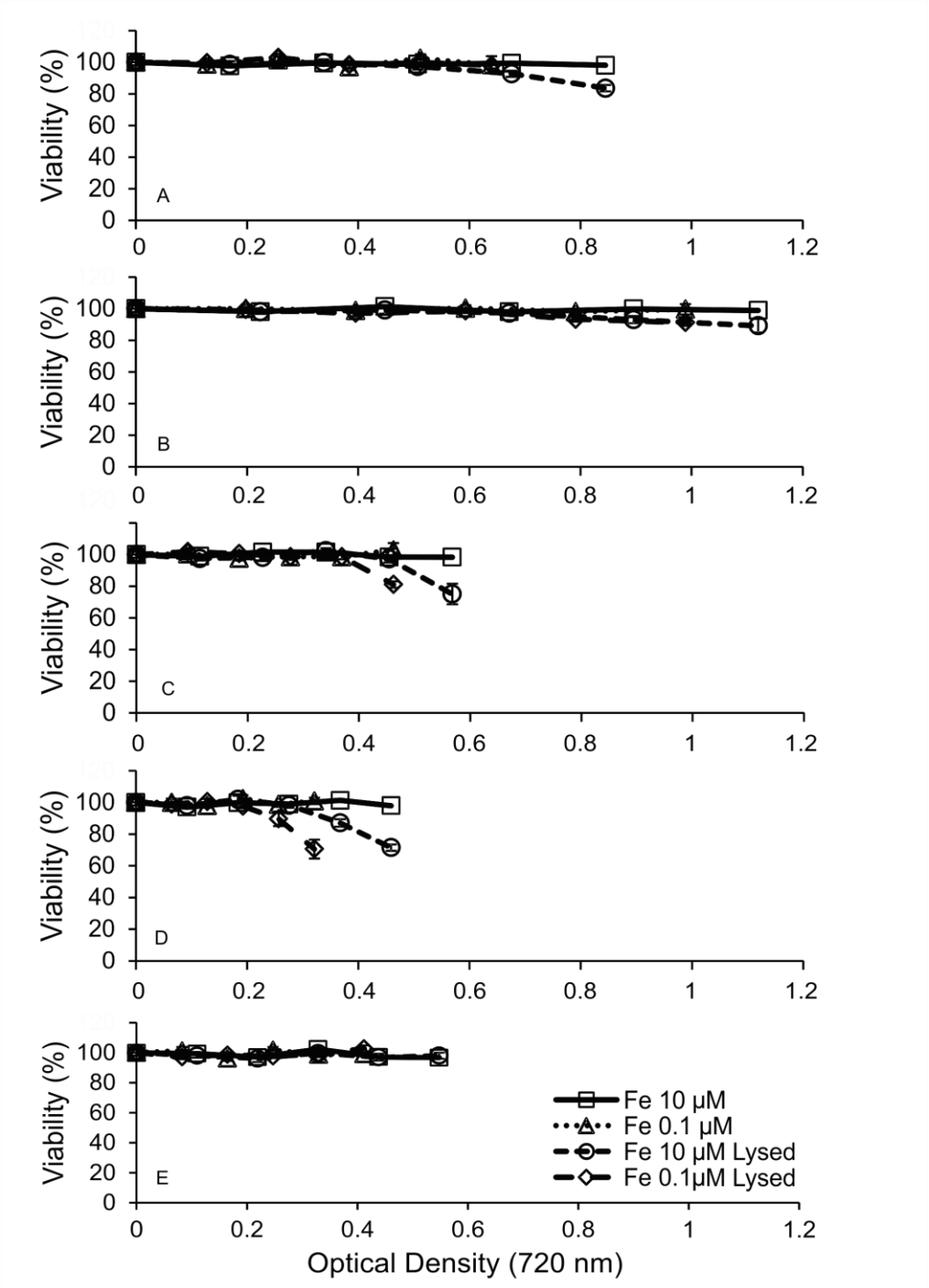

Figure 3.17. The effect of cyanobacterial metabolites produced in stationary phase was assessed with the ELA. Extracts from five isolates ((a) CPCC 124, *M. aeruginosa*, (b) CPC 299, *M. aeruginosa*, (c) CPCC 64, *A. flos-aquae*, (d) CPCC 543, *A. flos-aquae*, (e) CPCC 631, *Apha. Schindlerii)* and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

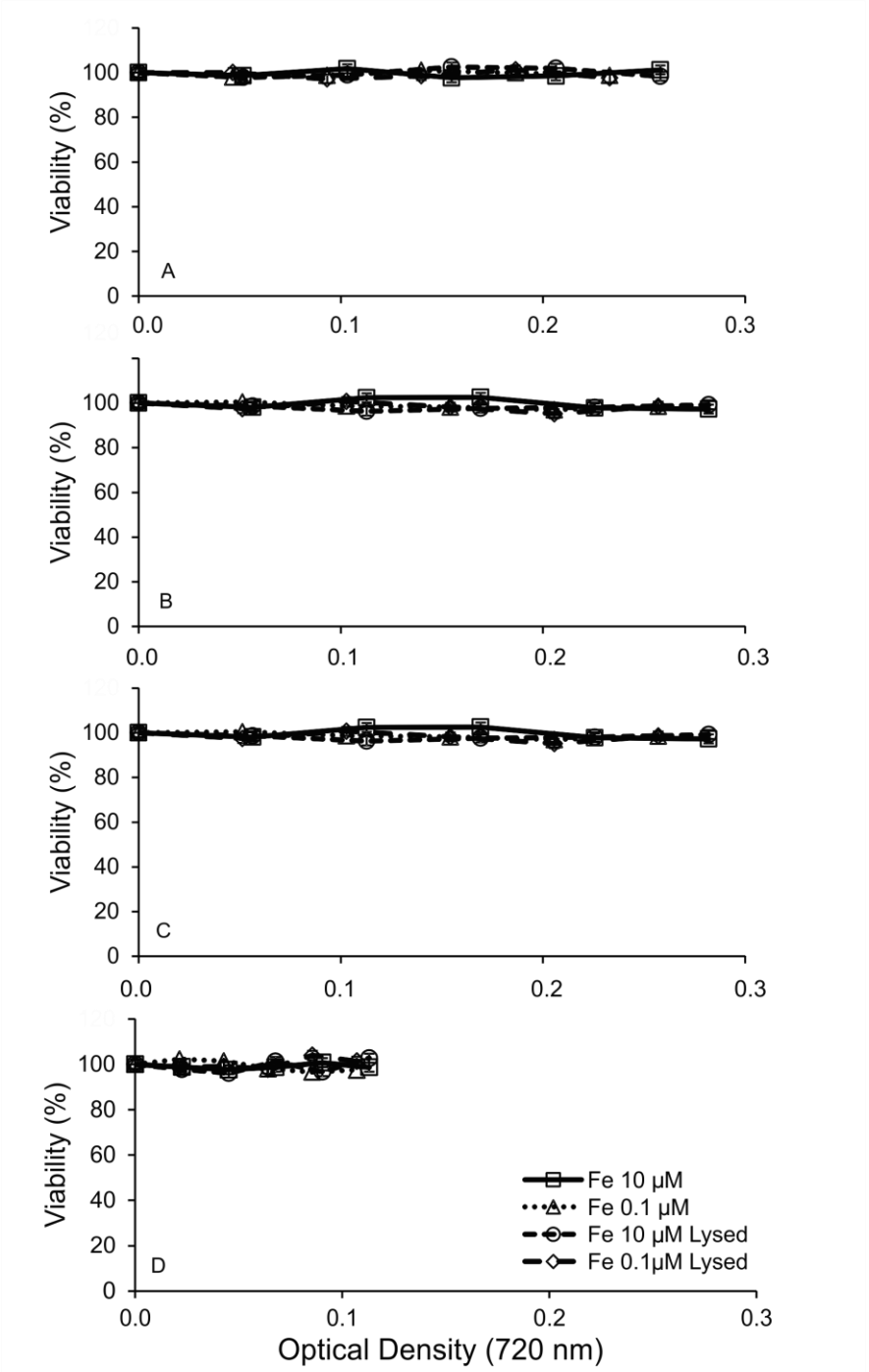

Figure 3.18. The effect of chrysophyte metabolites produced in exponential phase was assessed with the ELA. Extracts from four isolates ((a) CPCC 495, *Synura petersenii*, (b) CPCC 422 *Synura uvella*, (c) CPCC 392, *Dinobryon sp.* (d) CPCC 276, *Uroglena sp.*) and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

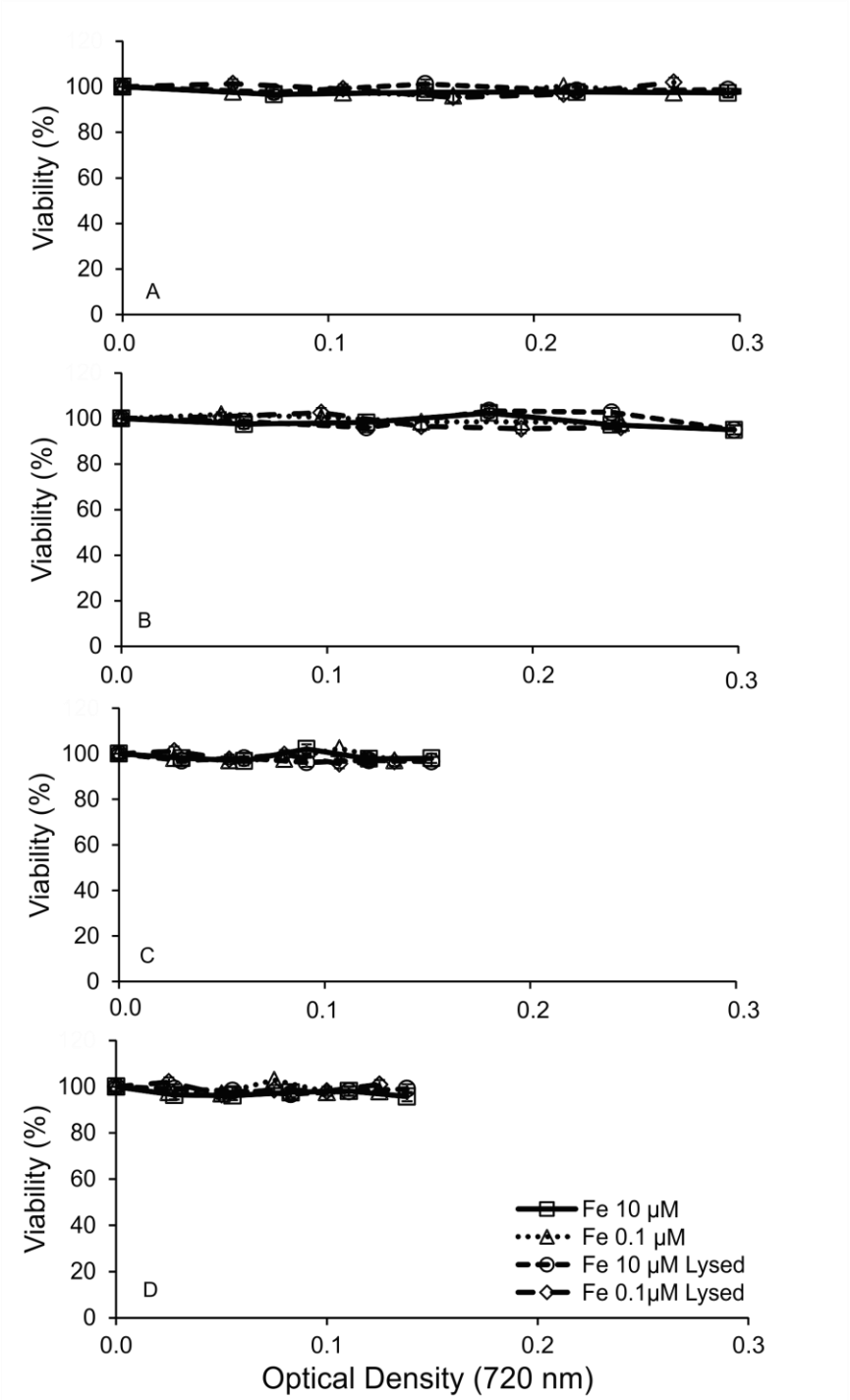

Figure 3.19 The effect of chrysophyte metabolites produced in stationary phase was assessed with the ELA. Extracts from four isolates ((a) CPCC 495, Synura petersenii, (b) CPCC 422 Synura uvella, (c) CPCC 392, Dinobryon sp. (d) CPCC 276, Uroglena sp.) and four treatments (Fe 10 µM, Fe 0.1 µM, Fe 10 µM lysed, and Fe 0.1 µM lysed) were tested (mean viability ± standard error of the mean, n = 3). Exposure solutions were evaluated at concentrations equivalent to 100, 80, 60, 40, 20, and 0% of the culture density, as expressed by optical density at 720 nm.

3.4 Discussion

Freshwater algae produce a large number of toxic, noxious, and bioactive metabolites, and selecting one specific test to quantify these effects is seemingly impossible. Using a bioassay, rather than an extraction and evaluation test, may provide useful information on both the general toxicity of a sample, and on the directed regulation of toxins (Fent, 2001).

Two tests using dramatically different cell types, the gill cell line (RCA, metabolic activity) and the red blood cell bioassay (ELA, membrane integrity), were chosen to evaluate the cytotoxic effects of both individual compounds and of extracts from algal cells grown under specific conditions. These two tests are complementary and provide an opportunity to evaluate samples on multiple cell structures.

Despite the application of seven microcystin variants and nodularin at a concentration that exceeded environmentally measured averages (Table 3.4), the RCA proved to be insensitive to detecting these compounds (Figure 3.8). Fish and mammalian cell lines generally show a wide range of sensitivities to these hepatotoxins, although no mammalian or fish cell line has been shown to be sensitive to microcystins at environmentally measured concentrations (McDermott et al., 1998; Chong et al., 2000; Pichardo et al., 2005, 2006, 2007). This repeated insensitivity may be due to the nature of the cell-based assays, which are primarily tests for acute cytotoxic effects, while microcystins, nodularins and a variety of other cyanotoxins generally show effects over periods of long-term (chronic) exposure (Schindler 1987; Chen et al., 2009; El Ghazali et al., 2010).

The RCA also proved to be predominantly insensitive to the noxious taste and odour compounds tested (Figure 3.9). With the exception of the moderate losses in viability observed in the heptadienal trials, the investigated compounds caused negligible decreases in viability at average environmental concentrations (Table 3.4). Although these compounds do not have the extensive history with bioassay work as is seen with microcystins, the documented qualities of these compounds as potential dermal irritants makes these results somewhat unexpected (Graham et al., 2008). However, these compounds are generally recognized for their ability to produce foul odours, rather than their ability to adversely affect surrounding biota (Watson, 2003).

The significant decreases in viability observed in the RCA trials involving bioactive metabolites from cyanobacteria cultures have shed light on several key areas of cyanobacteria metabolite production and storage. The most consistent result observed was the lack of response seen from exposure solutions derived from exponential phase relative to those from stationary phase (Figure 3.10, 3.11). Despite a reduction in viability seen in the high (10 μM) Fe treatments in the exponential phase sample from isolate CPCC 64 (*A. flos-aquae)*, the remaining four isolates showed no losses in viability from any sample derived from exponential growth phase. This suggests that either the cytotoxic/bioactive compounds are not being produced in the exponential phase, or that they are being produced but are not present at high enough quantities to have a significant impact on the RCA. This result is opposite to my predicted outcome, and does not support the findings from similar studies that evaluated cyanobacterial toxin production rates in exponential phase (Schatz et al., 2007; Kaplan et al., 2012).

Another observation from the cyanobacteria testing was the propensity for lysed samples (intracellular + extracellular material) to result in greater decreases in RCA viability relative to the non-lysed samples (extracellular material only) of the same treatment (Figure 3.10, 3.11). This observation supports my prediction, as well as previous work suggesting that the proportion of bioactive material that is retained inside the cell over the course of its growth is greater than the proportion that is excreted to the extracellular environment (prior to senescence) (Park et al., 1998). This may have played a role in the differences observed in the exponential and stationary phase tests, as the accumulation of intracellular compound would increase proportionally with the age of the cells (Park et al., 1998).

The role of Fe was also observed to be a contributing factor in the cytotoxicity of the bioactive metabolites taken from cyanobacteria cultures. Contrary to my expectations that nutrient limitation increased toxicity, it was generally the Fe-replete (10 μM) treatments that resulted in the greatest reductions in RCA viability (Figure 3.10). Cells grown under the elevated Fe concentration repeatedly proved to provide the most cytotoxic samples, providing support for the claim that it is under optimum growth conditions, rather than nutrient limitation, that bioactive compound synthesis is at its peak (Kaebernick & Neilan, 2001). This result suggests that the Fe-limited conditions that

favour microcystin production are not necessarily ideal for the production of a wider variety of bioactive compounds as has been suggested by previous work (Alexova et al., 2011; Kaplan et al., 2012). Furthermore, it is likely that the bioactive products responsible for the loss in viability in the RCA are derivatives of molecules produced by photosynthetic processes (Kaebernick & Neilan, 2001). Fe-limited treatments may decrease the cells' photosynthetic efficiency and therefore limit the production of these molecules.

Aside from the large declines in viability observed in the stationary phase, high (10 μM) Fe, lysed treatment of algal isolate CPCC 495 (*S. petersenii)*, no decline in viability was observed in any other chrysophyte-derived treatment (Figure 3.12, 3.13). The loss in viability seen in isolate CPCC 495 suggests similar patterns to that of the cyanobacteria tests — high (10 μM) Fe conditions and intracellular storage may be factors in their production of the bioactive compounds — however, these findings are not supported by any of the other three chrysophyte isolates tested.

The overall lack of response seen in the RCA when exposed to mixtures isolated from chrysophyte cultures was likely a result of an inability to grow cultures to sufficient densities, and there may be potential for this assay to be used in the future with these isolates. Concentration of cell cultures to increase density was considered following the growth experiments of the chrysophyte cultures; however, these techniques led to a level of complexity that would remove many of the advantages gained by using cell-based assays.

The relative lack of sensitivity of the ELA towards the toxic and noxious compounds investigated indicates that the selected compounds do not cause hemolysis or cytotoxic responses based on membrane disruption at environmentally relevant concentrations (Figure 3.14, 3.15). Only at extremely high concentrations of the selected noxious compounds were detectable rates of lysis observed, often at concentrations more than five orders of magnitude beyond the environmental measured levels (Figure 3.15, Table 3.4). These results for the toxins tested confirm previous work by Grabow et al. (1982), Henning et al., (1992) and Zhang et al. (2011) that detected hemolysis only at extremely high exposure concentrations. These results also

Similarly, an overall lack of hemolytic response was observed when applying the cyanobacteria and chrysophyte culture-derived samples (Figure 3.16-3.19). The exceptions were the moderate (~30%) lysis observed in the lysed treatments of isolate CPCC 543 (*A. flos-aquae*) in both exponential and stationary phase tests, as well as the lysed treatments of CPCC 64 (*A. flos-aquae*) in stationary phase. This observation supports the finding of freshwater cyanobacteria producing hemolytic compounds, even though full erythrocyte lysis was not achieved at the highest tested concentration (Wang et al., 2007; Zhang et al., 2011). These results, when combined with the information gained from the toxin observations, generally support the finding that microcystins are not the key factor in the hemolysins produced by cyanobacteria (Henning et al., 1992).

3.5 Conclusions

The RCA and ELA were ineffective at detecting responses to compounds at or below environmental average concentrations (*H1*). Significant declines in viability were not observed in any toxin trial up to three orders of magnitude above environmentally observed averages. Analysis of noxious compounds revealed similar insensitivity with both assays, although declines in viability were observed at concentrations greater than 2 orders of magnitude above environmentally observed averages. This study was the first to investigate the effects of all of these compounds using either the ELA or RCA.

Conversely, the RCA proved to be very sensitive to undefined algal metabolites produced by the cyanobacteria isolates tested (*H2*). This is a novel result and is the first evidence of extracts from cyanobacterial cultures being used against the RCA to evaluate cytotoxicity. Samples derived from stationary phase growth experiments showed a tendency to result in greater decrease in viability of the RCA relative to their counterparts derived from exponential phase growth experiments.

The above observation counters the predicted result and indicates that the production of secondary metabolites and other bioactive compounds capable of producing cytotoxic effects may be favoured. My prediction hinged on the increased rate of production of bioactive compounds in exponential phase being the major factor in decreasing cell viability. My results indicate that it is not the rate of production but the build-up of these compounds inside the cell that is more closely related to cytotoxic

responses (leading to larger decreases in viability being observed in stationary phase tests).

As predicted, exposure solutions derived from lysed cultures (intracellular + extracellular compounds) generally resulted in decreases in RCA viability relative to solutions derived from non-lysed samples (extracellular compounds only). It is likely that the cytotoxic and bioactive compounds responsible for the observed decreases in RCA viability are primarily stored intra-cellular.

Cyanobacteria isolates grown in Fe-replete (10 μM) conditions generally were responsible for greater decreases in viability of the RCA, suggesting that the production of cytotoxins is greater in these conditions than in Fe-deplete (0.1 μM) medium. Comparing this finding to the established link between Fe-deplete conditions and the stimulation of microcystin production suggests that the monitoring of microcystin levels in natural systems is likely not a good indicator of the overall levels of cytotoxic and/or bioactive compounds present in a sample, although it is still an important parameter to monitor in terms of drinking water quality.

The RCA proved to be a sensitive technique for the assessment of production of cytotoxins in laboratory cultures, although not ideally sensitive to relevant concentrations of the toxin and noxious compounds tested. Cultures were grown to densities above those normally observed in natural systems, so adaptation to field studies may require a concentration step. Despite the repeated use of the ELA for marine and freshwater algal research the assay was not ideal for use with the toxins, noxious compounds, or isolates selected for this study. Concentration of algal samples (via SPATT bags, dialysis bags, etc.) would be a necessary step to making this assay suitable for use with isolates that produce low levels of hemolysins.

3.6 References

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Chapter 4
General Conclusions

4.1 Scientific Findings

The two aims of this thesis were to (1) review the current state of knowledge in terms of toxic, noxious, and/or bioactive compound production by bloom forming freshwater algae; and (2) investigate the use of two cell-based assays designed to evaluate the cytotoxic effects of these compounds. My review of freshwater toxins and noxious compounds produced by cyanobacteria and chrysophyte algae in Chapter 2 is a unique synthesis of information not currently found in the literature. By combining research from a variety of sources and assessing the totality of the information in one place the stark differences in the mode of action, risk of exposure, and genera responsible for production of each class of compound are easily identified. Through this review the importance of the emerging group of compounds known as bioactive compounds emerged. Little research has been done to this point with regards to these compounds, and the details provided in the review summarize the latest information available. The importance of bioactive compounds on cytotoxicity and the inhibition of cellular processes were further demonstrated in some of the results from my experimental work in Chapter 3. This review provides a resource for aquatic researchers and as it provides the latest information on the subject of freshwater algal toxicology.

My investigation into suitable cell-based bioassays established that the RCA and ELA were ineffective at detecting individual toxic or noxious compounds at concentrations at or below environmental averages (*H1)*. Declines in cell viability were observed at concentrations that greatly exceed environmentally measured concentrations. This outcome suggests that these assays are not ideal for analysis of these particular compounds in isolation (Figure 4.1).

However, the RCA proved to be sensitive to metabolites produced by the algal isolates tested, specifically those produced by cyanobacteria (*H2)*. Complete loss of viability was observed in RCA tests against all cyanobacteria isolates collected during stationary phase growth under high (10 μM) Fe, lysed treatments. Additionally, all

cyanobacteria isolates except CPCC 64 (*A. flos-aquae*) and 543 (*Apha. schindlerii*) showed losses in viability when extracts from stationary phase growth under high (10 μM) Fe, non-lysed samples were tested. These results suggest that the harmful metabolites responsible for decreased in RCA viability are being produced at higher concentrations under high (10 μM) Fe conditions relative to low (0.1 μM) Fe conditions. This may be due to the increased capacity of the cyanobacteria to photosynthesize at higher rates under Fe replete conditions, allowing for a continued production of metabolites within the cell (Rueter & Ades, 1987). These results also suggest that the RCA is a tool that is sensitive enough to be used as a part of a sampling regimen for cyanobacteria blooms events, and would be helpful in identifying the biological hazards presented by cyanobacteria bloom events (Figure 4.1).

Furthermore, when comparing the results of the RCA tests with cyanobacteria extracts and the negative results observed in the toxin and noxious compound trials, it is apparent that there must be another group of compounds being produced by the cyanobacteria isolates. Based on the findings in Chapter 2, it is possible that it is the bioactive compounds produced by the cyanobacteria isolates that are the key players in the observed losses in RCA viability. Although no data is available with regards to the production of these bioactive compounds with each isolate tested, it is likely that they are in part responsible for observed losses in viability.

In contrast, ELA proved to be insensitive to the mixtures of compounds produced by the algal isolates tested (*H2).* Slight declines in viability were observed in cyanobacteria isolates CPCC 124 (*M. aeruginosa*), 64 (*A. flos-aquae*), and 543 (*Apha. schindlerii*), specifically in the high (10 μM) Fe, lysed treatments. Complete loss of viability was not observed in any treatment, even at the highest concentrations tested. This result does not mean that the algal isolates were all unable to produce of hemolytic compounds, but rather that they were likely produced at concentrations too low to induce toxicity.

Declines in RCA viability were observed when extracts from chrysophyte isolate CPCC495 were tested. Similar to the outcomes of the cyanobacteria trials, the high (10 μM) Fe, lysed treatment resulted the largest decline in viability. All other results

involving the extracts from chrysophyte isolates had no declines in viability, and this result may have been a product of the relatively low culture density that was achieved.

4.2 Management Implications

Comparing my findings to the established concepts of individual compound assessments raises concerns in terms of traditional monitoring strategies. My results show that monitoring of individual compound concentrations (such as microcystins) in natural systems is likely not a good indicator of the overall levels of cytotoxic and/or bioactive compounds present in a sample. My results suggest that the complex mixtures of bioactive compounds produced by cyanobacteria may have additive and or synergistic effects that cannot be accounted for via the analysis of individual compounds. Further development of affordable but biologically comprehensive methods of testing are essential for expanding understanding of how the complex mixtures of toxic and noxious compounds act together in open systems. Cell-based assays such as the RCA and ELA are generally not sensitive enough to include as part of routine analysis for compound-specific identification, but may have value as secondary tools to assess algal toxicity when trying to assess the biological risk posed by a cyanobacteria bloom event (Figure 4.1).

4.3 Future Research Directions

Further investigations into the use of cell-based assays to evaluate the toxic, noxious and/or bioactive compounds produced by bloom-forming freshwater algae are necessary to determine the most effective biological analysis of these compounds as they occur in mixtures. The ELA and RCA represent two of the most likely candidates for further use as their ability for detection has been documented here and in other publications (Wang et al., 2007; Dorantes-Aranda et al., 2011). Expanding the search for cell lines that may not have been previously investigated for this type of application, and exploring the ability of these cell lines to respond to a range of compounds produced by freshwater algae is essential in identifying new methodologies. Putting an emphasis on assays that employ high throughput technologies is also important for future work, as this allows for maximization of the number of samples that can be analyzed per unit effort.

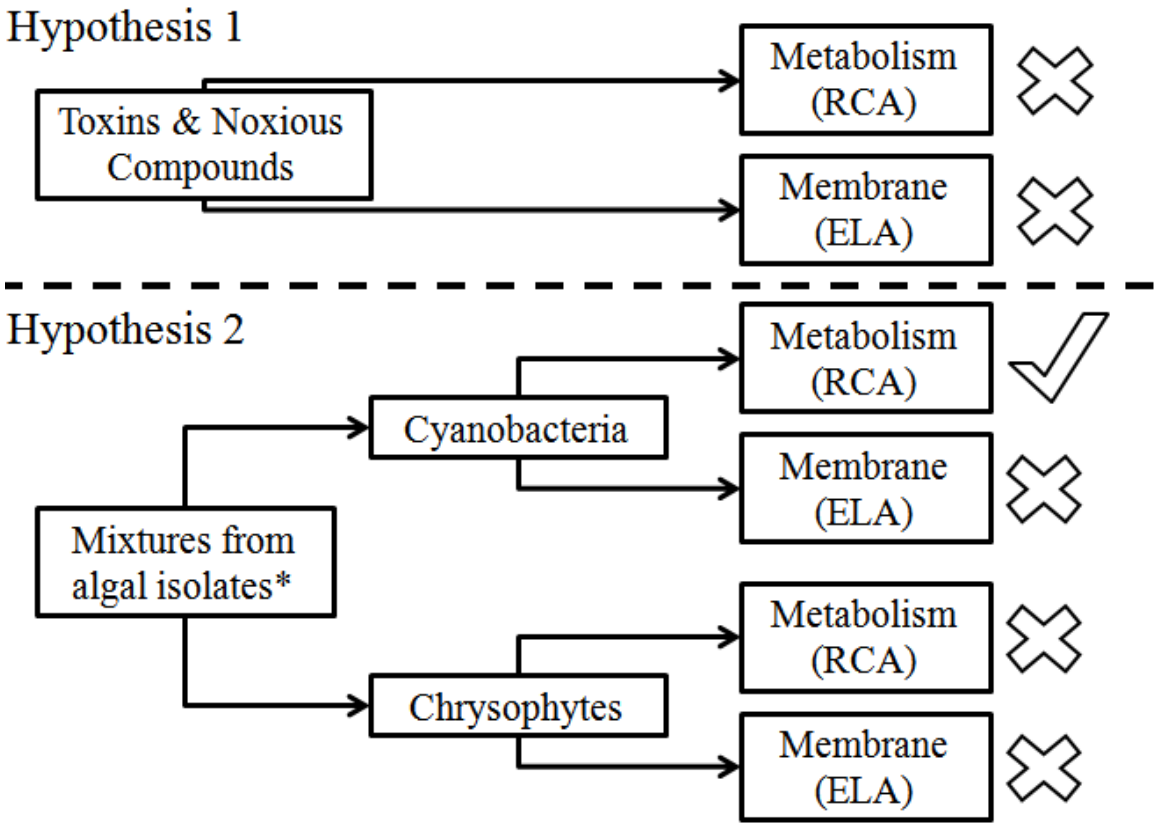

Figure 4.1. Flow chart illustrating the suitability of cell based assays to be used as screening tools for toxic and noxious compounds produced by cyanobacteria. X indicates test insensitive for application, and √ indicates that test is sensitive for application.

4.4 References

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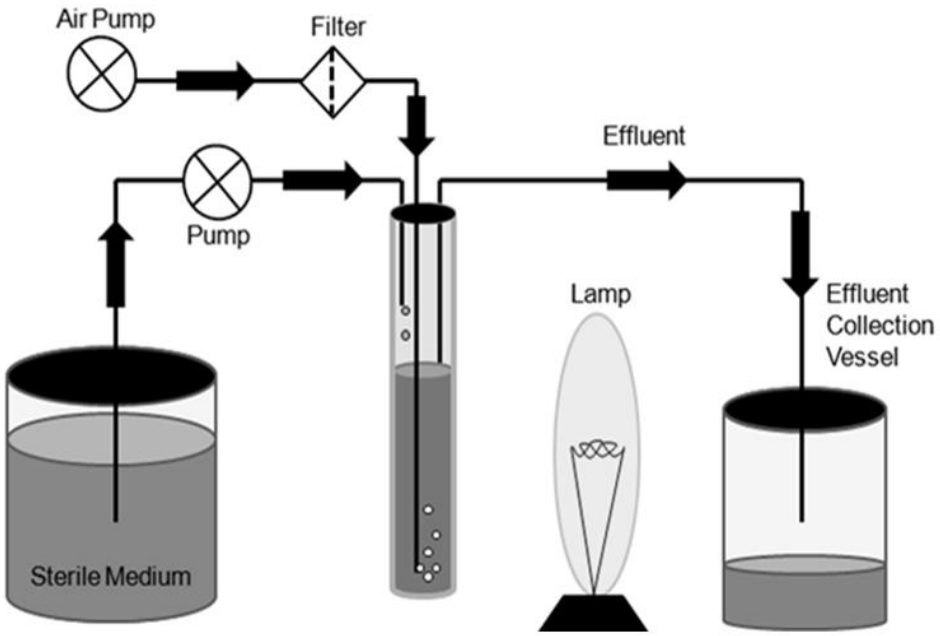
Appendix A



Schematic diagram of the chemostat apparatus used for cyanobacteria exponential growth at high (10 µM) and low (0.1 µM) Fe treatments. This design, specifically when used with a variable speed pump, allows the optimization and maintenance of cultures in exponential growth phase. This is made possible by determining the maximum growth rate (μ_{max}) of the culture and adjusting the dilution rate to match this value. Diagram not to scale.

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