The relative effects of the herbicide atrazine on selected microalgae

By

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Abstract

Cyanobacterial blooms are often associated with eutrophication of lakes and waterbodies which degrade the water quality due to chronic and episodic inputs of nutrients, water stratifications and climatic changes. Increasing terrestrial application of photosynthesis-inhibiting herbicides that enter water bodies during/after heavy rain, can affect the photosynthetic capacity and growth of phytoplankton at sub-lethal concentrations. As herbicide sensitivity of phytoplankton varies among species, their presence can alter phytoplankton community structure to favour more tolerant species, or particular groups such as cyanobacteria which are considered more tolerant of photosynthesis-inhibiting herbicides. This study examined the potential for photosynthesis-inhibiting herbicides to promote cyanobacterial blooms in temperate lakes and waterways. The most commonly applied triazine herbicide, atrazine, was used due to its solubility, mobility and persistence in temperate environments. The relative effects of atrazine on the growth of selected planktonic green algae and cyanobacteria (primarily bloom-forming *Anabaena* species) were investigated using laboratory mono-cultures and two-species competition cultures.

In the second chapter, the relative tolerance to atrazine of some common freshwater green algae (*Selenastrum capricornutum, Desmodesmus asymmetricus* and *Chlorella protothecoides)* and cyanobacteria of the genus *Anabaena*, particularly *Anabaena circinalis* were compared in single-species assays using *in-vivo* fluorescence estimation of growth rates. While the green algae species examined displayed higher intrinsic growth rates than *Anabaena* strains, their relative tolerance to atrazine (50 – 250 µg L^{-1}) expressed as EC_{50} was of similar magnitude and range (72-140 µg L^{-1}) compared to the seven *Anabaena* strains (59 - 111 µg L^{-1}) under light and temperature conditions typical of temperate mid-latitude summer conditions. However, atrazine tolerance varied significantly among the 10 species examined but there was no significant difference in mean atrazine tolerance between the two groups, the cyanobacteria and green algae indicating that the selective effects of atrazine operate at a species/strain level rather than more generally favouring cyanobacteria over green algae.

The third chapter adapted and tested a high through-put microplate-based approach as a rapid and reliable phytoplankton herbicide sensitivity assay that could be used to examine the influence of herbicides on the growth of green algae and cyanobacteria in two-species competition cultures. The assay was based on *in-vivo* fluorescence quantification of chlorophyll a and phycocyanin. Minimum detection limits and correlations of cell concentration and fluorescence were established for two species of eukaryotic green algae and seven *Anabaena* strains. Calibration curves were established for the seven species examined and the detection limits and ranges were sufficient for reliable detection and simultaneous estimation of cyanobacteria and green algal growth rates in two-species competition laboratory cultures. Two-species competition culture experiments were carried out using *A. circinalis* grown with the green algae *Selenastrum capricornutum* or *Desmodesmus asymmetricus*. The growth rate of *A. circinalis* strains showed a 20% increase in exponential growth rate compared to mono-culture controls, whereas the green algal species growth rate was reduced by 13-17%, indicating that allelopathic interactions may alter the selective effects of herbicides on phytoplankton community structure.

In the fourth chapter, relative inhibition of the green alga, *Desmodesmus asymmetricus* and the cyanobacterium *A. circinalis* by atrazine was examined at different combinations of light (high = 100, low =30 µmole photon m^{-2} s^{-1}) and temperature (high = 24°C ±1 and low= 18±1°C) when grown separately or in two-species competition cultures. When grown separately, *A. circinalis* showed similar or higher tolerance (EC_{50}) to atrazine as *D. asymmetricus* and maintained an increasingly higher growth rate with increasing atrazine concentration under all conditions, except at low light and high temperature where the growth rate of *D. asymmetricus* was higher at atrazine concentrations >150 µg L^{-1}. When grown in competition, *A. circinalis* was favoured in the presence of atrazine under high light conditions regardless of temperature, and *D. asymmetricus* was favoured by the presence of atrazine (or equally tolerant) under low light regardless of temperature. Overall, the presence of atrazine favoured *A. circinalis* at high light with the largest relative effect at low temperature. This may explain how temperate mid-latitude summer blooms of *Anabaena circinalis* can maintain their relative community dominance during declining autumn temperatures in lakes and rivers.

The fifth chapter used two-species competition cultures with different relative starting concentrations of *D. asymmetricus* and *A. circinalis* to determine whether the outcome of green algae/cyanobacteria growth competition could be reversed by atrazine starting from scenarios of different relative dominance (4:1, equal, or 1:4 starting concentration of each species). In the absence of atrazine, *D. asymmetricus* dominated 10 day growth competition experiments from scenarios from both dominant and equal starting concentration, whereas *A. circinalis* dominated only in cultures in which it started with 1:4 dominance. In the presence of low concentrations of atrazine (10-60 µg L^{-1}), *A. circinalis* dominated over *D. asymmetricus* regardless of the species dominance at the start of the experiment. The relative patterns of growth in the experiments suggested that the dominant factor during exponential growth phase (first 5-6 days) was inhibition of both species by atrazine but more severe inhibition for *D. asymmetricus*. After day 5 inhibition of *D. asymmetricus* by the allelopathic activity of *A. circinalis* became the dominant factor. These experiments show that the allelopathic activity of *A. circinalis* and low concentrations of atrazine (10µg L^{-1}) combine reverse growth competition outcomes even from a position of green algal dominance, and indicate a mechanism by which low concentrations of herbicides can shift algal communities toward cyanobacterial dominance in temperate mid-latitude lakes and rivers.

The influences of photosynthetic-inhibiting herbicides in combination with other adaptive physiological strategies/mechanisms that promote cyanobacterial blooms are also discussed.

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Table of contents

List of Tables

List of Figures

Chapter 1

General Introduction

1.1 Introduction

The effect of toxicants such as herbicides on aquatic species is an increasing ecological problem worldwide. Herbicides which are a sub-class of pesticides are used or targeted to protect plants from damaging influences such as weeds, disease and pests (US-EPA 2007). These herbicides are found alone or in mixtures in the aquatic ecosystems in agricultural landscapes which may result in undesirable side effects on non-target aquatic biota (Ronday et al., 1998; Liess and Schulz, 1999). Due to their, direct effects on these microorganisms and indirect effects on the trophic system, there is a considerable challenge in regulating their use and evaluating their potential impact on the ecosystem.

Most agricultural herbicides are applied during intensive farming, and residues can be found in adjacent lakes and rivers especially after heavy rain (Krieger et al., 1988; Richards and Baker, 1993). Herbicides are often applied in close proximity to water bodies and are liable to affect non-target aquatic plants, including microalgae after runoff through leaching or accidental spills. This can have a detrimental effect on the aquatic primary producers which are the most sensitive species in the freshwater systems and may exert a negative impact on the ecosystem structure and productivity (Gustavson et al., 2003). Some major impacts include reduction in algal taxa, displacement of sensitive species such as the chlorophytes by more tolerant species or groups (e.g. cryptophytes and cyanobacteria), and subsequent changes in food availability and quality for grazers, and ultimately an impact on the entire food web (Hamilton et al., 1988; Lürling and Roessink, 2006). Water quality and chemistry is also indirectly affected by herbicide inhibition of the photosynthetic phytoplankton such as increase in ammonium, dissolved carbon and nitrate levels, and reduction in oxygen and chlorophyll levels (Hamilton et al., 1989; Juttner et al., 1995).

1.2 Existing approaches for ecotoxicity assessment

Ecotoxicological studies can provide an understanding of the impact of anthropogenic toxicants on species abundance, diversity, productivity, community structure and shifts and nutrient cycling (Preston, 2002). Regulatory authorities and legislation have been set up to control and reduce herbicides usage and undesirable impacts on the environment, primarily based on toxicity data generated in the laboratory ecotoxicology bioassays (Norton et al., 1992; Wijngaarden et al., 2005).

Ecotoxicology bioassays are commonly based on single-species culture approaches to assess relative sensitivity or tolerance to particular chemicals. Even though they represent individual species which respond to a toxicant in isolation, the toxicity estimate of single-species test can still provide a preliminary generalization of how species may respond to such toxicant in a community assemblage (Wijngaarden et al., 2005).

The Bottle test method, where algal toxicity data is used to assess the effect of chemicals and effluents, was first developed in early 1970's to provide information on environmental safety of chemicals and effluents to meet the growing problem of eutrophication (National Eutrophication Research, 1971). Additional methods and standards have since been developed by various organisations such as the American Society for Testing and Materials (ASTM), American Public Health Association (APHA), International Organization for Standardization (ISO), the Organization for Economic Cooperation and Development (OECD), and the US Environmental Protection Agency (EPA) to improve the test procedures (Lewis, 1990).

Typically, these methods evaluate the growth inhibition of a set of recommended algal species to a chemical in a nutrient enriched media under controlled conditions. The concentration of the test chemical resulting in a proportional reduction in the algal population (usually 50%) is referred to as the EC_{50} (Lewis, 1990; Nyholm, 1990). However, due to variations of algal tolerance to the same chemicals, several algal species from a broad range of taxonomic representation are recommended in the laboratory testing or bioassay (Blanck et al., 1984). To enhance the predictive ability of these methods, some recommend that these laboratory-derived single species need field validation by incorporating ecological factors to the laboratory tests (Lewis, 1990).

Larsen et al., (1986) indicated the EC_{50} values from microcosm studies could provide a reasonable estimate of species tolerance toward atrazine. These differential tolerances among species could also provide authority bodies with basic information on the possible species dominance or community shifts under specific light and temperature conditions.

Traditionally, single-species toxicity methods have been assessed by the standardized algal bottle test methods, however with increasing demands for such tests, 96-well microplate approaches have been incorporated into many studies. This approach can be used as screening test for phytotoxicity to increase the efficiency and the processing of samples compared to the bottle test methods (Blaise et al., 1986; Thellen et al., 1989).

The toxicity on any single compound on any single organism varies significantly with environmental conditions. In addition, species interactions could play major roles in shifting community structure by favouring one species over another (De Bernardi, 1981; Preston, 2002). The large variations in reported EC_{50} values in the literature may also depend on the exposure period and the parameters measured, such as photosynthetic capacity, growth rate or biomass (Pannard et al., 2009). However, these so called “first tier” test systems have been debated and there is a need for the testing system to reflect the effects of toxicant in the real world situations (Liess, 2002). Therefore, micro- and mesocosm studies are considered important to validate single species studies of toxicants (DeNoyelles et al., 1982; Boxall et al., 2002; Wijngaarden et al., 2005).

Many researchers also recommend that ecotoxicological studies need to consider indirect effects and species interactions (Lampert et al., 1989; Boxall et al., 2002; Wijngaarden et al., 2005) so that toxicity estimates are more relevant to natural systems.

1.3 Photosynthesis inhibiting herbicides

Photosynthesis inhibiting herbicides such as triazines, act to interfere with photosynthesis. Light energy absorbed by chlorophyll molecules and light-harvesting complexes induce pigment excitation and trigger a chain of oxidation-reduction events. Maintaining the electron-transport process between the Photosystem II and Photosystem I (PS I) and the formation of NADPH and ATP is necessary for CO_2 fixation and other biochemical processes in algal cells (Fai et al., 2007). Once PS II absorbs light and received by the first electron acceptor or the (Q_A) which is tightly bound to the D2 protein, it is not able to accept another electron until it has passed it onto a subsequent electron carrier (Q_B) at the D1 protein (Maxwell and Johnson, 2000). Herbicides of the triazine family block electron transfer between the two photosystems by competing with binding at plastoquinone II in the reaction centre, the (Q_B) at the D1 protein. At low concentration of atrazine or low light, the herbicide can reduce photochemical process and growth rate by reducing carbon incorporation as electron transport reduced without altering cell viability (Murdock and Wetzel, 2011). Debloise et al., (2013) confirmed that atrazine interfere directly with phytoplankton capacity to convert light energy into biologically available energy such as ATP and NADPH. The relationship between photosystem inhibitor herbicides and growth rate and biomass was found consistent and linear and suited to measure the sub-lethal impacts of PS II inhibiting herbicides on microalgae (Magnusson et al., 2008). At high atrazine concentrations or high light, excess excitation energy leads to the generation of triplet chlorophyll, especially to cells which are not pre-acclimated to high light intensities. If not quenched by carotenoids which are efficient free-radical scavengers that protect the reaction center, this may induce the formation of singlet oxygen which induces lipid peroxide formation (Dodge, 1982; Powles, 1984; Heber et al., 2006; Fai et al., 2007). Damage of photosystem II due to photoinhibition occurs at both the donor site (water-splitting complex) and the acceptor site (Q_A) and/or (Q_B) acceptors (Powles, 1984; Jones et al., 2003).

The presence of triazines thus provides an opportunity for more tolerant species or groups to dominate over less tolerant species, leading to community shifts toward groups or species that have a higher tolerance (Lürling and Roessink, 2006). Single species laboratory bioassays have indicated that the green algae are less tolerant to inhibitors of photosystem II (PS II) than the cyanobacteria (Abou-Waly et al., 1991; Fairchild et al., 1998). Mesocosm studies also have shown that the presence of herbicides can favour cyanobacteria compared to green algae (Hamala and Kollig, 1985; Brock et al., 2004). Other environmental and seasonal variables (e.g. changes in light, temperature or nutrients, and species interactions) may also influence the relative tolerance of different species (Berard et al., 1999).

1.4 Community effects of photosynthetic inhibiting herbicides

A range of studies have indicated that the presence of photosynthetic inhibiting herbicides can shift community structure toward cyanobacteria (Hamala and Kollig, 1985; Gustavson et al., 2003; Lürling and Roessink, 2006). Changes in algal community structure have been reported in mesocosms and pond experiments presumably due to the selective toxicity of these herbicides altering species composition. For example, green algae, diatoms and cryptophytes were significantly lower in control microcosms compared to mesocosms contaminated with 100 μgL^{-1} of atrazine, while small unicellular cyanobacteria increased in relative abundance (Hamala and Kollig, 1985). However, in another study, the same atrazine concentration eliminated cyanobacteria but had little effect on diatoms (Herman et al., 1986).

At low concentrations of photosynthetic inhibiting herbicide, 2 μgL^{-1} of metribuzin severely affected chlorophytes while cyanobacteria and diatoms were stimulated in natural community study (Gustavson et al., 2003).

Resistance to triazine herbicides is possible and is thought to be caused by mutations that decrease binding of the herbicide within the thylakoid membrane (Pfister et al., 1979). Induced community tolerance has also been observed in contaminated biofilms exposed to pulses of the herbicide diuron. These communities appeared to be less impacted during second or third pulses as the photosynthetic efficiency of the chronically exposed microalgae was more tolerant to diuron than the non-exposed communities (Tlili et al., 2008; Tlili et al., 2011). However, this induced tolerance to the PS II inhibitors varies with environmental conditions (Berard and Benninghoff, 2001). Tolerance is also believed to be due to the heterotrophic behaviour of cyanobacteria or switch in energy-acquisition to PS I, which compensates for the inhibition of photosynthesis by PS II inhibitors (Seguin et al., 2001).

1.5 Allelopathic interactions

Community structures are also regulated by ability of certain species to produce allelochemicals which can inhibit the growth of species competing for limited resources (Graneli et al., 2008). Lake Eutrophication is usually associated with cyanobacterial blooms which alter the nutrients balance and the algal species that can compete successfully for available growth limiting nutrients have the potential to become dominant and form blooms. It has been suggested that the ecological role of allelopathy is to maintain cyanobacterial dominance after critical cell concentrations have been reached due to the environmental factors; therefore, other environmental factors likely also play a critical role in cyanobacterial bloom formation and interspecific competition (Suikkanen et al., 2004).

Interactions with other species and competition for nutrients and light resources within algal communities have also been suggested to be important factors influencing community responses to photosynthesis-inhibiting herbicides (Berard et al., 1999a). Further studies reported inhibition of the green alga, *Chlorella vulgaris* was three-fold greater in nanocosm community tests than in single-species laboratory tests (Berard et al., 2003). The growth of the cyanobacterial species, *Oscillatoria limnetica* was also not stimulated in monoculture microcosms and remained unaffected in natural uni-algal culture (Berard et al., 1999).

1.6 Atrazine

Atrazine is a triazine herbicide widely applied in agriculture to control annual grasses and broad-leaved weeds in selected vegetables and cereal crops, sugarcane, corn, canola, and forestry. It is applied as part of pre- and post-planting stages of forest plantations and to inhibit growth of weeds (Graymore et al., 2001). However, it is moderately water-soluble and weakly sorbed to soil (See Appendix 1 for the physicochemical characteristics of atrazine) which makes atrazine a relatively mobile herbicide that can leach to ground water or be transferred to surface water during spraying or heavy rains. As a result, atrazine has been more frequently detected in groundwater than any other herbicide (e.g. Belluck et al., 1991). Climatic conditions also contribute to atrazine persistence in temperate climates; dissipation rate decreases at low temperatures and herbicides half-life can be extended for years in rivers and lakes (Graymore et al., 2001; Kookana et al., 2010).

The ecological effects of atrazine on the phytoplankton assemblage have been widely debated and vary considerably in the literature, reflecting the complexity and the fragility of the natural ecosystem. For example, field studies by Solomon et al., (1996) considered that ecological effects of atrazine on the ecosystem to be considered at >50 µg L^{1}. However, Huber, (1993) claimed that 20 µg L^{-1} has no effect on photosynthesis whereas DeNoyelles et al., (1982) reported inhibition of some species at concentrations as low as 1 µg L^{-1}. Bérard et al., (1999) considered atrazine could have significant effects at concentrations as low as 10 µg L^{-1}.

The published data on atrazine effect on phytoplankton usually use the 50% damage level with varying measurement parameters and endpoints (Table 1.1). These values vary according to exposure period to atrazine, concentration, species sensitivities, and endpoint measurements. Although most of these values are above the trigger value which is set at 13 µg L^{-1} for the protection of aquatic species in Australia, sensitivity of microalgae to atrazine can be affected by other abiotic factors and exposure period (Wightwich and Allison, 2007). The range of atrazine

Table 1.1 Growth rate inhibition, EC_{50} values of published data on eukaryotic green algae and prokaryotic cyanobacteria toward atrazine.

Species	**EC_{50} (µg L^{-1})**	**Duration (days)**	**Reference**
Selenastrum capricornutum	283	3	Abou-Waley et al., 1991
Selenastrum capricornutum	214	7	Abou-Waley et al., 1991
Selenastrum capricornutum	50	3	Solomon et al., 1991
Scendesmus subspicatus	21	11	Solomon et al., 1996
Scenedesmus quadricauda	38-49	1	Larsen et al., 1986
Chlorella vulgaris	25	11	Solomon et al., 1996
Chlorella sp.	72.9	7	Tang et al., 1997
Anabaena flos-aquae	58	3	Abou-Waley et al.,1991
Anabaena flos-aquae	766	7	Abou-Waley et al.,1991
Anabaena inaequalis	100	12	Stratton, 1984
Anabaena cylindrical	3600	12	Stratton, 1984
Anabaena cylindrical	178	1	Larsen et al., 1986

concentration was found between 0.04 – 14 μgL^{-1} in NSW rivers, while in Tasmanian rivers and streams it ranged between less than 0.01 to 53000 μgL^{-1} during the period of 1989-1992 (Davies et al., 1994)

Natural phytoplankton communities are controlled primarily by physical and chemical factors. Phytoplankton community structure and composition are primarily determined by temperature, light, nutrients, mixing and winds events, grazing, and anthropogenic inputs (Klarer and Millie, 1994; Ke et al., 2008; Paerl et al., 2011), therefore, atrazine tolerance and the outcome of growth competition in the presence of atrazine would be expected to vary with changing light, temperature and nutrients (Mayasich et al., 1987; Lampert et al., 1989; Berard and Benninghoff, 2001; Pannard et al., 2009)

1.7 Blooms of *Anabaena*

One of the ubiquitous freshwater cyanobacteria that occur throughout Europe, North America, Asia, and Australia is the genus, *Anabaena,* an akinete-forming nitrogen-fixing species in the order Nostocales (Thompson et al., 2009). In Australian lakes and rivers, the species *Anabaena circinalis* can form large and persistent blooms that are sometimes neurotoxic due to their ability to produce the compound saxitoxin (Humpage et al., 1994). Areas affected by *A. circinalis* blooms include the Murray-Darling and the lower Murray River blooms between 1995 - 1997, the Myall Lakes system in 1999, Craigbourne Dam (Tasmania) in 1997 and summer blooms in Lake Trevallyn (Tasmania) in 2007-2009 (Humpage et al., 1994; Negri and Jones, 1995; Bobbi, 1997; Dasey et al., 2005; McCausland, 2011).

1.8 Monitoring contaminants/ herbicides in the aquatic system

Given the potential for herbicide contamination to alter aquatic community composition, monitoring herbicide concentration is an important ongoing challenge for environmental agencies. The universally accepted technique for monitoring is spot sampling that relies on collecting discrete samples from a waterbody at a particular time followed by a laboratory-based extraction and determination of analyte of interest (Mills et al., 2007; Kingston et al., 2000). Herbicide contamination in aquatic systems is usually episodic and highly variable in space and time as the highest concentration is associated with the first rainfall after application. Thus a need for monitoring over longer time periods makes passive samplers one of the promising tools (Vrana et al., 2005; Kot-Wasik et al., 2007). (See Appendix 1, laboratory testings of passive sampler in atrazine uptake in ultra-pure water and river water).

1.9 Study outline

A range of published studies have indicated that cyanobacteria are more tolerant of photosynthesis-inhibiting herbicides and that contamination by such herbicides can alter algal community composition in favour of cyanobacteria, particularly in community level micro- and meso-cosm studies. This study investigates this hypothesis using single-species laboratory culture models and two-species competition experiments to examine the combined effect oftraizine herbicides and allelopathic interactions on growth competition between cyanobacteria and eukaryotic microalgae.

1.9.1 Research approach

For the purposes of this study, the most widely applied triazine herbicide, atrazine, was chosen, because it is highly soluble in water and exhibits long half-lives in soil and water (Solomon et al., 1996), especially at the lower temperatures characteristic of temperate and cool-temperate regions. The culture experiments and competition models utilized a selection of common freshwater green algae (*Desmodesmus* and/or *Scenedesmus*) and cyanobacteria of the genus *Anabaena*, a common and globally distributed bloom-forming genus ranging from high-latitude cool-temperate to low-latitude tropical waterways. In particular, the study focused on *Anabaena circinalis*, a species commonly responsible for neurotoxic (saxitoxin, STX) blooms in Australian freshwater lakes and inland waterways.

Growth studies were conducted and evaluated in two formats, 50 mL tubes and a 96-well microplate with quantification of green algae and *Anabaena* adapted from Gregor and Marsalek (2005). The approach allowed two-species growth competition experiments, under varying environmental conditions that incorporate the combined effect of atrazine and allelopathic interactions between cyanobacteria and green algae.

The experiments carried out in this study are summarized below:

Chapter 2: Relative effects of atrazine on the growth of selected microalgal species.

The chapter examines the effect of atrazine on a selected group of green algae and cyanobacteria in single-species culture using 50 mL test tubes at a fixed light and temperature to determine the comparative extent of growth inhibition by atrazine. The hypothesis tested is whether atrazine tolerance of cyanobacteria of the genus *Anabaena* differs from that of other common species of green algae.

Chapter 3: Development of a high-throughput platform to measure cyanobacterial and eukaryotic algal growth in two-species competition cultures.

This chapter developed a method to measure growth of green algae and cyanobacteria in 96-well microplate format. Single species calibration curves were determined and simultaneous detection of cyanobacteria and green algae was assessed.

Chapter 4: Effect of seasonal light and temperature combinations on atrazine inhibition (EC_{50}) of cyanobacteria and green algae.

The effects of seasonal changes in light and temperature on growth inhibition of green algae and cyanobacteria by atrazine were examined. A combination of single-species and two-species and culture experiments were carried out utilizing the developed microplate method, allowing the assessment of the contribution of allelopathic interactions to differing tolerance of cyanobacteria and green algae to atrazine. The hypothesis tested was whether light, temperature and allelopathic interactions alter the effects of atrazine on growth competition outcomes.

Chapter 5: Effect of the herbicide atrazine on growth and competition between *Anabaena circinalis* and green algae, *Desmodesmus asymmetricus*.

The two species were grown in two-species competition culture experiments in the presence of different concentrations of atrazine (10-60 µg L^{-1}) and three different initial relative cell concentrations. The hypothesis tested was whether atrazine, in combination of allelopathic interactions, can alter growth competition outcomes starting from different relative species dominance scenarios: cyanobacterial dominance, green algal dominance and equi-dominance.

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Chapter 2

Relative effects of atrazine on growth of selected microalgal species

2.1 Introduction

The impact of the widespread use of commercially available chemicals for agricultural and industrial purposes is of serious concern around the world due to their toxic effect on non-target organisms and their effect on the structure and function of aquatic ecosystems (DeLorenzo et al., 2001). Of particular importance is the lack of information on guidelines and basis for regulatory agencies. Therefore, quick and sensitive test procedures are essential for assessing the potential of these toxicants on non-target species within the freshwater environment. Both short- and long-term assessments can be used to assess both acute and chronic responses and lethal or sub-lethal effects.

One of the main potential impacts of herbicides in aquatic environments is that they may alter the algal community composition, and in particular, there is evidence to suggest that herbicide contamination may alter the micro-algal community toward dominance of cyanobacteria (Bérard et al., 1999; Gustavson et al., 2003; Lürling and Roessink, 2006); however, A number of studies have shown that microalgae can differ in their tolerance to herbicides.

Several laboratory studies have shown that cyanobacteria have a higher tolerance to the photosynthesis-inhibiting triazine herbicides (atrazine and simazine) than microalgae (Abou-Waly et al., 1991; Peterson et al., 1997; Fairchild et al., 1998; Lürling and Roessink, 2006). Field studies and mesocosm experiments with natural communities also indicate that triazines may shift community structure toward cyanobacteria-dominance rather than a chlorophyte-dominated community (Bérard et al., 1999; Gustavson et al., 2003). In other microcosm studies 100 μg L^{-1} atrazine, inhibited the chlorophytes more than cyanobacteria (Hamala and Kolig, 1985). However, other studies of freshwater mesocosms showed that atrazine concentrations of 100 μg L^{-1} completely eliminated cyanobacteria while most diatoms (Bacillariophyceae) and green algae (Chlorophyta) species remained viable (Herman et al., 1986). In a similar mesocosm studies, Seguin et al., (2002) found that chlorophyceae was more tolerant to atrazine than cyanobacteria.

Atrazine was introduced in the 1950s primarily to control weeds in agricultural crops such as corn, sorghum wheat and soybeans (Fedtke, 1982; Braden et al., 1989) and remains one of the most widely used herbicides world-wide, especially in North America (Steinberg et al., 1995; Pannard et al., 2009). Because it is not tightly bound to soil particles and is water soluble, it is usually transported from the point of application to streams and sub-surface drainage via surface runoff (Muir et al., 1978; Solomon et al., 1996; Bruce et al., 2000). The persistence of atrazine in the aquatic environment varies considerably, from a half-life of several days (Kosinski, 1984) up to several months (Wauchope et al., 1992; Davies et al., 1994). Despite this wide variation, there is general agreement that atrazine persists longer under cool, dry condition and stable pH environment. Consequently, it is banned in many cool climate countries which possess fine textured soils (Kruger et al., 1996; Graymore et al., 2001). In Australia, the rate of dissipation of atrazine in subtropical regions such as Queensland was 2-3 times faster than sites located in the colder temperate climate of Tasmania in which atrazine residue was still detectable even year after application (Kookana et al., 2010). The range of atrazine concentration in Tasmanian streams and rivers between 1989- 1992 were 0.01 to 53000 µg L^{-1} (Davies et al., 1994).

Given the fact that atrazine can persist in water for a long priod especially in cold climate region, it's very likely the co-occurrence of *Anabaena* and the herbicide in the same water body. Since the detected atrazine concentrations ranged from 0.01 to 53000 µg L^{-1} in Tasmanian Rivers (Davies et al., 1994) and 0.04 to 14 µg L^{-1} in NSW rivers (Wightwick and Allinson, 2007); this can pose a threat to the aquatic environment especially that the trigger value for the protection of aquatic species in Australia is 13 µg L^{-1} (Wightwick and Allinson, 2007).

While most of the published data on photosynthetic inhibitor herbicides such as atrazine indicated a higher tolerance of the cyanobacteria than the green algae (Lürling and Roessink, 2006; Li, 2010; Singh et al., 2011) the range of algal species examined remains relatively low, and there is limited data on the effect of this herbicide on most common bloom forming freshwater cyanobacteria.

Blooms of toxic cyanobacteria are a common occurrence in Australian rivers and inland lakes and are responsible for livestock deaths and deteriorating water quality (Falconer, 2001). The most widespread and abundant genus is *Anabaena*, with the most prominent toxic species being the neurotoxic species *Anabaena circinalis*. This species forms regular blooms in a wide range of temperate and tropical rivers and reservoirs (Baker and Humpage, 1994; Bowling and Baker, 1996).

Most major Australian river catchments have already experienced substantial shifts in land use toward intensive agriculture, including crops where herbicide application is an established and regular practice (Kookana et al., 1998; Wightwick and Allinson, 2007).

Studies have reported that herbicides have been regularly detected in Australian surface and groundwater in which triazine herbicides are the most frequently detected herbicide (Davies et al., 1994; Elliott and Hodgson, 2004; Wightwick and Allinson, 2007). Movement of herbicide residues into the river systems may thus be a factor promoting blooms of *Anabaena circinalis* and other cyanobacteria in Australian rivers.

Using atrazine as an example, this chapter aims to determine whether common-bloom forming *Anabaena* species and *Anabaena circinalis* in particular, are more or less tolerant than common freshwater green algae to growth suppression by triazine herbicides.

2.2 Materials and Methods

2.2.1 Cultures and culture conditions

Eight of the ten strains of microalgae used in this study were supplied by CSIRO Collection of Living Microalgae (http://www.marine.csiro.au/microalgae/collection.html). These strains include three species of green algae (Chlorophyceae), and three species (7 strains) of *Anabaena* (Cyanophyceae) (Table 2.1).

Table 2.1 Isolation details of the algal strains used in this study.

Species	Strain	CSIRO Code	Source Locality	Isolator, Date
Chlorophyceae				
Selenastrum capricornutum	UTEX 1648	CS-327	River Nitelva, Akershus, Norway	Skulberg 01/01/95
Desmodesmus asymmetricus		CS-899	Tasmania, Australia	
Chlorella protothecoides	CCAP	CS-41	Aquaculture centre collection, NCMCRS	
Cyanophyceae				
Anabaena circinalis	ACBU01	CS-337/01	Burrinjuck Dam, NSW, Australia	C. Bolch 18/11/93
Anabaena circinalis	ACMB01	CS-537/02	Mt Bold Reservoir, SA, Australia	W. Van Dok, 01/01/93
Anabaena circinalis	ACCD17	CS-545/17	Craigbourne Dam, Tas, Australia	I. Jameson, 05/06/97
Anabaena circinalis	ACCR02	CS-533/02	Canning River Perth, WA, Australia	S. Blackburn
Anabaena circinalis	AC03		Lake Trevallyn, Tas, Australia	C. Bolch, 19/3/2008
Anabaena cf *spiroides*	ASCR05	CS-546	Canning River Perth, WA, Australia	I. Jameson, 05/06/97
Anabaena sp.	AN01		Lake Trevallyn, Tas, Australia	C. Bolch, 19/3/2008

All species were grown in MLA medium (Bolch and Blackburn, 1996) a modified version of ASM-1 (Gorham et al., 1964). For MLA medium, the source of phosphate was dipotassium hydrogen phosphate and the source of carbon and buffering capacity was improved by the addition of sodium hydrogen carbonate (Table 2.2). A number of the trace metal salts were also varied from ASM-1 medium whilst retaining equivalent trace metal composition between the MLA and ASM-1 media. Concentrated stocks of nutrients, trace metals and vitamins were prepared in 18 MΩ cm^{-1} water (Barnstead, Thermo Scientific, USA) and filter-sterilized through 0.2 µm cellulose acetate syringe filters (Minisart, Sartorius Corp., USA) and stored at 4°C until used to prepare MLA medium. Growth medium was prepared by addition of concentrated nutrients to autoclave-sterilized 18MΩ cm^{-1} water (UHQ water, Barnstead, Thermo Scientific, USA) (Table2.2).

All stock cultures were maintained in 100 mL Erlenmeyer flasks in a controlled environment incubator (Model HDISCB, Williams Refrigeration Ltd, , Melbourne, Australia) at 18±1 °C and a light intensity of 65 µmole photon $m^{-2}s^{-1}$ with a 12:12 h light: dark cycle.

2.2.2 Herbicides

Approximately 1000µg of Analytical grade atrazine (98.8% purity, Chem-Service, Pennsylvania 19381) was weighed and dissolved in 900 mL of autoclaved UHQ water under continuous agitation for 48 hours with periodic ultrasonication (Unisonics Pty Ltd, Sydney, Australia). No organic solvent or carrier solvent was used to avoid any possible side effects on the algae growth. Atrazine solution was then filtered through a 0.45 µm cellulose acetate membrane filter (Advantec MFS, USA), MLA medium components added, and the solution made up to a final volume of 1 litre.

Analysis of the prepared stock solution of MLA media with the dissolved atrazine was performed on a GBC HPLC system (GBC Scientific Equipment Pty Ltd, Victoria, Australia) consisting of LC1150 HPLC pump, LC1205 UV/Vis detector and LC1650

Table 2.2 Nutrient composition of MLA medium used in experiments with cyanobacterial and green algal species (pH=8.5 – 9.0) (Bolch and Blackburn, 1996).

Nutrient	Concentration (µM)
Salts/nutrients	
$MgSO_4.7H_2O$	200
$NaNO_3$	2000
K_2HPO_4	200
H_3BO_3	40
Micronutrients	
Na_2EDTA	12.8
$FeCl_3.6H_2O$	5.8
$NaHCO_3$	0.07
$MnCl_2.4H_2O$	0.02
$CuSO_4.5H_2O$	0.04
$ZnSO_4.7H_2O$	0.08
$CoCl_2.6H_2O$	0.04
$Na_2MoO_4.2H_2O$	0.02
Additional salts/nutrients	
H_2SeO_3	0.01
$CaCl_2.H_2O$	200
$NaHCO_3$	2011
Vitamins	
Thiamine HCl	0.29
Biotin	0.002
Vitamin B12	0.00036

Advanced Autosampler operated by Winchrom Lite software version 1.0, 2007-2008. Injection volume was 50 µL with a mobile phase consisting of acetonitrile:UHQ water (60:40 v/v) with separations on a SUPELCOSIL LC-PAH column for polyaromatic hydrocarbons (Catalogue no 58229, column size 25 cm x 4.6 mm x 5 µm). Atrazine concentration in the media was determined from a calibration curve using external standards of atrazine dissolved in UHQ water containing 5% acetonitrile at a concentration ranging from 100– 2000 µg L^{-1}. Detection of analyte occurred at 220 nm.

2.2.3 Toxicity studies

Growth experiments with the algal cultures were carried out at concentrations of atrazine ranging from 50µg L^{-1} to 250µg L^{-1} prepared by quantitative dilutions of the stock MLA solution containing 1000 ug L^{-1} atrazine (range determined from preliminary experiments). Prior to the experiment, cultures were maintained in log-phase growth by regular sub-culture in 100 mL Erlenmeyer flasks. To initiate a growth experiment, one mL inocula of each strain was diluted into a 50 mL screw-capped borosilicate glass tube (Kimax, USA) containing MLA medium containing atrazine, mixed thoroughly and one mL of the dilution used to inoculate tubes containing 40 mL of MLA medium prepared with different concentrations of atrazine. Five concentrations of atrazine were tested in accordance with OECD guidelines (Guideline 201, 1981). All experiments included controls that contained no atrazine (Figure 2.1), and all species/concentration treatments were replicated three times.

Growth rate was estimated daily by *in-vivo* fluorescence using a Turner Fluorometer (model 10-A-005-CE Fluorometer; Turner Biosystems, CA, USA). This method, measures the fluorescence of the algal pigment, chlorophyll a which is also proportional to the activity of algal pigment or the cell biomass. Growth and photosynthesis are tightly coupled in phytoplankton and cyanobacteria, therefore the change in fluorescence can be used as a proxy for growth rate. In addition, photosynthesis and fluorescence responses to herbicides have also been shown to

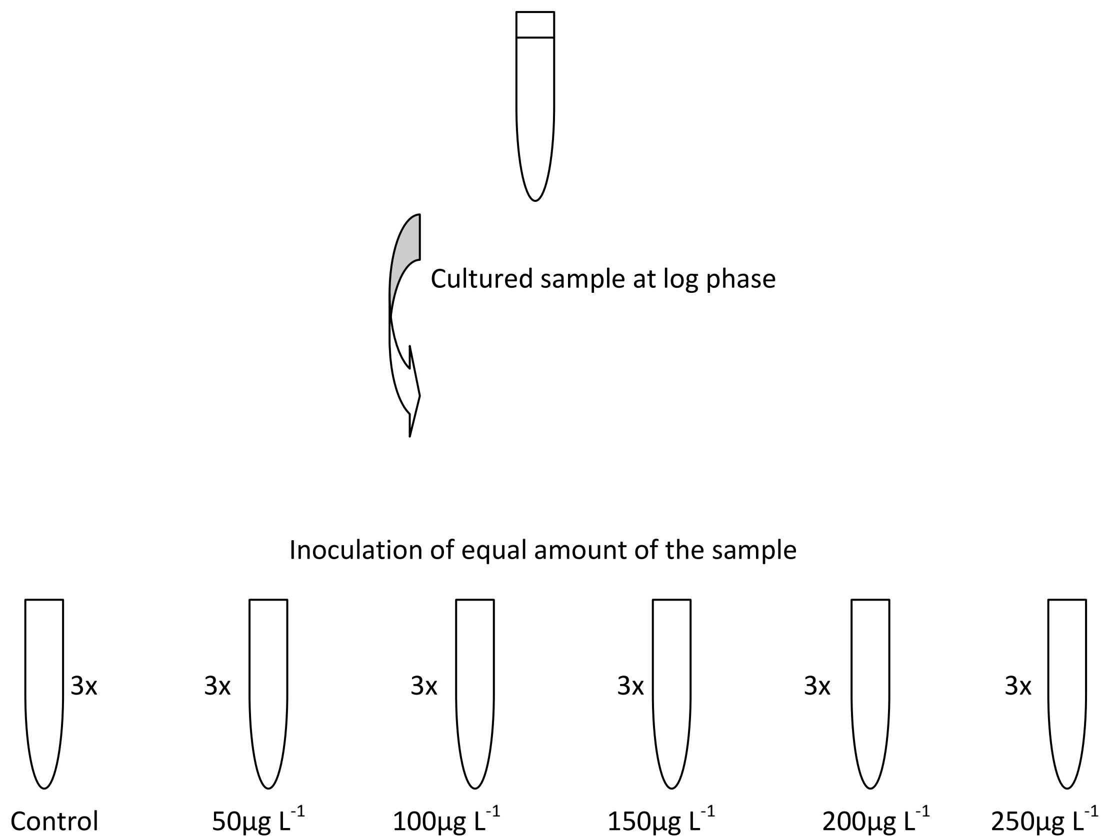

Figure 2.1 Inoculation of tubes containing growth media prepared with different concentrations of atrazine ranged from 50µg L^{-1} to 250µg L^{-1}. One tube was inoculated at a time at each concentration, and replicated three times.

be highly correlated to changes in growth rate (Magnussen et al. 2008). Fluorescence was determined at inoculation and daily for seven days. Before measurements, tubes were mixed gently by inversion, and returned to the incubator at random positions to minimize positional effects including minor light and temperature variation during the experiment.

2.2.4 Data analysis

The exponential growth rate of each replicate was determined during exponential phase growth only according to the following formula:

Growth rate (μ) = ($\ln N_{t2}$ - $\ln N_{t1}$)/ Δt

> Where N_{t2} is the population size at the end of the time interval (t_2) and N_{t1} is the population size at the beginning of time interval (t_1), and Δt is the length of time interval (t_2- t_1) (Andersen, 2005).

The exponential growth rate for each replicate at each concentration was expressed as a proportion of the mean growth rate relevant to the no-atrazine control, the relative growth rate plotted against atrazine concentration for each replicate, and a linear or log-linear curve fitted to the data for each replicate. The EC_{50} concentration (concentration at which growth was reduced to 50% of control) was calculated for each replicate from the equation of the curve and the mean EC_{50} for each species determined from the three replicate EC_{50} values.

Comparison of the mean EC_{50} between the green algae and cyanobacteria was compared using a time-blocked design of a one-way analysis of variance (ANOVA) using SPSS statistics 17.0.

2.3 Results

2.3.1 Atrazine concentration

The concentration of prepared atrazine in MLA medium was determined based on the linear formula generated from the standard curve of atrazine standards dissolved in UHQ water containing 5% acetonitrile. The replicated concentrations: 100, 500, 1000, and 2000 µg L^{-1} (Figure 2.2), and the calculated atrazine concentration were 1140 ± 13 µg L^{-1} from a sample of atrazine dissolved in MLA.

2.3.2 Toxicity studies

All 10 species listed on table 2.1 showed a curvi-linear reduction in the logarithmic growth rate with increasing atrazine concentration (Figure 2.3). The green algae displayed higher growth rates in the no-atrazine control than any of the *Anabaena* species examined, and this pattern was maintained at all atrazine concentrations examined. The green algae displayed steeper decline in growth rates as atrazine concentration increased, especially for *Chlorella protothecoides* and *Desmodesmus asymmetricus*. The calculated mean of EC_{50} and EC_{10} values and their growth response to atrazine is indicated in (Figure 2.4) and (table 2.3) for the 10 algal species.

The time factor showed statistically no significant differences (F=1.92, df=2,18, and P=0.175) in which inoculation or treatment was replicated three times at each concentration of atrazine. By comparing the EC_{50} values between the different 10 species grown under the light and temperature indicated previously, there were significant differences based on the microalgal species (F=3.52, df=9,18, and P=0.011).

Tolerance to atrazine as indicated by (Figure 2.5) which varied significantly among the ten species (F=3.52, df=9,18, and P=0.011). The green algae species, *Selenastrum capricornutum* was significantly more tolerant of atrazine than the other nine species, while *Anabaena circinalis* ACCR02, *A circinalis* AC03 and *Anabaena spiroides* ACSR05 were less tolerant. The tolerance of the green algae *Chlorella protothecoides* and *Desmodesmus asymmetricus* did not differ

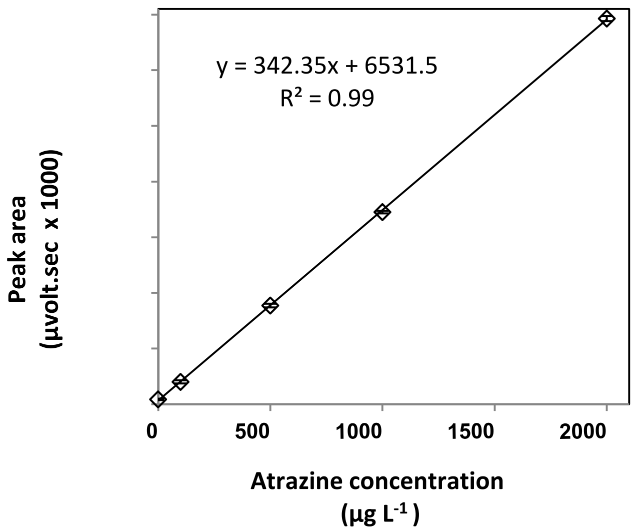

Figure 2.2 Calibration curve for GBC HPLC analysis of atrazine (± SE).

significantly from *Anabaena circinalis* strains ACBU01, ACMB01 and ACCD17 and the *Anabaena* sp. AN01.

When comparing the two groups (Figure 2.6), no significant difference to atrazine tolerance expressed as EC_{50} values (F =2.74, df =1, 26 and P=0.11). The mean EC_{50} value of eukaryotic green algae showed a wider range than the prokaryotic cyanobacteria.

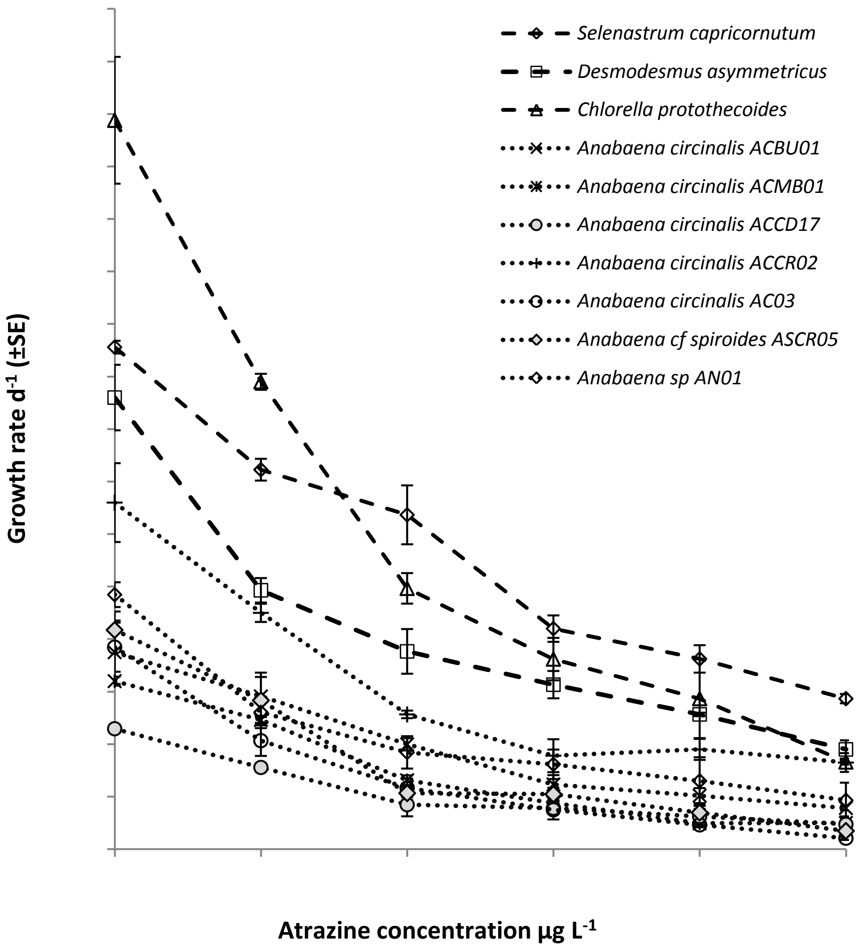

Figure 2.3 Effect of atrazine on 10 species tested of green algae and cyanobacteria at a light intensity of 65 µmole photon $m^{-2}s^{-1}$ and 18.0 ±1.0 °C, over a period of 7 days. All 10 species showed a gradual decrease with increasing atrazine in a curved rather than a linear relationship. All treatments retained a positive growth rate (±SE) even at 250 µg L^{-1}.

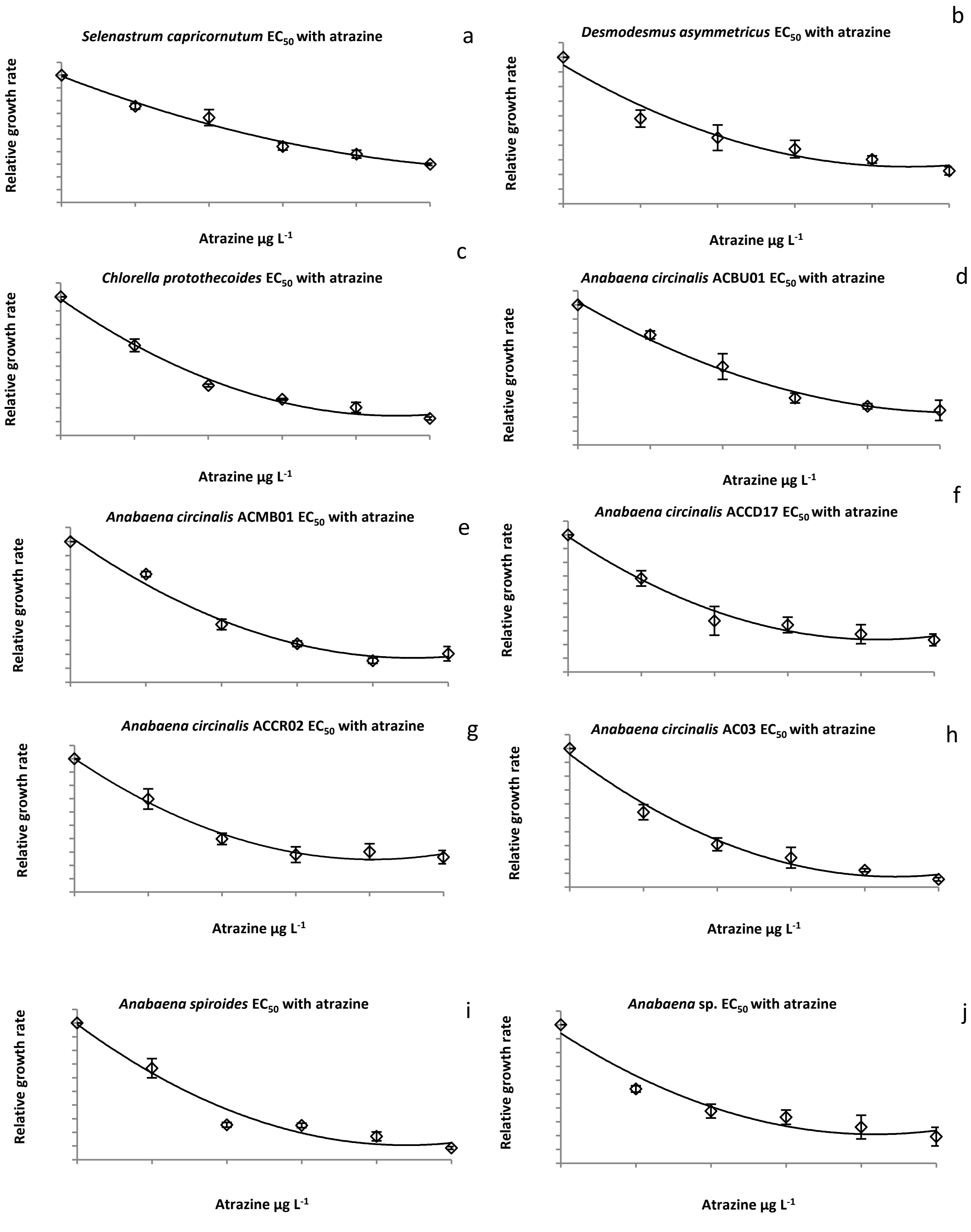

Figure 2.4 Growth responses of 10 algal species tested to increasing atrazine concentration. Relative growth rate (±SE) is expressed as a proportion of corresponding no-atrazine controls. The curve shown is fitted to mean growth rate data, however, mean EC_{50} values were calculated from log-linear curves fitted for each independent replicate.

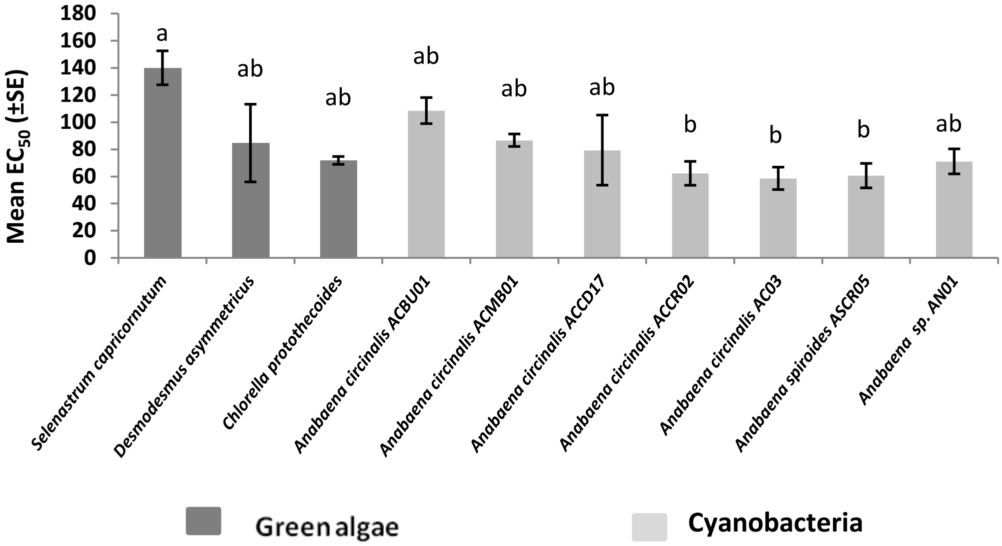

Figure 2.5 The comparative tolerance of 10 species/ strains tested of green algae and cyanobacteria to atrazine (mean EC_{50} ± standard errors). Different letters indicate significantly different means ($p<0.05$).

Table 2.3 Growth rate inhibition, EC_{50} and EC_{10} values (±SE) of the ten species/ strains of green algae and cyanobacteria tested toward atrazine.

Species	**EC_{50} ($\mu g\ L^{-1}$)**	**EC_{10} ($\mu g\ L^{-1}$)**
Selenastrum capricornutum	139.9± 12.5	20.3±6.9
Desmodesmus asymmetricus	84.4±28.5	8.6±3.7
Chlorella protothecoides	71.7±2.9	5.66±2.8
Anabaena circinalis ACBU01	108.46±9.6	20.7±6.96
Anabaena circinalis ACMB01	86.56±4.6	16.7±3.86
Anabaena circinalis ACCD17	79.31±25.8	10.5±5.1
Anabaena circinalis ACCR02	62.18±8.8	6.9±3.5
Anabaena circinalis AC03	58.49±8.3	5.1±1.2
Anabaena cf *spiroides* ASCR05	60.48±9.1	7.8±4.4
Anabaena sp. AN01	70.99±9.2	4.7±1.2

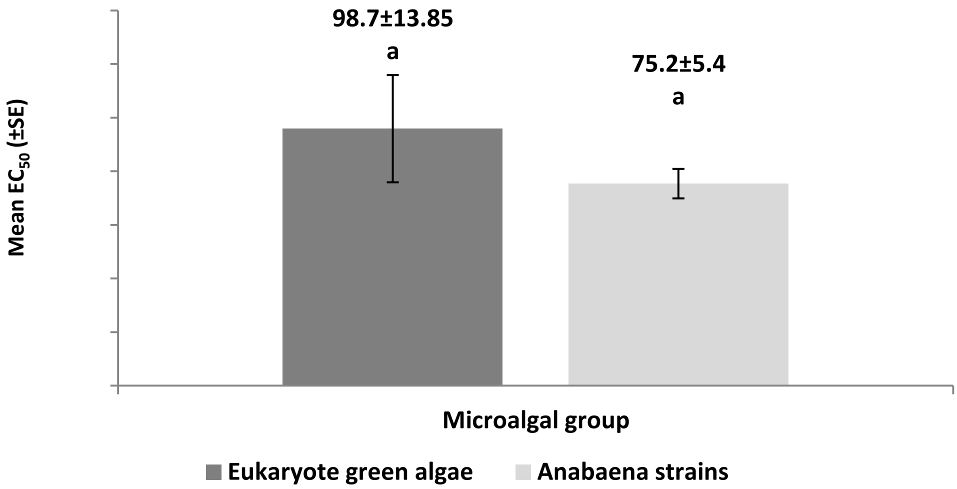

Figure 2.6 The comparative tolerance to atrazine between the two groups of algae tested (EC_{50}± standard errors). Paired non-capital letters indicate no significant difference ($p<0.05$).

2.4 Discussion

The data presented here indicate that strains of the common bloom-forming cyanobacterium *Anabaena* and *A. circinalis* display a similar range of tolerance to atrazine (59- 111 µg L^{-1}) as three common green algae (72- 140µg L^{-1}). Based on comparative suppression of growth (expressed as EC_{50}), the findings do not support the assertion that green algae are less tolerant to herbicides in general (Hollister and Walsh, 1973; Tang et al., 1997; Li, 2010) or that cyanobacteria are more tolerant of triazine herbicides (Stratton, 1984; Abou-Waly et al., 1991; Fairchild et al., 1998; Singh et al., 2011). The data here are consistent with previously published atrazine tolerances of green alga (*Selenastrum capricornutum)* (Abu-Waley et al 1991, US-EPA 2003) that used Chlorophyll a content as a proxy for growth to determine the effect of atrazine on the freshwater algae expressed as EC_{50} value over time. Other EC_{50} values for atrazine attained for *Chlorella* by (Tang et al 1997) are also similar to this study using a similar testing protocol (Table 2.4).

The published range of EC_{50} for atrazine for cyanobacteria shows considerable variation. For example, for *Anabaena flos-aquae* published values range from 58 - >3000 µg L^{-1} (Table 2.5) in which chlorophyll a was measured fluorometrically *in-vitro* in the former and *in-vivo* in the latter using different atrazine concentration. In this study, the EC_{50} values of *Anabaena* strains differ from the published values however these strains have not been used in any previous herbicide toxicity studies. Calculated EC_{50} values for either the green algae or *Anabaena* species also vary depending on different testing protocols (Stratton 1984).

Published data for tolerance to individual herbicides show considerable variation. Some differences may be due to differences in the light and temperature conditions used, incubation period, to the method used to estimate biomass or growth rate, or differences in growth performance in different formats (e.g. low volume plates or larger volume tubes). Some variation in values may also be associated with incubation times and low sampling frequency (e.g. 3 day versus 7 day experiments). Estimating growth rate from two timepoints over a standardized time period (e.g. Abou-Waly et al., 1991) or multiple-points (Stratton, 1984; Fairchild et al., 1998) also potentially integrates substantial lag- and stationary-phase periods evident at some herbicide concentrations, confounding growth response to the herbicide with the effect of culture acclimation and/or nutrient limitation. The period of exponential growth in the experiments here varied considerably, from as long as 8 days to as short as 3 days depending on the concentration of atrazine. As growth rates were estimated from regressions of no fewer than 3 sample points, the relationships and EC_{50} estimations presented here are unlikely to be confounded by these factors. While no consistent difference in tolerance could be demonstrated between *Anabaena* species and green algae, the substantial (almost two-fold) differences in tolerance indicates that atrazine contamination can still have significant species/strain selective effects on algal communities at even the lowest concentrations used here (50 µg L^{-1}) with relative reduction in growth varying from 21-46% among the species/strains examined here (Figure 2.5).

In Australian ground water and surface water, triazine herbicides contamination is usually detected at high concentration after application and heavy rainfall in areas where broad acre application of herbicides is used to control weed growth. In NSW, atrazine has been detected at concentrations ranging between 0.04 to14 µg L^{-1} (Wightwick and Allinson, 2007) while concentrations detected in Tasmanian rivers and streams range from <0.01 µg L^{-1} to 53000 µg L^{-1} and from <0.01 µg L^{-1} to 478.5 µg L^{-1} for atrazine and simazine, respectively, during the period 1989-1992 (Davies et al., 1994). However, Davies may have made an error because 53 mg L^{-1} is almost twice the solubility in fresh water and it was perhaps 53 µg L^{-1}.

Most of the detected atrazine and simazine which was principally used by the forestry industry were from samples collected from streams draining forestry plantations. In 1997, the manager of public forest resources in Tasmania (Forestry Tasmania) ceased using triazine herbicides and instead shifted to use of glyphosate and/ or metsulfuron-methyl to control weed growth during establishment of tree seedlings. The triazine herbicides including atrazine, simazine and hexazinone were responsible for a total of 85.5% of herbicide detected in Tasmanian surface fresh waters during the years of 1993-2003 with concentration ranging from >0.5 µg L^{-1}

Table 2.4 Comparison of growth rate inhibition, EC_{50} values to other published data on eukaryotic green algae toward atrazine.

Species tested	**EC_{50} (μg L^{-1})**	**Reference**	**Notes**
Selenastrum capricornutum	140	This study	7-day EC_{50}, (Biomass/Chl a)
Desmodesmus asymmetricus	84	This study	7-day EC_{50}, (Biomass/Chl a)
Chlorella protothecoides	72	This study	7-day EC_{50}, (Biomass/Chl a)
Selenastrum capricornutum	214	Abou-Waly et al., 1991	7-day EC_{50}, (Chlorophyll a)
Selenastrum capricornutum	4	US-EPA, 2003	4-day EC_{50}, (cell numbers)
Selenastrum capricornutum	150	US-EPA, 2003	4-day EC_{50}, (Chlorophyll a)
Selenastrum capricornutum	34-53	Larsen et al., 1986	24 hr EC_{50}, (^{14}C uptake)
Scenedesmus quadricauda	38-49	Larsen et al., 1986	24 hr EC_{50}, (^{14}C uptake)
Scenedesmus quadricauda	171	Tang et al., 1997	7-day EC_{50}, (Chlorophyll a)
Chlorella vulgaris	94	Fairchild et al., 1998	4-day EC_{50}, (Chlorophyll a)
Chlorella sp.	72.9	Tang et al., 1997	7-day EC_{50}, (Chlorophyll a)
Chlorella pyrenoidosa	300	Stratton, 1984	14-day $EC_{50,}$ (cell numbers)
Chlorella pyrenoidosa	1000	Stratton, 1984	14-day $EC_{50,}$ (Biomass/Chl a)

Table 2.5 Comparison of growth rate inhibition, EC_{50} values to other published data on prokaryotic cyanobacteria toward atrazine:

Species tested	**EC_{50} ($\mu g\ L^{-1}$)**	**Reference**	**Notes**
Anabaena circinalis	59-111	This study	7-day EC_{50}, (Biomass/Chl a)
Anabaena sp.	71	This study	7-day EC_{50}, (Biomass/Chl a)
Anabaena spiroides	61	This study	7-day EC_{50}, (Biomass/Chl a)
Anabaena flos-aquae	58	Abou-Waly et al., 1991	3-day EC_{50}, (Chlorophyll a)
Anabaena flos-aquae	766	Abou-Waly et al., 1991	7-day EC_{50}, (Chlorophyll a)
Anabaena flos aquae	>3000	Fairchild et al., 1998	4-day EC_{50}, (Chlorophyll a)
Anabaena variabilis	100	Stratton, 1984	12-day EC_{50}, (^{14}C uptake)
Anabaena variabilis	5000	Stratton, 1984	12-day EC_{50}, (growth rate/Chl a)
Anabaena inaequalis	300	Stratton, 1984	12-day EC_{50}, (^{14}C uptake)
Anabaena inaequalis	100	Stratton, 1984	12-day EC_{50}, (growth rate/Chl a)

to > 20 μgL^{-1} (Elliott and Hodgson, 2004). These values, are of similar order of the EC_{50} values of species examined here, and sufficient to suppress algal growth and have potentially selective effects. The effect of the atrazine on aquatic organisms is related to the persistence of atrazine in the environment due to its physicochemical characteristics. In nature atrazine can undergo, chemical degradation as pH changes (Tomlin, 1994) or photo-degraded to less dangerous molecules by sunlight (Lányi and Dinya, 2003). In Tasmanian Rivers, residues of atrazine decreased with time from a median of 8.1 to 3.8 μg L^{-1} after one month, then to a median of 0.3 μg L^{-1} after 13-15 months after spraying (Davies et al., 1994) which shows the persistent nature of the herbicide in colder temperate regions like Tasmania (Kookana et al., 2010). Although such low concentrations may not pose permanent damage to aquatic environment (Huber, 1993; Solomon et al., 1996), seasonal variations and changing parameters of light and temperature could alter atrazine tolerance of freshwater algae which could have ecological impact on the species composition in the aquatic system (Bérard et al., 1999). Therefore, not only *Anabaena* tolerance may increases but also growth could be stimulated in the presence of low concentration of atrazine in water body due to changes in seasonal variations as indicated by Bérard et al., (1999). This tolerance is also believed to be due to the heterotrophic behaviour of cyanobacteria or high D1 regenerations (Bérard et al., 1999a; Seguin et al., 2001). In previous studies, Kawamura et al., (1978) indicated that the proportions of PSII and PSI varies depending on the growth conditions of *Anabaena* strains; therefore, under weak light, the numbers of PSII reaction centers of cyanobacteria was markedly smaller than PSI. A similar adaptive capability was found in cyanobacteria and in the presence of the photosynthetic inhibitor herbicide in which PS I was higher than PS II under high light condition (Koenig, 1990). These evidences suggest that these reaction centers are a variable component which may undergo phase shifts in cyanobacteria.

This study indicates that atrazine can have influence on community structure but doesn't necessarily favour the cyanobacteria over green algae. From Table 2.5, the growth rate inhibition is lower than previoulsy published values perhaps because of the relatively low-temperature of 18±1 °C compared to other studies. Berard et al., (1999a) has indicated growth inhibition by atrazine of the cyanobacterium, *Oscillatoria limnetica* was lower when experiments were performed at low temperatures.

This study concludes that the variation and tolerance to atrazine among strains of cyanobacteria is similar to that among the green algae. While presence of atrazine can create a shift among species there is no evidence from the experiments carried out here, that atrazine favours cyanobacteria over other groups of microalgae.

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Chapter 3

Development of a high-throughput platform to measure cyanobacteria and eukaryotic algal growth in two-species competition cultures

3.1 Introduction

Bioassays are important for assessing the toxicity, sub-lethal effects and bioconcentration of pollutants on aquatic environments. They also serve in evaluation and registration of pesticides and herbicides in many countries. Traditionally, microalgae are used in standard toxicity assays using flask methods (OECD, 1981; Fazio et al., 1993). However, the increases in ecotoxicological testing demands have prompted the need for more efficient, cost-effective and less labour intensive methods.

Growth experiments with microalgae typically use manual counts by haemacytometer which is time consuming and requires skills and patience. While electronically particle counters and flow cytometers are less labour intensive, both have difficulty with cells that form colonies or chains. As an alternative, multi-well plastic microplates are widely used platform for bioassays and are a practical format for high through-put algal procedures (Blaise et al., 1986; Lukavský, 1992). For algal bioassays, higher sensitivity and improved growth rates can be obtained using 24-well microplate formats due to increased culture volume (Geis et al., 2000). Other improvement includes the use of non-static microplates, and growth medium or nutrient additions but increases the labour and cost of bioassays (Radetski et al., 1995). These improvements show enhanced algal growth rate in microplates with no effect on the outcome of growth inhibition tests (Thellen et al., 1989).

Fluorescence is considered one of the most sensitive techniques to measure chlorophyll and accessory pigments even in natural environment in linear relationship (Lorenzen, 1966). Microplate-readers equipped with fluorescence detection can thus be configured to detect *in-vivo* fluorescence of chlorophyll a and other accessory pigments as a proxy for total biomass or cell concentration (Sieracki et al., 2005). Depending on their pigment composition of the cells, different phytoplankton groups can also be identified based on their absorption and fluorescence emission spectra. *In-vivo* fluorescence of photosynthetic pigments present in phytoplankton cell offers a potential way to determine total phytoplankton amounts and detections, however, the differentiation of eukaryotic green algae is based on chlorophyll a fluorescence while prokaryotic cyanobacteria on phycocyanine fluorescence (excitations/emission spectra) (Gregor et al., 2005). Phycocyanin is considered an accurate and useful variable to quantitavily measure cyanobacteria than chlorophyll a (Ahn et al., 2002). These pigments are excited at different wavelength with green algae at the lower range (400-530 nm) and the cyanobacteria at a relatively higher range (550-630 nm) (Vincent, 1983; Mihaylova et al., 2003; Gregor and Marsalek, 2005).

The purpose of this chapter is to evaluate and calibrate a previously published high throughput approach for simultaneous *in-vitro* quantification of green algae and cyanobacteria (Gregor and Marsalek, 2005) that can be used to examine the effect of herbicides on growth competition between green algae and cyanobacteria. Quantification sensitivity and range are determined for two green algae; *Selenastrum capricornutum* and *Desmodesmus asymmetricus* and five strains of the cyanobacterium *Anabaena circinalis* and the performance of the method examined in two-species competition cultures between green algae and cyanobacteria.

3.2 Materials and Methods

3.2.1 Algal strains and culture conditions

Seven strains of microalgae including 2 strains of green algae (*Selenastrum capricornutum,* and *Desmodesmus asymmetricus*) and 5 strains of *Anabaena* species (*Anabaena circinalis* ACMB01*, Anabaena circinalis* ACCD17*, Anabaena circinalis* ACBU01*, Anabaena circinalis* ACCR02*,* and *Anabaena circinalis* AC03) were obtained from the Australian National Algal Culture Collection (CSIRO, Marine and Atmospheric Research, Hobart, Australia) and cultured in MLA medium (Bolch and Blackburn, 1996) as described in Chapter 2. The stock cultures were maintained in 100 mL Erlenmeyer flasks containing 50 mL of growth medium at a constant irradiance of 65 µmole photon $m^{-2}s^{-1}$ with an 18: 6 h light: dark cycle and a temperature of 21±2 °C.

Prior to calibration experiments, cultures were grown to late-logarithmic growth phase under the conditions above as estimated using *in-vivo* fluorescence measured using a Turner Fluorometer (model 10-A-005-CE; Turner Biosystems, CA, USA). Flasks were swirled manually once a day to limit cell clumping and cell deposits on the glass flask. One mL sub-samples were retained and fixed with lugol's iodine and cell concentration was estimated in triplicate using a Sedgwick-Rafter chamber.

3.2.2 Calibration for detection of single algal species

Preliminary testing of sensitivity of detection and quantification of microalgae was carried out in polystyrene 96-well microplates, using a TECAN Genios plate reader (TECAN Sales Switzerland AG, Mannedorf, Switzerland). Initial studies included comparisons of white-walled and black-walled multi-well plates (Greiner bio-one, Kongress Industrielle Zelltechnik, Lubeck, Germany, Catalogue no 655 090 and 655 098, Flat bottom) with either top- or bottom-reading of fluorescence. Proportional serial dilutions (in MLA medium) of the late-logarithmic phase cultures were prepared in clear-walled 24-well microplates (TPR, Switzerland, product no. 92424; 50% dilution for green algae, 40% for cyanobacteria), and 260 µL of each dilution transferred to duplicate wells of 96-well micro-plates. All plates included duplicate blank wells containing MLA medium only.

To determine reproducibility of the instrument detection, *in-vivo* fluorescence was determined in triplicate with the plate-reader set to the parameters described by Gregor and Marsalek (2005): Excitation wavelength: 485 nm (20 nm bandwidth) for eukaryotic green algae pigments (Chloropyhll a and carotenoids) and 595 nm (20 nm bandwidth) instead of (590 nm) for cyanobacterial pigments (Phycocyanin); emission wavelength 670 nm (25nm bandwidth) with a wider bandwidth to fit the emission wavelength of both chlorophyll a (685nm) and phycocyanin (645nm); gain: 80; integration time 40 µsec; Lag time: 0; number of flashes: 3.

Minimum sensitivity (lower detection limit) was determined for each species by comparison of lowest dilutions with media-only controls using student's t-test. (i.e. as the lowest concentration statistically higher mean fluorescence, alpha value = 0.05).

3.2.3 Simultaneous detection and quantification of green algae and cyanobacteria

Cultures of one green algal species, (*Desmodesmus asymmetricus*) and one *Anabaena* species (*Anabaena circinalis* ACCR02) were grown to logarithmic-phase at 21 ± 2 °C and continuous illumination of 65µmole photon PAR $m^{-2}s^{-1}$. These cultures were then diluted to approximately equal cell concentrations (determined by the *in-vivo* fluorescence from the single species calibration curve) and mixed at various ratios (see Figure 3.1).

A 96-well plate was then inoculated with 260 µL of each 2-species mixture and *in-vivo* fluorescence measured at 670nm (excitation at 595nm for cyanobacteria and 485nm for green algae) using the TECAN microplate reader. Mean fluorescence was calculated from three readings and a calibration curve for two-species detection established by plotting the ratio of chlorophyll a to phycobilin fluorescence versus the relative cell concentration of the green algae to cyanobacterium.

3.2.4 Two-species mixed culture studies

The growth of the eukaryotic green algae, *Desmodesmus asymmetricus* and *Selenastrum capricornutum,* was examined alone and in mixed culture with either of the two cyanobacteria strains, *Anabaena circinalis* ACCR02 and *A. circinalis* ACBU01. *In-vivo* fluorescence of chlorophyll a and other accessory pigments such as phycocyanin has been used as aproxy of cell concentration (Sierack et al., 2005) with a linear relationship even in their natural environment (Lorenzen, 1966). The four species were grown in MLA medium, and then 260 µL of each culture inoculated at equal *in-vivo* fluorescence which is equivalent to 5:1 ratio in cell

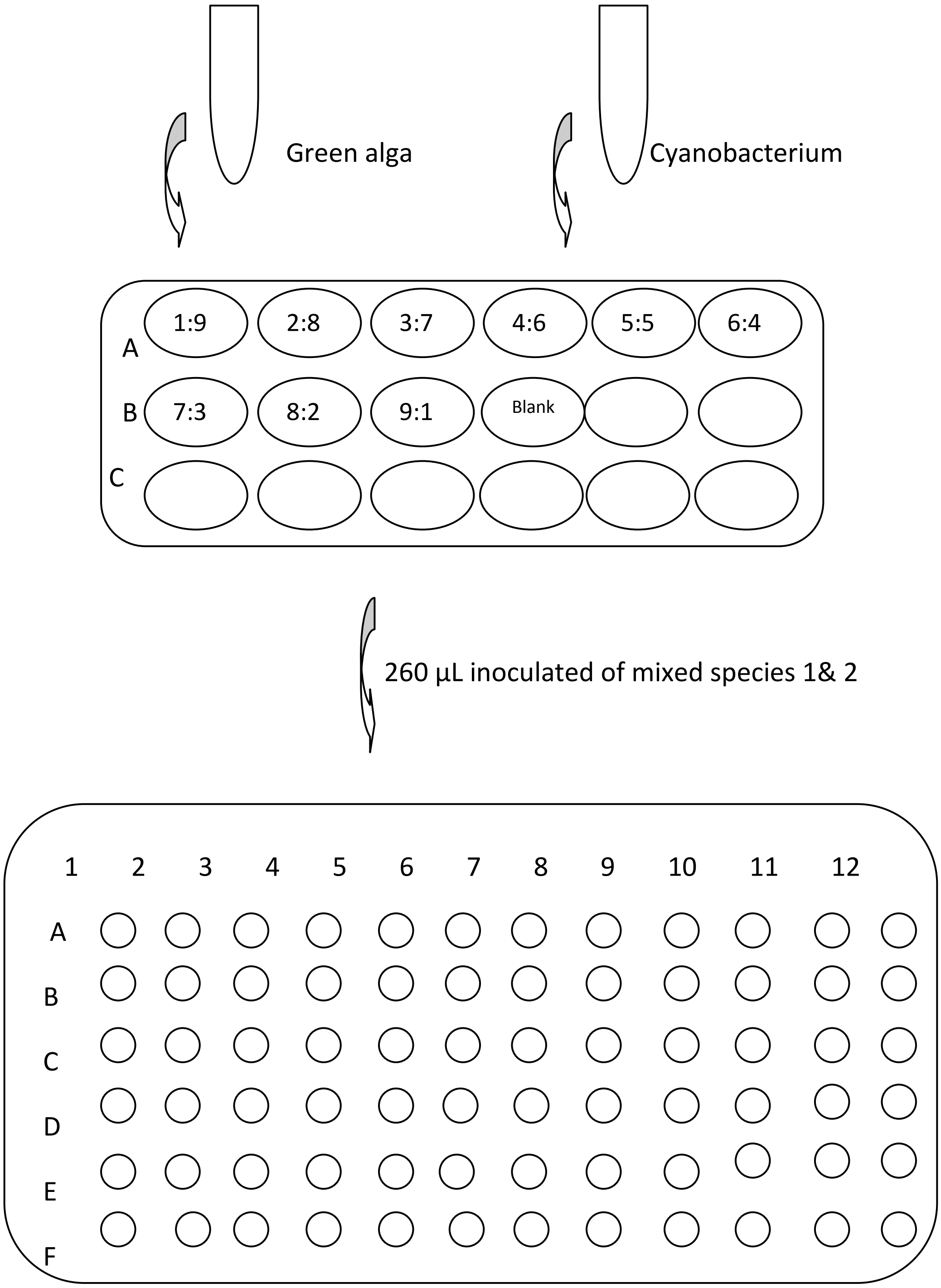


Figure 3.1 Preparation of two-species mixtures, species 1 (green algae) with species 2 (cyanobacteria) in 24-well plates.

concentration of *Anabaena* to green algae. The transferred inoculum to multi-well plates was either as single species, or as a mixture of both (130 µL of species 1 - green algae plus 130 µL of species 2 – cyanobacteria). The plate was then incubated at 21 ± 2 °C with continuous illumination at 65 µmole photon $m^{-2}s^{-1}$, (cool white, and 11 watts). Readings of fluorescence intensity of both species in the plate cultures was estimated daily as described earlier.

3.3 Results

Measurement in both white and black 96 well plates resulted in a linear relationship of fluorescence and cell concentration; however, much greater *in-vivo* fluorescence intensity was detected in white plates compared with black plates (Figure 3.2). At cell concentrations of 8.75 x10^5 (±1.20 x 10^5) cells mL^{-1}, the fluorescence intensity of the cyanobacteria strain *Anabaena circinalis* ACCD17 in white plates was almost 8 times higher than in black plates. However, the background fluorescence from the medium blank was three times higher in the white plates.

Top-mode reading in white plates yielded higher *in-vivo* fluorescence intensity than bottom-mode for *Anabaena circinalis* AC03 (Figure 3.3); the medium-only blank was almost identical in either read mode.

Due to the higher fluorescence detected, white plates (Greiner Bio-One) and top-read mode were chosen for all subsequent growth studies.

3.3.1 Single microalgal species detection

For *Desmodesmus asymmetricus* and *Selenastrum capricornutum* there was a linear correlation (r^2 = 0.99) of fluorescence intensity (485 nm, top read mode) with cell concentration (cells mL^{-1}) (Figure 3.4).

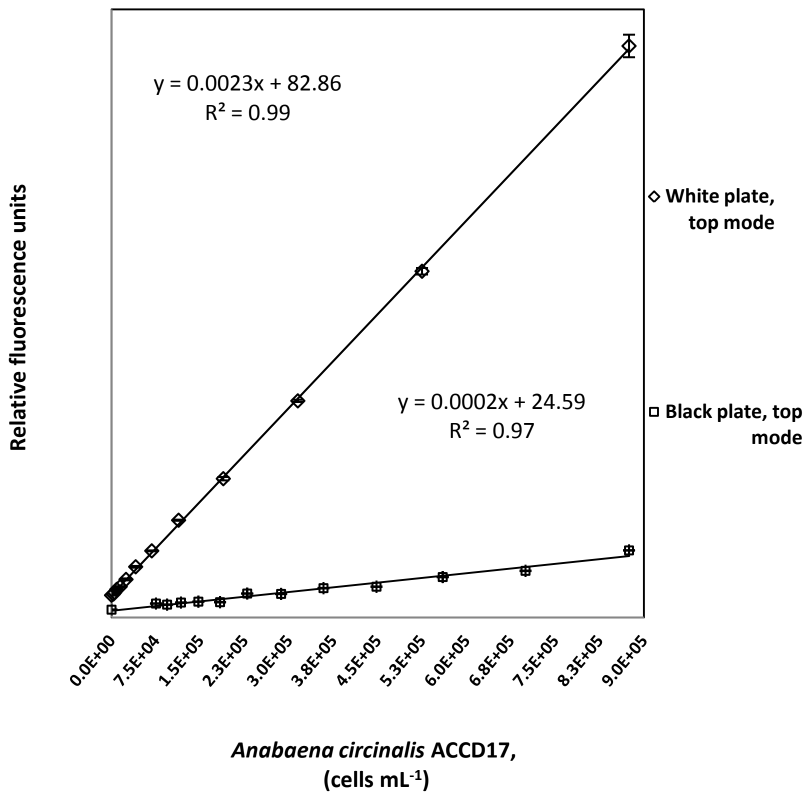


Figure 3.2 Comparison of fluorescence intensity (±SE) of serial dilutions of *Anabaena circinalis* ACCD17 in top-read mode using white versus black 96-well plates.

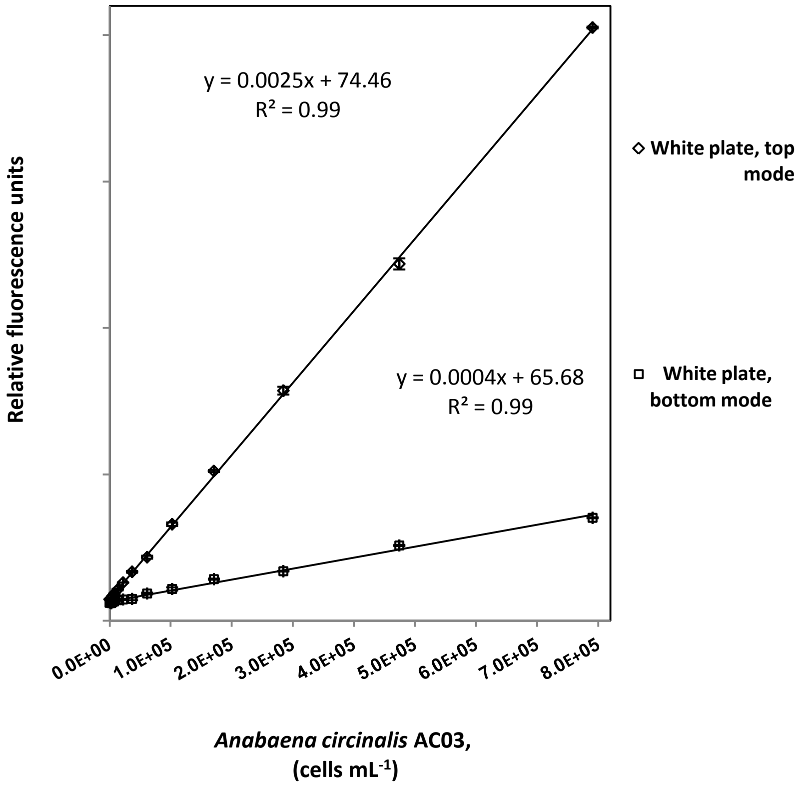
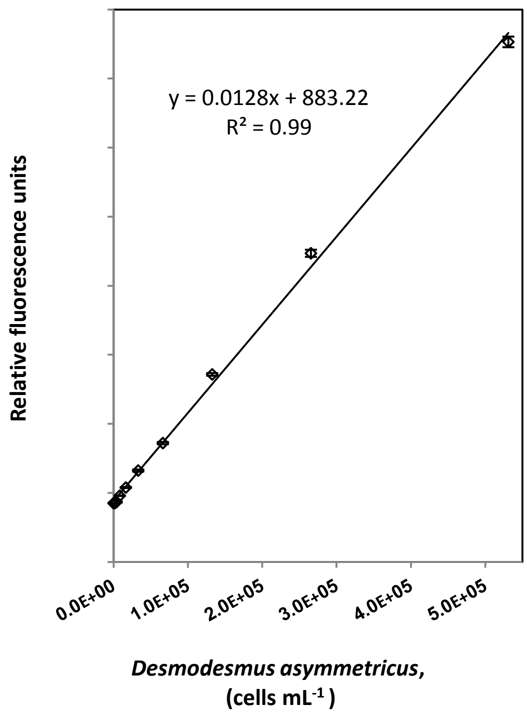


Figure 3.3 Comparison of fluorescence intensity (±SE) of serial dilutions of *Anabaena circinalis* AC03 in top-read versus bottom-read mode using 96-well white plates.

a



b

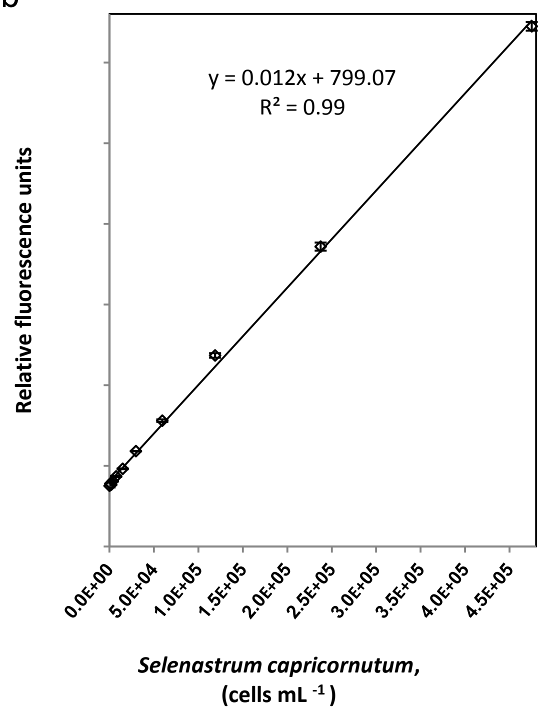


Figure 3.4 Relationship between cell concentration (cells mL^{-1}) and *in-vivo* fluorescence (485nm excitation) (±SE) from 96-well white plates for two green algal species. a) *Desmodesmus asymmetricus*; b) *Selenastrum capricornutum*. Linear equation of best fit is shown.

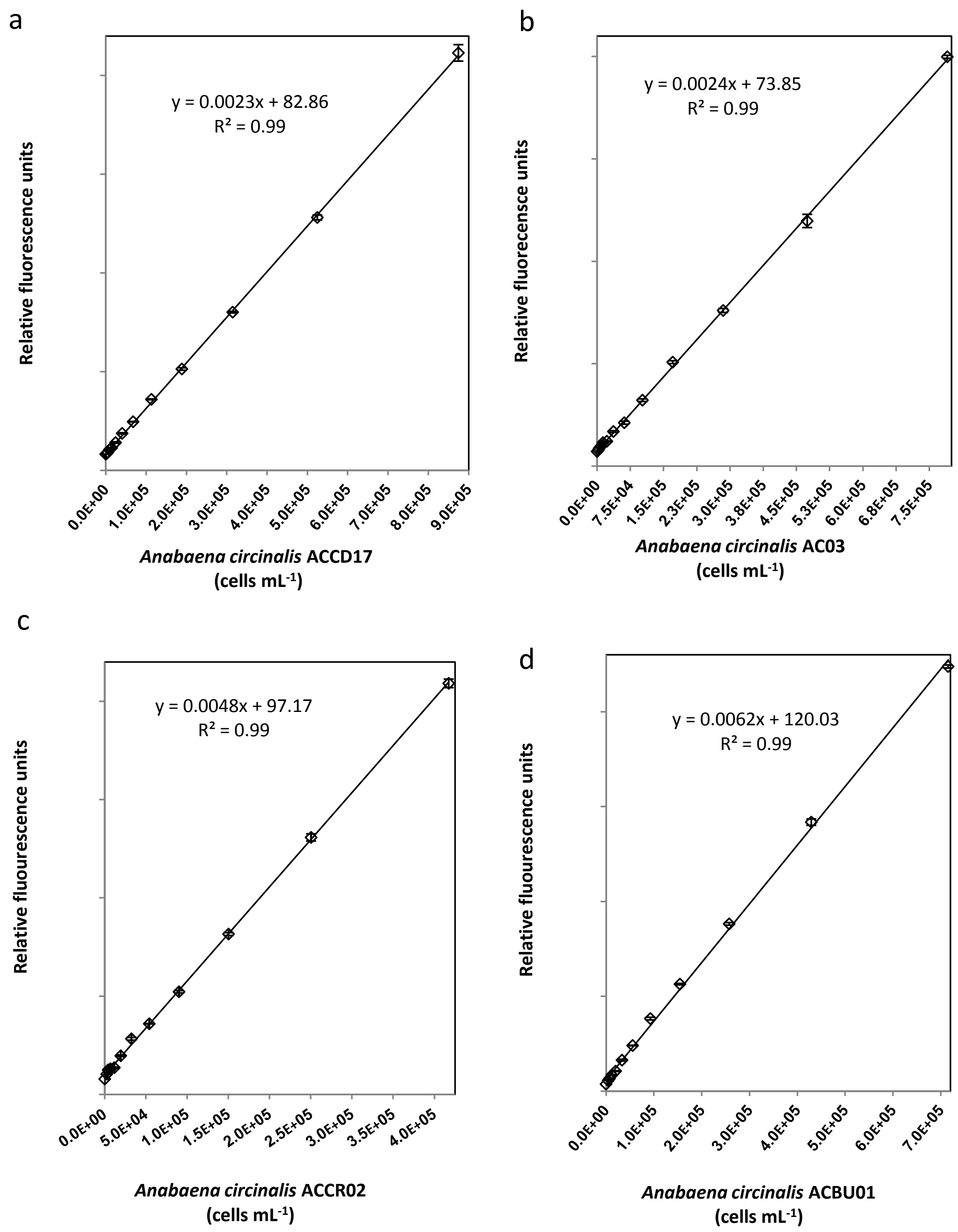
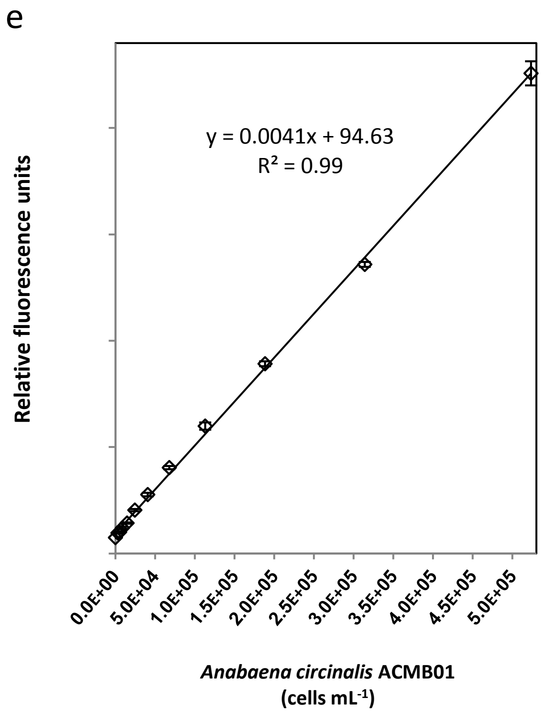


Figure 3.5 Relationship between cell concentration (cells mL^{-1}) and *in-vivo* fluorescence (595 nm excitation) (±SE) from 96-well white plates for five *Anabaena* species. a) *Anabaena circinalis* ACCD17; b) *Anabaena circinalis* AC03*;* c) *Anabaena circinalis* ACCR02*;* d) *Anabaena circinalis* ACBU01. Linear equation of best fit is shown.



Continue Figure 3.5 and e) *Anabaena circinalis* ACMB01.

Table 3.1 Detection limits of green algae and *Anabaena* cells for TECAN Genios fluorescence using 96-well microplate.

Species	**Lower detection limit (cells mL^{-1})**	**Upper linear range (cells mL^{-1})**	**Linear formula**
Desmodesmus asymmetricus	8.3×10^3	5.31×10^5	$y = 0.0128x + 883.22$
Selenastrum capricornutum	7.4×10^3	4.75×10^5	$y = 0.0120x + 799.07$
Anabaena circinalis ACCR02	2.5×10^3	4.17×10^5	$y = 0.0048x + 97.17$
Anabaena circinalis ACBU01	4.3×10^3	7.16×10^5	$y = 0.0062x + 120.03$
Anabaena circinalis AC03	4.8×10^3	7.91×10^5	$y = 0.0024x + 73.85$
Anabaena circinalis ACMB01	5.3×10^3	5.23×10^5	$y = 0.0041x + 94.63$
Anabaena circinalis ACCD17	5.3×10^3	8.75×10^5	$y = 0.0023x + 82.86$

The *in-vivo* fluorescence intensity response for green algae was much higher at 485 nm (Figure3.4) compared to *Anabaena circinalis* (at 595 nm) which also showed a linear relationship between fluorescence intensity and cell concentration (r^2>0.99) (Figure3.5). Table 3.1 compares the fluorescence responses among all the green algae and *Anabaena* species.

The reliable minimum detection (lowest dilution significantly different from the medium blank) was less for *Anabaena circinalis* strains than the green algae examined (Table 3.1). The detection limit for *Anabaena circinalis* strains ranged from 2.5 x 10^3 - 5.3 x 10^3 compared to 7.4 x 10^3 and 8.3 x 10^3 cells mL^{-1} for the green algae, *Selenastrum capricornutum* and *Desmodesmus asymmetricus,* respectively (Table 3.1).

3.3.2 Simultaneous detection and quantification of green algae and cyanobacteria

Simultaneous fluorescent detection (485 and 595nm excitation) from *Anabaena circinalis* ACCR02 and *Desmodesmus asymmetricus* cultures diluted to a range of different ratios from solutions of equal cell concentrations (Figure 3.6) indicate that the relative fluorescence at (595/485nm) is correlated with cyanobacterial/green algae cell ratio and followed a saturating curve best modelled by a 2nd order polynomial (r^2=0.99).

3.3.3 Single species and two-species growth studies

Both green algae and *Anabaena circinalis* strains examined showed positive growth when grown separately in the 96-well plate format (Figure 3.7). The mean exponential growth rate of both *Selenastrum capricornutum* and *Desmodesmus asymmetricus* was reduced by 13-17% and the latter was significantly reduced (t=4.9, df=4; P=0.008) when grown in mixed culture with *Anabaena circinalis*. In contrast, *Anabaena circinalis* strains exhibited a 20-21% increase in mean growth

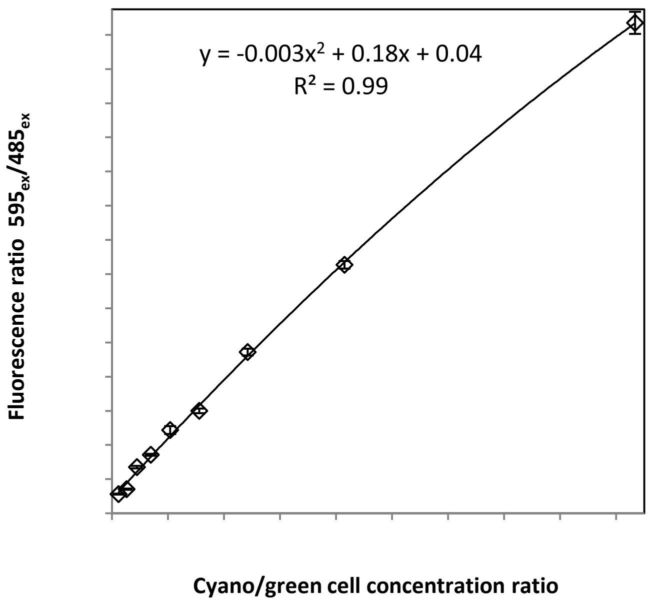
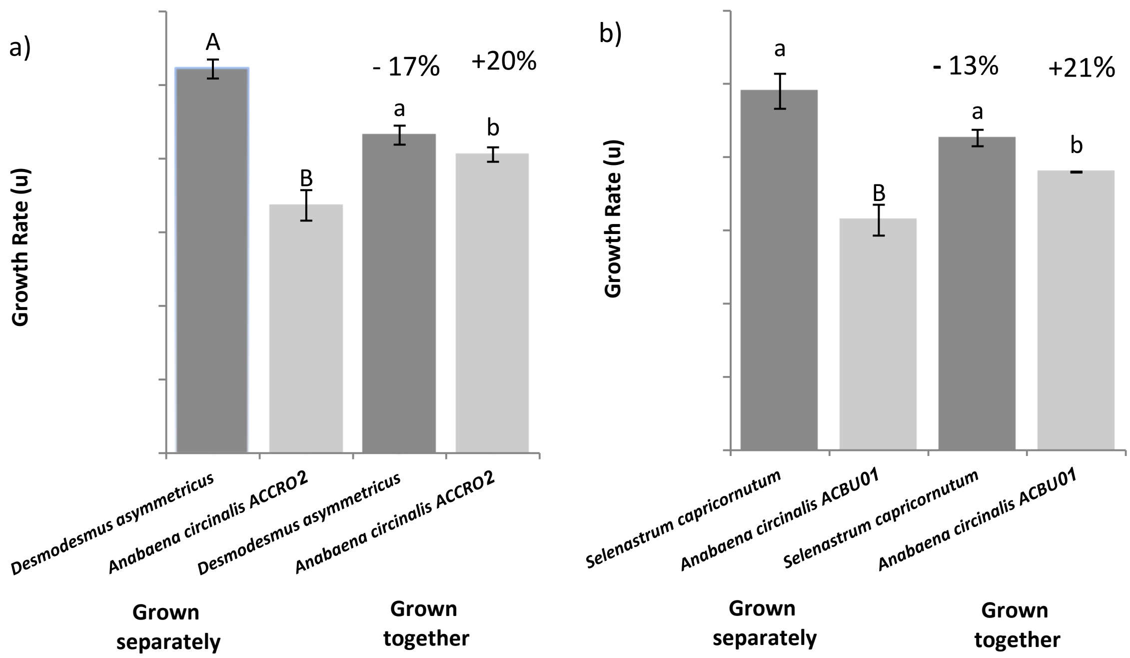


Figure 3.6 Correlation of the fluorescence ratio of 595/485 nm (±SE) with the cyanobacteria/ green algae cell counts per mL (mixtures of *Anabaena circinalis* ACCR02/*Desmodesmus asymmetricus*)



Figures 3.7 Growth rates (±SE) of green algae and *Anabaena circinalis* alone and in mixed green algal/cyanobacterial two-species cultures in 96-well microplate at 21±2 °C and irradiance 65µmole photon $m^{-2}s^{-1}$. a) *Desmodesmus asymmetricus* and *Anabaena circinalis* ACCR02 grown separately and together. b) *Selenastrum capricornutum* and *Anabaena circinalis* ACBU01 grown separately and together. Significant differences (p <0.05) in growth rate are indicated by capital versus non-capital letters. Paired non-capital letters indicate no significance difference.

rate when grown with a green algal species compared to when grown separately (t=-3.0, df=4; P=0.039) and (t=-3.1, df=4; P=0.036) (Figure 3.7).

3.4 Discussion

The experiments described here demonstrate that simultaneous fluorescent detection, of cyanobacteria and green algae is both achievable and reliable in low volume (260µL), high throughput format using a fluorescent plate reader. The detection limits of cells of both algal groups offers opportunity for the utilisation of this method in detecting and differentiating low quantities of cyanobacteria or bloom-forming *Anabaena* which could be missed by traditional methods using microscope when they exist with other species like the green algae. Furthermore, this method could be applied in measuring growth rate in competition experiments between two species. The growth rates were higher using microplate method compared to test tube probably due to different light and temperature parameters (table 3.2). The growth studies undertaken also show that the detection and plate format used here can be a reliable platform for short-term (3-5 days) growth experiments with uni-cellular algae, and competition experiments between eukaryotic algae and cyanobacteria.

The optical and physical properties of the plates produced different results in fluorescence intensity or sensitivity when measuring fluorescence using the TECAN microplate reader. The higher the pigment concentration of the plastic the lower the background and auto-fluorescence or optical crossover between well. Therefore, the coloured non-transparent wall plates reduce well-to-well crosstalk and improved sensitivity compard to clear-walled plates (Sieracki et al., 2005). The higher fluorescence detected from white walled plates may be due to absorption of the fluorescence emission by the black pigment of the plate wall.

The clear flat bottom of the well provided algae with good light penetration and growth of the microalgae was possible in only 260 µL culture volumes in the 96-well microplates.

Our data indicate that white-walled plates are the most suitable for detecting *in-vivo* pigment fluorescence, particularly for algal growth experiments where the increased signal is critical for reliable detection at low cell concentration directly after inoculation. While more concentrated inoculums may overcome this limitation, this would significantly reduce the time period over which logarithmic growth would be possible (due to nutrient limitation), decreasing the capacity to collect data from which to reliably estimate growth rate.

The *in-vivo* fluorescence intensity of the eukaryotic green algae represented by the *Desmodesmus asymmetricus* and *Selenastrum capricornutum* was much higher than the *Anabaena* species using microplate reader since the chlorophyll a pigments are the major light harvesting pigments in photosystem II (Vincent, 1983; Beutler et al., 2002).

The interference between the two pigment estimates of the green alga and the cyanobacterium when mixed together was insignificant. When excited at 485 nm, the green algal fluorescence intensity was insignificantly different ($p>0.05$) for both ratios of 1:1 and 1:4 of cell concentrations of (*Desmodesmus* and *A. circinalis*). When excited at 595 nm, the cyanobacterial fluorescence intensity was also similar and insignificantly different ($p>0.05$) when mixed with the green alga strain at either 1:1 or 4:1 ratios of cell concentrations of (*Desmodesmus* and *A. circinalis*). As indicated by Heaney, (1978), although cyanobacteria contain chlorophyll a mainly associated with photosystem I, the chlorophyll a content is much lower than phycocyanin in photosystem II (Heaney, 1978).

Schubert et al. (1989) showed that chlorophyll a in three cyanobacterial species when excited with blue light at 420-490 nm showed very little fluorescence. Light was most efficiently used at wavelengths between 580-670 nm as photosynthetic activities peak in term of oxygen production in the cyanobacteria. This pattern of fluorescence efficiency or intensity was seen in the *in-vivo* fluorescence of *Anabaena circinalis* ACCR02 at 485 nm which was 0.25 of the fluorescence intensity at 595 nm; in another word the ratio of fluorescence intensity of chlorophyll a to

Table 3.2 Growth rates (±SE) of green algae and cyanobacteria cultured in 260µL microplate well and 50-mL tube at light intensity of 65µmole photon $m^{-2}s^{-1}$.

Species	**Growth rate (microplate)** **21±2°C, continuous light**	**Growth rate (flask)** **18±1.0°C,12:12h (D:L) cycle**
Selenastrum capricornutum	0.85±0.022	0.96±0.01
Desmodesmus asymmetricus	1.04±0.026	0.86±0.62
Anabaena circinalis ACBU01	0.63±0.0.04	0.37±0.06
Anabaena circinalis ACCR02	0.67±0.04	0.66±0.07

phycocyanin in the *Anabaena* sp. was 1 to 4. The ratio of chlorophyll a to phycocyanin in cyanobacteria is approximately 1 to 3 (Owens, 1991).

Phycocyanins are considered an accurate and useful indicator in quantitative measuring of cyanobacterial bloom (Ahn et al., 2002). The detection limits of the cyanobacteria used in this study was just above 2000 cell mL^{-1} for *Anabaena* sp. The observed differences in fluorescence per cell and in the cell-fluorescence relationship among different *Anabaena circinalis* strains may be related to either differences in pigment content per cell, or differing cell morphology. For example, some strains form long coiled filaments, and others are shorter clusters of cells.

The correlations between the ratio of fluorescence excited at 595 nm and 485 nm and cell concentrations of cyanobacteria to green algae indicates that this detection method and platform is a simple and useful approach for rapid detection of cyanobacteria and eukaryotic algae. From the data shown in Table 3.1, this method can provide more reliable detection of *A. circinalis* at cell concentrations above approximately 2000 cells mL^{-1}.

The reduced growth rates of both green algae when grown with *Anabaena circinalis* may be due to direct inhibition by *Anabaena circinalis*. The production on growth-inhibiting chemicals (allelopathy) has been reported for other species of cyanobacteria. For example, strains of *Oscillatoria* have been shown to produce synergistic cyclic peptides (Portoamides) containing unusual modified amino acids that inhibit a range of other cyanobacteria and green algae (Leão et al., 2009). Interestingly the inhibition was evident with both *A. circinalis* ACBU01 (toxic) and *A. circinalis* ACCR02 (non-toxic) indicating that the allelochemical is not saxitoxin and that the allelopathic activity against the two green algae is not related to saxitoxin production. Similar allelopathic properties have also been demonstrated in other non-toxic strains of cyanobacteria (Suikkanen et al., 2004).

The increased growth rate of *A. circinalis* when grown in the presence of green algae also indicates that there are other chemical/biological interactions operating in the two-species cultures, not evident from comparative single-species bioassays. These findings reinforce the importance of species interactions as a critical factor influencing competitive outcomes in natural populations. The increase in growth rates especially for the *Anabaena* strains compared to chapter 2 is likely due to change in temperature parameters. Since, these microalgae cultured at 21 °C and at continuous light, *A. circinalis* ACBU01 attained growth rate of 0.63±0.04 compared to 0.37±0.06 cultured in test tube at 18 °C and non-continuous light (table 3.2).

The data presented here, show that multi-well plates are a suitable robust and convenient format for algal growth studies even at low volumes. Growth of both eukaryotic algae and cyanobacteria can be easily monitored using the microplate reader in few minutes over numerous numbers of wells, allowing experiments with complex experimental designs and high levels of replication. Furthermore, this method allows monitoring of the growth pattern in mixed culture systems allowing examination of possible allelopathic interactions and competition that exist in natural environments.

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Chapter 4

Effects of seasonal light and temperature combinations on atrazine inhibition (EC_{50}) of cyanobacteria and green algae

4.1 Introduction

In the presence of available nutrients, phytoplankton growth is predominantly regulated by available light and water temperature. Species able to sustain higher growth rates under the prevailing light/temperature conditions changes will, in theory, become the dominant species in a water body. Toxicants such as photosynthetic inhibitory herbicides can also act as a major modifier of the species composition due to their differential effect on different phytoplankton species. They can act as additive/synergistic or as an antagonistic factor as they influence the metabolic process in phytoplankton (Yu, 2001).

One of the key target modes of action of herbicides is the blocking of photosystem II complex by competing with the plastoquinone-binding site or the D1 protein (Fairchild et al., 1998). At high concentrations, this will cause photoinhibition of the photosynthetic apparatus by blocking the electron flow at photosystem II and deactivation of the D1 protein; thus slow down or ceases the driving photochemical process of photosynthesis (Mayasich et al., 1987; Powles, 1984).

The photoinhibition process is characterised by reduced electron transfer system, photophosphorylation and also reduces chlorophyll fluorescence. The sensitivity of algae to photoinhibition depends largely on species susceptibility, selection process, abiotic factors such as light and temperature, and toxicant concentration (Huse and Nilsen, 1989). It has been indicated that the kinetics of photoinhibition is temperature independent while the kinetics of recovery is temperature dependent. This is based on results from toxicity studies of selected species of cyanobacteria which showed increased tolerance to the photosynthetic inhibitors at higher temperature, suggesting that the recovery is reliant on the rate of regeneration of the D1 protein (Huse and Nilsen, 1989; Wunschmann and Brand, 1992; Roos and Vincent, 1998). Although light is an important factor for autotrophic microorganisms to carry out photochemical process; exposure to high light intensities above the saturation level could cause photoinhibition in the algal cells due to excessive irradiance especially in the absence of effective thermal energy dissipation (Skogen et al., 1986).

Most phytoplankton achieve their maximal rate of replication due to increased metabolic rate at higher temperature (25-35 °C) as temperature plays a major role influencing the phytoplankton bloom and seasonal succession (Abrantes et al., 2006; Reynolds, 2006). This could favour algal tolerance to the herbicide through enhanced recovery of photosystem II apparatus from photoinhibition, therefore the tolerance of algae to herbicides might be expected to vary seasonally, especially in higher latitude temperate environments that experience wide seasonal temperature and light fluctuation.

Previous laboratory studies of selected algal groups indicate both light and temperature affect atrazine tolerance (Mayasich et al., 1987). Microcosm studies have also indicated that light and temperature affect the relative tolerance to photosynthetic inhibiting herbicides and that the response in uni-algal cultures differs from that observed in mixed phytoplankton populations, perhaps due to allelopathic effects that alter tolerance to the herbicide (Bérard et al., 1999; Berard et al., 1999a).

Utilizing the microplate method developed in Chapter 3, the relative tolerance to atrazine of *A. circinalis* and the green alga *D. asymmetricus* is examined at different seasonal light and temperature combinations experienced in temperate water bodies. Experiments are carried out with the two species grown separately and also in competition to determine how interactive effects between the two species influence their relative seasonal tolerance to the herbicide.

4.2 Materials and Methods

Two strains of freshwater microalgae, the green alga, *D. asymmetricus* and the cyanobacterium *A. circinalis* (ACCR02) were maintained in MLA medium in 100 mL Erlenmeyer flasks with continuous light was provided by cool white fluorescent lamps (Cool white, 18W WPT218/T8 fluorescent tubes). Prior to the experiment, cultures were transferred and pre-adapted for five days at two temperatures (low =

18±1, high = 24±1°C) and two light intensities (low = 30, high = 100 µmole photon $m^{-2}s^{-1}$) combinations in an orthogonal design.

For the experiment, cultures were acclimatised for 2 weeks at each of the experimental light and temperature combinations. Cultures were mixed with MLA medium prepared to contain 5 different final atrazine concentrations (250, 200, 150, 100 and 50 µg L^{-1}) to achieve a standard starting cell concentration of 1.0 x10^4 cells mL^{-1}. Controls containing no atrazine were also prepared. All atrazine concentrations were conducted in triplicate in 96-well, white-walled microplate with a final culture volume of 260 µL. Experiments carried out with both species grown together used equal starting concentrations of each species (5.0 x10^3 cells mL^{-1}). Growth rates were estimated using *in-vivo* fluorescence of chlorophyll-a (excitation at 485nm for *Desmodesmus*) and/or phycocyanin (excitation at 595nm for *Anabaena*) from daily measurements taken with a TECAN GENios fluorescent microplate reader as described in Chapter 3.

The calculated EC_{50} values were determined from the exponential growth rate for each replicate at each concentration, expressed as a proportion of the relevant no-atrazine control, and the relative growth rate plotted against atrazine concentration for each replicate. A curve was fitted and the EC_{50} calculated from the curve equation for each replicate, and mean EC_{50} values determined from the replicate EC_{50} values.

Mean EC_{50} value for each strain of green algae and cyanobacteria in each treatment was compared using a two-way analysis of variance (ANOVA) using SPSS statistics version 19.0. A paired t-test was performed to compare the mean EC_{50} value of growth conditions (grown mixed vs. separate) at each light and temperature combination.

4.3 Results

When grown separately in the absence of atrazine (no atrazine controls), *A. circinalis* maintained a 7-14% higher growth rate than *D. asymmetricus* under all light and temperature conditions. However, when the two species were grown together, the growth rate of *A. circinalis* was reduced (15.3 to 23.8%) under both high light combinations and the low light, low temperature combination; the growth rate of *D. asymmetricus* was increased under all conditions (3.4 to 12.3%), except at high light and high temperature. However, the *A. circinalis* gained a higher growth rate ratio compared to the *D. asymmetricus* in the low light high temperature combination in the absence of atrazine (Figure 4.5) (Table 4.1).

The relative growth rates of *A. circinalis* and *D. asymmetricus* varied with increasing atrazine concentration (Figures 4.3- 4.6).

When grown separately at high light, high temperature combinations *A. circinalis* maintained a higher growth rate than *D. asymmetricus* at all concentrations of atrazine tested, particularly at concentrations in excess of 100 μg L^{-1} (Figure 4.3). However when grown together, growth rates of both species was more similar across the entire range of atrazine concentrations examined with *A. circinalis* favoured only at intermediate concentrations (100-150 μg L^{-1}) (Figure 4.3).

At high light and low temperature, grown separately, *A. circinalis* maintained a higher growth rate than *D. asymmetricus*at all atrazine concentrations. However, when grown together the growth rate of *D. asymmetricus* was similar to *A. circinalis* at 50 μg L^{-1} of atrazine. At concentrations above 50 μg L^{-1} of atrazine, *A. circinalis* exhibited a much higher growth rate than *D. asymmetricus* (Figure 4.4).

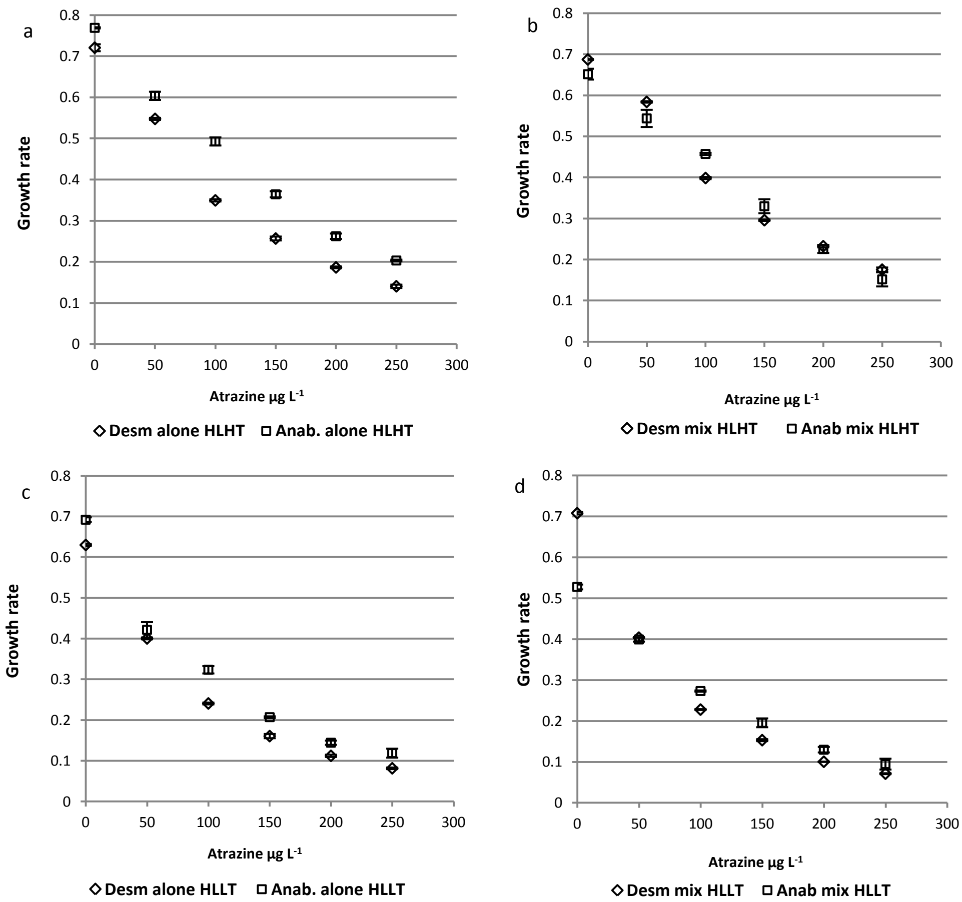

Figure 4.1 (a-d) Absolute growth rates (± SE) of *A. circinalis* and *D. asymmetricus* at different atrazine concentrations at high light combinations grown separately and mixed: a) *A. circinalis* and *D. asymmetricus* cultured separately at high temperature, b) *A. circinalis* and *D. asymmetricus* cultured mixed together at high temperature, c) *A. circinalis* and *D. asymmetricus* cultured separately at low temperature, d) *A. circinalis* and *D. asymmetricus* cultured mixed together at low temperature.

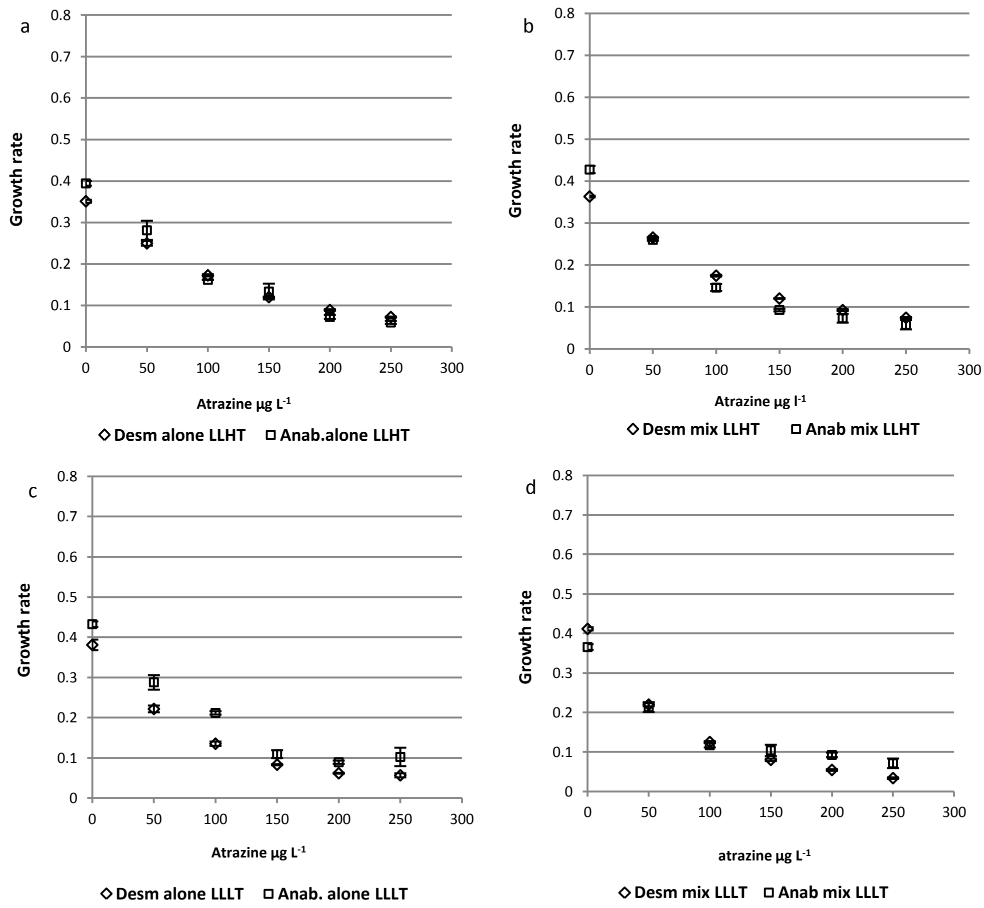

Figure 4.2 (a-d) Absolute growth rates (± SE) of *A. circinalis* and *D. asymmetricus* at different atrazine concentrations at low light combinations grown separately and mixed: a) *A. circinalis* and *D. asymmetricus* cultured separately at high temperature, b) *A. circinalis* and *D. asymmetricus* cultured mixed together at high temperature, c) *A. circinalis* and *D. asymmetricus* cultured separately at low temperature, d) *A. circinalis* and *D. asymmetricus* cultured mixed together at low temperature.

Table 4.1 Relative growth rates (±SE) of *A. circinalis* and *D. asymmetricus* grown alone and together in the absence of atrazine at different light and temperature combinations. The two light intensities (LL= 30, HL= 100 µmole photon $m^{-2}s^{-1}$) and two temperatures (LT= 18±1, HT= 24±1 °C)

Light/Temp. Conditions	Growth rate ratio (*A.circinalis/D. asymmetricus*) Alone	together	Growth rate ratio (together/alone) *A. circinalis*	*D. asymmetricus*	% change *A. circinalis*	% change *D. asymmetricus*
HLHT	1.07±0.01	0.95±0.02	0.85	0.95	-15.3	-4.6
HLLT	1.10±0.01	0.75±0.01	0.76	1.12	-23.8	+12.3
LLHT	1.12±0.03	1.18±0.02	1.08	1.03	+8.4	+3.4
LLLT	1.14±0.02	0.89±0.01	0.85	1.08	-15.5	+8.0

Generally, the growth rate of both species was lower in the two low light combinations (Figure 4.2). However, at low light high temperature with no atrazine, the growth rate of *A. circinalis* was higher than *D. asymmetricus* when grown separately or together (Figure 4.2).

The growth rates were more inhibited by atrazine in the low light combinations as indicated by Figure 4.2. In term of relative growth rate, the presence of atrazine at concentrations in excess of 50 µg L^{-1} favoured *D. asymmetricus*, which maintained a higher growth rate than *A. circinalis* grown either separately or together with *D. asymmetricus* (Figure 4.5).

When grown separately at low light and low temperature, the presence of atrazine favoured *A. circinalis* at all concentrations examined (Figure 4.6). When grown together, *D. asymmetricus* grew marginally faster than *A. circinalis* at low concentrations of atrazine but at concentrations of 150 µg L^{-1} and above, *A. circinalis* was able to sustain much higher growth rates than *D. asymmetricus* (> 2-fold difference at 250 µg L^{-1}) (Figure 4.6).

When comparing the effect of light and temperature on each species grown alone or mixed, two-way ANOVA analysis indicated both species was significantly affected by the two variables (Tables 4.3 and 4.4). The interaction between light and temperature was also significant for both species whether grown separately or together, except for *D.asymmetricus* grown separately.

Comparison of change in tolerance/ EC_{50} values of *A. circinalis* and *D.asymmetricus* grown mixed to separately using t-test at the different light and temperature combinations used in this experiment are summarized in table (4.2)."

Comparison of EC_{50} values for atrazine shows that both species were most tolerant of atrazine when grown at higher temperature (Table 4.2). When grown separately at low temperature, a reduction in light reduced the atrazine tolerance of *D. asymmetricus* but not the tolerance of *A. circinalis*. When grown together at high light and high temperature atrazine tolerance of both species increased significantly

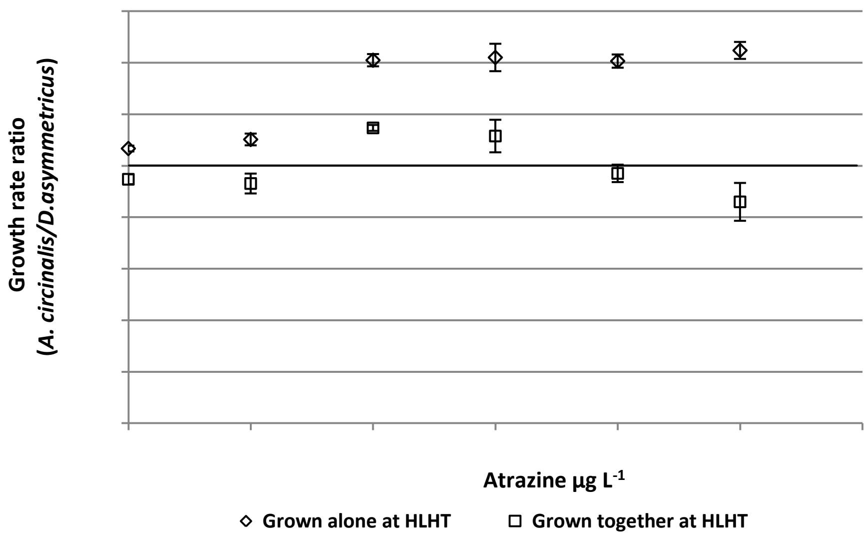

Figure 4.3 Relative growth rates (±SE) of *A.circinalis/D. asymmetricus* at different atrazine concentrations grown separately and together at high light high temperature. Ratios above 1.0 indicate *A. circinalis* growth rate was higher than *D. asymmetricus*.

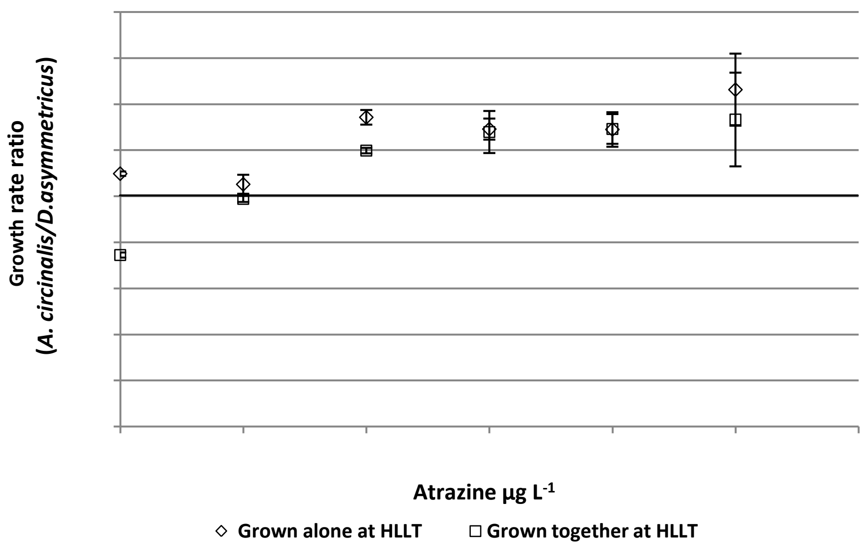

Figure 4.4 Relative growth rates (±SE) of*A.circinalis/D. asymmetricus* at different atrazine concentrations grown separately and together at high light low temperature. Ratios above 1.0 indicate *A. circinalis* growth rate was higher than *D. asymmetricus*

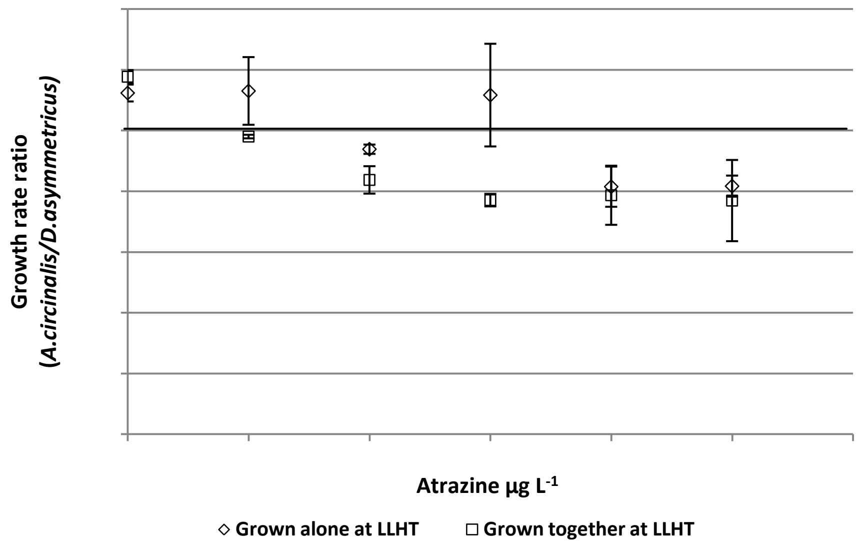

Figure 4.5 Relative growth rates (±SE) of*A.circinalis/D. asymmetricus* at different atrazine concentrations grown separately and together at low light high temperature. Ratios above 1.0 indicate *A. circinalis* growth rate was higher than *D. asymmetricus*

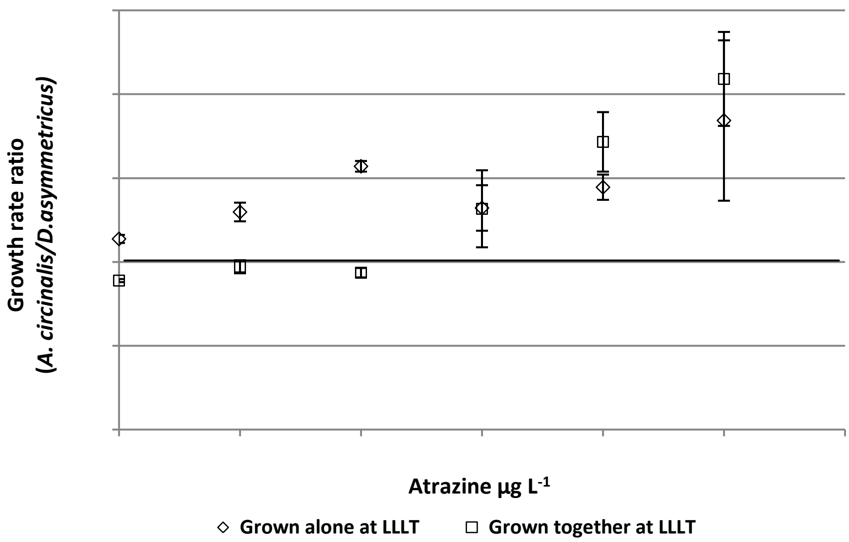

Figure 4.6 Relative growth rates (±SE) of*A.circinalis/D. asymmetricus* at different atrazine concentrations grown separately and together at low light low temperature. Ratios above 1.0 indicate *A. circinalis* growth rate was higher than *D. asymmetricus*

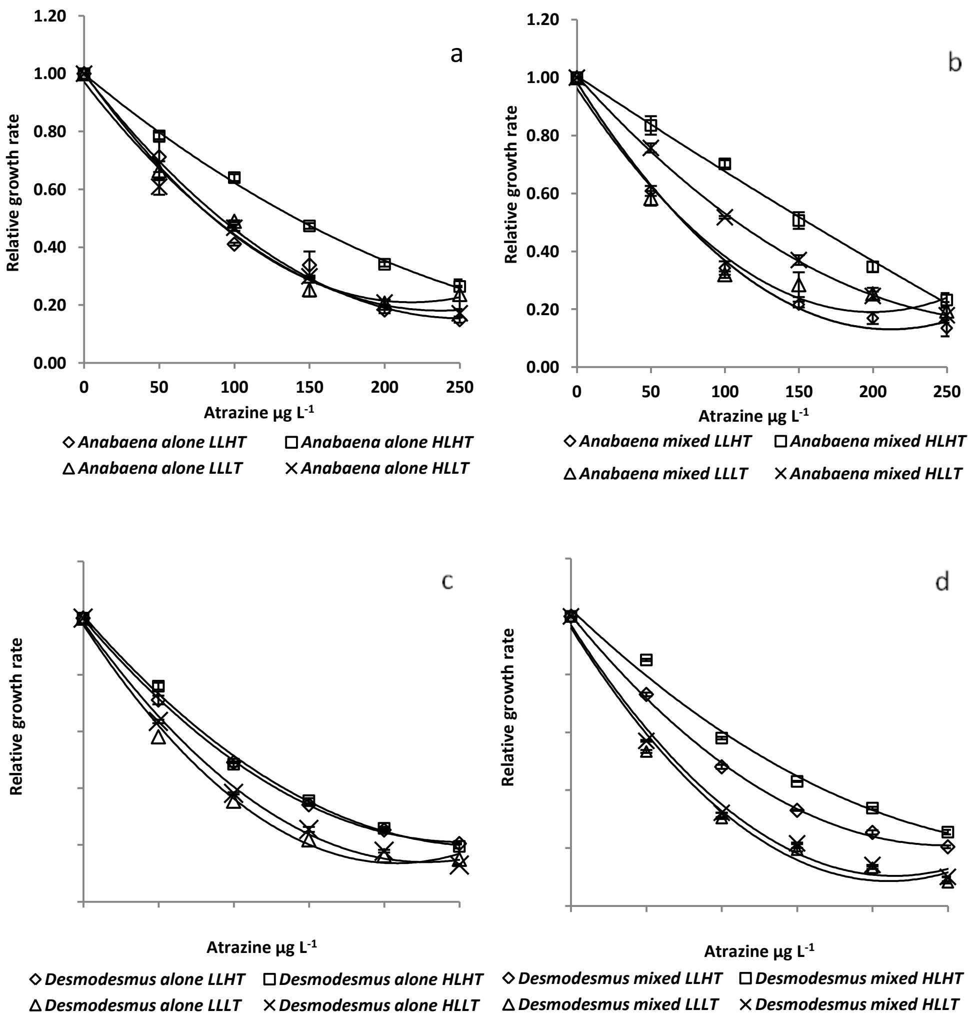

Figure 4.7 The growth response of the *A. circinalis* and *D. asymmetricus* to different concentrations of atrazine at different light and temperature combinations: a) *A. circinalis* ACCR02 cultured separately, b) in mixed culture with *D. asymmetricus*, c) *D. asymmetricus* cultured separately, and d) in mixed culture with *A. circinalis.* Relative growth rate (± SE) is expressed as a proportion of corresponding no-atrazine controls. The curve shown is fitted to mean growth rate data, with mean EC_{50} values calculated from EC_{50} for each independent replicate.

Table 4.2 Comparative atrazine tolerance expressed as EC_{50} (±SE) of *A. circinalis* and *D. asymmetricus* grown in separate or mixed culture. The two light intensities (LL= 30, HL= 100 µmole photon $m^{-2}s^{-1}$) and two temperatures (LT= 18±1, HT= 24±1 °C)

	EC_{50} Separate (µg L^{-1})			EC_{50} Together(µg L^{-1})			% change in tolerance (EC_{50} together/EC_{50}Separate)	
Conditions	***A. circinalis***	***D. asymmetricus***	**EC_{50} ratio AC/DA**	***A. circinalis***	***D. asymmetricus***	**EC_{50} ratio AC/DA**	***A. circinalis***	***D. asymmetricus***
HL HT	140.5 ± 2.2	102.6 ± 2.7	1.37	155.8 ± 4.6	131.5 ± 0.8	1.18	110.9*	128.2*
HL LT	85.9 ± 3.0	78.6 ± 1.0	1.09	108.3 ± 0.4	69.0 ± 0.2	1.57	126.1*	87.8*
LL HT	91.2 ± 6.7	98.9 ± 1.6	0.92	71.8 ± 1.6	98.0 ± 0.9	0.73	78.7	99.1
LL LT	85.7 ± 2.7	70.9 ± 0.5	1.21	73.0 ± 1.5	65.3 ± 0.7	1.12	85.2*	92.1*

*Significant difference for EC_{50} values for each species when grown mixed vs. alone at p= 0.05

(10.9 – 28.2%) compared to grown separately, for both *A. circinalis* ($t=-6.1$, $df=2$, $p=0.026$) and *D. asymmetricus* ($t=-8.36$, $df=2$, $p=0.014$).

When grown together at high light, low temperature combination, *A. circinalis* showed increased (26.9%) atrazine tolerance, while *D. asymmetricus* showed a 12.2% decrease in tolerance.

At low light, high temperature, tolerance to atrazine did not change significantly for both species when grown together compared to grown separately ($t=2.59$, $df=2$, $p=0.12$) and ($t=0.286$, $df=2$, $p=0.80$).

Table 4.3 Analysis of variance for the EC_{50} values with atrazine of *A. circinalis* ACCR02 and *D. asymmetricus* grown alone in 96-well microplate at light intensities of (30 and 100 μmole photon $m^{-2}s^{-1}$) and temperature (18±1 and 24±1 °C).

Species	Factor	Degrees of freedom	F value	Significance
Anabaena circinalis ACCR02, alone	Light	1,8	36.7	<0.001
	Temperature	1,8	53.8	<0.001
	Light*Temperature	1,8	35.8	<0.001
Desmodesmus asymmetricus, alone	Light	1,8	11.4	<0.001
	Temperature	1,8	53.8	<0.001
	Light*Temperature	1,8	35.8	0.27

Table 4.4 Analysis of variance for the EC_{50} values with atrazine of *A. circinalis* ACCR02 and *D. asymmetricus* grown mixed together in 96-well microplate at light intensities of (30 and 100μmole photon $m^{-2}s^{-1}$) and temperature (18±1 and 24±1 °C).

Species	Factor	Degree of freedom	F value	Significance
Anabaena circinalis ACCR02, mix	Light	1,8	543.8	<0.001
	Temperature	1,8	81.5	<0.001
	Light*Temperature	1,8	91.2	<0.001
Desmodesmus asymmetricus, mix	Light	1,8	692.9	<0.001
	Temperature	1,8	4554.3	<0.001
	Light*Temperature	1,8	438.3	<0.001

4.4 Discussion

Both light and temperature were found to have significant and important interactive effects on overall atrazine tolerance of the cyanobacterium *A. circinalis* and the green alga, *D.asymmetricus.* The changes in absolute and relative tolerance to atrazine when grown alone compared to mixed culture also demonstrated that species interactions are an important factor modifying relative tolerance to atrazine. Importantly, the absolute and relative effect of these interactions varied under different light and temperature conditions. The relative changes were substantial enough to reverse the outcome of growth competition in competition cultures, and thus may potentially alter competitive outcomes in natural communities.

Using a single species culture approach, *A. circinalis* was favoured under all conditions; however, this doesn't match with what we typically see in natural populations as phytoplankton community structure is controlled by biotic and abiotic factors as well as the absence of competitor or predators which determine the outcomes of species tolerance (Lampert et al., 1989; Preston, 2002; Berard et al., 2003). The presence of sediments, nutrients and other different toxicants will also determine the difference in tolerance in laboratory tests and the natural aquatic system (Pratt et al., 1990; DeLorenzo et al., 2001). This stresses the need for more complex toxicity studies which can account for species interactions rather than single monoculture studies in which the interactive impact of herbicide and the environmental factors on the microorganism will be excluded.

The experimental outcomes of this work (Table 4.5) indicated that the green alga, *D.asymmetricus* was favoured under all but low light, high temperature conditions in the absence of atrazine when species interactions were included. In natural aquatic systems, *A. circinalis* blooms don't tend to dominate under all conditions or seasons. Scheffer et al. (1997) found that green algae dominated over cyanobacteria in Dutch shallow turbid lakes under conditions of high light and nutrient availability. In Australian rivers, the chlorophytes and diatoms dominante in temperate regions which are related to flow conditions, turbidity and nutrient concentration (Hotzel and Croome, 1996). However, many cyanobacteria are buoyant and can access light in turbid waters, and have lower saturating light intensity for photosynthesis than chlorophytes (Kromkamp, 1987; Scheffer et al., 1997), this can give them an advantage in warmer waters under low light conditions. In this experiment, *A. circinalis* ACCR02 cultured in microplate wells attained growth rate of 0.77±0.001 at 24 °C and light intensity of 100μmole photon $m^{-2}s^{-}1$ compared to 0.67±0.04 cultured at 21°C and light intensity of 65μmole photon $m^{-2}s^{-1}$ in chapter 3. These results are consistent with Paerl and Huisman, (2008) in which cyanobacterial growth is thought to be favoured over eukaryotic phytoplankton at temperatures in excess of 20 °C. In natural environment this is relevant, particularly when higher temperatures lead to thermal stratification of the water column and increasing in phosphorus recycling from sediments (Pettersson et al., 2003; O'Neil et al., 2012). *Anabaena* species are nitrogen-fixers that require high light; therefore, the interaction of mixing and cellular buoyancy in turbid Australian rivers is considered a major cause of *A. circinalis* blooms. However, recent studies have also demonstrated that *A. circinalis* is capable of N_2-fixation even under light-limited conditions providing a significant advantages over other species (McCausland, 2003).

When grown together, relative growth rate comparisons indicated that the presence of atrazine favoured *A. circinalis* in three out of four light and temperature combinations even at low atrazine concentrations of 150 μg L^{-1} (Table 4.5). At 250 μg L^{-1} atrazine, *A. circinalis* was able to sustain much higher growth rate than the *D. asymmetricus* even at low temperature and low light (18°C and 30 μmoles photons PAR m^{-2} s^{-1}) (Figure 4.6). This suggests that atrazine may favour *A. circinalis* over green algae particularly in temperate areas with relatively low water temperatures. Blooms of this species were recorded in 1997-1998 in Craigbourne Dam, Tasmania (Bobbi, 1997) even at temperatures as low as 6.5°C during July, collapsing only when the water body experienced increased wind-driven turbulence in September. This indicates that the presence of herbicides such atrazine could act as a possible stimulus for the *A. circinalis* bloom which can extend to the period of cold seasons especially with light availability during the prolonged water residency. This is supported by Bérard et al., (1999, 2003) who showed that cyanobacteria not only tolerated low concentrations of atrazine (10 µg L^{-1}) but were stimulated during both summer and fall (autumn) in Lake Geneva.

Cyanobacterial dominance in the natural mixed community could also be influenced by the combined added effects of atrazine and the allelopathic compounds produced by some species that target competing species and may have similar activity as herbicides. Algal allelopathic compounds are solvent soluble and capable of reaching the thylakoid membrane where photosynthesis occurs (Leflaive and Ten-Hage, 2007). Recent studies have also discovered that some cyanobacteria produce cyclic peptides with unusual modified amino acids that inhibit green algae during different stages of their growth depending upon the species of the cyanobacteria (Suikkanen et al., 2004; Leão et al., 2009). However, for these compounds to reach significant and effective concentrations in natural water bodies, high residence times and water column stratification are required to ensure that any exudates won't be diluted (Leflaive and Ten-Hage, 2007).

The outcomes of the experiments suggest that the presence of atrazine can potentially shift the community dominance toward *A. circinalis* and that species interactions are a significant additional factor. The data explains the differences noted between culture-based and mesocosm community studies (Bérard et al., 1999; Seguin et al., 2001; Berard et al., 2003).

When considering the effect of atrazine based on tolerance expressed as EC_{50}, the experimental outcomes indicate that *A.circinalis* is more (or equally) tolerant of atrazine than *D. asymmetricus* when interactions are not considered (grown separately). However, when species interactions are considered (grown in competition), changes in atrazine tolerance favours *A. circinalis* under high light regardless of temperature, and *D. asymmetricus* under low light, regardless of temperature (Table 4.6).

Table 4.5 Summary of the experimental outcomes based on relative growth rate comparisons of the two species at the different light and temperature parameters. (AC= *A. circinalis*, DA = *D. asymmetricus*, HL=100, LL=30 µmole photon $m^{-2}s^{-1}$and HT=24±1, LT=18±1 °C).

Conditions	**No atrazine No multi-species**	**No atrazine + multi-species**	**+ Atrazine(50) + multi-species**	**+ Atrazine(100) + multi-species**	**+ Atrazine(150) + multi-species**
HL HT	AC	DA	DA	AC	AC
HL LT	AC	DA	AC=DA	AC	AC
LL HT	AC	AC	AC=DA	DA	DA
LL LT	AC	DA	AC=DA	AC=DA	AC

However, if considering relative change in atrazine tolerance (EC_{50}), *A. circinalis* gains the most advantage relative to *D. asymmetricus* at high light and low temperature combinations due to a reduction in tolerance of *D. asymmetricus* and a substantial increase in tolerance of *A. circinalis* (Table 4.2). The observed changes in tolerance to atrazine may explain why temperate blooms of *A. circinalis* can sometimes persist in temperate water bodies even when water temperatures decline well below 20 degrees in late summer and autumn (March-May in southern hemisphere). For example, blooms of *A. circinalis* in Lake Trevallyn (Tasmania) in 2007 were initiated in January when lake water temperatures of 22-24 °C coincided with strong thermal stratification and relatively high inputs of phosphorus. Once established, the bloom persisted until May of the same year even though lake water temperatures had declined to 10-12 °C. The bloom was eventually terminated due to increased river flows that may have weakened stratification (McCausland, 2011). In natural samples, cyanobacteria, especially *A. circinalis* were able to maintain significant rates of photosynthesis (62% of maximum rate at 10 °C) during spring in temperate Lake Mendota, Wisconsin, USA (Konopka and Brock, 1978), indicating that growth may still be significant at temperatures typically thought to be unfavourable for cyanobacteria. Laboratory studies of *Anabaena* strains Isolated from Oued Mellah Lake in Morocco indicate maximum growth rate at high light and high temperature, however their occurrence is also noted at low light and low temperature of greater than 10 °C allowing *Anabaena* to blooms to dominate in spring and remain dominant until the beginning of autumn (Sabour et al., 2005).

While herbicide contamination may shift community dominance toward cyanobacteria, other factors assist the persistence of *A. circinalis* blooms in temperate lakes. Firstly, the ability to germinate from akinetes in resuspended sediments to the euphotic zone due to winds or animal foraging behaviour that causes sediment disturbances throughout the year at water temperatures between>10 to 35 °C, (Baker, 1999; Baker and Bellifemine, 2000)

Table 4.6 Summary of the experimental outcomes based on EC_{50} comparisons grown separately and together at different light and temperature parameters. (AC= *A. circinalis*, DA = *D. asymmetricus*, HL=100, LL=30 µmole photon $m^{-2}s^{-1}$and HT=24±1, LT=18±1 °C).

Conditions	No multi-species (Grown separately)	Multi-species (Grown together)
HL HT	AC	AC
HL LT	AC=DA	AC
LL HT	AC=DA	DA
LL LT	AC	AC=DA

The findings of this study are particularly relevant for mid- latitude environments where light and temperature conditions vary considerably from season to season. Despite relatively low water temperatures, contamination of waterways with atrazine (and other triazine herbicides) may increase due to a combination of increased rain and surface runoff to waterways during spring and autumn, and the increased half-life (and longer persistence of atrazine) at lower temperatures (Kookana et al., 2010). The shifts in relative atrazine tolerance and the effect of atrazine on growth competition could favour cyanobacterial blooms in temperate lakes and rivers such as Tasmania.

This experiment shows the importance of multi-species effects for studies of herbicide toxicity. The outcomes of species competition between the cyanobacterium, *A. circinalis* and the green alga *D. asymmetricus* at a range of light and temperature combinations was significantly altered by the presence of atrazine. The study indicates that the presence of triazine herbicides such as atrazine can act in combination with a range of other environmental factors including multi-species effects, and may not only promote initiation of blooms of cyanobacteria, but also provide a selective advantage that leads to extended bloom dominance during declining temperatures in temperate lakes.

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Chapter 5

Effect of the herbicide atrazine on growth competition between the cyanobacterium, *Anabaena circinalis* and the green alga, *Desmodesmus asymmetricus*

5.1 Introduction

Natural fresh water bodies and rivers are typically dominated by chlorophytes and diatoms, and cyanobacterial dominance is rare, especially under non-limiting nutrient conditions in slow to moderately flowing rivers (Hotzel and Croome, 1996; Fabbro and Duivenvoorden, 2000). However, changes in physical, chemical and biological factors such as elevated water temperature, high light penetration, low river flows or high water residence times, high pH, or changes in nutrients inputs and grazing, can shift community dominance toward cyanobacteria. Global climatic change and warmer temperatures has been cited as a major factor associated with increases in harmful cyanobacterial blooms and research has linked higher temperatures with increasing growth of the toxic strains of cyanobacteria rather than non-toxic strains (Davis et al., 2009). Higher temperatures are also associated with increasingly persistent thermal stratification in water bodies, and light penetration in shallow water has been identified one of the major factors increasing germination of benthic akinetes (resting cells) of *A. circinalis* species (Baker, 1999; Tsujimura and Okubo, 2003; Thompson et al., 2009). Stratified conditions allow germinated cells to attain maximum growth rate due to the buoyancy capabicity of many cyanobacteria, allowing them to maintain a position near the surface (Mitrovic et al., 2001)

A recently highlighted but poorly understood mechanism promoting cyanobacterial dominance is the effect of photosynthetic-inhibitor herbicides that enter the water from agriculture and forestry activities in the catchment (e.g. Lampert et al., 1989; Lürling and Roessink, 2006). These contaminants find their way to water bodies and rivers during heavy rain and there is considerable evidence that they can influence algal community composition by favouring species with higher resistance to the effects of the herbicides (Lürling and Roessink, 2006).

One proposed mechanism that favours cyanobacteria is that they are better able to maintain synthesis of additional light-harvesting complexes and maintain photosynthetic function in the presence of herbicides (Hatfield et al., 1989). Another is that cyanobacteria are also reported to produce a variety of bioactive allelochemical substances to regulate growth of other microorganisms in the surrounding water (Suikkanen et al., 2004). In some cases, these allelochemicals appear to have similar photosynthesis-inhibiting activity to herbicides, especially when environmental conditions are sub-optimal such as nutrient limitation, or unfavourable light and temperature combinations (Leflaive and Ten-Hage, 2007). Previous studies have suggested that the allelopathic activity of cyanobacteria at low cell densities of (10^3-10^4 cells mL^{-1}) is less frequent (Leão et al., 2009).

In Chapter 3, preliminary competition testing indicated that high concentrations of *A. circinalis* inhibited the growth of green algae. It is possible that these natural growth inhibitors may act synergistically with other photosynthesis-inhibiting herbicides to suppress growth of competing algal species. This chapter examines this hypothesis using two-species (cyanobacteria and green algae) competition cultures at different relative starting densities. The combined effects of cyanobacterial allelopathy and atrazine concentrations that is relevant to natural environment (10-60 µg L^{-1}) on growth competition between green alga, *D. asymmetricus* and the cyanobacterium *A. circinalis* will be examined.

5.2 Materials and Methods

Two strains of microalgae, the green alga, *D. asymmetricus* and the cyanobacterium, *A. circinalis* ACCR02 were maintained in log phase at the high light high temperature parameters used in Chapter 4. The strains were cultured in MLA media in 100 mL Erlenmeyer flasks at constant light intensity of 100 µmole photon $m^{-2}s^{-1}$ by cool white fluorescence light (CLIPSAL WPT218 cool white fluorescent, 2 x 18 watts) at a temperature of 24 ±1 °C. The temperature chosen for the experiment matches the water surface temperature recorded during blooms of *A. circinalis* in temperate lakes and reservoirs (e.g. Trevallyn Dam, 22 -24 °C; McCausland, 2011).

A concentration of 75, 37.5 and 12.5 µg L^{-1} of atrazine in MLA was prepared from a stock MLA medium solution containing 1000µg L^{-1} of atrazine. Inocula of 0.5 mL of acclimatised culture were added to 2 mL of prepared atrazine-containing media in 24-well microplates to yield final atrazine concentrations of 60, 30 and 10 µg L^{-1}. Cell concentrations of inocula were estimated by *in-vivo* fluorescence for each species (Chapter 3) to yield the relative starting concentrations of each experiment (Table 5.1); at equal concentrations, or with either species having a 4:1 relative dominance.

Growth of both species was estimated using *in-vivo* fluorescence from day 0 (inoculation) for 10 days using a TECAN Genios plate reader with the excitation wavelength of 485 nm for green algae and 595 nm for the *Anabaena* strain (described in detail in Chapter 3).

The increase in cell concentrations of *A. circinalis* and the *D. asymmetricus* in the control and the different atrazine treatments (10, 30, and 60 µg L^{-1}) were compared using paired T-test at the end-points at day 10 as well as at day 6. Growth rates were calculated from *in-vivo* fluorescence data according to the methods outlined in Chapter 2. *Post-hoc* analysis was performed by using one-way ANOVA analysis to compare the growth rates and maximum cell concentrations at the different atrazine treatments.

Table 5.1 Starting cell concentrations of each species mixed together in the competition experiment.

Cells ratio (cells mL^{-1})	*D. asymmetricus*	*A. circinalis*
Low/Low (1:1)	5000	5000
High/Low (4:1)	20000	5000
Low/High (1:4)	5000	20000

5.3 Results

In the absence of atrazine (control), *D. asymmetricus* dominated when grown with equal or with higher starting cell concentration than the *A. circinalis* (Figure 5.1). Final cell concentrationsat day 10 of *D. asymmetricus* were significantly higher than *A. circinalis* starting from 4:1 *D. asymmetricus* dominance or 1:1 equal starting cell ratio. However, starting from cyanobacterial dominance of 1:4 cell ratios of *A. circinalis*, the maximum cell concentrations of both species at day 6 and day 10 were not significantly different (Figure 5.1). The growth rate of *D. asymmetricus* was significantly reduced when starting from *A. circinalis* 1:4 dominance compared to equal starting cell concentrations of each. The growth rate of *A. circinalis* was not significantly different among the three starting cell concentration combinations (Figure 5.2).

The presence and increasing concentration of atrazine had a significant effect on the outcomes of the growth competition experiments. This is evident both in changes in exponential growth rates of treatments and also differences in final cell concentrations of the species (Figures 5.2, 5.3). In the presence of 60 µg L^{-1} of atrazine, *A. circinalis* sustained higher relative growth rates compared to *D. asymmetricus* at all cell concentrations combinations (Figure 5.3). The relative exponential phase growth rate of the two species (*A. circinalis/D. asymmetricus*) did not change significantly with increasing atrazine concentration; with the exception of cultures starting with a cell ratio dominated 1:4 by *A. circinalis.* At the highest atrazine concentration (60 µg L^{-1}), *A. circinalis* had a higher exponential growth rate than *D. asymmetricus* (Figure 5.3).

A consistent decline of *D. asymmetricus* was observed when *A. circinalis* exceeded 8×10^5 cells mL^{-1}; from day 5-6 at equal starting ratio; from day 6-7 starting from *D. asymmetricus* dominance; and day 4-5 starting from *A. circinalis* dominance (Figure 5.4). Maximum cell concentrations achieved by *D. asymmetricus* also consistently declined with increasing atrazine concentration, especially when starting from *A. circinalis* dominance (Figure 5.5).

The growth curves of both species at different atrazine concentrations are shown in Figure 5.4. Patterns of growth and competitive outcomes were significantly altered by increasing atrazine concentration under all three starting cell ratios examined (Figure 5.4). At all three starting cell ratios, maximum and final cell concentrations of *D. asymmetricus* descreased with increasing atrazine concentration, while final cell concentration of *A. circinalis* increased with increasing atrazine (Figure 5.4). Compared to the competitive outcomes in no atrazine controls (see Fig 5.1), competitive outcomes were reversed at atrazine concentrations $\geq 30\ \mu g\ L^{-1}$ at all starting cell ratios, including 4:1 dominance by *D. asymmetricus*. At atrazine concentrations of $10\ \mu g\ L^{-1}$, competitive outcomes were not reversed either from starting with *D. asymmetricus* dominance or 1:1 equal dominance, however, starting with *A. circinalis* dominance, *A. circinalis* achieved a higher cell concentration after day 6 despite its lower growth rate from days 1-4.

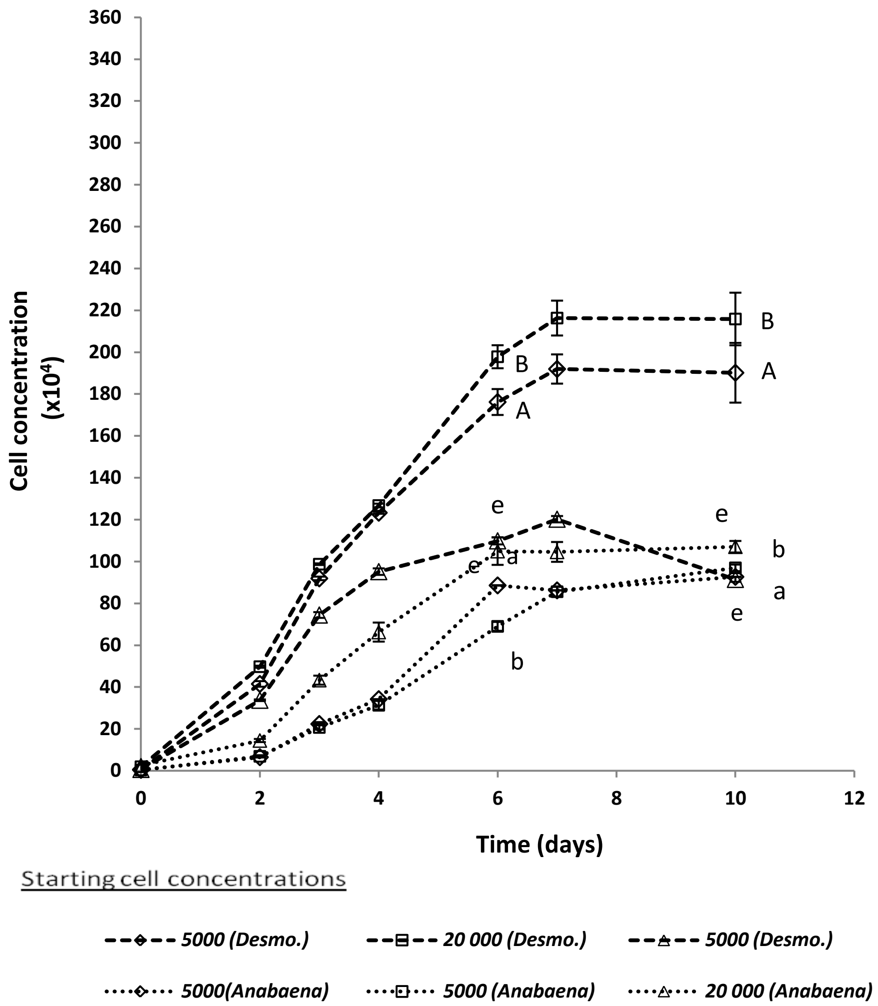


Figure 5.1 Cell concentrations of *D. asymmetricus* and *A. circinalis* when grown together without atrazine (±SE). Significance differences (p <0.05) in cell concentration at day 6 and day 10 are indicated by capital versus non-capital letters. Paired non-capital letters indicate no significance difference.

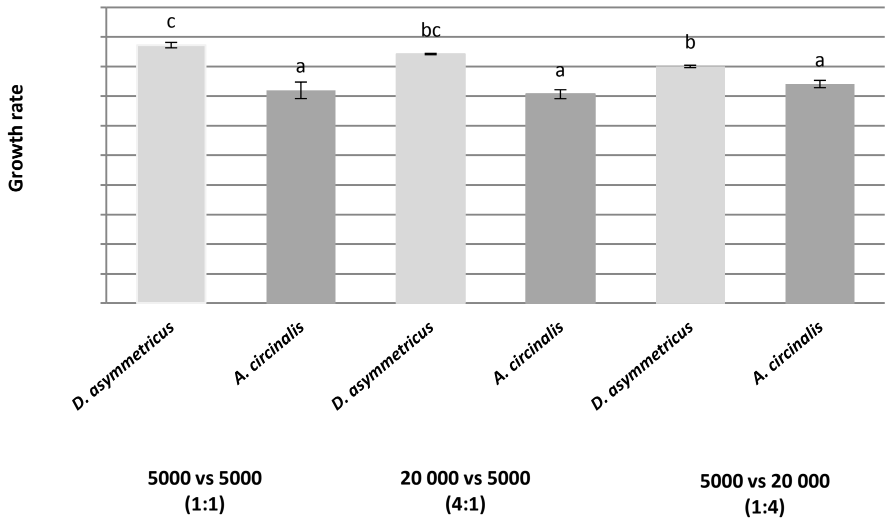


Figure 5.2 The exponential phase growth rates (±SE) of *D. asymmetricus* and *A. circinalis* grown together at different cell densities in no-atrazine controls. Different letters indicate significantly different means ($p<0.05$). Paired letters indicate no significant difference.

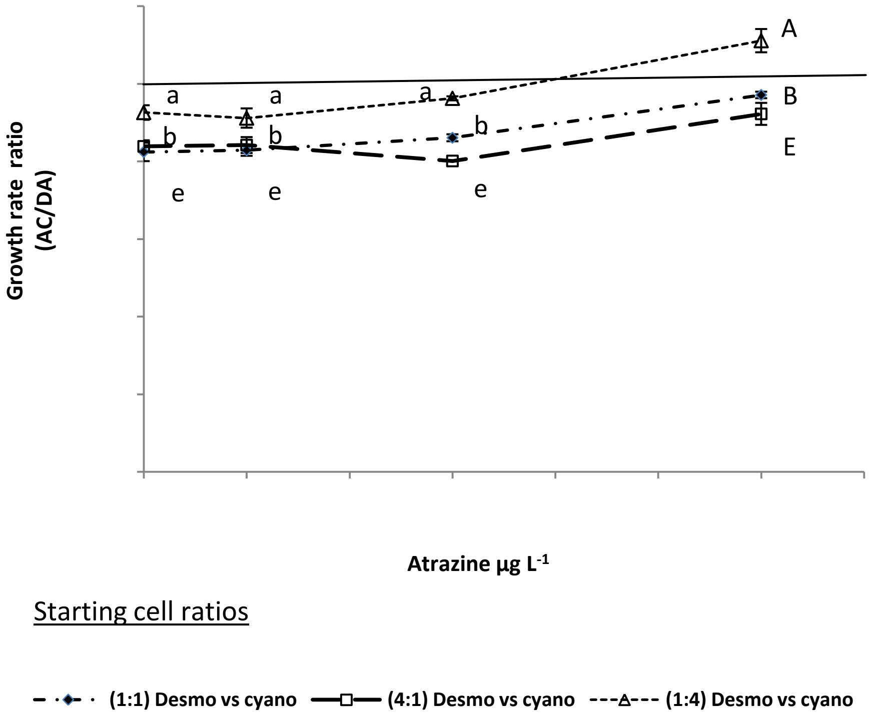
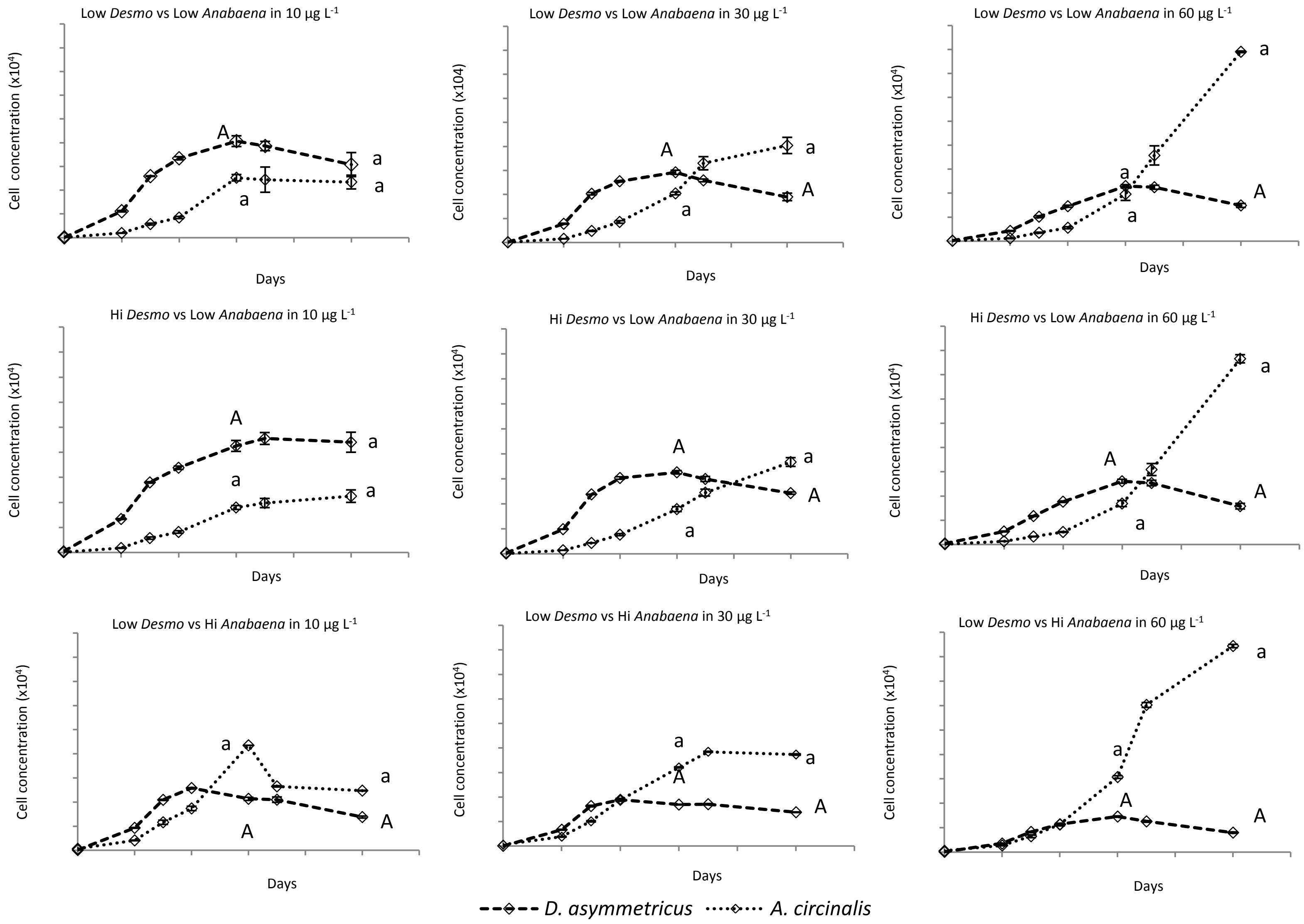


Figure 5.3 Relative growth rates(± SE) of (*A. circinalis*/*D. asymmetricus*) grown together at the three different starting cell concentrations in the presence of atrazine at 24°C and 100 µmoles photons PAR m^{-2} s^{-1}. Ratios above 1.0 indicate *A. circinalis* growth rate was higher than *D. asymmetricus*. Significance differences ($p<0.05$) among the different atrazine concentrations at each starting cell ratios are indicated by capital versus non-capital letters.

Figure 5.4 Growth curves (± SE) of *D. asymmetricus* and *A. circinalis* grown together at equal starting cells inocula of (low *Desmo*. and low *Anabaena*), green algae dominance (high *Desmo*. and low *Anabaena*) and cyanobacteria dominance (low *Desmo*. and high *Anabaena*) in the presence of atrazine concentration of 10, 30, and 60 μgL^{-1} at constant irradiance of 100 µmole photon m^{-2} s^{-1} and a temperature of 24±1 °C. Significant differences ($p<0.05$) between *D. asymmetricus* and *A. circinalis* indicated by capital versus small letters at for each species at day 6 and 10.


Low Desmo vs Low Anabaena in 10 µg L-1
Cell concentration (x10⁴)
Days
Low Desmo vs Low Anabaena in 30 µg L-1
Cell concentration (x104)
Days
Low Desmo vs Low Anabaena in 60 µg L-1
Cell concentration (x10⁴)
Days
Hi Desmo vs Low Anabaena in 10 µg L-1
Cell concentration (x10⁴)
Days
Hi Desmo vs Low Anabaena in 30 µg L-1
Cell concentration (x10⁴)
Days
Hi Desmo vs Low Anabaena in 60 µg L-1
Cell concentration (x10⁴)
Days
Low Desmo vs Hi Anabaena in 10 µg L-1
Cell concentration (x10⁴)
Days
Low Desmo vs Hi Anabaena in 30 µg L-1
Cell concentration (x10⁴)
Days
Low Desmo vs Hi Anabaena in 60 µg L-1
Cell concentration (x10⁴)
Days
D. asymmetricus
A. circinalis

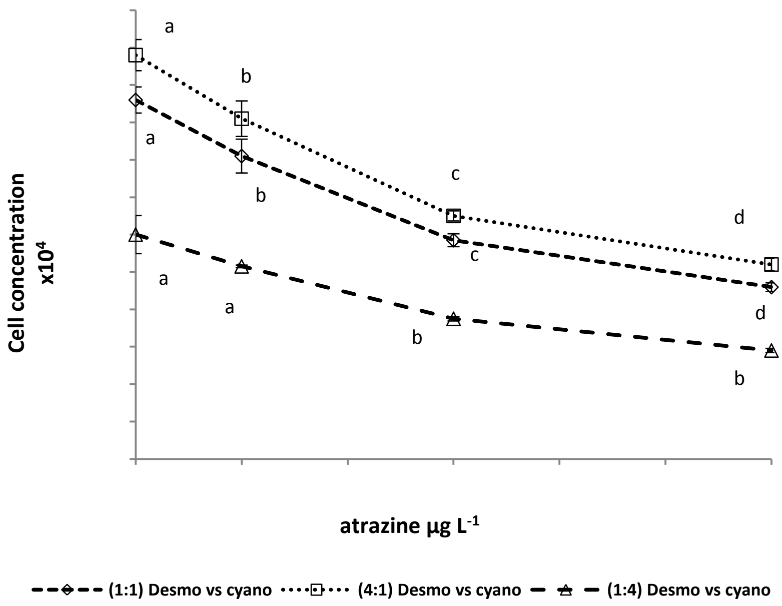


Figure 5.5 Maximum cell concentrations (± SE) of *D.asymmetricus* co-existed with the different combination of cell ratio of *A. circinalis* at different atrazine treatments. Significance designated by different letter at p<0.0 at each treatment of each combination. Paired letters indicate no significant difference in cell concentration between treatments of each combination.

5.4 Discussion

The presence of atrazine had a significant effect on growth competition between *D. asymmetricus* and *A. circinalis* at all three starting combinations tested. All concentrations of atrazine favoured *A. circinalis* compared to no-atrazine controls, whether starting from a position of *A. circinalis* dominance or *D. asymmetricus* dominance, and in all but two cases resulted in *A. circinalis* dominance by the end of the experiment.

Relative starting dominance would be expected to alter algal competition in a batch culture experiment due to competition for limited nutrients; however the differential effects of atrazine on the two species can reverse the competition outcomes. In no atrazine controls, *D. asymmetricus* maintained dominance at equal and/ or higher starting cell concentrations but starting from *A. circinalis* dominance the final cell concentrations were similar due to lower growth rate of *A. circinalis*. Hu and Zhang, (1993) reported the important role of the initial cells density on algal competition for limited nutrients as *Anabaena flos-aquae* displaced the diatoms when grown at higher cell density and when nitrate at short supply for both species.

The growth inhibiting effect of increasing atrazine appears to be primarily evident up to day 6 of the experiment. The growth rate of *D. asymmetricus* is increasingly reduced by higher atrazine concentrations, whereas the growth rate of *A. circinalis* was much less inhibited during exponential phase. After day 6, cell concentrations of *A. circinalis* increased substantially in treatments probably to phase shift to PSI to overcome the PS II inhibitory where *D. asymmetricus* has been inhibited by atrazine, while *D. asymmetricus* declined in these treatments. Koenig (1995) observed a similar increase in cell density after 6 day of icubations in the presence of sub-lethal concentration of atrazine (20 -210 µg L^{-1}).

Earlier experiments in Chapter 3 of this thesis indicated that *A. circinalis* exhibited allelopathic inhibition of *D. asymmetricus* when grown in competition cultures. The inhibition of *D. asymmetricus* by A. *circinalis*, noted in Chapter 3 was also noted in this experiment. Consistent and severe inhibition, leading to a decline in *D.*

asymmetricus was evident when *A.circinalis* concentrations approached or exceeded 8x10^5 cells mL^{-1}. A similar decline was evident in no-atrazine controls, but only in treatments starting with *A. circinalis* dominance, and only after cell concentration exceeded 1 x10^6 cells mL^{-1}. This suggests that *A. circinalis* allelopathic inhibition and atrazine combined either additively or synergistically to inhibit *D. asymmetricus*, or that atrazine may act on to increase the production or activity of *A. circinalis* allelochemicals. There is a general agreement that algal allelochemical activity increases with increasing cell density (Graneli et al., 2008; Tillmann et al., 2007). It appears that the allelopathic factor produced by *A. circinalis* also only has significant inhibitory activity when at sufficiently high cell concentration, approaching 10^6 cells mL^{-1}. Nutrient deficiency is also known increase the release of allelopathic substances by cyanobacteria (Ray and Bagchi, 2001). The pattern of inhibition in these experiments indicates that allelopathic activity of *A. circinalis* is likely produced and exported into the medium in late-exponential or stationary phase, as are other cyanobacterial secondary metabolites such as saxitoxins (Negri et al., 1997). Alternatively, competition for nutrients or a nutrient imbalance has also been suggested to trigger allelochemical production (Graneli and Johansson, 2003)

Similar allelopathic activities have been identified in other freshwater cyanobacteria, also associated with high cell densities or during cyanobacterial blooms. The allelochemicals are directed against a range of biochemical processes of competing organisms in the same habitat. For example, cyanobacterin inhibits other cyanobacteria and green microalgae, fischerellin targets photosystem II (Leao et al., 2010). Some cyanobacteria are also known to suppress the growth of competitors owing to the release of photosynthetic-inhibitors or antibiotics (Chauhan et al., 1992). More recently cyclic peptide allelochemicals (Portoamides) containing unusual modified amino acids have been shown to inhibit the green alga, *Chlorella vulgaris* (Leao et al., 2010). However, it has also been suggested that allelopathy may only rarely operate in natural systems where cell densities are often relatively low (Leao et al., 2009).

The decline of the *D. asymmetricus* especially after day 6 in the presence of atrazine is also associated with an increase in growth of *A. circinalis*, especially at higher atrazine concentrations. Similar stimulatory response to atrazine is known from other cyanobacteria. For example, a continuous increase in cell density of *Anacystis* with increasing in atrazine was observed by Koenig (1995), only decreasing at concentrations above 210 µg L^{-1}. In addition, Murdock and Wetzel (2011) have observed increase in cell biovolume of the same cyanobacterium incubated at 10-100 µg L^{-1} over the same time frame of 6 days. Therefore, they suggested that the algal structural assemblage and its compositional changes are based on the physiological response of individual algal cells to these pollutants which stimulate some cells and inhibit others.

In high light, cyanobacteria have increased in D1 protein synthesis, while in high light and in presence of sublethal concentration of herbicide, phycocyanin synthesis was increased (Koenig, 1990). In contrast, the eukaryotic green algae exposed to high irradiance in the presence of herbicide, are more prone to photoinhibition and the destruction of photosynthetic pigments. Herbicides will block electron flow through photosystem II which normally used for NADP reduction, therefore, promotes the productions of excited chlorophyll molecules which are not able to transfer their energy to the reaction centre. This causes the spontaneous formation of triplet chlorophyll which reacts to molecular oxygen to form singlet oxygen. The build up of these reactive oxygen species in the photosystem apparatus result in lipid preoxidation and the photooxidation of the photosynthetic pigments (Powles, 1984).

In related studies, Weiner et al., (2007**)** found that among four algal species cultured in the presence of atrazine, the macromolecular changes were observed in the cyanobacteria. The total carbon uptake was the highest in the cyanobacteria at the highest atrazine concentration 88 µg L^{-1} where the increase of the carbon uptake in the cyanobacteria was for carbon allocation into protein that is particularly important for the production of protein subunits D1 and D2 while cholorphytes protein synthesis was significantly decreased**.** This indicates the cyanobacteria are capable of synthesizing protein and cell growth in the presence of atrazine concentrations of 88 µg L^{-1} and may explain the increase noted in the experiment especially an atrazine concentration of 60 µg L^{-1}. Magnusson et al., (2012) suggested that a metabolic shift to higher rate of heterotrophy could be interpreted to compensate for the high stress by the PS II inhibitory effect. The increase in cell numbers in *Anabaena circinalis* after day 6, may also be explained due to atrazine degredation or may have become less bioavailable as the cell numbers increased, yet phase shift from PS II to PS I has been a possible explanation to overcome atrazine inhibitory effects on PS II. Yates and Rogers, (2011) provided such explanation of the golden algal blooms in Lakes of Texas, USA and suggested that the heterotrphic, atrazine was not only promoting the growth of bloom-forming golden algae but also enhanced toxin production due to the algal's inability to photosynthesis.

The adaptative capability of cyanobacteria by reorganization of the photosynthetic apparatus and pigment profile to counteract the damage to the photosynthetic apparatus system by the herbicide provided them with similar measurable rates of photosynthetic electron transport as the controls which contained no herbicide during a 5-day subculturing of cyanobacterial cells (Hatfield et al., 1989). These adaptive capabailities probably provided them with a better photosynthetic capacity in the presence of photosynthetic inhibitor herbicide as indicated by Lürling and Roessink (2006) in which he observed a significantly higher reduction in growth rate of the green alga, *Scenedesmus* compared to cyanobacterium, *Microcytis* in 100 µg L^{-1} of the herbicide, metribuzin compared to the control. This is comparable to my results in which the growth rate reduction of *D. asymmetricus* was 26 % compared to the control in the presence of 60 µg L^{-1} atrazine while the *A. circinalis* reduction in growth rate was only 12%.

Therefore, cyanobacteria are probably better adapted to carry-out photosynthesis process in the presence of atrazine and carry out complete metabolic pathways required to synthesize protein or production in excess of D1 protein to counteract for the impaired photosynthetic pathways. In contrast, the decrease of

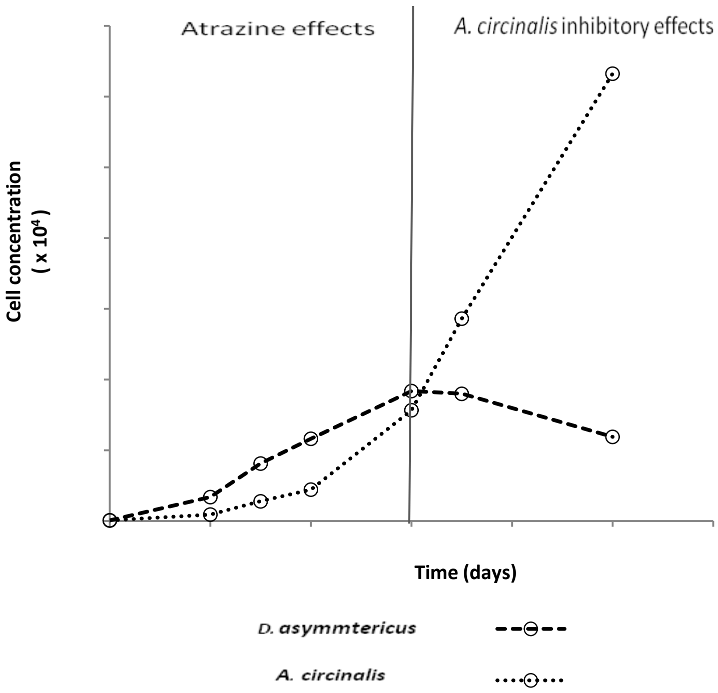


Figure 5.6 Conceptual model of growth curve of the *D. asymmetricus* and *A.circinalis* when grown at the presence of atrazine. Day 0-6: Atrazine inhibition dominant. Exponential growth of both species occurs with available nutrients; *D. asymmetricus* growth suppressed by atrazine. Day 6-10: Allelopathic inhibition of *D. asymmetricus* by *A. circinalis* becomes dominant effect leading to a decline in *D. asymmetricus*. Allelopathic activity continues exacerbated by nutrient limitation. Death/lysis of *D. asymmetricus* may also release nutrients for continued growth of *A.circinalis*.

macromolecules and the increase in low molecular weight macromolecules in green algal cells is indication that these cells are unable to complete the metabolic pathway required to produce whole macromolecules (Weiner et al., 2007).

A conceptual model of the two-species culture competition is shown in Figure 5.6. These experiments indicate that the relatively low concentrations of atrazine can alter growth competition to favour cyanobacteria over green algae. The data suggest that effect of atrazine is primarily evident during the first 3-5 days while both species were experiencing exponential growth. From day 6 onward, allelopathic inhibition of *D. asymmetricus* becomes the dominant factor increasingly favouring *A. circinalis*, even from a starting scenario of *D. asymmetricus* dominance. As suggested by O'Neil et al., (2012) cyanobacteria have highly refined strategies for accessing phosphorus from organic compounds and they occur as a sequence of events in temperate ecosystems. Therefore, cyanobacterial blooms are likely to precede as non-cyanobacterial phytoplankton are succeeded or die and their biomass will be remineralized into organic forms which most cyanobacteria are well adapted to exploit. This study support Chalifour and Juneau (2011) findings in which they hypothesized that even in the presence of atrazine, cyanobacterial bloom will be prompted by higher water temperature. Therefore, atrazine and other photosynthesis-inhibiting triazine herbicides may thus play a role in enhancing *Anabaena* blooms in freshwater.

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Chapter 6

General discussion and summary

6.1 Overview of thesis

This study introduces important additions to ecotoxicological approaches used to evaluate herbicide tolerance and draws some important findings on the effect of photosynthetic-inhibitor herbicides on the microalgal community structure. While their effects on microalgae are influenced by physical factors (light, temperature, nutrients, pH, turbidity and inputs), this work demonstrates that species interactions are an important element of understanding how communities respond to such toxicants. A singles-species assay approach can provide information about the effective concentration range (eg. nano-, micro-, or milligrams per litre) for each species; however, this work shows that relative sensitivity/tolerance studies alone are insufficient to predict changes in growth competition and thus community-level changes.

While there is a considerable literature examining the effect of nutrients, stratification and other physical/chemical factors promoting cyanobacterial blooms, few previous studies have examined how the PS II inhibitors herbicides may promote blooms, particularly blooms of the common bloom forming, *A. circinalis*. Therefore, this investigation makes an important contribution to understanding phytoplankton community responses to the most widely used herbicide, atrazine.

The major findings of this study are summarized as follows:

Chapter 2: The relative atrazine tolerance range of green algae and cyanobacteria was found to be not significantly different. Tolerance varied substantially among species and strains of *Anabaena* and *Anabaena circinalis* and the three green algae examined indicating that atrazine could influence community structure; however, there was no evidence that it should consistently favour cyanobacteria.

Chapter 3: This chapter adapted a multi-well plate fluorescence approach for high-throughput algal growth and toxicity studies and particularly growth competition assays between green algae and cyanobacteria. The method developed allowed herbicide tolerance experiments that incorporate possible allelopathic interactions detected in two-species cultures of green algae and the cyanobacterium *Anabaena circinalis*.

Chapter 4: Atrazine tolerance of *A. circinalis* and *D. asymmetricus* varied significantly under different combinations of light and temperature. When grown together, presence of atrazine favoured *A. circinalis* under 3 of 4 combinations of light and temperature, particularly at lower temperatures and high light characteristic of mid-latitude autumn, potentially explaining how bloom forming *A. circinalis* can persist in temperate lakes for long periods even at sub-optimal temperatures.

Chapter 5: Using two-species competition cultures of *A. circinalis* and the green alga, *D. asymmetricus*, this chapter demonstrated that atrazine at concentrations as low as 10-60 µg L^{-1} could alter growth competition outcomes to favour cyanobacterial dominance, even starting from green algal dominance. The growth patterns indicated that the combined effect of atrazine growth inhibition and allelopathic suppression by *A. circinalis* led to *A. circinalis* dominance.

The ability to examine the combined effect of herbicide and allelopathic interactions demonstrates the limitations of single-species approaches to toxicity assessment laboratory assays as a means of predicting community-level responses. Assessment of relative effects from monoculture assays over the same concentrations cannot be used to predict natural community effects. This study supports European legislation directives that recommend pesticide approvals include mesocosm studies (Seguin et al., 2002). The study also indicates that species interactions are likely to explain why field- and mesocosm-toxicity assessment outcomes differ substantially from single-species laboratory toxicity data (Brock et al., 2004).

The study also shows that herbicide-induced shifts in species composition of natural aquatic systems are not simply due to species with high herbicide tolerance outcompeting less tolerant species (Pinckney et al., 2002). In fact, the mechanisms are much more complex and driven by a series of interrelated factors. For example, Pannard et al., (2009) showed that low doses of the PS II inhibiting herbicides stimulate cyanobacterial tolerance over the green algae particularly under higher nutrient supply such as increased phosphorus. Cyanobacterial blooms are complex events driven by multiple factors occurring simultaneously rather than a single environmental driver (Havens, 2008; Heisler et al., 2008). Below, I summarize these factors in relations to our finding in this experiment:

6.2 Major factors of cyanobacterial bloom forming

1) Temperature:

Temperature plays major role in determining the rate of photosynthesis and cyanobacterial growth by controlling the enzymatic rates and improves nitrogen fixation (Jellison and Melack, 1993). Optimal growth of cyanobacteria is usually above 25 °C (Robarts and Zohary, 1987; Sabour et al., 2005), and at these higher temperatures cyanobacteria grow faster and compete better than the diatoms and eukaryotic algae (Paerl et al., 2011). Photoinhibition increases as temperature go below or above the optimum temperature (Torzillo and Vonshak, 1994).

Elevated temperature is considered the driving force for thermal stability of the water column especially in reduced flow rivers. Long and persistent periods of thermal stratification also causes increase the phosphorus recycling from anoxic sediments in the hypolimnion (Pettersson et al., 2003; Ulen and Weyhenmeyer, 2007). Temperature elevation also reduces the density of the epilimnion which allows the buoyant cyanobacteria such *Anabaena* species to exploit the stratified layer for atmospheric carbon and nitrogen, while it causes others non-buoyant non-motile species to sink faster (Klemer and Konopka, 1989; Walsby et al., 1997)

In our studies, higher temperatures and light increased tolerance toward atrazine in both species tested; However, the relative atrazine tolerance of *A. circinalis* increased most (compared to *Desmodesmus asymmetricus*) at high light and low temperature, and may explain the dominance of *Anabaena* even at lower water temperature (<20 degrees) in temperate lakes. This indicates the importance of seasonal variation in the selective action of the herbicides in the natural aquatic system. For example, Bérard et al., (1999) found cyanobacteria were not only tolerant to atrazine in the summer season but were stimulated in atrazine contaminated microcosms.

2) Light:

Light is one of the major factors controlling not only cyanobacterial growth but distributions and assemblage as they response in correlation to irradiance intensity and the pigment content (Oliver and Ganf, 2000). The growth requirement for light within the cyanobacterial species varies, for example in shallow eutrophic lakes, low-light adapted species such as *Oscillatoria*, *Lyngbya* and non-nitrogen fixers will dominate in wind mixed turbid conditions, while calm conditions lead to stable water columns and a dominance of high light adapted cyanobacteria such as *Microcystis* and nitrogen fixing groups such as *Anabaena* (Reynolds, 1984). *A. circinalis* requires high irradiance (light saturation at 90 µmole photon $m^{-2}s^{-1}$) to grow and fix nitrogen and it gains access to such conditions through light-mediated buoyancy from gas vesicles (McCausland et al., 2005). In Chapter 4, *A. circinalis* was favoured under high light conditions regardless of temperature.

3) Mixing and stratification:

In Australian rivers, the initiation of *A. circinalis* blooms is usually associated with establishment of persistent thermal stratification for approximately 14 days (Sherman et al., 1998). This indicate that *A. circinalis* is a cyanobacterium that requires high irradiance to grow and water residency is very important factor for their dominance as it provides opportunity to gain access to light due to their buoyancy (McCausland et al., 2005). The growth and dominance of *A. circinalis* in the Barwon-Darling river in 1991, Australia was related to water residence for a period above 5 days due to low river discharge, allowing near maximum growth rate (Mitrovic et al., 2001; Mitrovic et al., 2003). This thesis indicates that concentrations of atrazine as low as 10 -60 µg L^{-1} could shift community structure in favour of cyanobacteria. Given the long half-life of atrazine in temperate environments, high water residence times in lakes and rivers can lead to prolonged exposure of phytoplankton to herbicides such as atrazine, increasing the risk of cyanobacterial blooms.

4) Nutrients:

Nutrient inputs especially through agricultural development and runoff during heavy rains have contributed to lake changes in altering the nutrient ratios of rivers and catchments which lead to disruptions of algal species favouring sometimes the less desirable species, the cyanobacteria (Howarth et al., 1996; Perakis, 2002). The ratio of different concentration of nutrients can play an important role in species growth and succession in the aquatic system (Sommer, 1993). Since many bloom forming cyanobacteria can utilize nitrogen in the organic form (O'Neil et al., 2012), Smith, (1983) suggested that cyanobacterial blooms may be caused by low ratio of N: P ratios. However, data from 99 of temperate zone’s lakes correlated the blooms with increases in total phosphorus, suggesting that phosphorous loadings are a better predictor for cyanobacterial dominance (Downing et al., 2001; Havens, 2008). Nutrient deficiency has been implicated as a factor promoting the production of allelochemicals, by lysing other competitor species may scavenge released nutrients for their own growth (Ray and Bagchi, 2001; Graneli et al., 2008). In Chapter 5, the allelopathic activity of *A. circinalis* combined with growth inhibition by atrazine was able to reverse outcomes of growth competition with the green alga, *D. asymmetricus*. The allelopathic activity was the dominant effect at high cyanobacterial cell concentrations (approaching 10^6 cells mL^{-1}). While the effects may be simply concentration dependent (Schmidt and Hansen, 2001; Tillmann et al., 2007); it is also possible that nutrient limitation increases production of the allelochemicals. Highly stratified water columns and high residence times that allow *A. circinalis* to form highly concentrated surface populations would further enhance allelochemical concentrations favouring increased growth suppression of other species and increasing dominance.

5) Grazing

Grazing on the phytoplankton community takes place mainly during the spring when the water is dominated by the edible phytoplankton species and in a lesser amount during the summer or clear water period when it is dominated by the more resistant species presumably as result of selection occurred during the earlier grazing season (Oliver and Ganf, 2000). Cyanobacteria are considered a poor source of food for zooplankton due to their size, low digestibility, toxin production and allelopathic compounds (Lampert, 1987; Carmichael, 2001; Lürling and Roessink, 2006). Therefore, significant disappearances of crustacean zooplankton and grazers have been found during cyanobacterial dominance (Haney, 1987; Ke et al., 2008). In eutrophicated freshwaters, species of the genera *Oscillatoria* and *Anabaena* are considered the most evenly distributed toxin producing species that can affect the populations of the natural grazers and other aquatic biota (Dokulil and Teubner 2000). Recent studies have discovered many of these toxins including the previously unidentified toxin (BMAA) which is potentially produced by a broad range of cyanobacterial genera and the gene required for anatoxin-a biosynthesis in *Anabaena circinalis* strain was also described (O'Neil et al., 2012). The toxins contained in these strains could play role in suppressing the grazers in the aquatic system; therefore, *A. circinalis* may dominate under optimal or nutrient-limited conditions through a combination of suppression of other phytoplankton species as indicated in Chapter 3 and 5 as well as in suppression of grazers.

6) Anthropogenic inputs and herbicides:

In broad terms these inputs which occur during heavy rain and runoffs include all the effluents associated with agricultural inputs and intensive farming, such as pesticides, herbicides, suspended solids as well as nutrient inputs which have already been addressed. Water bodies receive runoff from agricultural fields treated with herbicides such as atrazine, especially during the post-application periods which may directly affect the most susceptible organisms within these system, the phytoplankton, periphyton, and macrophytes (Solomon et al., 1996). Most of these pollutants are associated with suspended solids combined with dissolved organic contaminants that find their way to catchment and rivers in flow events, during land clearing, flooding or heavy rain. Therefore, in many cases there is a positive correlation with rainfall, turbidity, and nutrient loads (Panta, 2011), and all of which interact in shifting the phytoplankton community toward the cyanobacterial blooms in Australia (Bormans et al., 2004; Bormans et al., 2005). Agricultural herbicides and fertilizers are applied routinely during the year and their effects in changing the community structure of the phytoplankton taxa are, in theory based on the differential response or growth to these chemicals (Krieger, 1984; Klarer and Millie, 1994). More resistant species within the community may also develop tolerance through changes in genetic composition (Kasai, 1999; Kasai and Hanazato, 1995).

Studies in this thesis do not support the hypothesis that cyanobacteria are generally more tolerant of atrazine than green algae, but instead indicate that tolerance varies considerably among species and strains of both algal groups (Chapter 1). Abiotic and biotic factors such as light, temperature and species interactions have substantial influences on relative atrazine tolerance (Chapters 3, 4) and, under a range conditions, may combine to favour cyanobacterial dominance over green algae (Chapter 5). Therefore, the observed community-level effects appear to arise from more complex mechanisms than species-level differences in relative herbicide tolerance.

6.3 Future research

Cyanobacterial tolerance of the herbicide, atrazine needs to be further investigated to understand the protective mechanism in which the herbicide exerts selective pressure on sensitive species rather than the tolerant ones. This probably can be achieved through molecular and genetic studies of the protein components of the photosynthetic apparatus. The role of the D1 protein regenerations to carry out the photosynthetic process to compensate for the presence of herbicide presence need to be elucidated, while the assumption that some species switch to heterotrophic metabolic pathway as an alternative way is still premature and warrant for more investigations.

Results of this study support the role of the photosynthetic-inhibitor herbicide in promoting the cyanobacterial abundance and subsequently their blooms, especially when taking account of species interaction and environmental factors. Therefore, when considering anthropogenic inputs as major causes of cyanobacterial blooms, our data indicate that the effects of herbicide inputs must be considered along with nutrients inputs (TN and TP) for promoting and extending the cycles of cyanobacterial blooms. However, the mechanism or the causes that lead to increase in cell concentration and abundance of *Anabaena circinalis* in the presence of atrazine need further investigations.

Furthermore, the breakthrough in molecular methods in detection, identification and genetic elucidation of toxins of some neurotoxin-producing cyanobacterial strains of *Anabaena circinalis* will probably contribute in understanding the allelopathic activities and defence mechanisms exerted by these species to compete and initiate dominance in the community structure which eventually leads to lake eutrophications, water quality deteriorations, and threaten the aquatic biodiversity system.

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Appendix 1

Laboratory testings of passive sampler in atrazine uptake in ultra-pure water and river water

A1.1 Introduction

Herbicides or chemical weed killers are one of the major achievements of modern agriculture. Their increased use in agricultural practices leads to their occurrences in water bodies and underground waters due to leaching or runoff. Most of these herbicides find their way to waterbodies during heavy rain, particularly the first rain after application (Graymore et al., 2001; Solomon et al., 1996)

Since its introduction in 1950, atrazine has become one of the most widely used herbicides in agriculture to control weeds in North America (Pannard et al., 2009). In Australia it is considered one of the most prolific herbicides with over 3000 tonnes used annually for weed control and broad-acre agriculture (Kookana et al., 2010). Atrazine was used by Forestry Tasmania to reduce competition between weeds and eucalypts plantation during the spring and winter spraying until 1995 (Elliott and Hodgson, 2004). However, spraying can cause atrazine leaching into streams and waterbodies, especially after heavy rain, with detected concentrations ranging from 0.01 – 53000 µg/L (Davies et al., 1994). Based on physical and chemical characteristics (Table A1.1) atrazine is considered a relatively mobile herbicide with moderate water solubility and having a $\log K_{ow}$ of less than 4 is considered a relatively polar compound.

Traditionally, monitoring and detection of pollutants or herbicides are usually performed by grab or spot sampling. However, this could be costly and miss episodic pollution events, especially during heavy rains. Therefore, the passive sampler approach could be a promising tool to overcome these problems. The passive sampling method is based on the diffusional flow of analyte molecules in aqueous medium to a receiving phase containing a porous adsorbent in which accumulation of analyte in passive sampler follows first-order kinetics (Vrana et al., 2005). This is characterized by an initial linear phase of accumulation of analyte, followed by a curvilinear and then stationary or equilibrium phase.

Table A1.1. Physical and Chemical properties of atrazine: (Solomon et al., 1996)

Item	Characteristics
CAS number	**1912-24-9**
Chemical name	**2-Chloro-4-ethylamino-6-isopropyl-amino-1-s triazine**
Molecular weight	**215.70 gm/mol**
Molecular formula	$C_8H_{14}N_5Cl$
Partition coefficients	
1- Log K_{ow}	**2.68 at 25°C**
2- Log K_{oc}	**1.96-2.5**
pK_a	**1.68**
Melting point	**175-177 °C**
Water solubility	**33 mg l^{-1} at 22 °C**
Vapour pressure	**2.89 X 10^{-7} mmHg at 25 °C**
Henry's law constant	**2.48 X 10^{-9} atm m^3 mol^{-1}**
Hydrolysis	**pH 5-9 at 25 °C**

Passive sampling has not been considered as a monitoring method in the Water Framework Directive of the European Union because method validation and documentation inaccordance with EN ISO/IEC-17025 standards has not been established yet (Smedes F. et al., 2010). However a review of the feasibility of passive samplers as an alternative monitoring technique considers passive sampling as one of the complementary methods that can be used for monitoring "particularly given the fact that determining annual average concentration is one of the main objectives of the WFD".

a) Principle of passive samplers

Passive sampling typically consists of a receiving phase containing a bound solid-phase material with high affinity for organic pollutants, separated from the aquatic environment by rate-limiting diffusion membrane. They rely wholly on the partitioning of organic compounds from the aqueous to receiving phase by means of diffusion through suitable membrane. Sampling rates are dependent on intrinsic factors such as physicochemical properties of the analytes and passive sampler design, particularly the surface area of the exposed disk and the rate of permeation through the diffusion-limiting membrane. Extrinsic factors include water temperature, turbulence and biofouling (Tran et al., 2007).

The relationship between the mass of an analyte in the receiving phase and the concentration in the aqueous environment after an exposure time has been formulated as follows:

$M_D=C_WR_{DW}(t)$

Where M_D is the mass of analyte accumulated in the receiving phase (ng), C_W is the analyte concentration in the aqueous environment (ng/mL), R_{DW}, the device sampling rate (mL/d) and t is the exposure time (d). For most devices operating in the kinetic mode, the sampling rate is affected by the water turbulence, temperature and biofouling (Tran et al., 2007; Stephens et al., 2009; Kingston et al., 2000).

b) Types of Passive samplers

There are two types of passive samplers, one is used to monitor inorganic pollutants such metals and elements while the other types for organic pollutants monitoring. The latter, includes the samplers with potential use for aquatic environment monitoring such as the Semi-Permeable Membrane Devices, SPMD, the Polar Organic Chemical Integrative Sampler, POCIS, to monitor hydrophilic contaminants such as pesticides, hormones and personal-care products and Chemcatcher for sampling non-polar and polar pollutants by changing the diffusion-limiting membrane (Kot et al., 2000; Vrana et al., 2005; Kot-Wasik et al., 2007).

Kingston et al., (2000) developed two novel sampling systems based on their log octanol/water partition coefficient values; a sampler for polar organic compounds with log K_{ow} between 2 and 4 and the other for non-polar organic compounds with log k_{ow}> 4. The passive samplers are characterized by having the same solid-phase material of C_{18} Empore disk as a receiving phase but fitted with different rate-limiting membrane materials, polysulfone for the polar and polyethylene for the non-polar analytes.

To address the issue of hydrophilic organic contaminants such as pesticides, pharmaceuticals, and personal care products, the polar organic chemical integrative sampler (POCIS) was specifically designed to sequester hydrophilic compounds from water in the United Kingdom (Alvarez et al., 2004). In Australia, passive sampling devices have been assessed for the sampling of polar herbicides used for agricultural purposes, including five non-ionised herbicides (simazine, atrazine, diuron, clomazone and metolachlor) and four phenoxy acid herbicides (Tran et al., 2007). In this study, styrenedivinylbenzene receiving phases Empore SDB-XC and the more hydrophilic Empore SDB-RPS were compared with different polar and non-polar diffusive membranes.

c) Uptake rate of the (SDB-RPS) with PES membrane sampler:

In order to check the suitability of such samplers to monitor the herbicide, atrazine in the aquatic system, we designed a passive sampler with hydrophilic receiving phase with a styrenedivinylbenzene copolymer sorbent (SDB-RPS) covered with polyethersulfone membrane (PES).

A1.2 Materials and Method

1) Uptake by SDB-RPS Empore disk covered with PES membrane in spiked ultrapure water with 10 ppb atrazine at 15 ±2 °C.

A) Chemicals

Solvents used were HPLC grade methanol (HiPerSolv, BDH, Sydney, Australia), AR grade acetone (Chem supply, Sydney Australia), and Analytical grade atrazine (98% purity, Chem-Service, Pennsylvania 19381). For the mobile phase Acetonitrile (HPLC grade, Supragradient, Barcelona, Spain) and ultrapure water (18 MΩ cm^{-1}, Barnstead, Thermo Scientific, USA) was used. The mobile phase was degassed by filtration through 0.45 µm GH hydrophilic polypropylene filters from Pall life.

B) Passive samplers

Passive samplers consisted of a 47 mm diameter 3M Empore SDB-RPS reversed phase sulfonated disk (Part # 12145026, Agilent Technology) and in the case of a membrane covered disk, the 3M Empore SDB-RPS disk was covered with two 47 mm, 0.2 µm pore size hydrophilic polyethersulfone membrane filters (Part # 60301, Life Science). A 3M Empore SDB-RPS disk, with or without PES membrane filters, was placed between two aluminium disks which had an outside diameter of 70 mm and an exposed area of a diameter of 40 mm and held together by three stainless steel bolts, with the external edge of the sampler wrapped with PTFE tape to prevent solution from entering along the edges.

Atrazine uptake experiments were carried out in 600 mL glass jars with the passive sampling device suspended in the 600 mL glass jars by nylon line in the vertical position. Uptake experiments were conducted in triplicate and maintained at constant agitation (100 rpm) by a mechanical shaker (Orbit Shaker, Lab-Line) at temperature of 15 ±2 °C. Passive samplers were deployed in 10 ppb atrazine spiked in ultrapure water for 1, 3, 5, and 7 days with the deployment solution replaced daily to minimise the atrazine concentration change. On removal of passive samplers from the deployment solutions, the PES membrane filters if used were removed, and the Empore SDB-RPS disk was placed on an all glass filtration apparatus and rinsed with 2x 5mL ultrapure water. Elution of the accumulated atrazine was then achieved with 2x 5mL acetone and 2x 5mL methanol, which was then evaporated to dryness under a gentle stream of air, and reconstituted with 1 ml mobile phase under sonication (Unisonics Ultrasonic Cleaner FXP8, Sydney, Australia) for 15 min. After sonication, the 1 mL sample was transferred to 2 mL screw vials (part # 5183-4428).

C) Analysis

Analysis was achieved using a GBC HPLC system (GBC Scientific Equipment Pty Ltd, Victoria, Australia) with a LC1150 HPLC pump, LC1205 UV/Vis detector and LC1650 Advanced Autosampler using Supelcosil LC-PAH column (Supelco 58229, 25cm x 4.6mm id 5 µm) and with an injection volume 50 µL with mobile phase (40:60 H_2O:CH_3CN), read at 220 nm.

Calibration and analyte concentration was determined using linear regression from atrazine external standards (stock solution of 20.0 ppm diluted in 5% methanol) diluted at different range of concentrations (0.0, 1.0, 5.0, 10.0, 20.0 ppm).

2) Uptake by naked SDB-RPS Empore disk in spiked ultrapure water with 10 ppb atrazine at 15 ±2 °C.

The same procedures are applied as mentioned above but without using the diffusive membranes covering the empore disk.

3) Uptake by naked SDB-RPS Empore disk in spiked unfiltered river water with 10 ppb atrazine at 21 ±2 °C.

Following the same procedure as that used for passive sampling of atrazine in ultrapure water, a passive sampling experiment was undertaken using water from the South Esk River, collected on the 19-10-2011 at the Gorge Basin in 4 L plastic bottles. The water was passed through a coarse strainer (estimate the sieve size 0.5 - 1.0 mm) to remove large particulates and stored at 4°C prior to use. For the uptake experiment the South Esk River water was spiked to 10 ppb atrazine with deployment solutions replaced daily and a temperature of 21 ± 2 °C maintained throughout the experiment. Samplers were retrieved at 1, 3, 5, and 7 day deployments and rinsed on retrieval to remove any dirt from the disk surface.

A1.3 Results and Discussion

1) Uptake by SDB-RPS Empore disk in spiked ultrapure water with 10 ppb atrazine covered with PES membrane at 15 ±2 °C.

Figure (A1.1) shows the uptake of atrazine by SDB-RPS disks covered with PES membranes in 10 ppb atrazine spiked solution in ultrapure water over a 7 day deployment. The rate of accumulation of the analyte by the receiving phase was approximately linear during the first five days of deployment, but it decreased at day 7 as the disk probably reached saturation. The calculated sampling rate is 0.044 L/day or 44 mL/day, and with an instrument detection limit at 1 ng this provides the ability to measure environmental concentrations at 0.001/0.044 = 0.023μg/L for one day deployment, or 0.003μg/L for a 7 day deployment.

2) Uptake by naked SDB-RPS Empore disk in spiked ultrapure water with 10 ppb atrazine at 15 ±2 °C.

The rate of accumulation of atrazine from a 10 ppb atrazine spiked solution in ultrapure water by the naked RPS disks showed a linear and higher uptake rate over the 7 day deployment period (Figure A1.2)

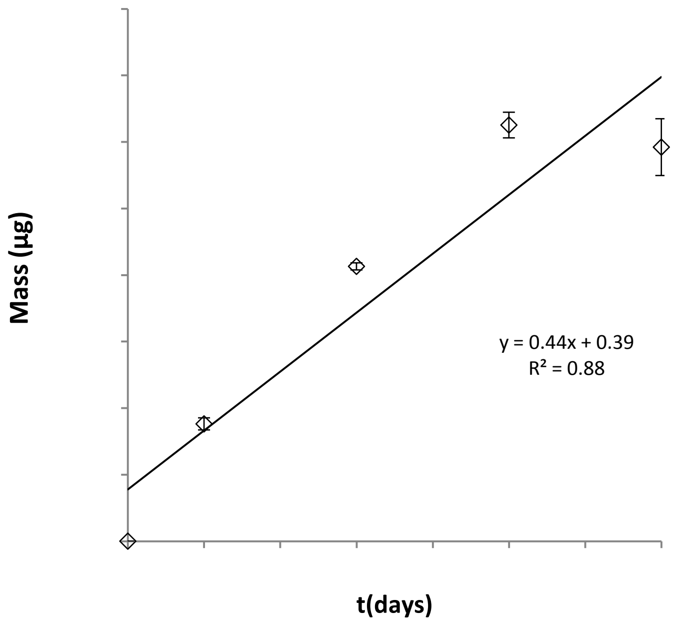

Figure A1.1 Uptake of atrazine at 10 ppb in ultra-pure water by RPS disks covered with PES membrane at 15 ±2 °C during 7 days deployment

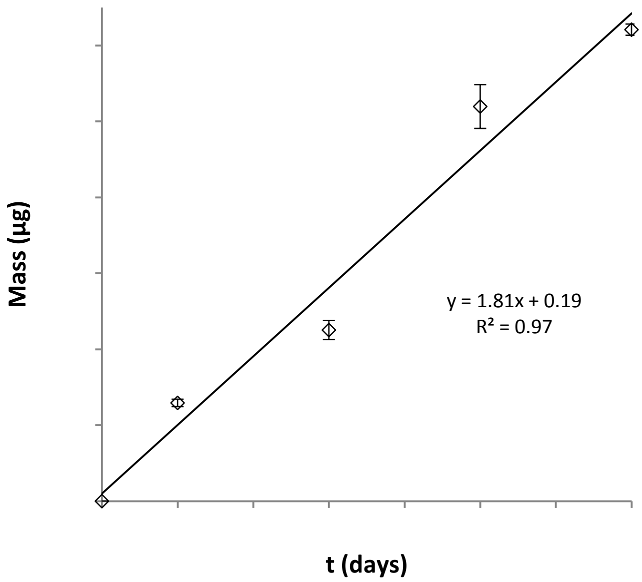

Figure A1.2. Uptake of atrazine at 10 ppb in ultra-pure water by naked RPS disks at 15 ±2 °C during 7 days deployment

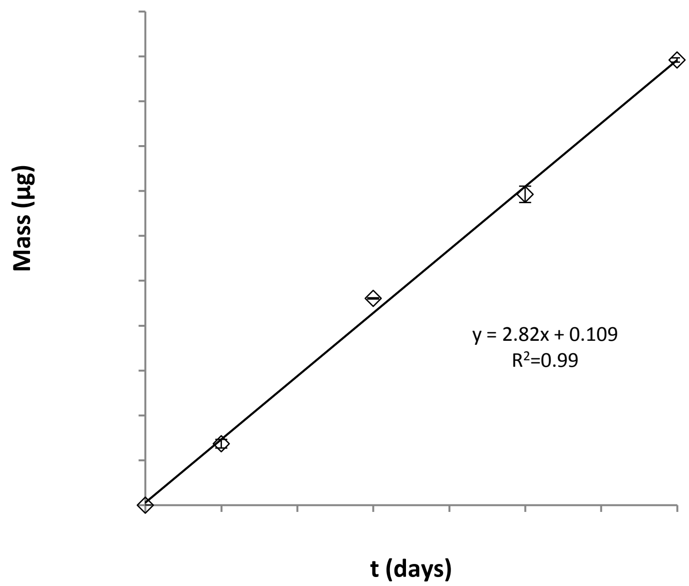

Figure A1.3. Uptake of atrazine at 10 ppb in unfiltered river water by naked RPS disks at 21 ±2°C during 7 days deployment

From Figure (A1.2) the sampling rate can be calculated to be 0.180 L/ day or 180 mL/ day, which will improve the environmental detection limit for a one-day deployement to 0.006 µg/L.

3) Uptake by naked SDB-RPS Empore disk in spiked unfiltered river water with 10 ppb atrazine at 21 ±2 °C.

When deployed in South Esk River water spiked at 10 ppb atrazine the SDB-RPS Empore disk passive sampler again showed linear uptake over the 7 day period, with 19±0.09 µg of atrazine accumulated in the sampler after 7 days (Figure A1.3). The mass accumulation was almost doubled at day 7 at 21 ±2 °C compared to the ultrapure water spiked with 10 ppm atrazine at 15 ±2 °C with a sampling rate of 0.28 L/day or 280 mL/day.

The uptake rate is affected by the presence of diffusive membrane and temperature during constant shaking or turbulence. The membrane can act as a barrier between the aqueous layers and sorbent of receiving phase, for example, the ratio of the amount of atrazine absorbed or accumulated by diffusion membrane compared to Empore disk was 1:1.2 after 6 day of deployment (Tran et al., 2007)

In calibration experiment, Kingston et al., (2000) has indicated that atrazine uptake using sampler with polysulfone membrane was less affected by stirring speed instead, the polysulfone membrane was diffusion-limited for the atrazine uptake. In our study, the accumulation of atrazine from ultra-pure water into the naked disk passive sampler was almost four times higher than the PES covered disk sampler during a 7 day deployment. Using similar disks of SDB-RPS covered with polyethersulfone membrane, naked disk RPS passive sampler was found to be three times greater in mass uptake of atrazine (29 ng) compared to (8.8 ng) of the membrane-covered disk during 7 days of field deployment (Stephens et al., 2009).

The effect of temperature in the increase in sampling rate of the membrane-covered empore disk has been indicated by Kingston et al., (2000) in which Arrhenius plots with linear regression of atrazine showed increase in sampling rate with increase in water temperature over a range of 4 – 20 °C. This variation of uptake due to temperature increase

is consistent with our findings as the uptake rates and analyte accumulations of atrazine in naked (SDB-RPS) disks increased from 12µg at 15 °C to approximately 20 µg at 21 ±2 °C after 7 days of deployment. In the diffusion gradients in thin films, DGT, temperature can have an effect on the uptake by the sorbents as it influences the diffusion coefficient and solution viscosity; therefore, increase in temperature can alter the sorbents or gel properties and causes increase the process of mass transport and the diffusive flux of ions (Zhang and Davison, 1995).

These variations stress the importance of abiotic factors in sampling rate that could affect passive samplers performance during the winter season and summer season; therefore calibrations is very important for such factors before deployment.

Therefore, passive samplers have the potential to monitor herbicides in general and atrazine in specific at low concentration that can alter the algal community structure for extended period of five or seven days by the hydrophilic disk SBD-RPS in naked form and the covered membrane. Although uptake is greatly improved using the naked one and could improve the detection limits, however, adding a layer of diffusive membrane could protect the sampler from biofouling especially in stagnant water which probably could also extend the deployment period.

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