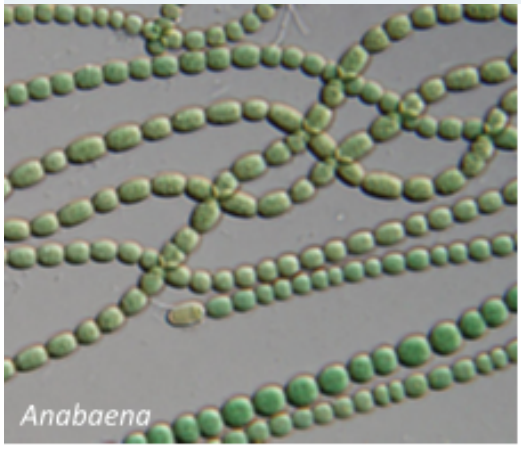
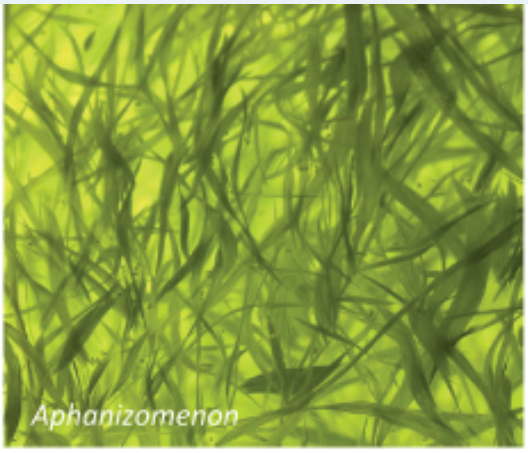
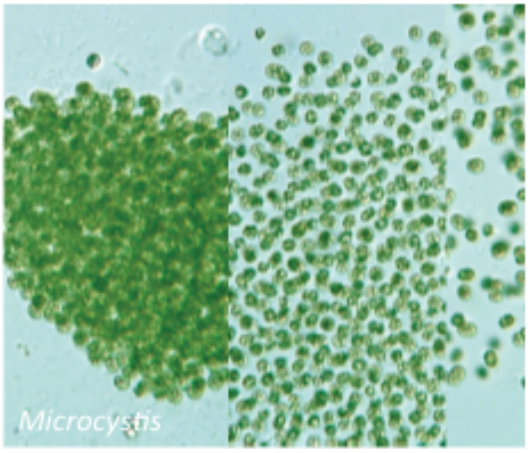
Factors Affecting Growth of Cyanobacteria

With Special Emphasis on the Sacramento-San Joaquin Delta







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SCCWRP Technical Report 869

Factors Affecting the Growth of Cyanobacteria with Special Emphasis on the Sacramento-San Joaquin Delta

Prepared for:
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and
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EXECUTIVE SUMMARY

A world-wide increase in the incidence of toxin-producing, harmful cyanobacterial blooms (cyanoHABs) over the last two decades has prompted a great deal of research into the triggers of their excessive growth. Massive surface blooms are known to decrease light penetration through the water, cause depletion of dissolved oxygen following bacterial mineralization of blooms, and cause mortality of aquatic life following ingestion of prey with high concentrations of toxins. Additionally, humans coming in contact with the water may develop digestive and skin diseases, and it may affect the drinking water supply.

The Central Valley Regional Water Quality Control Board (Water Board) is developing a science plan to scope the science needed to support decisions on policies governing nutrient management in the Delta. Blooms of cyanoHABs are one of three areas, identified by the Water Board, that represent pathways of potential impairment that could be linked to nutrients. The Water Board commissioned a literature review of the factors that may be contributing to the presence of cyanoHABs in the Delta. The literature review had three major objectives:

1) Provide a basic review of biological and ecological factors that influence the prevalence of cyanobacteria and the production of cyanotoxins;
2) Summarize observations of cyanobacterial blooms and associated toxins in the Delta;
3) Synthesize literature to provide an understanding of what ecological factors, including nutrients, may be at play in promoting cyanobacterial blooms in the Delta.

This review had four major findings:

#1. Five principal drivers emerged as important determinant of cyanobacterial blooms in a review of the global literature on factors influencing cyanobacteria blooms and toxin production. These include: 1) Water temperature, 2) Water column irradiance and water clarity, 3) Stratified water column coupled with long residence times, 4) Availability of N and P in non-limiting amounts; scientific consensus is lacking on the importance of N: P ratios as a driver for cyanoHABs, and 5) Salinity regime.

#2. Existing information is insufficient to fully characterize the threat of CyanoHABs to Delta ecosystem services because cyanoHABs are not routinely monitored. Based on existing data, the current risk to Delta aquatic health is of concern and merits a more thorough investigation. This observation is based total microcystin levels found in Delta fish tissues that are within the range of sublethal effects to fish as recently reviewed by the California Office of Environmental Health Hazards (OEHHA 2009), and dissolved toxin concentrations that occasionally exceed both the OEHHA action level and the World Health Organization (WHO) guideline of 1000 ng L^{-1} in certain "hotspots" of the Delta.

#3. Comprehensive understanding of the role of nutrients vis-à-vis other environmental factors in influencing cyanoHAB presence in the Delta is severely hampered by the lack of a routine monitoring program. Drawing on available information on the five factors influencing cyanoHABs, we can conclude the following:

- Temperature and irradiance appear to exert key roles in the regulation of the onset of blooms. Cyanobacteria require temperatures above 20°C for growth rates to be competitive with eukaryotic phytoplankton taxa, and above 25°C for growth rates to be competitive with diatoms. In addition, they require relatively high irradiances to grow at maximal growth rates.
- It appears that N and P are available in non-limiting amounts in the Delta; moreover, concentrations, or ratios, do not change sufficiently from year–to–year in order to explain year–to–year variation *Microcystis* biomass or occurrence. Therefore the initiation of *Microcystis* or other cyanoHAB blooms are probably not associated with changes in nutrient concentrations or their ratios in the Delta. However, as with all phytoplankton blooms, once initiated, cyanoHABs cannot persist without an ample supply of nutrients.
- Salinity is controlling the oceanward extent of cyanobacteria blooms in the Delta, but salinity gradients do not explain the spatial distribution of cyanoHABs in the Delta. Notably, salinity regime is not a barrier to toxin transport, as cyanotoxins have been detected in SF Bay.
- Turbidity, low temperatures, and higher flows during most of the year are likely restricting cyanobacteria blooms to the July-August time period.

#4. Climate change and anthropogenic activity associated with land use changes have the potential to alter cyanoHAB prevalence in the future. Climate change will likely result in warmer temperatures and increased drought, the latter of which could result in reduced flows, increased residence time and water column stability leading to higher light availability in the Delta. Both temperature and reduced flows would presumably result in a greater prevalence of cyanoHABs. It's noteworthy that phytoplankton biomass and primary productivity are depressed relative to available nutrients in the Delta, so it's unclear what the effect of modifying nutrient loads will have on frequency and intensity of cyanoHAB occurrence in the future.
Given these findings, two major science recommendations are proposed:

R1: Implement Routine Monitoring of CyanoHABs. DWR is currently conducting a monitoring program which routinely samples many of the variables of interest known to influence cyanoHABs. Comprehensive cyanoHAB monitoring should be added as a component to this program. To begin, a work plan should be developed which specifically scopes the needed changes in the program to comprehensively monitor cyanoHABs. This report details specific components that should be considered in this workplan. The workplan should also consider monitoring needed to develop and calibrate an ecosystem model to further investigate controls

on primary productivity and phytoplankton assemblage (see R2 below). The workplan should be peer-reviewed by subject matter experts. After an initial period of 3-5 years, the monitoring data should be used to comprehensively report on the status and trends of cyanoHABs and the factors that favor bloom occurrence in the Delta.

R2: Develop an Ecosystem Model of Phytoplankton Primary Productivity and HABs Occurrence to further Inform Future Risk and Hypotheses on Factors Controlling CyanoHABs. Because nutrients are not currently limiting cyanobacterial blooms, it is critical that an improved understanding is gained of the factors that are controlling phytoplankton primary productivity in the Delta, since increased phytoplankton growth could lead to increased risk of cyanoHAB blooms. To inform management action moving into the future, an ecosystem model of phytoplankton primary productivity and HABs occurrence should be developed. This model should have the capability to provide information on primary productivity and biomass as well as planktonic food quality and transfer of carbon to higher trophic levels. To step into model development, three actions should be taken: 1) examine existing models already available to determine suitability for this task, 2) utilize existing data to explore, to the extent possible, the relationships between chlorophyll a, phytoplankton composition, climate variables *et al.* factors. This analyses should inform hypotheses that can be tested through model development as well as potential future scenarios, and 3) a work plan should be developed that lays out the modeling strategy, model data requirements, and implementation strategy.

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1. Introduction, Purpose and Organization of the Review

1.1 Background and Context

The Sacramento–San Joaquin River Delta, is an inland river delta and estuary approximately 1300 square miles in size, found in Northern California. Formed at the western edge of the Central Valley by the confluence of the Sacramento and San Joaquin Rivers, the Delta is a key component of the State's water resource infrastructure and a region that is rapidly urbanizing, yet serves as critical habitat or fish, birds and wildlife. Water from the 45,000 square mile Delta watershed fuels both local and statewide economies, including important agricultural commodities. The Delta is widely recognized as in "crisis" because of human effects on the environment and competing demands for the Delta's resources. The consequences of these competing demands include point and non-point discharges, habitat fragmentation and loss, modified flow regimes, introduction of non-native species, all of which combine to threaten ecosystem health, including the continued decline of threatened and endangered species

In 2009 the California legislature passed the Delta Reform Act creating the Delta Stewardship Council. The mission of the Council is to implement the coequal goals of the Reform Act and provide a more reliable water supply for California while protecting, restoring, and enhancing the Delta ecosystem. The Council wrote and adopted a Delta Plan in 2013 to implement these goals. Chapter 6 of the Delta Plan deals with water quality and contains recommendations to implement the coequal goals of the Delta Reform Act. Recommendation # 8 states, in part, "…the State Water Resources Control Board and the San Francisco Bay and Central Valley Regional Water Quality Control Boards (Water Board) should prepare and begin implementation of a study plan for the development of objectives for nutrients in the Delta … by January 1, 2014. Studies needed for development of Delta… nutrient objectives should be completed by January 1, 2016. The Water Boards should adopt and begin implementation of nutrient objectives, either narrative or numeric, where appropriate, in the Delta by January 1, 2018. Potential nutrient related problems identified in the Delta Plan for evaluation are:

1) Decreases in algal abundance and shifts in algal species composition,
2) Increases in the abundance and distribution of macrophytes, including water hyacinth and Brazilian waterweed,
3) Increases in the magnitude and frequency of cyanobacterial blooms

To provide better scientific grounding for the study plan, the Water Board commissioned two literature reviews centered on these three potential areas of impairment. This document provides a synthesis of literature on cyanobacterial blooms in the Delta. Technical Adivory Group and Stakeholder comments on the review are provided in Appendices B and C, respectively.

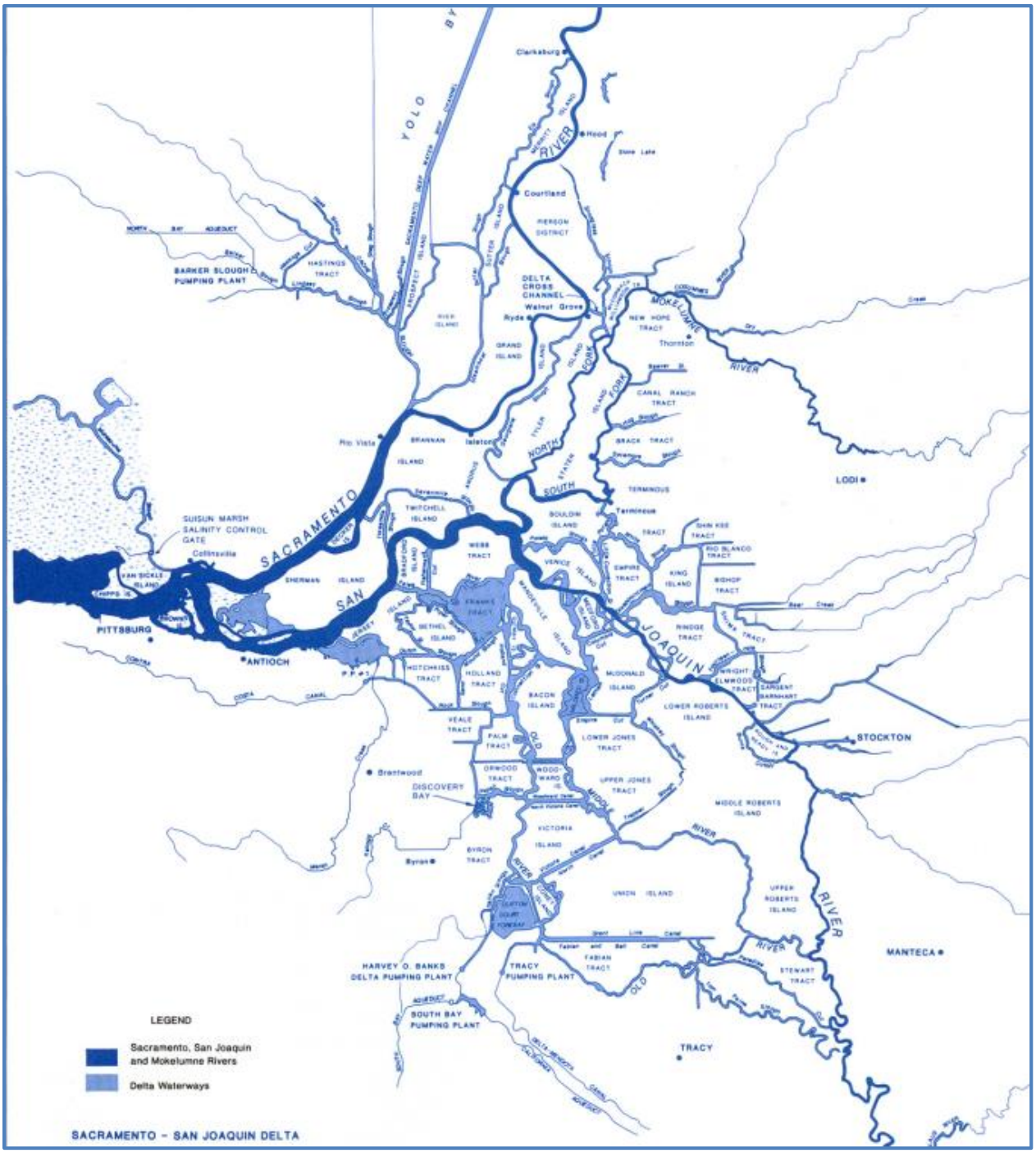

Figure 1.1. The Sacramento-San Joaquin Delta Region.

1.2 Goal and Organization of Cyanobacterial Literature Review

The goal of the cyanobacterial literature review is to synthesize available information to provide insight into cyanobacterial blooms in the Delta. The review had three major objectives:

1) Provide a basic review of biological and ecological factors that influence the prevalence of cyanobacteria and production of cyanotoxins;
2) Summarize observations of cyanobacteria blooms and associated toxins in the Delta;
3) Synthesize literature to provide an understanding of what ecological factors, including nutrients, may be at play in promoting cyanobacteria blooms in the Delta.

This review, and the recommended next steps, will contribute to a science plan to determine whether or how to proceed with the development of nutrient objectives for the Delta. The document is organized as follows:

Section 1: Introduction, Purpose and Organization of the Review

Section 2: Basic Biology and Ecology of Cyanobacteria

Section 3: Factors Influencing Cyanobacterial Blooms and Toxin Production

Section 4: Prevalence of CyanoHABs and Potential for Effects on Ecosystem Services in the Delta

Section 5: Synthesis of Factors Influencing CyanoHABs Presence and Toxin Production in the Delta

Section 6: Recommendations

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2. Basic Biology and Ecology of Cyanobacteria

2.1 Overview

Cyanobacteria are a versatile group of bacteria that were the ancient colonizers of Earth and the photosynthetic ancestors of chloroplasts in eukaryotes such as plants and algae. As pioneers of photosynthesis, cyanobacteria were responsible for oxygenating Earth's atmosphere 2.5 billion years ago. In addition to being photosynthetic, cyanobacteria can differentiate into specialized cell types called heterocysts and fix nitrogen (N), exhibit gliding mobility, and tolerate a wide range of temperatures as evidenced by their ability to thrive in hot springs and ice-covered Antarctic lakes. Cyanobacteria also produce an array of bioactive compounds, some of which possess anti-microbial, anti-cancer and UV protectant properties. However, a subset of these bioactive compounds is highly toxic to humans and wildlife.

Blooms of cyanobacteria that produce these toxins, collectively known as harmful cyanobacterial algal blooms (cyanoHABs), has garnered a great deal of attention due to their increased occurrence in recent decades (Chorus and Bartram 1999, Carmichael 2008, Paerl and Huisman 2008, Hudnell 2010). The geographical distribution of these blooms has also increased with blooms appearing in areas previously unaffected (Lehman *et al.* 2005, Lopez *et al.* 2008). CyanoHABs can have major negative impacts on aquatic ecosystems. Toxins produced by cyanobacteria can lead to mortality in aquatic animals, waterfowl and domestic animals (Havens 2008, Miller *et al.* 2010). Moreover, toxins in drinking water supplies can pose a variety of adverse health effects and therefore require expensive treatment options such as filtration, disinfection, and adsorption with activated carbon (Cheung *et al.* 2013). In addition to the threat of toxins, oxygen depletion due to organic matter decomposition following the die-off of blooms can result in massive fish kills. CyanoHABs can also lead to revenue losses and impact local economies by reducing business in affected water bodies during the peak of tourism season. Considerable costs are associated with mitigation of blooms and lake restoration (Dodds *et al.* 2009).

The San Francisco Bay Delta is an area where cyanoHABs were previously undetected but have become commonplace since early 2000 (Lehman *et al.* 2005). In addition to providing a home for several species of pelagic fish and other wildlife, the Delta serves as a critical source of drinking water, and freshwater for irrigation of farms, to communities locally as well as farther south including the Los Angeles Metropolitan Water District. In concert with the occurrence of cyanoHABs, concentrations of the toxins they produce have been detected in the water and in higher trophic levels including zooplankton and fish (Lehman *et al.* 2010). The purpose of the following sections summarizes the basic biology of cyanobacteria beginning with classification, light harvesting, carbon metabolism, buoyancy regulation, nitrogen metabolism, cellular N:P ratios and toxin production, in order to build fundamental concepts that are later utilized in the review.

2.2 General Characteristics

2.2.1 Classification, Distribution and Akinete Production

Classification

Traditionally, morphological traits have been used to subdivide the cyanobacteria into five sub-groups (Rippka *et al.* 1979). The major division is between cyanobacteria that are single celled and/or colonial and those that grow filaments (Table 2.1). Each category contains a mixture of marine and freshwater species. In the former category are the Group I Croococcales including the freshwater *Microcystis* and *Synechocystis*, and the marine *Synechococcus* and *Prochlorococcus*. Group II Pleurocapsales include *Pleurocapsa* and *Xenococcus* (Table 2.1). The filamentous algae, Groups III, IV, and V, are further subdivided into the Oscillatoriales that produce only vegetative cells, including the freshwater planktonic *Planktothrix* species, the benthic *Oscillatoria* and *Lyngbya* species, as well as the marine *Trichodesmium* sp. (Table 2.1). Group IV, the Nostocales, contain filamentous algae that differentiate into heterocysts and fix N_2. This group includes *Aphanizomenon*, *Anabaena*, *Nostoc* and *Cylindrospermopsis* (Table 2.1). Additionally, the Nostocales is known for differentiation into resting cells called akinetes during unfavorable conditions. Group V, the Stigonematales include species with filaments that grow in complex branching patterns.

Table 2.1. Cyanobacterial groupings based on morphological traits. Adapted from Rippka *et al.* 1979.

Croococcales Unicellular, reproduce by binary fission		GROUP 1	*Gloeothece (N)* *Synechococcus* *Microcystis* *Synechocystis* *Prochlorococcus* *Prochloron*
Pleurocapsales Unicellular, reproduce by multiple fission		GROUP 2	*Pleurocapsa* *Staniera (N)* *Xenococcus (N)*
Filamentous chain (trichome) forming; reproduce by random trichome breakage, hormogonia, germination of akinetes	Trichome composed of vegetative cells	**Oscillatoriales** 1 plane division GROUP 3	*Lyngbya (N)* *Oscillatoria (N)* *Phormidium* *Prochlorothrix* *Trichodesmium (N)*
	In the absence of fix d N, trichome contains heterocysts; some produce akinetes	**Nostocales** 1 plane division GROUP 4	*Aphanizomenon* *Anabaena* *Cylindrospermum* *Nodularia* *Nostoc*
		Stigonematales Division in more than 1 plane GROUP 5	*Chlorogleopsis* *Fisherella*

It was originally thought that N_2 fixation primarily existed in the Nostocales which had the ability to differentiate into heterocyst cells. More recent investigations tracking the *nifD* and *nifH* gene diversity has uncovered that N_2 fixation occurs in a range of unicellular, non-filamentous cyanobacteria dispersed throughout the five original groups first proposed by Rippka *et al.* (1979). These species are indicated by an (N) after their name in Table 2.1. Depending on which functionality of the cyanobacteria is emphasized, recent gene-based groupings of cyanobacteria have created as many as ten different sub-categories (Turner *et al.* 1999, Tomatini *et al.* 2006). However, there appears to exist no general consensus over the best manner in which to categorize the cyanobacteria based on functionality and marker genes. Most cyanobacteria are planktonic and are dispersed throughout the five groups. The benthic cyanobacteria are found mainly in the Oscillatoriales subgroup. The toxic cyanoHAB-forming cyanobacteria are mostly freshwater planktonic species dispersed throughout groups I, III and IV and include the N_2 fixing genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsin*, and *Nodularia*; the benthic N_2 fixing genera *Lyngbya* and some *Oscillatoria*; and the non-N_2 fixing genera *Microcystis* and *Planktothrix* (Paerl and Paul 2012).

Akinete formation

Akinetes are the resting cells produced by the Nostocales in order to survive adverse environmental conditions such as cold and desiccation (Tomatini *et al.* 2006). Akinete cells maintain low levels of metabolic activity (Thiel and Wolk 1983, Sukenik *et al.* 2007), are dispersed in sediments (Baker 1999, Kim *et al.* 2005, Rucker *et al.* 2009), and are distinguishable from vegetative cells by their larger size (Figure 2.1). They germinate in response to improved environmental conditions such as light and temperature (Baker and Bellifemine 2000, Karlsson-Elfgren *et al.* 2004, Yoshimasa and Nakahara 2005, Kaplan-Levy *et al.* 2010) and provide an inoculum of Nostocales vegetative cells to the water column from the sediments where the akinete “seed bank” may remain viable for decades (Stockner and Lund 1970, Livingstone and Jaworski 1980). Therefore, eradication of Nostocales from a system once it has become “infected” is very difficult.

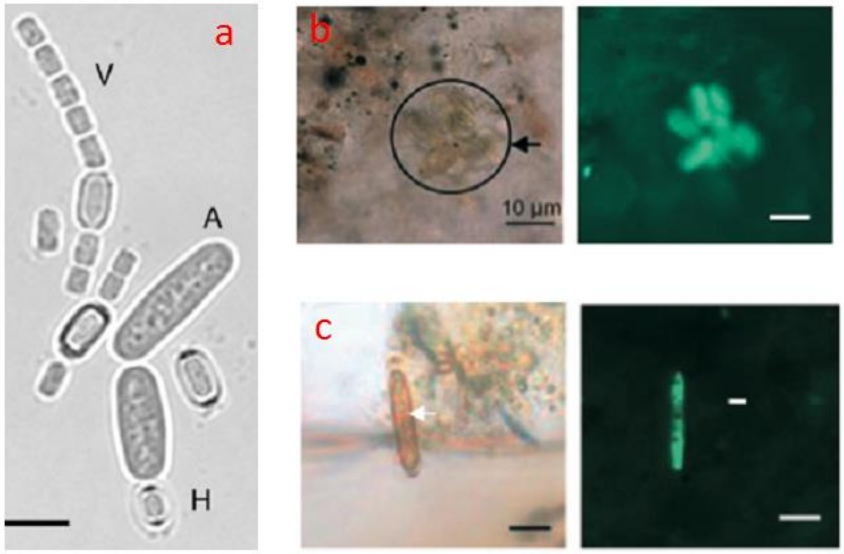

Figure 2.1. Akinetes of a) Anabaena cylindrica culture grown in medium without nitrogen; A=akinete; H=heterocyst; V=vegetative cell (picture from Tomatini *et al*. 2006), b) Anabaena lemmermanni, and c) Cylindrospermopsis raciborskii in lake sediments under light microscopy and hybridized with probe under fluorescence microscopy; scale bar is 10µm (pictures from Ramm *et al*. 2012).

2.2.2 Light Harvesting, Photosynthesis and Carbon Fixation

Cyanobacteria are distinct from all other algae in that most of them possess two light harvesting systems (as opposed to one). Maintaining two light harvesting system is costly in terms of protein and N requirements and manifests strongly in their cell biology. For example, the extra protein requirement means that cyanobacteria have a high tissue nitrogen:phosphorus (N:P) ratio and a high N requirement for growth (discussed below). Despite this, light harvesting is necessary in photosynthetic organisms to 1) collect light energy from the sun and 2) convert it to chemical energy in the form of electrons and ATP that can be used to power carbon fixation.

Light harvesting pigments and photosynthesis

Light harvesting is performed by chlorophyll *a* (Chl *a*) pigment molecules that are associated with two photosystems (PSI and PSII) that comprise the centers of the photosynthetic process which starts with the liberation of an electron from the splitting of water and ends with the production of ATP. Sitting in each of the photosystems is a specialized Chl *a* molecule that initiates the flow of electrons through the electron transport chain that eventually powers ATP synthesis. The other Chl *a* molecules, 40 and 90, together with 12 and 22 carotenoid pigment molecules, in PSI and PSII respectively, funnel light energy to the reaction core (DeRuyter and Fromme 2008). This complex of Chl *a* and carotenoid pigment molecules, coordinated by a large number of proteins, is very similar in its structure to the light-harvesting complex (LHC)

embedded into the thylakoid membranes of vascular plants and eukaryotic phytoplankton (Fromme *et al.* 2001, 2002).

What makes the cyanobacteria unique is that they have a second light harvesting antenna complex peripheral to the thylakoid membrane that is water soluble (e.g. not membrane bound). This pigment complex, comprised of pigmented proteins arranged in rods fanning out from a core attached to the thylakoid membrane, called the phycobilisome (PBS), is what gives cyanobacteria their name (Grossman *et al.* 1993, Grossman 2003). Similar to the carotenoid pigments mentioned above, the PBS chromophores absorb light inbetween the Chl *a* absorption peaks of 440nm and 670nm (Grossman *et al.* 1993). Interestingly, the PBS proteins are not exclusive to cyanobacteria; they also occur in photosynthetic eukaryotes.

Up to 50% of cyanobacterial cellular protein content is bound in the PBS complex taking a large proportion of the cell's resources, particularly its nitrogen (N) allocation. Therefore, under stress condition such as N starvation, the entire PBS can be degraded within a few hours and the N can become reused within the cell (Sauer *et al.* 1999). When conditions improve, the PBS will be re-synthesized and re-assembled (Collier and Grossman 1994, Grossman *et al.* 2001).

Carbon fixation

The ATP produced and the electrons liberated during photosynthesis are used to power the fixation of carbon into sugars in the Calvin Cycle. They are also used to reduce oxidized sources of N to ammonia during N assimilation (discussed below). The primary and rate-limiting enzyme in carbon fixation is Rubisco which catalyzes the first step in the Calvin Cycle. To deal with the rate-limiting nature of Rubisco, cyanobacteria have evolved specialized structures called carboxysomes. In addition to housing Rubisco, the carboxysomes contain a number of other enzymes that help concentrate CO_2 in its vicinity to speed its reaction rate (Kaplan and Reinhold 1999). Cyanobacteria fix carbon to provide the skeletons needed to assimilate N into amino acids and build protein and cellular biomass; fixed carbon can also be used to accumulate carbohydrate storage products (carbohydrate ballasting) in order to make the cell heavier during buoyancy regulation.

2.2.3 Buoyancy Regulation

One distinct advantage of many cyanobacterial genera such as *Microcystis*, *Planktothrix*, *Anabaena* and *Aphenizomenon* is their ability to regulate their buoyancy by a combination of producing gas vesicles and carbohydrate storage products (Oliver 1994, Beard *et al.* 1999, Brookes *et al.* 1999). The former renders them positively buoyant whereas the latter does the opposite (Walsby 1994, 2005). The carbohydrate storage products are derived from C-fixation and the amount produced varies depending on the species and on irradiance (Howard *et al.* 1996, Visser *et al.* 1997, Wallace and Hamilton 1999). At an irradiance that is specific to each species and strain, the amount of carbohydrate storage product will perfectly balance the upward lift

created by the gas vesicles and the cyanobacteria will become neutrally buoyant (Walsby *et al.* 2004). In addition to producing and storing the carbohydrates, cyanobacteria also consume the storage products to produce energy.

By regulating the amount of carbohydrate storage products consumed, cyanobacteria control their vertical position in the water column (Thomas and Walsby 1985, Konopka *et al.* 1987, Wallace and Hamilton 1999). Models demonstrate that filamentous cyanobacteria can sink or float at speeds up to 0.3 m per day in order to position them at a depth where irradiance is such that it maximizes their growth potential (Walsby 2005). These speeds are only achievable for filaments of a certain size and weight; picocyanobacteria and small filaments do not have enough momentum to respond by vertical repositioning to changes in irradiance (Walsby 2005). Of course, carbohydrate production, therefore buoyancy regulation, is affected by nutrient availability; nitrogen starved cells have excess carbohydrate stores and tend to lose buoyancy more easily than nutrient sufficient cells (Klemer *et al.* 1982, Brookes *et al.* 1999, Brookes and Ganf 2001).

2.2.4 Nitrogen Metabolism

Cyanobacteria use a wide variety of N sources for growth including ammonium (NH_4^+), nitrate (NO_3^-), nitrite (NO_2^-), urea, amino acids, cyanate, and several species are also capable of dinitrogen gas (N_2) fixation to satisfy their cellular N demand. Below we discuss the pathways of N transport, metabolism and assimilation, and their regulation.

Ammonium transport and assimilation of N into amino acids

Being a charged molecule, NH_4^+ cannot diffuse freely into the cell and has to be transported via active transport. Transport of NH_4^+ into cyanobacteria (as well as in eukaryotic algae) occurs via the Amt family of transporters. These transporters are either expressed constitutively or differentially depending on external N concentrations. At environmental concentrations, most of the NH_4^+ is transported into the cell via the high-affinity transporter Amt1 encoded by the gene *amt1* (Muro-Pastor *et al.* 2005).

Before it can be assimilated, all N sources, whether N_2, NO_3^- or organic N containing molecules, first have to be converted to NH_4^+. The NH_4^+ is then assimilated into amino nitrogen through the GS/GOGAT pathway. The primary NH_4^+ assimilating enzymes in cyanobacteria (as well as in vascular plants and eukaryotic algae) are glutamine synthetase (GS) and glutamate synthase (also called glutamine-2-oxogluterate-amido transferase, GOGAT) acting in concert to aminate 2-oxogluterate (2-OG). Photosystem I (PSI)-reduced ferredoxin (Fd_{red}) is typically used as a reductant in this reaction:

GS: Glutamate + NH_3 + ATP → Glutamine +ADP +P_i
GOGAT: Glutamine + 2-OG + 2[H] → 2 Glutamate
GS/GOGAT: 2-OG + NH_3 + ATP + 2[H] → Glutamate + ADP +P_i

An alternate route of NH_4^+ assimilation involves the enzyme glutamate dehydrogenase (GDH) but it's postulated that this occurs only during select conditions such as stationary growth:

GDH: 2-OG + NH_3 + 2[H] → Glutamate + H_2O

In all photosynthetic cells the link between the carbon (C) and N cycles in the cell occurs at the GS/GOGAT reactions because the two key ingredients in N assimilation is 1) 2-OG derived from carbon fixation, and 2) Fd_{red} derived from PSI. GOGAT (and also GDH) will not proceed without their presence, which avoids wasteful consumption of glutamine, and ensures that even in the presence of excess N, assimilation will not proceed unless an adequate supply of C skeletons is available (Flores and Herrero 2005, Muro-Pastor *et al.* 2005).

Nitrate transport and reduction to $NH_4^{\pm}$

As NO_3^- is also a charged molecule it's transported into the cell via active transport. Cyanobacteria use two different transport systems. Most freshwater species, including *Anabaena*, *Synechocystis* and *Gloebacter*, use the high affinity ATP-binding cassette (ABC) transporter NrtABCD (Flores *et al.* 2005). Most marine species (*Synechococcus* and others) take up NO_3^- and NO_2- via the major facilitator superfamily transporter NrtP, also a high-affinity transporter (Flores *et al.* 2005). Some species also have a NO_2^--specific transporter NIT (Maeda *et al.* 1998). Nitrate uptake is tightly regulated by the external concentration of NH_4^+; when NH_4^+ becomes available, cells cease NO_3^- uptake and switch to use NH_4^+ which is preferred. This process is regulated at the level of NO_3^- uptake (Flores and Herrero 1994). In addition, CO_2-fixation (regulated by irradiance) is required to maintain active NO_3^- uptake, a regulatory link that ensures that the product of NO_3^- reduction (ammonium) can be incorporated into carbon skeletons (Luque and Forchhammer 2008).

Reduction of NO_3^- to NH_4^+ is a two-step process catalyzed by the enzymes nitrate reductase (NR) and nitrite reductase (NiR). The power for the reduction reaction, in the form of 2 electrons for NR and 6 electrons for NiR, is provided by Fd_{red} via PSI providing a strong link between the light reactions and NO_3^- use by the cell (Flores *et al.* 2005).

In cyanobacteria, the genes encoding NR, narB, and Nir, nirA, and the NO_3^- transporter NrtP, are typically clustered in the same operon. An operon is a unit that tells the cells to transcribe a sequence of genes simultaneously. In cyanobacteria, the transcription of operons associated with N metabolism is tightly regulated by the transcription factor NtcA (discussed below).

The only cyanobacteria discovered to date that is not able to use NO_3^- is *Prochlorococcus* which lives in the open ocean. While it was initially thought that some species could assimilate NO_2^-, sequencing of their genomes demonstrates that they all lack the *nirA* genes and therefore cannot reduce NO_2^- (Garcia-Fernandez *et al.* 2004).

Urea transport and metabolism

Many, but not all, cyanobacteria can use urea as a source of N for growth. Because urea is not a charged molecule it diffuses freely into the cell; however, environmental concentration are not such that diffusion can supply the needed concentration of urea for the urease enzyme (based on its K_m). Both in freshwater and marine cyanobacteria, an ABC-type active transport system specific for urea has been identified (Valladares *et al.* 2002). The subunits of this transporter are encoded by the five genes urtA-E. In *Anabaena*, the urea transporter genes are in the same NtcA-activated promoter and subject to metabolic repression by NH_4^+ (Valladares *et al.* 2002).
Urea is metabolized to two molecules of NH_3 and CO_2 by the enzyme urease, also called urea amidohydrolase (Mobeley *et al.* 1995). The urease enzyme is well-conserved throughout the bacteria and eukaryotic organisms and consists of two small and one large subunit encoded by at least seven genes, three which encode the structural subunits (*ureA, ureB, ureC*) and the other four (*ureD, ureE, ureF, ureG*) encoding accessory polypeptides required for the assembly of the nickel metallocenter (Collier *et al.* 1999, Palinska *et al.* 2000).

Amino acid transport

All cyanobacteria tested to date have at least one transport system for amino acids. These transporters appear to have broad specificity (i.e. they can transport more than one type of amino acid) and different species have different combinations of transporters (Herrero and Flores 1990, Montesinos *et al.* 1997). For example, freshwater *Synechocystis* sp. has four different amino acid transporters, including the ABC transporter Nat for glutamine and histidine, the ABC transporter Bgt for basic amino acids, and two glutamate-specific transporters GHS and Gtr (Quintero *et al.* 2001). Once in the cell, cyanobacteria possess a variety of deaminase enzymes that can deaminate the amino acids to NH_3 which then enters the GS/GOGAT pathway.

Cyanate transport and metabolism

Cyanobacteria, including freshwater and marine species, can use cyanate (a toxin) as a N source for growth since they have the genes encoding a transporter (*cynA, cynB, cynC*) and the gene encoding the cyanase enzyme (*cynS*) which hydrolyzes cyanate to NH_3 and CO_2 (Kamennaya and Post 2011). In freshwater cyanobacteria, these genes are repressible by NH_4^+ suggesting that they are under NtcA regulation.

Nitrogen fixation

Arguably the most expensive (energetically speaking) source of N for cyanobacteria is molecular dinitrogen gas (N_2). Nitrogen fixation, the process of reducing N_2 to NH_3, is catalyzed by the nitrogenase enzyme. The nitrogenase has two subunits. The first is the dinitrogenase subunit which catalyzes the reduction of N_2 to NH_4^+, composed of the NifD and NifK polypeptides encoded by the *nifD* and *nifK* genes. The dinitrogenase contains an iron-molybdate active site and two iron-sulfur clusters. The second is the dinitrogenase reductase subunit (NifH polypeptide

encoded by the *nifH* gene) which contains a central iron-sulfur cluster whose function it is to donate electrons derived from ferredoxin to dinitrogenase. Reduction of N_2 to NH_3 requires 8 electrons and 15 molecules of ATP in the following reaction:

$$N_2 + 8[H] + 16ATP \rightarrow 2\ NH_3 + H_2 + 16ADP + 16P_i$$

It was recently discovered that under conditions of molybdate limitation, some *Anabaena* species express an alternative nitrogenase containing a vanadium-iron cofactor instead of the molybdate-iron cofactor (Thiel 1993, Boison *et al.* 2006). Both these variants require iron cofactors to function and N_2 fixation cannot proceed under iron-limiting conditions.

The nitrogenase enzyme is very sensitive to oxygen (O_2), and O_2 is evolved as a byproduct of the water-splitting reactions at photosystem II (PSII), requiring the nitrogenase enzyme to be kept separate from PSII. Accordingly, freshwater cyanobacteria have evolved heterocysts (Wolk *et al.* 1994). These are specialized cells where PSII is inactivated, the PBS antenna proteins are degraded, and energy to power the cell is derived from cyclic electron flow around PSI. Rates of respiration in these cells are also high to scavenge any O_2. The ATP and reductant needed for N_2 reduction is generated by carbohydrate metabolism inside the heterocyst. The carbohydrate is synthesized in the non-heterocyst, vegetative cells flanking the heterocyst and transported inside. In turn, NH_3 produced inside the heterocyst is exported to the vegetative cells in the form of amino acids (Wolk *et al.* 1994). However, many species of cyanobacteria that fix N_2 do not form heterocysts; these species either separate N_2 fixation from photosynthesis in time (e.g. by fixing N_2 at night such as *Lyngbya aestuarii* and *Crocosphaera watsonii*) or in different regions of filaments as is hypothesized to be the case for *Trichodesmium* sp. (Frederiksson and Bergman 1997).

Because nitrogen fixation is such an energy expensive process, from the formation of the heterocysts to the reduction of N_2, it is tightly regulated by NtcA and is only induced under N starvation and in the absence of any other fixed N source (Herrero *et al.* 2004).

Regulation of nitrogen metabolism

As evident from the preceeding sections, the transcription factor NtcA (encoded by the gene *ntcA*) regulates most of the cyanobacterial genes associated with nitrogen uptake and assimilation, and is therefore considered the master regulator of N metabolism (Herrero et al 2004). NtcA binds to and activates the operons for heterocyst differentiation, N_2 fixation, NO_3^- uptake and reduction, urea uptake and hydrolysis, and glutamine synthetase to mention a few. In other words, none of the genes related to N metabolism are transcribed and their enzymes synthesized unless NtcA binds to their promoter in the genome (Luque *et al.* 1994, Wei *et al.* 1994, Forchammer 2004, Luque and Forchammer 2008). The exception to this rule are some NH_4^+ transport proteins which are not under NtcA control and are transcribed constitutively, i.e.

always “on” (Herrero *et al.* 2001). NtcA also controls signaling proteins that fine-tune cellular activities in response to fluctuating C/N conditions (Herrero *et al.* 2001).

NtcA is under negative control by NH_4^+, meaning that when NH_4^+ is detectable by the cell, *ntcA* gene transcription is repressed (Herrero *et al.* 2001, Lindell and Post 2001). There is an inverse relationship between NH_4^+ concentration and *ntcA* expression in all cyanobacteria tested to date, with basal levels of *ntcA* expression observed in the presence of high external NH_4^+ concentrations and maximal levels of *ntcA* expression observed under N starvation (Frias *et al.* 1994, Lindell *et al.* 1998, Lee *et al.* 1999, Sauer *et al.* 1999, Lindell and Post, 2001). Ammonium regulates expression of *ntcA* via 2-OG which is synthesized in the Calvin cycle and consumed in the GS/GOGAT cycle. Thus 2-OG is at the crossroads between C and N metabolism and is ideally suited to “sense” NH_4^+ concentrations (Vazquez-Bermudez *et al.* 2002, Tanigawa *et al.* 2002, Forchammer 2004).

The repression of *ntcA* expression by NH_4^+ places NH_4^+ at the top of the hierarchy of N substrates utilized and assimilated by cyanobacteria. The order in which N substrates other than NH_4^+ is assimilated differs depending on species. For example, in N_2 fixing cyanobacteria, NH_4^+ represses both N_2 fixation and NO_3^- assimilation. Nitrate, in turn, represses N_2 fixation. Therefore N_2 fixation is at the bottom of the hierarchy in some cyanobacteria (Ramasubramanian *et al.* 1994). But in others such as marine *Trichodesmium* sp., NO_3^- does not repress N_2 fixation genes and the process of N_2 fixation is on a more even footing with NO_3^- assimilation (Post *et al.* 2012).

2.2.5 Cellular Nitrogen:Phosphorus (N:P) Requirement

In 1958 Redfield published his discovery that phytoplankton particulate matter was composed of N and P in a molar ratio of 16, similar to the ratio of dissolved N:P in the water (Redfield 1958). Redfield suggested that the ratio of dissolved N:P in the ocean was driven by the remineralization of phytoplankton particulate matter, a theory which has since taken hold (Falkowski 2000, Geider and LaRoche 2002). Given that the average N:P ratio was discovered to be 16 in phytoplankton, it was deduced that under nutrient limiting conditions phytoplankton would become limited by N at dissolved N:P less than 16 and limited by P at dissolved N:P ratios greater than 16.

Shortly after Redfield’s discovery of the universality of the N:P ratio of 16, investigators turned to phytoplankton cultures to examine how closely phytoplankton cellular N:P ratios varied around 16. Parsons *et al.* (1961) published the first investigation demonstrating variability in cellular N:P ratios depending on the phytoplankton species. Subsequent investigations noted that diatoms and dinoflagellates tended to have cellular N:P ratios below 16 whereas chlorophytes and cyanobacteria typically had ratios above 25 (Geider and LaRoche 2002; Ho *et al.* 2003; Quigg *et al.* 2003; Klausmeier *et al.* 2004; Hillebrand *et al.* 2013; Figure 2.2). This difference

among the taxa stems from slight variations in macromolecular composition of the phytoplankton, principally in their ratio of protein, the largest store of N in the cell, to nucleic acids, the largest store of P in the cell (Terry *et al.* 1985, Falkowski 2000, Elser *et al.* 2000, Geider and LaRoche 2002). As mentioned above in section 2.2.2, cyanobacteria have two light-harvesting complexes requiring a greater association of proteins with the light-harvesting pigments compared with eukaryotic cells which only have one light harvesting complex (Raven 1984, Geider and LaRoche 2002). The "excess" protein associated with the peripheral phycobilisomes substantially increase the cellular N:P ratios of cyanobacteria. Once it was realized that that there were significant departures in the cellular N:P ratio depending on taxa, it also became clear that the ratio of N:P uptake differed with respect to taxa and that this was a major basis of resource-based competition among taxa (Rhee 1978). That phytoplankton take up N:P in proportion to their tissue composition was subsequently confirmed in culture experiments (Droop 1974, Elrifi and Turpin 1985, Tett *et al.* 1985, Quigg *et al.* 2003, Leonardos and Geider 2004). In other words, phytoplankton do not take up nutrients according to the ratio that occurs in water, but rather the ratio dictated by the macromolecular composition of their tissues.

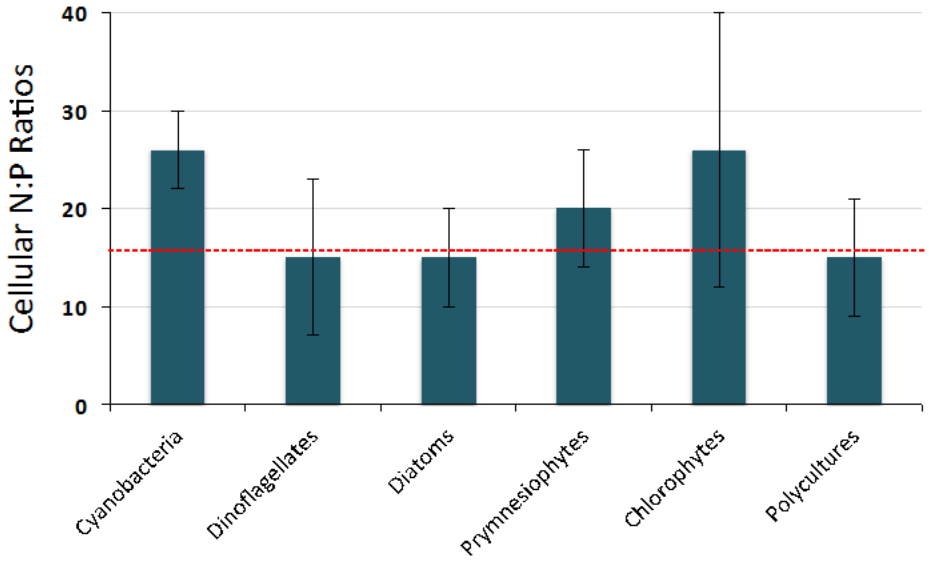

Figure 2.2. Cellular N:P ratios (mole:mole) in different phytoplankton taxa. Dashed red line indicates the average phytoplankton cellular N:P ratio of 16, also called the Redfield ratio. Data from Hillebrand *et al.* 2013.

Tissue N:P composition is not a fixed trait and phytoplankton are able to adjust it, within certain limits, in order to keep growing when environmental conditions change for the suboptimal. When limited for a nutrient, uptake of the non-limiting nutrient can proceed for a while skewing cellular ratios. But, severe limitation by one nutrient will eventually prevent the uptake of the other, non-limiting nutrient, even when the other is present in excess. This quirk of nature

constrains the extent to which cellular ratios vary (Droop 1974, Tett *et al.* 1985, Leonardos and Geider 2004, Hillebrand *et al.* 2013). For example, a summary of nearly 50 phytoplankton studies demonstrates that the N:P ratio of P-limited phytoplankton converge around 28 and the N:P ratio of N-limited phytoplankton converges around 16 (Hillebrand *et al.* 2013).

Irradiance may also change the cellular N:P ratio through its influence on the cellular protein content (LaRoche and Geider 2002). Pigments (Chl *a* and light harvesting antenna pigments) are bound in pigment-protein complexes rich in N that increase as irradiance decreases, and decrease under high light as cells reduce the size of the light harvesting complex to avoid photodamage (Wynne and Rhee 1986, Falkowski and LaRoche 1991, Nielsen 1992, Leonardos and Geider 2004). The irradiance-dependent change in N:P ratios is even more pronounced among cyanobacteria due to the greater association of protein with the phycobilisome than in the eukaryotic light harvesting complex (Raven 1984, Geider and LaRoche 2002).

In contrast with limiting nutrient concentrations or changes in irradiance, changes in the medium N:P ratio when nutrient concentrations are in excess of demand was found not to affect cellular N:P ratios in phytoplankton in early experiments (i.e. Tilman *et al.* 1982, Tett *et al.* 1985, Reynolds 1999, Roelke *et al.* 2003, Sunda and Hardison 2007) and has not been pursued by the scientific community.

2.2.6 Toxin Production

Cyanobacteria produce a large variety of toxins with a number of different actions in animals and humans leading to significant health risks and drinking water issues globally (c.f. Chorus and Bartram 1999, Chamichael 2008, Cheung *et al.* 2013). The toxin-producing cyanobacteria, and the suite of different toxins that each species produces, is discussed below.

Toxin-producing taxa

The cyanobacterial toxins were named according to the species that they were originally discovered in and isolated from. For example, microcystin was discovered in *Microcystis aeruginosa* and anatoxin was originally isolated from *Anabaena*. However, most cyanobacteria produce several different types of toxins, with the exception of nodularin which is only produced by *Nodularia spumigena.*

The toxin most widely produced by different cyanobacterial taxa is the recently discovered neurotoxin Beta-N-methylamino-L-alanine (BMAA, Cox *et al.* 2005). This is followed by the microcystins which are produced by nine different taxa (Table 2.2). Chief among the microsystin producing taxa are *Microcystis* (the toxin was originally isolated from *Microcystis aeruginosa*), followed by *Planktothrix* and *Anabaena*. Another widely distributed toxin is anatoxin-a, which is produced by eight different cyanobacterial taxa, principally *Anabaena*, the genus from which the toxin was originally isolated.

Table 2.2.Toxins produced by cyanobacteria. Based on data from Cox *et al.* 2005, Sivonen and Borner 2008, Cheung *et al.* 2013.

	Microcystin	Nodularin	Cylindro-spermopsin	Anatoxin-a	Anatoxina(S)	Saxitoxin	Dermatotoxin	BMAA
Microcystis	X							X
Planktothrix	X			X		X		X
Anabaena	X		X	X	X	X		X
Nostoc	X							X
Anabaenopsis	X							
Radiocystis	X							X
Synechococcus	X							X
Phormidium	X			X				X
Oscillatoria limosa	X			X				
Oscillatoria				X			X	
Nodularia		X						X
Cylindro-spermopsis			X			X		X
Aphanizo-menon			X	X		X		X
Raphidiopsis			X	X				X
Cylindro-spermum				X				X
Lyngbya						X	X	X
Shizothrix							X	
Umezakia natans			X					

Anabaena species, including *flos-aquae*/ *lemmermannii*/ *circinalis*, may be the most toxically versatile of all the cyanobacteria as they can produce all the toxins, including BMAA, microcystins, cylindrospermopsin, anatoxin-a, anatoxin-a(S) and saxitoxins, save nodularin (Table 2.2). Nodularin is only produced by *Nodularia spumigena*. Another versatile toxin producer is *Aphanizomenon flos-aquae* which produces BMAA, cylindrospermopsin, anatoxin-a and saxitoxins (Table 2.2). *Planktothrix* also produces four different toxins including BMAA, microcystins, anatoxin-a and saxitoxins. The cyanobacteria *Cylindrospermopsis raciborskii* from whence cylindrospermopsin was originally isolated also produces saxitoxins (Table 2.2). Benthic cyanobacteria are also versatile when it comes to toxin production. For example, *Oscillatoria limosa* can produce microcystins as well as anatoxin-a while *Lyngbya wollei* can produce saxitoxins and dermatotoxins (Table 2.2).

Toxin types and their biosynthetic pathways

The toxins produced by cyanobacteria can be divided into three main groups: hepatotoxins that damage the liver of the organisms ingesting them, neurotoxins that cause respiratory arrest, and dermatoxins that cause rashes and inflammations. Each is discussed separately below.

Hepatotoxins. The most well-known hepatotoxins are microcystins and nodularin which are serine/threonine protein phosphatase inhibitors (Table 2.3). A large variety of different microcystins (close to 80) have been identified, with the most toxic being microcystin-LR. These cyclic heptapeptides contain seven amino acids, including a unique beta amino acid ADDA (MacKintosh *et al.* 1990, Yoshizawa *et al.* 1990). In contrast with microcystins, only a few varieties of nodularin have been identified (Yoshizawa *et al.* 1990). The toxicity of cyanobacterial toxins is typically measured by injecting them into mice and calculating the lethal dosage to half the population (LD_{50}; Table 2.3).

Biosynthesis of the microcystis and nodularin peptides occurs by non-ribosomal peptide synthases (NRPS) and polyketide synthases (PKS) found mainly in bacteria (Welker and von Dohren 2006). Both of these enzyme classes are needed for both the microcystin and nodularin biosynthesis pathways which have been sequenced from a number of cyanobacterial species including *Microcystis*, *Planktothrix* and *Anabaena* (Borner and Dittman 2005). For example, the *mcyA*, *mcyB* and *mcyC* genes encode the NRPS that synthesize the pentapeptide portion of microcystins. The *mcyD*, *mcyE*, *mcyF* genes encode the PKS which synthesize the ADDA amino acid unique to microcystins. Finally, the *mcyF*, *mcyG*, *mcyH*, *mcyI*, *mcyJ* genes encode the proteins that tailor and transport specific microcystins (Table 2.3). Similarly, the *nda* gene cluster specific to nodularin encode the NRPS and PKS synthases as well as the tailoring and transport proteins (Table 2.3). Although not verified through functional investigations, the cylindrospermopsin gene cluster, encoding the genes *cyrA*, *cyrB*, *cyrC*, has recently been characterized in *Aphanizomenon flos-aquae* (Stuken and Jakobsen 2010).

Table 2.3. Common cyanobacterial toxins. ND: Not determined.

Toxin	Chemical Class	Action	Effect	LD_{50}	Reference	Gene Name	Gene Reference
Micro-cystins	Cyclic heptapeptides; 80 variants; microcystin-LR is most toxic	Serine/threonine protein phosphatase (1 and 2A) inhibitors	Hepatotoxin; damages liver	50 µg kg^{-1}	MacKintosh *et al.* 1990, Yoshizawa *et al.* 1990	*mcyA-I*	Tillett *et al.* 2000, Christiansen *et al.* 2003
Nodularin	Cyclic pentapeptide; only a few variants identified	Serine/threonine protein phosphatase 1 and 2A inhibitor	Hepatotoxin; damages liver	50 µg kg^{-1}	Yoshizawa *et al.* 1990	*ndaA-I*	Moffitt and Neilan 2004
Cylindro-spermopsin	Cyclic guanidine alkaloid	Protein synthesis inhibitor	Hepatotoxin/Cytotoxin; affects liver as well as kidney, spleen, thymus and heart	200 µg kg^{-1} at 6 days 2000 µg kg^{-1} at 24 hrs	Runnegar *et al.* 1994, Terao *et al.* 1994, Ohtani *et al.* 1992	*cyrA-C*	Stuken and Jakobsen 2010
Anatoxin-a	Alkaloid	Competitive inhibitor of acetyl choline	Neurotoxins: causes death by respiratory arrest	200-250 µg kg^{-1}	Devlin *et al.* 1977, Carmichael *et al.* 1990, Skulberg *et al.* 1992	*ana*	Mejean *et al.* 2010
Anatoxin-a(S)	Phosphate ester of cyclic N-hydroxyguanine	Anticholin-esterase	Neurotoxins: causes death by respiratory arrest	20 µg kg^{-1}	Carmichael *et al.* 1990	*ana*	Mejean *et al.* 2010
Saxitoxins	Carbamate alkaloids; the most potent are saxitoxins and neosaxitoxins	Sodium channels blocker	Neurotoxin	10 µg kg^{-1}	Sivonen and Jones 1999	*stxA-Z*	Kellmann *et al.* 2008
BMAA	Non-protein amino acid		Neurotoxin: linked with neuro-degenerative diseases (e.g. Parkinson's Dementia Complex)	ND	Cox *et al.* 2005	ND	
Dermato-toxins	Aplysiatoxins	Protein kinase C activators	Dermatotoxin: tumor promoters; dermatitis and oral/ gastrointestinal inflammations	ND	Mynderse *et al.* 1977, Fujiki *et al.* 1990	ND	

Neurototoxins. By far the most potent toxins are the neurotoxin saxitoxin that causes paralytic shellfish poisoning (PSP) syndrome and respiratory arrest in humans and animals. This neurotoxin is produced both by cyanobacteria and dinoflagellates and is an alkaloid that acts as a sodium channel blocker. Another alkaloid neurotoxin, anatoxin-a, competitively inhibits acetyl choline, and a variant, anatoxin-a(S), acts as an anti-cholinesterase (Devlin *et al.* 1977, Myderse *et al.* al 1977, Carmichael *et al.* 1990, Sivonen and Jones 1999). The LD_{50} of these toxins vary from 200-250 µg kg^{-1} in the case of anatoxin-a, 20 µg kg^{-1} in the case of anatoxin-a(S), to 10µg kg^{-1} in the case of saxitoxins (Table 3). The gene clusters encoding the saxitoxin biosynthesis and anatoxin biosynthesis pathways were very recently elucidated via functional homology and each contains 20 or more genes (Kellmann *et al.* 2008, Mejean *et al.* 2010). The recently discovered neurotoxin BMAA, a non-protein amino acid that is potentially linked to neurogenerative diseases such as Parkinson Dementia Complex (PDC), is produced in almost all cyanobacteria tested to date (Cox *et al.* 2005).

Dermatotoxins. Benthic cyanobacteria, including *Lyngbya*, *Oscillatoria* and *Schizothrix*, produce a number of different toxins including aplysiatoxins, debromoaplysiatoxins and lyngbyatoxin-a. These toxins are protein kinase C activators that cause dermatitis and oral and gastrointestinal inflammations, and can also promote tumor formation (Mynderse *et al.* 1977, Cardellina *et al.* 1979, Fujiki *et al.* 1990). The pathways and genes involved with the production of the dermatotoxins have yet to be elucidated.

Potential functions of toxin production

Interestingly, researchers have not been able to determine the purpose of toxin production in cyanobacteria, or under what conditions toxins are most likely to be produced (Sivonen and Borner 2008). Moreover, under environmental conditions cyanobacteria that produce toxins co-exist with cyanobacteria of the same genus that do not produce toxins; it's unclear whether the possession of, or lack of, the toxins confers an ecological advantage (Sivonen and Borner 2008, Baxa *et al.* 2010).

Despite these complications, several explanations for the potential function of toxin production exist. Originally it was thought that cyanotoxins acted as allelochemicals and that their secretion into the surrounding water would suppress the growth of competitors (Keating 1977, Keating 1978, Flores and Wolk 1986, Klein *et al.* 1995). But, when the distribution of toxins, such as microcystins, was compared between cells and the surrounding medium using immunodetection combined with electron microscopy, most of the toxin was found to be cell-bound (Rapala *et al.* 1997, Wiedner *et al.* 2003, Tonk *et al.* 2005, Gerbersdorf 2006). Because, live (i.e. non-lysed) cyanobacteria do not secrete the toxins they produce it is doubtful that they act as allelopathic chemicals. Consistent with this notion, most investigations that demonstrate allelopathic effects do so at concentrations of extracted toxins far above what is ecologically relevant, leading

investigators to conclude that the ability of cyanobacterial toxins to work as allelophathic chemicals appears unlikely (Babica *et al.* 2006, Berry *et al.* 2008, Holland and Kinnear 2013).

One explanation that is gaining ground is that the primary role of toxins is probably not to be toxic (Llewellyn 2006). Rather, investigators are hypothesizing that toxins may be produced to protect the cells from abiotic stresses. For example, microcystins are produced during all phases of growth but the greatest accumulation typically occurs under conditions that support optimal growth, including growing under optimal light levels (Sivonen and Jones 1999, Wiedner *et al.* 2003). Several lines of evidence point towards increases in irradiance as being a trigger for microcystin production. These include accumulation of intraceullular microcystin-LR with increased irradiance, the association of intracellular microcystins with the thylakoid membranes, and increased microcystis gene expression with increased irradiance (Kaebernick *et al.* 2000, Tonk *et al.* 2005, Borner and Dittman 2005, Gerbersdorf 2006). As such, it makes sense that microcystins are produced across a number of cyanobacterial taxa, such as *Microcystis*, *Anabaena*, and *Planktothrix*, that grow well in high-light environments (Paerl and Paul 2012).

Microcystins may also be implicated in preventing iron-stress by acting as siderophores to scavenge iron (Utkilen and Gjolme 1995, Lyck *et al.* 1996), an idea supported by the discovery that the iron-regulator factor Fur binds to the genes that produce microcystins in cyanobacteria (Martin-Luna *et al.* 2006). As such, microcystin production may provide an advantage to cyanobacteria in early stages of iron-limiting conditions (Alexova *et al.* 2011, Holland and Kinnear 2013) vis-à-vis eukaryotic competitors (Molot *et al.* 2014).

Another potential role for cyanotoxins is to act as a grazing deterrent (Burns 1987, Gilbert 1996). However, recent research using *Microcystis aeruginosa*, has demonstrated that it's not the toxic microcystins that deters Daphnia from grazing *M. aeruginosa* but other substances it produces. In other words, the substances causing toxicity and deterrence are not identical and the non-toxic substances may be much important in terms of grazing deterrence (Rohrlack *et al.* 1999, 2003).

While the toxic substances are by far the most well-known, there are hundreds of other, secondary metabolites similar in structure to the toxins that are produced by cyanobacteria. Just as the toxins, these cyclic or linear peptides may not be needed for growth but may serve protective functions. For example, the grazing deterrents discussed above belong to a class of depsipeptides called microviridins (originally isolated from *Microcystis viridis*) and has since their isolation been found in a range of cyanobacteria (Rohrlack *et al.* 2003). These secondary metabolites may also have important pharmacological applications. An alkaloid produced by *Nostoc*, called nostocarboline, is a cholinesterase inhibitor which has an effect comparable to galanthamine, a drug approved for Alzheimer's disease (Becher *et al.* 2005). Also isolated from *Nostoc* is a compound called cyanovirin-N which has antiviral activity and is under development as an antiviral agent against HIV (Boyd *et al.* 1997, Bolmstedt *et al.* 2001).

3. Factors Influencing Cyanobacterial Blooms and Toxin Production

The world-wide increase in the incidence of cyanoHABs such as the N_2 fixing genera *Anabaena*, *Aphanizomenon*, *Cylindrospermopsin*, and *Nodularia*; the benthic N_2 fixing genera *Lyngbya* and some *Oscillatoria*; and the non-N_2 fixing genera *Microcystis* and *Planktothrix* has prompted a great deal of research into the conditions that favor the growth of these species (Chorus and Bartram 1999; Carmichael 2008; Paerl and Huisman 2008; Hudnell 2008, 2010; O'Neill *et al.* 2012; Paerl and Paul 2012). These conditions typically include favorable salinity, ample supply of nutrients, calm water and stratified conditions, plenty of irradiance and warm water temperatures (Figure 3.1). In contrast, the most successful strategies to mitigate blooms of cyanoHABs include reducing the supply of nutrients, increasing the flow of water to promote mixing and destratify the water column (Figure 3.1). In the following sections, we will focus on the conditions that are favorable for the growth of the cyanoHAB genera.

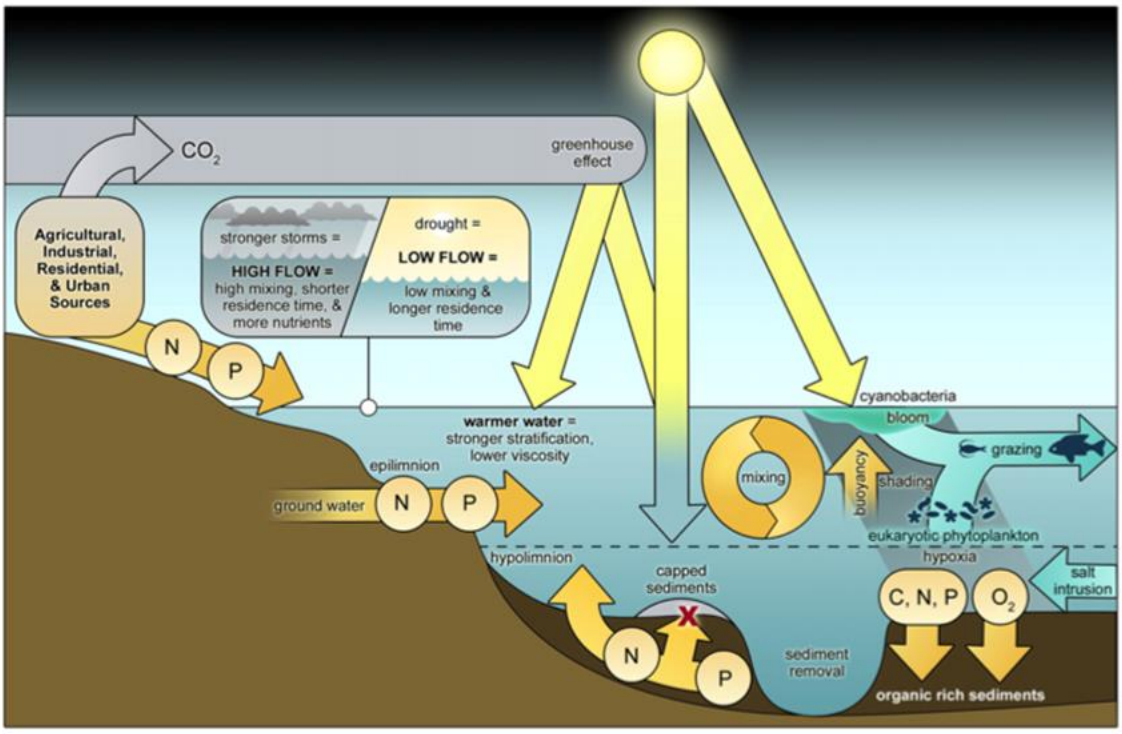

Figure 3.1. Conceptual model of factors affecting cyanobacteria blooms including warmer water, drought and decreased flow, decreased mixing, increased residence time, and increased N and P inputs from agricultural, industrial and urban sources. From Paerl *et al*. 2011.

3.1 Salinity

Most harmful algal bloom-forming and toxin-producing cyanobacteria (cyanoHABs) are freshwater species. In contrast, marine cyanobacteria such as *Prochlorococcus*, *Synechococcus* sp. and *Trichodesmium* sp. are not toxic and do not form cyanoHABs. However, laboratory investigations of freshwater cyanoHAB species demonstrate that these have quite wide salinity

tolerance ranges. For example, the least tolerant, *Cylindrospermopsis* only thrives up to 2.5 ppt salinity, but the most tolerant, *Anabaenopsis* and *Nodularia* spp., thrive at salinities from 5-20 ppt (Moisander *et al.* 2002). *Microcystis aeruginosa* tolerates up to 10 ppt salinity without a change in its growth rate compared to that on freshwater (Tonk *et al.* 2007). What these studies suggest is that given optimal growth conditions, these species could also bloom in brackish-water regions. Indeed, recent decades have witnessed a spread in the geographical extent of these species into the mesohaline (5-15 ppt) reaches of coastal systems (Paerl and Paul 2012). For example, blooms of *Microcystis aeruginosa* have occurred in the Baltic Sea (Maestrini *et al.* 1999) and the San Francisco Estuary (Lehman *et al.* 2013) suggesting 1) that factors other than salinity are regulating their geographical distribution and that 2) those factors are currently changing to allow cyanoHAB growth to occur in regions where they previously did not exist. In summary, salinity may not be the strongest "barrier" in terms of restricting the occurrence and geographical distribution of toxic cyanoHABs.

3.2 Nutrient Concentrations and Ratios

As with other photosynthetic phytoplankton, given optimal temperatures and irradiance, cyanobacterial biomass accumulation is directly proportional to the amount of nutrients (N and P) available in the water column. Therefore, strategies to reduce the accumulation of cyanoHAB biomass and severity of their blooms frequently focus on reductions of nutrient concentrations (Paerl 2008).

3.2.1 Influence of N and P Loadings and Concentrations in Stimulating Cyanobacterial Growth

Cyanobacterial growth in freshwater systems (rivers and lakes), which tend to become limited by P sooner than by N, is frequently linked with excessive P loading (Likens 1972, Schindler 1977, Edmondson and Lehman 1981, Elmgren and Larsson 2001, Paerl 2008, Schindler *et al.* 2008). In contrast with freshwater systems, estuarine and marine systems tend to be more sensitive to N loading (Figure 3.2), and eutrophication due to cyanobacterial growth is frequently linked with excessive N loading (Ryther and Dunstan 1971, Nixon 1986, Suikkanen *et al.* 2007, Paerl 2008, Conley *et al.* 2009, Ahn *et al.* 2011).

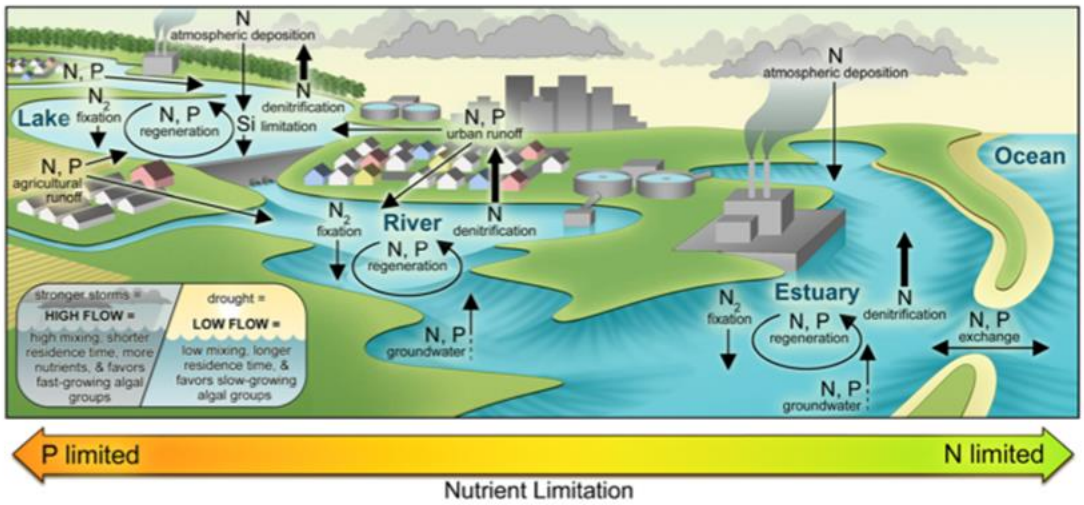

Figure 3.2. Conceptual diagram of interaction of nutrient inputs, cycling processes, and limitation of primary production along the freshwater to marine continuum. From Pearl *et al.* 2014b.

However, both non-point and point source nutrient contributions, such as agriculture and wastewater effluent, tend to increase N and P concentrations simultaneously (Paerl and Paul 2012, Paerl *et al.* 2014b). For example, human population growth-induced intensification of wastewater discharge and agriculture has led to hypereutrophication of China's third largest lake, Taihu (Qin *et al.* 2007). Increased nutrient loads, combined with low water column depth and increased water temperatures, has led to an explosive growth of cyanobacteria and a change in total phytoplankton community composition from being mainly diatom-dominated to being dominated by *Microcystis aeruginosa* (Qin *et al.* 2010, Paerl *et al.* 2014a). Bioassay experiments during summer months when cyanobacterial biomass is at its maximum, and nutrient concentrations at a minimum, demonstrate that N and P exert equal control over biomass accumulation in this system (Paerl *et al.* 2014a).

In general, dominance of both N_2-fixing and non-N_2 fixing cyanobacteria such as *Aphenizomenon flos aquae*, *Nodularia spumigena*, *Microcystis aeruginosa* and *Cylindrospermopsin raciborskii*, have increased world-wide in concert with increased loads of both N and P (Chapman and Schelske 1997, Jacoby *et al.* 2000, Gobler *et al.* 2007, Burford *et al.* 2006, Burford and O'Donahue 2006, Hong *et al.* 2006, Suikkanen *et al.* 2007, O'Neill *et al.* 2012).

3.2.2 Influence of Changes in N:P Ratios on Stimulation or Limitation of Cyanobacterial Growth

At low and intermediate nutrient loadings, reduction in only N or P may be sufficient to control blooms of cyanobacteria. But with elevated loadings of both N and P, reduction of only one type of nutrient can lead to an imbalance in the N:P ratio of the water column potentially leading to a

worsening of the cyanoHAB problem, or even lead to a eukaryotic HAB problem (Smith 1983; Paerl 2008; Pearl *et al.* 2011, 2014b).

Low nutrient concentrations

Pioneering studies by Smith (1983, 1990) predicted that phytoplankton community composition would be dominated by cyanobacteria when N:P ratios were < 15, and by eukaryotic phytoplankton when N:P ratios > 20. This was because many nuisance freshwater cyanobacteria that fix N_2 were hypothesized to thrive at very low ambient concentrations of fixed N, therefore at N:P < 15. In comparison, growth rates of eukaryotic phytoplankton that could not fix N_2 were predicted to slow down at N- limiting concentrations, resulting in eukaryotic species becoming outcompeted at N:P < 15. At N:P > 20, growth rates of eukaryotic phytoplankton would not be limited by N and therefore they could dominate phytoplankton community composition (Smith 1983, 1990). These predictions suggested that one could control growth of cyanobacteria by increasing the dissolved N:P ratio above 20. Consequently, many investigators who study lakes with low to intermediate nutrient loadings advocate for reductions in "P only" as a way to control cyanobacterial growth (Schindler 1977, Schindler *et al.* 2008). However, increasing the dissolved N:P ratio >15 becomes less important as a way to control cyanobacterial growth at high concentrations of nutrients, for a number of reasons, including: 1) nutrient concentrations are high relative to biomass and non-limting; 2) the prevalence of N_2 fixation in N_2-fixing cyanobacteria is not as great as initially hypothesized; 3) the cellular N:P ratio of cyanobacteria, and their N requirement, is high; 4) analysis of lake data by several investigators have demonstrated that absolute concentrations of N and P are more important in supporting blooms of N_2 fixing cyanobacteria rather than specific ratios of dissolved N:P.

High and non-limiting nutrient concentrations

In order for changes in nutrient ratios to affect phytoplankton growth, nutrient concentrations must be so low (relative to the phytoplankton biomass) that either P or N will eventually limit their growth rates. In the last decades, both N and P loadings have increased to the point that they exceed the assimilative capacity of the resident phytoplankton in many systems (Chapman and Schelske 1997, Jacoby *et al.* 2000, Burford *et al.* 2006, Burford and O'Donahue 2006, Hong *et al.* 2006, Gobler *et al.* 2007, Suikkanen *et al.* 2007, Paerl 2008, Paerl *et al.* 2011, Dolman *et al.* 2012, O'Neill *et al.* 2012, Paerl and Paul 2012, Paerl *et al.* 2014a). Therefore, changes in the N:P ratio have little effect on the growth of any of the phytoplankton taxa present in the water column (Paerl 2008, Davidson *et al.* 2012, but see also Glibert *et al.* 2011 with respect to diatoms).

Prevalence of N_2 fixation

An assumption that must be met in order that N_2 fixing cyanobacteria dominate the community at low N:P ratios (and N limiting conditions) is that they mostly use N_2 gas rather than fixed N for growth. However, investigations demonstrate that the proportion of the N demand of N_2

fixers that is met by N_2-fixation is typically less than 25% (Levine and Lewis 1987, Findlay *et al.* 1994, Laamanen and Kuosa 2005). For example, in Baltic Sea phytoplankton communities dominated by the N_2 fixers *Aphanizomenon flos aquae* and *Nodularia spumigena*, less than 20% of N utilization is due to N_2 fixation under N-limiting conditions (Sorensson and Sahlsten 1987; Berg *et al.* 2001, 2003; Laamanen and Kuosa 2005). As mentioned in section 2.2.4, N_2 fixation is repressed in the presence of NH_4^+; culture studies of the N_2 fixing cyanobacterium *Cylindrospermopsis raciborskii* demonstrate that N_2 fixation is shut down in the presence of NH_4^+ and that it's competitive for fixed N (Sprosser *et al.* 2003, Moisander *et al.* 2008). Based on a wide range of investigations, the assumption that most of the N demand of cyanobacteria is met by N_2 fixation does not hold.

Cellular N:P composition

As discussed above (Section 2.2.5), the cellular N:P requirement of cyanobacteria is greater than any other eukaryotic group due to the large protein demand of the peripheral light harvesting antennae. At N-limiting conditions, cyanobacteria would need to provide most, if not all, of their N demand by N_2 fixation in order to meet their high tissue N demand. This would lead to a sharp divide in the distribution of genera that fix N_2 from those that do not; the latter group would be much better suited to dominate high N:P ratio (>25) than low N:P ratio environments. On the flip side, many genera of eukaryotic phytoplankton, such as diatoms and dinoflagellates, have relatively high tissue P requirements and have cellular N:P ratios <16 (Geider and LaRoche 2002, Quigg *et al.* 2003, Hillebrand *et al.* 2013) rendering them better suited for environments with N:P <16 (Arrigo *et al.* 1999, Mills and Arrigo 2010). Based on their cellular N:P ratios, cyanobacteria are better suited to dominate high N:P ratio systems (>25) and some eukaryotes low N:P ratio systems (<16) which is opposite of the conclusions reached by Smith (1983).

Confounding factors

Because the height of a phytoplankton bloom, including blooms of N_2 fixers, frequently coincides with a depletion in N and N:P <15, it is often assumed that the major control on the cyanobacteria is the nutrient ratio, rather than the other way around. Additionally, there may be time lags between nutrient uptake and increased biomass such that a correlation between the two variables at a given point in time may not imply causality. Blooms of N_2 fixers also coincide with a warm, stratified water column coupled with adequate or high irradiance. Because all these parameters (warm water, high irradiance, stratification, depletion of N, overall increase in Chl *a*) occur in concert, it's difficult to separate out the impact of nutrients from other co-occurring environmental variables in order to quantify the most important effect on increases in cyanobacterial biomass. Investigations that separate out the effect of changes in absolute concentrations from ratios, find that changes in absolute concentrations of nutrients, or changes in total Chl *a* biomass, are more strongly related to changes in cyanobacterial biomass than changes in the ratio of N:P (Trimbee and Prepas 1987, Downing *et al.* 2001, Dolman *et al.* 2012).

Meta analyses of Lake Studies

Consistent with the problems of assigning shifts in phytoplankton community composition to changes in N:P ratios described above, Trimbee and Prepas (1987) and Downing *et al.* (2001) demonstrated that changes in cyanobacterial biomass was more strongly associated with changes in the absolute concentrations of N and P than with changes in the dissolved N:P ratio in 99 different freshwater systems. In a study of 102 lakes in Germany, Dolman *et al.* (2012) found that the more enriched in both N and P the lakes were, the greater was their total cyanobacterial biomass. The cyanobacterial taxa that responded most to nutrient enrichment included *Planktothrix agardhii*, *Microcystis* and *Anabaenopsis*. Moreover, differences between cyanobacterial taxa were not consistent with the hypothesis that N fixing taxa were favored in low N:P conditions as the greatest biomass of *Aphaenizomenon* and *Cylindrospermopsis raciborskii* were found lakes with the greatest N:P ratios (Dolman *et al.* 2012).

3.2.3 Influence of Type of N on Growth of Cyanobacteria

As previously mentioned, NtcA is central in cyanobacterial N regulation and is under negative control by NH_4^+ (Section 2.2.4). Other than NH_4^+-transporters, transcription of all N related enzymes requires binding of the NtcA transcription factor in order to be transcribed. Therefore, uptake and metabolism of sources other than NH_4^+ does not take place unless NH_4^+ is at limiting concentrations (Lindell and Post 2001, Lindell *et al.* 2005). In contrast, NH_4^+ transporters are constitutively expressed, or always "on", regardless of external concentration of NH_4^+ (Berg *et al.* 2011). In addition, the *amt1* NH_4^+ transporter gene is one of the most highly expressed in cyanobacterial genomes. In the marine cyanobacteria *Synechococcus* and *Prochlorococcus*, *amt1* is expressed on par with, or at a greater level, respectively, than the gene encoding the C-fixation enzyme Rubisco (Berg *et al.* 2011). Considering the countless other critical processes happening within cells, it is noteworthy that the protein responsible for NH_4^+ uptake is one of the most abundant proteins in cyanobacteria.

Given that NH_4^+ exerts such a strong control over the use of other N sources in cyanobacteria, is the preference for NH_4^+ reflected in different rates of growth on different N sources? There is no clear answer to this question. From a theoretical perspective it should not be the case because the magnitude of reductant and ATP needed for carbon fixation dwarfs the energetic costs of N assimilation, even assimilation of "expensive" sources such as NO_3^- or N_2 gas (Turpin 1991). The type of N should not affect the rate of growth other than under conditions of very low irradiance where assimilation of NO_3^- may compete with carbon fixation for reductant and ATP, thereby lowering the growth rate (Turpin 1991). Culture investigations appear to bear this out as faster rates of growth are typically not observed when cyanobacteria are grown on NH_4^+ versus NO_3^- (i.e. Berman and Chava 1999, Hawkins *et al.* 2001, Post *et al.* 2012, Saker and Neilan 2001, Solomon *et al.* 2010). Differences in growth rates when growing on NO_3^- versus on NH_4^+ are frequently detected for individual strains (i.e. Saker and Neilan 2001), but there is no pattern that can be generalized with respect to cyanobacteria as a whole. Even within the same species,

some strains may be growing faster on NH_4^+ and some on NO_3^-, but the difference with N source in most cases is smaller than the difference in growth rate among different strains (Figure 3.3). Therefore, observations of fast growth of cyanobacteria using NH_4^+ in the field are most likely due to 1) factors that promote fast growth of cyanobacteria generally (i.e. high temperature and high irradiance) combined with 2) high enough availability of NH_4^+ such that NtcA is repressed and only NH_4^+ is taken up and utilized by the cell.

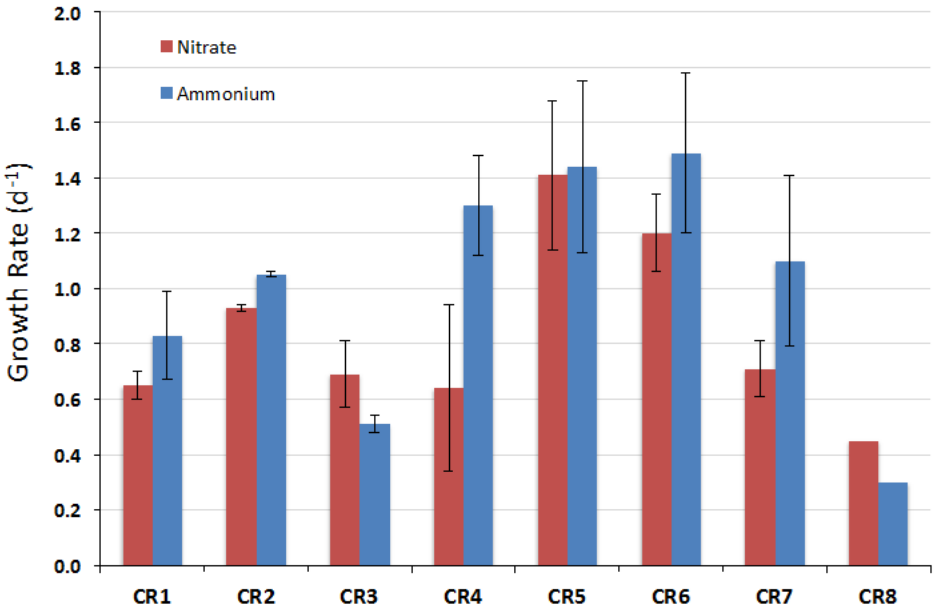

Figure 3.3. Difference in growth rates of *Cylindrospermopsin raciborskii* when growth on NO_3^- (red bars) versus NH_4^+ (blue bars) for eigth different strains. Data from Saker and Neilan 2001 and Stucken *et al.* 2014.

3.3 Irradiance and Water Clarity

Cyanobacteria have a distinct advantage with respect to other photosynthetic organisms in the amount of carotenoid pigments per cell volume (Section 2.2.2). These pigments serve a photoprotective function by dissipating excess light energy when required allowing cyanobacteria to be exposed to high irradiances without experiencing photoinhibition (Paerl *et al.* 1983, 1985). Recent investigations also demonstrate that the toxic peptides produced by cyanoHAB species accumulate in the thylakoid membranes potentially serving a role in photoprotection of the cells (Kaebernick *et al.* 2000, Borner and Dittman 2005, Gerbersdorf 2006). Interestingly, many cyanoHAB species are not strong competitors for light in a well-mixed environment due to their poor light absorption efficiency (Huisman *et al.* 1999, Reynolds 2006). Among the cyanoHAB species tested to date, *Microcystis* appears to possess the least efficient rate of photosynthesis for a given light intensity (Figure 3.4). The upshot of these traits

is that cyanobacteria grow ineffectively at low and mixed light, but very effectively when exposed to high light, particularly the toxic peptide-producing varieties (Huisman *et al.* 2004, Reynolds 2006, Carey *et al.* 2012).

Aided by their positive buoyancy, cyanobacteria such as *Microcystis*, can grow very close to the surface by tolerating irradiance levels that are inhibitory to other members of the phytoplankton community. As a result, these cyanobacteria can increase their cell densities past the point where they would ordinarily become light-limited by self-shading. Growing close to the surface can also help cyanobacteria avoid light limitation if there is a high concentration of suspended sediment matter in the water. In contrast, phytoplankton than are not positively buoyant can become shaded by the cyanobacteria growing at the surface (Carey *et al.* 2012).

In contrast with *Microcystis* and *Aphanizomenon*, other cyanoHAB species such as *Cylindrospermopsis raciborskii* and *Planktothrix* sp. are good competitors at low light. Cultures of *C. Raciboskii* can grow at optimal rates at very low irradiances (Briand *et al.* 2004, Dyble *et al.* 2006, Wu et al. 2009) and it grows well in deep water columns where it's exposed to fluctuating light levels as it mixes from the surface to the bottom (McGregor and Fabbro 2000, Burford and Donohue 2006, O'Brien *et al.* 2009). Not only is the rate of photosynthesis in *C. raciborskii* efficient at low irradiances, it's also efficient at high irradiances, making this a very versatile cyanoHAB species (Figure 3.4).

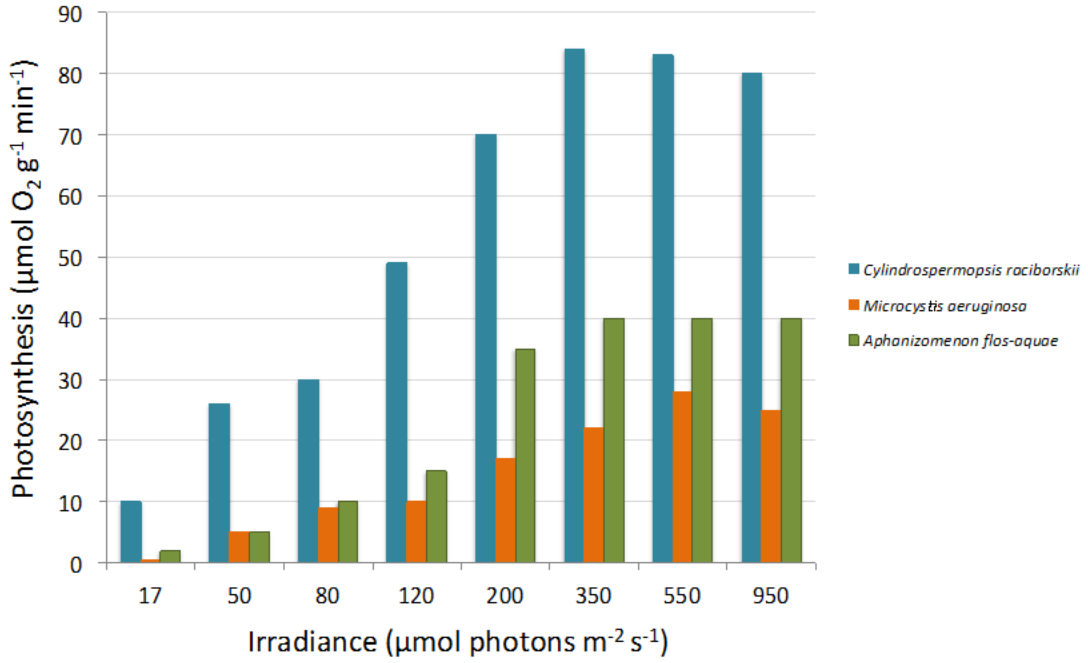

Figure 3.4. Photosynthesis as a function of irradiance in three cyanoHAB species. Data from Wu *et al.* 2009.

3.4 Factors Impacting Toxin Production and Degradation

While a large number of different toxins are produced by cyanoHAB species, the literature is heavily tilted towards investigations of factors impacting the production and degradation of microcystins. Therefore the information presented here is focused on microcystin-LR.

3.4.1 Toxin Production

Just as there is substantial discussion surrounding the purpose of toxin production in cyanobacteria, the conditions under which toxin production is enhanced is also vigorously debated. Previous studies have concluded that the greatest intracellular toxin concentrations are detected under favorable growth conditions, including high irradiance as discussed above, with maximal toxin production occurring at maximal rates of cell division and in late log phase (Watanabe and Oishi 1985, Orr and Jones 1998, Sivonen and Jones 1999, Van Der Westhuizen and Eloff 1985).

Investigations specifically focused on changes in nutrient concentrations and ratios, demonstrate that microcystin content reaches a maximum under maximum growth rates, regardless of medium N:P ratio, but that the microcystin content of the cells correlates with total cellular N and protein content (Lee *et al.* 2000, Vezie *et al.* 2002, Downing *et al.* 2005). These results make sense as the toxins, being peptides, require ample N in order to be synthesized. Consistent with this, total toxin production per cell decreases at N-limiting concentrations (Tonk *et al.* 2008).

Not only does toxin concentration per cell vary in strains that produce toxins (i.e. are toxigenic), but natural populations are typically comprised of a mix of toxigenic and non-toxigenic strains of the same species. It is also of interest to know whether the proportion of toxigenic:non-toxigenic strains within a population changes with nutrient concentrations or ratios. Laboratory culture investigations comparing growth of toxigenic and non-toxigenic strains of *Microcystis* demonstrated that toxigenic strains of *Microcystis* grew faster than non-toxigenic strains at N concentrations of 6000 µmoles L^{-1} and at N:P ratios >> 200 (Vezie *et al.* 2002). The reason for this is not clear, but could include microcystin conferring protection from NO_3^- toxicity in the toxin-producing strains at such unnaturally high concentrations of NO_3^-.

While results obtained with unnaturally high nutrient concentrations and ratios do not easily translate to natural systems, a nutrient enrichment bioassay investigation has demonstrated that toxigenic strains within a *Microcystis* population were promoted to a greater degree with N (and P) additions than non-toxigenic strains (Davis *et al.* 2010). However, the pattern of selective stimulation of toxigenic strains with increased nutrient concentrations is not evident in natural communities which typically exhibit a high degree of variability across small spatial scales in the proportion of toxigenic:non-toxigenic strains within a population. This variability appears not to be related to nutrient concentrations or ratios which do not exhibit the same spatial variability (Vezie *et al.* 1998, Baxa *et al.* 2010, Mbedi *et al.* 2005, Dolman *et al.* 2012).

3.4.2 Toxin Degradation

Together with labile dissolved organic carbon, toxins are rapidly degraded by the natural microbial community following sedimentation (and subsequent release of cellular material) of a cyanobacterial bloom (Jones *et al.* 1994, Rapala *et al.* 2005). In addition to non-specific degradation by the whole community, specific degradation of toxin peptides occurs due to bacteria belonging to the *Sphingomonadaceae* family (Bourne *et al.* 1996, 2001), and other more recently discovered families (Rapala *et al.* 2005, Yang *et al.* 2014). Bacteria that degrade microcystins may also degrade nodularin (Rapala *et al.* 2005). The predominance of these specialized bacteria in the microbial community may determine the length of time it takes (i.e. lag period) before bacterial degradation of toxins takes place. For example, Rapala *et al.* (1994) found the lag time decreased in waters with previous cyanobacterial blooms, compared with no previous cyanobacterial blooms, presumably due to a greater proportion of toxin-degradading bacteria in the former environment. Once degradation of toxin commences, it proceeds rapidly and toxin concentrations typically decrease in an exponential fashion (Figure 3.5), with a loss rate of 0.5 to 1 d^{-1}, corresponding to a half-life of only one day (Christoffersen *et al.* 2002, Jones and Orr 1994). While 95% of the toxins may be degraded within the first 3 days, a more recalcitrant fraction may remain for 20 days or more (Jones and Orr 1994). Other sinks for microcystin-LR include UV degradation (Tsuji *et al.* 1995), and adsorbtion onto clay particles (Morris *et al.* 2000). In the absence of bacteria, clay particles and UV light, microcystins are very stable in the environment and degrade slowly. At temperatures below 40°C the half-life of microcystin toxin increases to 10 weeks; this conservative estimate is used by the Office of Environmental Health Hazard Assessment to determine the risk of the toxin to wildlife (OEHHA 2009). Because there probably exists a great deal of variation in the relative importance of biological, chemical and physical processes in the degradation of microcystins depending on location, accounts in the literature regarding the half-life and recalcitrance of cyanoHAB toxins tend to be conflicting (i.e. Jones and Orr 1994, Gibble and Kudela 2014). Added to this uncertainty is the difference in toxin concentrations obtained using different methods of measurements (See Section 4.2.3 below).

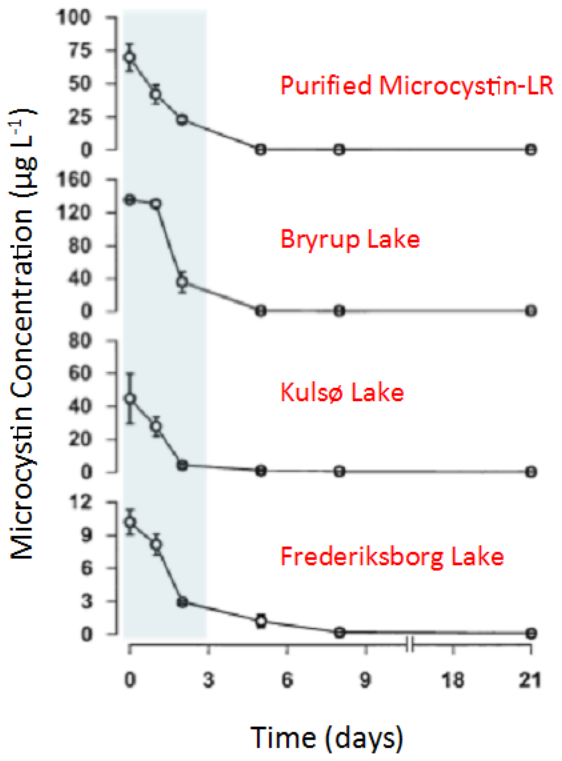

Figure 3.5. Concentration of dissolved microcystin-LR equivalents in bioassays as a function of time after addition of purified microcystin (top panel) or lysed bloom material (bottom 3 panels) to lake water containing natural microbial assemblages. Shaded area corresponds with time period of degradation of 95% of original microcystin concentration. Data from Christoffersen *et al.* 2002.

3.5 Temperature

Perhaps one of the most important factors in controlling the growth rate of cyanobacteria is temperature (Robarts and Zohary 1987, Butterwick *et al.* 2005, Reynolds 2006, Paerl and Huisman 2008). Cyanobacteria isolated from temperate latitudes (i.e. excluding polar regions) typically have temperature growth optima between 25 and 35°C (Reynolds 2006, Lurling *et al.* 2013). For example, in a survey of eight cyanobacteria the growth optima of two *Microcystis aeruginosa* strains were 30-32.5°C and that of *Aphanizomenon gracile* was 32.5°C. Lower growth temperature optima were observed in *Cynlindrospermopsis raciborskii* and *Planktothrix agardhii*, both at 27.5°C while *Anabaena* sp had an optimum of 25°C (Lurling *et al.* 2013). The optima of these freshwater HAB-forming cyanobacteria are greater than for marine cyanobacteria which typically have growth temperature optima ranging from 20-27.5°C (Breitbarth *et al.* 2007, Boyd *et al.* 2013).

Compared with other phytoplankton taxa, cyanobacteria typically demonstrate lower growth rates at colder temperatures and higher growth rates at higher temperatures. For example, diatoms typically have a 6-fold higher growth rate at 15°C, 3-fold higher growth rate at 20°C and

a similar growth rate at 25°C, compared with cyanobacteria (Figure 3.6). Growth rates of dinoflagellates typically peak at 25°C. Above 25°C both chlorophytes and cyanobacteria have faster growth rates than diatoms and dinoflagellates (Figure 3.6). The difference in the optimum growth temperatures of the various phytoplankton taxa is hypothesized to become increasingly important in determining phytoplankton community composition as global temperatures continue to increase above 20°C (Lehman *et al.* 2005, Paerl and Huisman 2008). For example, the acceleration of growth rate with a 10°C increase in temperature (Q_{10}) commonly varies from 1-4 for cyanobacteria and 1-3 for chlorophytes (Reynolds 2006). However, it varies from 4-9 for *M. aeruginosa*, the highest recorded for any phytoplankton (prokaryotic or eukaryotic) species (Reynolds 2006). These data suggest that in a mixed phytoplankton assemblage, all else being equal, cyanobacteria will be able to grow faster and outcompete other phytoplankton taxa as the temperature increases. With continued climate change and global warming, there's an increased risk that cyanoHABs will become increasingly competitive vis-à-vis diatoms which often dominate community composition in temperate regions.

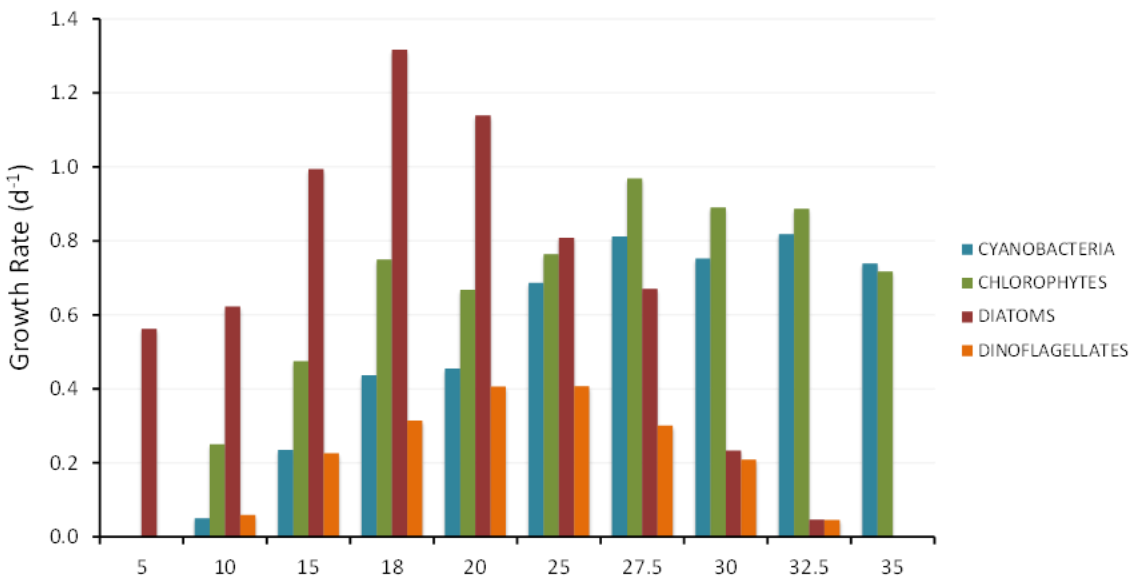

Figure 3.6. Changes in growth rate with temperature for diatoms (red ± 0.35 d^{-1}, T_{opt}= 20 ± 1.8 °C), Chlorophytes (green ± 0.21 d^{-1}, T_{opt}= 29 ± 3.8), Cyanobacteria (cyan ± 0.13 d^{-1}, T_{opt}=29 ± 4.5) and dinoflagellates (orange ± 0.1 d^{-1}, T_{opt}= 21 ± 2.8). Data from Kudo *et al.* 2000, Butterwick *et al.* 2005, Yamamoto and Nakahara 2005, Boyd *et al.* 2013, Lurling *et al.* 2013.

3.6 Stratification and Residence Time

3.6.1 Stratification

CyanoHAB blooms tend to occur during times of calm, stratified water columns (Huber *et al.* 2012). The degree of statification and water column stability increases with increased temperature, therefore stratification and temperature are closely linked (Paerl and Huisman

2008). The reasons that stratified conditions promote blooms of cyanobacteria are at least three-fold. First, growth rates will increase as a result of the increase in the temperature in the top layer of the water column. Second, cyanobacteria will remain in the top layer of the water column where irradiance is greater, and not become mixed down to the bottom and into lower light, allowing them to maintain higher growth rates. Third, stratification may be a sign of increased residence times (reduced flushing rates), which minimizes loss of cyanobacterial biomass from the system and allows cyanobacteria to use all the nutrients available in the water column (Jeppesen *et al.* 2007). In other words, it's likely that stratification does not directly promote cyanobacterial blooms, but rather it promotes blooms indirectly through increased temperatures, irradiance and reduced loss rates (Elliott 2010).

3.6.2 Residence Time

Because residence time is determined by the flushing rate, the direct effect of increased residence time is to decrease the loss rate of cyanobacteria (Romo *et al.* 2013). Indirect effects of residence time are the same as those for stratification; this is because residence time and stratification typically covary such that stratification is maximal when residence time is minimal, and vice versa. Studies that report on the effect of residence time suggest that cyanobacterial abundance, cell size and toxin concentration are positively related to increased residence time (Elliott 2010, Romo *et al.* 2013).

3.7 Other Factors

Additional to the above-mentioned factors, a number of others may influence cyanobacterial blooms including grazing by higher trophic levels and exposure to toxic compounds such as herbicides and pesticides. Grazing in the Delta region is dominated by *Corbicula fluminea* (Jassby 2008). It is not known to what extent *C. fluminea* impacts cyanoHAB species versus the rest of the phytoplankton community in the Delta. The same is true for grazing by zooplankton. Another factor that may differentially impact cyanoHAB species versus the rest of the phytoplankton community is resistance to herbicides and pesticides. Investigations demonstrate substantial variability in sensitivity to herbicides of cyanobacteria compared with other phytoplankton such as green algae and diatoms (Peterson *et al.* 1997, Lurling and Roessink 2006)

4. Prevalence of CyanoHABs and Potential for Effects on Ecosystem Services in the Delta

The Sacramento-San Joaquin Delta (hereafter Delta) is formed at the intersection of two of California's largest rivers, the Sacramento and the San Joaquin Rivers, and contains 700 miles of sloughs and waterways that drain 47% of the runoff in the State of California (Figure 1.1). The land surrounding the waterways is composed of 57 leveed island tracts, many of which provide wildlife habitat. In the Delta, freshwater from the rivers mix with saltwater from the San Francisco Bay; together the Bay and the Delta form the West Coast's largest estuary.

4.1 Ecosystem Services

The Delta region has many ecosystem services including agriculture, drinking water supplies, and wildlife habitat, all of which translate directly to the beneficial uses designated in the Water Board Basin Plan (Appendix A). The population surrounding the Delta region, numbering 500,000 people, is principally engaged in agriculture and produce crops that bring in revenues exceeding $500 million annually. While there is some local demand on the water from the Delta, most of the water is distributed via the State Water Project and Federal Central Valley Projects to the Central Valley to irrigate farmland and to provide drinking water to Southern California (http://www.water.ca.gov/swp/delta.cfm). According to the California Department of Water Resources, about two thirds of Californians and millions of acres of irrigated farmland rely on the Delta for their water. Besides acting as a source of drinking water, the Delta is a popular recreation spot and many people use it for sport fishing.

In addition to the human demand, the Delta supplies critical habitat to a large wildlife ecosystem and intersects migration paths for several fish species, including salmon, traveling between the Pacific Ocean and the Sacramento River and beyond. This habitat is in a fragile state with close to 20 of its endemic species listed as endangered. A recent and unexpected decline in four pelagic fish species including the endangered Delta Smelt and the Longfin Smelt, as well as juvenile-Striped Bass and Threadfin Shad, has caused concern among resource managers and renewed calls for conservation of the fragile Delta ecosystem (Sommer *et al.* 2007).

Set against this backdrop of competing resource use by human populations and wildlife, a new threat to Delta ecosystem services and designated beneficial uses is emerging in the form of toxic cyanoHABs. The impact of toxic cyanobacteria on the aquatic ecosystem differs widely depending on whether their density is low or high. At low concentrations, they are not dense enough to affect light penetration or dissolved O_2 concentration; therefore, they do not affect the growth of other members of the aquatic community. However, even at low concentrations toxins released (upon death and cell lysis, or by grazing) can accumulate in tissues of higher trophic levels (Lehman *et al.* 2010). At high densities, cyanoHABs increase the turbidity of the water column to the point where light penetration is severely restricted suppressing the growth of other

phytoplankton, macrophytes, and benthic microalgae (Jeppesen *et al.* 2007, Paerl and Paul 2012). CyanoHABs also can cause night-time dissolved oxygen depletion via bacterial decomposition and respiration of dense blooms which results in fish kills and loss of benthic fauna (Paerl 2004, Paerl and Fulton 2006). At dense concentrations, mortality to aquatic animals such as sea otters, birds and seals may result from liver failure following ingestion of prey with high concentrations of toxin, or coming into physical contact with the toxin (Jessup *et al.* 2009, Miller *et al.* 2010). Humans coming in contact with the water may develop digestive and skin diseases (Section 2.2.6) and it may affect the drinking water supplies (Cheung *et al.* 2013). In the following sections, cyanoHAB abundance and toxin levels in the Delta vis-à-vis published guidance on alert levels are summarized in order to place the threat of cyanoHABs in the Delta into context.

4.2 Prevalence and Trends of CyanoHABs in the Delta

Since 1999 blooms of the toxin producing cyanobacteria *Microcystis aeruginosa* in the Delta have been observed by the Department of Water Resources (DWR), and have been reported in the scientific literature. In the beginning, only blooms of *Microcystis* were observed; these were documented visually appearing as little flakes of lettuce in the water (Lehman and Waller 2003). Later investigations (post 2005) employing microscopic enumeration and molecular characterizations have documented blooms comprised of a mix of *Aphanizomenon* sp. and *Microcystis*, with *Anabaena* sp. also present in much smaller densities (Lehman *et al.* 2010, Mioni *et al.* 2012).

While environmental indicators such as salinity, turbidity, temperature, total phytoplankton biomass (as Chl *a*), and phytoplankton species composition are monitored on a monthly basis by DWR, surface concentrations of cyanobacteria and cyanotoxins, which require special sampling, are not routinely monitored. As such, the information on the chronology of cyanoHAB occurrences presented here is taken from a handful of publications and reports, and varies somewhat in geographical extent according to where the authors sampled. Because *Aphanizomenon* and *Anabaena* densities have only been documented for two time points, the following sections will focus on *Microcystis* biomass and microcystin toxin concentrations. Additionally, these sections will focus on aquatic health rather than human health whose risks may be better evaluated from sampling of surface scums.

4.2.1 Spatial Distribution of Microcystis throughout the Delta

The Central Delta, between Antioch and Mildred Island, is typically the region with the highest surface *Microcystis* and *Aphanizomenon* concentrations. In 2003, the stations with the greatest recorded abundance of Chl *a* due to *Microcystis* (as determined by horizontal surface tows with a 75-µm mesh plankton net) were Jersey Point (D16), Mokelumne River Mouth and Navigation Marker 13 in the San Joaquin River, followed by San Mound Slough, Mildred Island, (D29) and Rancho del Rio (D28) in Old River (Figure 4.1). In following years, greatest abundance of

Microcystis has repeatedly occurred in the same areas in the San Joaquin and Old Rivers (Lehman *et al.* 2008, Mioni *et al.* 2012, Lehman *et al.* 2013). In 2012, abundant *Microcystis* colonies were also observed in the South-East Delta region in the Turning Basin of the Stockton Shipping Channel (Spier *et al.* 2013). Moving west from Antioch into Suisun Bay, *Microcystis* abundance decreases substantially to almost non-detectable by Chipps Island (Lehman *et al.* 2005, 2008, 2010). The same holds true when moving north where abundances detected at Antioch decline to almost zero by Collinsville at the entrance of the Sacramento River (Figure 4.1).

Whether or not the spatial distribution of *Microcystis* and other cyanoHAB species is affected favorably or unfavorably by concentrations of herbicides entering the Delta as run-off, or from the Sacramento and San Joaquin Rivers is not known. Recent reports suggest that a broad swath of herbicides and fungicides associated with agriculture is present at concentrations high enough to affect aquatic life (Orlando *et al.* 2014). As such, the impact of herbicides common to the Delta in selectively promoting certain phytoplankton species, including possibly cyanoHAB species, may deserve greater attention.

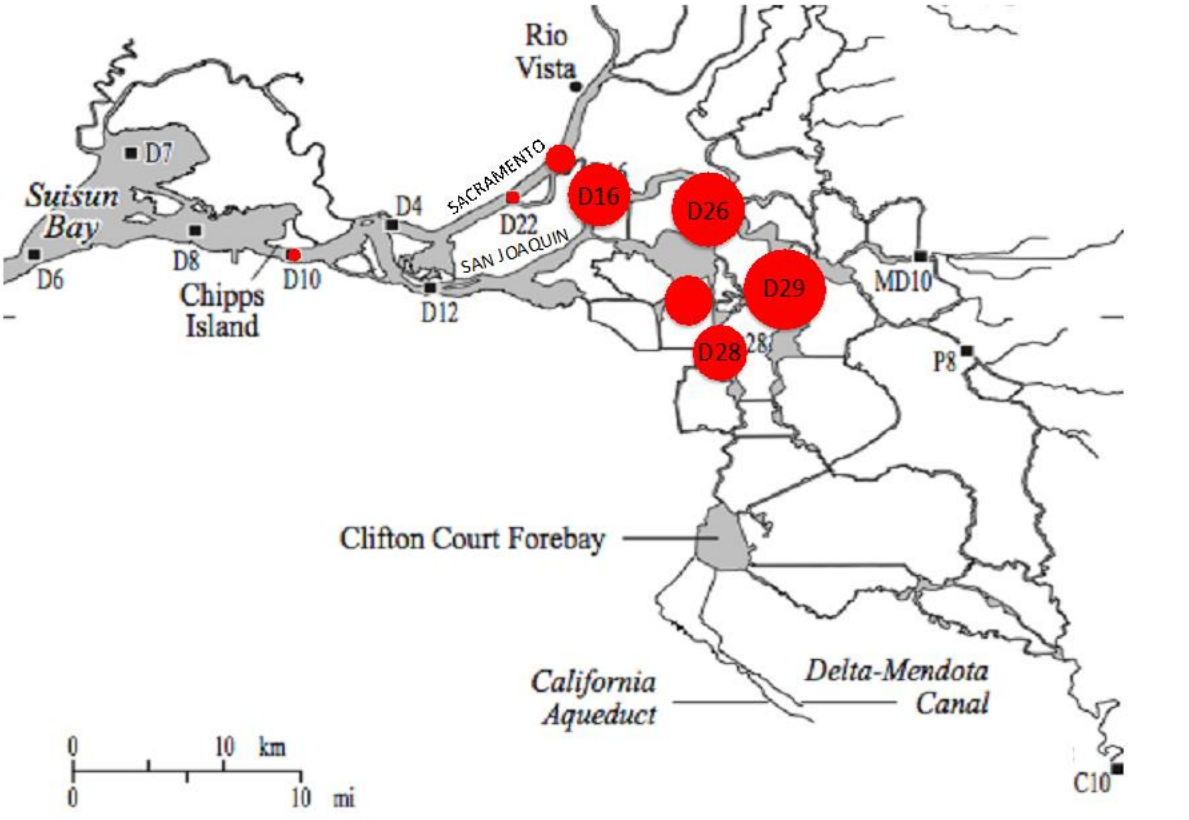

Figure 4.1. The Sacramento-San Joaquin Delta Region. Red bubbles mark locations with greatest Microcystis-associated surface Chl a concentrations (largest bubble=0.55 µg Chl a L^{-1}). Data from Lehman *et al.* 2005.

4.2.2 Interannual variability in Microcystis biomass in the Delta

Since 2003, *Microcystis* cell abundance in depth-integrated surface waters has varied from 4-40×10^3 cells mL^{-1} in the Delta (Lehman *et al.* 2008. The biomass (as surface Chl *a*) has also varied approximately 10-fold (Figure 4.2). Not only is *Microcystis* biomass patchy between years, its distribution in the years that it blooms is also variable. Even within a station, the distribution of *Microcystis* colonies is patchy, as evidenced by the low concentration of surface Chl *a*, sampled with horizontal net-tows normalized to total towed volume, which to date has not been above 0.6 µg Chl a L^{-1} (Figure 4.2). In the years following 2005, *Microcystis* was also present in the phytoplankton community together with *Aphanizomenon flos-aqua*, and to a lesser extent *Anabaena* sp. (Lehman *et al.* 2008, Mioni *et al.* 2012).

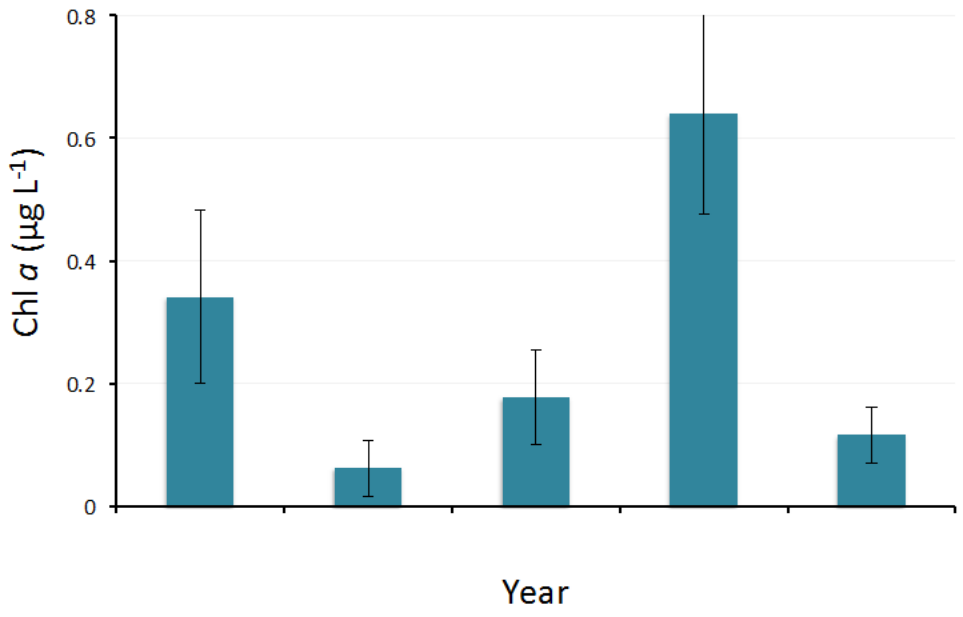

Figure 4.2. Interannual changes in surface Chl a due to abundance of Microcystis colonies. Means and standard deviations of 9 different stations in the San Joaquin River (Antioch (D12), Jersey Point (D16), Frank's Tract (D19), Potato Point (D26), Prisoners Point (D29), San Joaquin River at Turner Cut, Sand Mound Slough, Mildred Island, and Old River at Rancho del Rio (D28). Data from Lehman *et al.* 2005, 2013.

In addition to a high degree of horizontal variability, *Microcystis* cell densities and biomass also varies vertically in the water column, decreasing from the surface to almost zero at 1 m depth. The density of *Microcystis* in surface waters at the Central Delta Stations does not affect phytoplankton community composition in a measurable way. For example, at four stations where *Microcystis* dominated abundance of phytoplankton at the surface, the communities at 1m depth was a variable mix of different species of phytoplankton that was equally variable at stations containing no *Microcystis* in the surface. Rather than decreasing, the biomass of other

phytoplankton taxa increased in tandem with increasing *Microcystis* biomass (Lehman *et al.* 2010).

Compared with lakes widely recognized for severe CyanoHAB problems, *Microcystis* (and other cyanoHAB species) biomass appears low. For example, in Clear Lake spring and early summer Chl *a* concentrations average 11.5±8 µg Chl *a* L^{-1} but increase to 352±295 µg Chl a L^{-1} in the summer once *Microcystis* starts to bloom (Figure 4.4). Here, *Microcystis*-associated Chl *a* concentration is a factor of 100 to 1000 greater than it is in the Delta (Figure 4.4). One important caveat with respect to determining surface Chl *a* concentrations is that it depends on the method used to collect the surface Chl *a*. The difference between using a surface net tow (akin to what is used in Lehman *et al.* 2013) and a grab sample from the middle of a patch (akin to Mioni *et al.* 2012) can be close to be 100-fold, i.e. 0.2 µg Chl a L^{-1} versus 20 µg Chl a L^{-1}, respectively. This is because the former is an integrated measure and the latter is not, suggesting that the "coverage" of *Microcystis* colonies in surface waters of the Central Delta is around 1%. This is in sharp contrast with Clear Lake where surface Chl *a* is uniformly high (above 150 µg Chl a L^{-1}) at all stations during a bloom (Richerson 1994, Mioni *et al.* 2012).

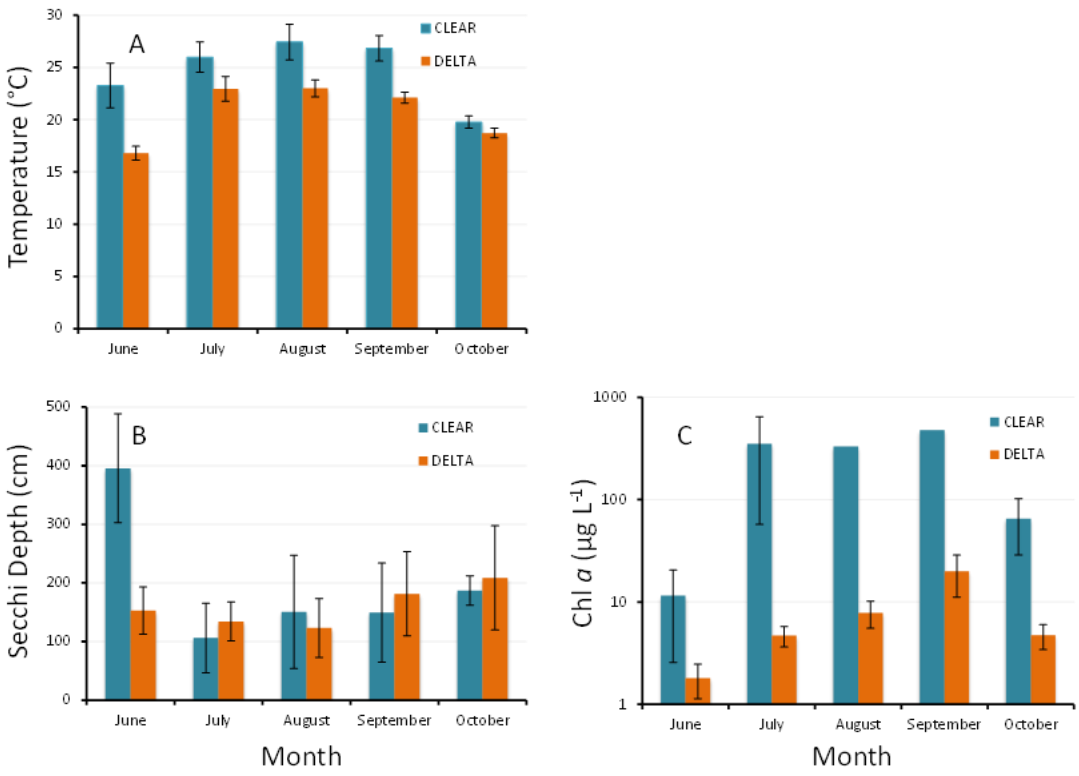

Figure 4.3. Comparison of environmental variables and Chl a in Clear Lake (Cyan) and the Delta (orange) using in-patch grab samples during the summer months of 2011. (A) Temperature, (B) Secchi disk depth, (C) Chl a. Data from Mioni *et al.* 2012.

4.2.3 Microcystin toxin concentrations in the Delta and San Francisco Bay

Given the number of different toxins produced by each cyanoHAB species, and the number of different genera present in Central California, one would expect a number of different toxins to be present in the water column. However, toxins other than microcystin are not frequently encountered (Kudela pers. com, Gibble and Kudela 2014). Based on the data available for the Delta, this section describes total microcystin concentrations and how they relate to *Microcystis* cell abundance.

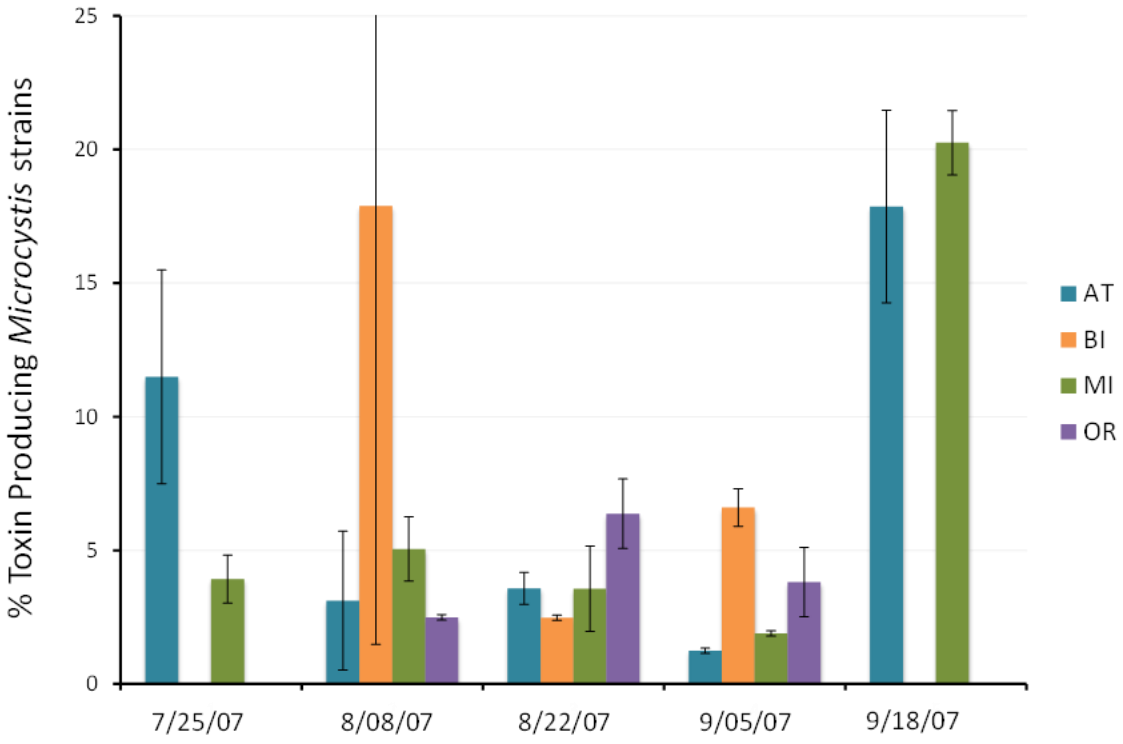

Figure 4.4. Percent toxin-producing strains in Microcystis assemblage at stations AT, Antioch (D12); BI, Brannan Island (D23); MI, Mildred Island; and OR, Old River at Rancho del Rio (D28). Data from Baxa *et al.* 2010.

Microcystis produces approximately 100-400 ng microcystin per µg Chl *a* in toxin producing strains (Sivonen and Jones 1999). Just as with other regions where *Microcystis* occurs, the strains that occur in the Delta are a mix of toxigenic and non-toxigenic strains (Baxa *et al.* 2010). Toxigenic strains generally comprise 2-20% of the total number of *Microcystis* strains present. This variation in the proportion of toxigenic strains is observed everywhere (i.e. at every station) and at all times (Figure 4.4). No single station stands out as consistently producing a greater proportion of toxigenic strains compared with other stations (Figure 4.4). Accordingly, total microcystin concentrations reflect total *Microcystis* cell abundance, typically varying from 10-50 ng L^{-1} (Lehman *et al.* 2008). However, in 2012 concentrations approaching 2000 ng L^{-1} were detected in the Stockton shipping channel during a *Microcystis* event (Spier *et al.* 2013).

In the Sacramento River, intermediate concentrations of total microcystins have been detected at a station close to Rio Vista (Brannon Island) where *Microcystis* cell abundance is low to non-detectable (Lehman *et al.* 2008, 2010). This station is connected via a channel to the San Joaquin River and the Frank's Tract area. Physical mixing of water directly from the San Joaquin River with brackish water at this station situated at the entrance to the Sacramento River may bring toxins but establishment of *Microcystis* populations may be prevented by the conditions in the Sacramento River including colder water, greater flow rates, mixing down to the bottom, and lower water clarity (Lehman *et al.* 2008).

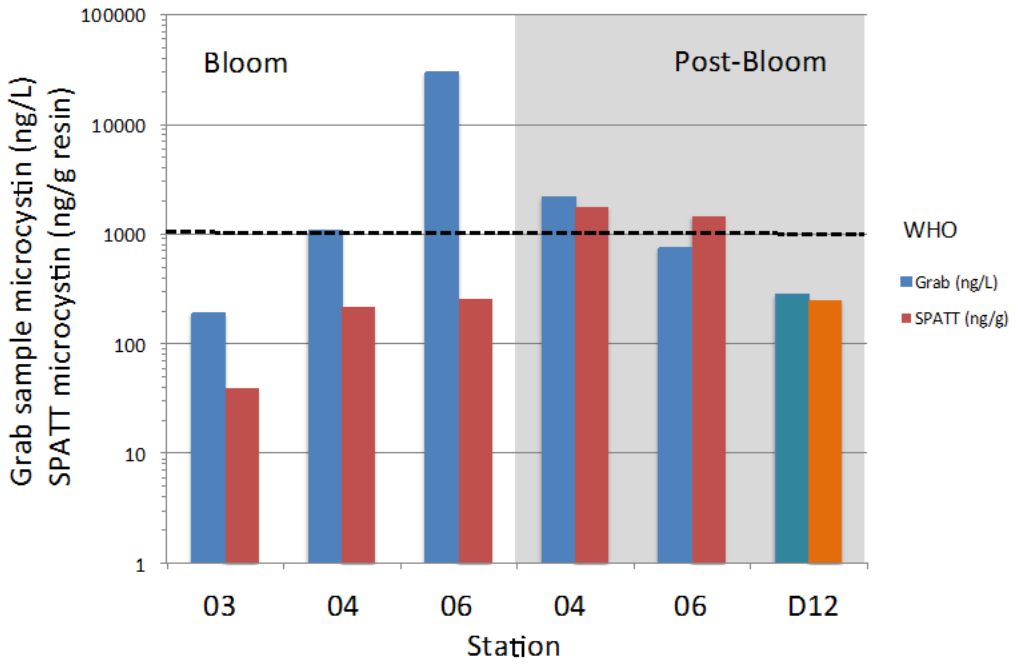

Figure 4.5. Microcystin toxin concentrations determined with grab samples (blue/cyan) and with SPATT resin (red/orange) at three stations in Clear Lake, during and after a Microcystis bloom, and at one station (D12, Antioch) in the Delta. Data from Mioni *et al.* 2012.

Microcystin toxin has also been detected at low concentrations throughout the Delta and the San Francisco Estuary using the novel Solid Phase Adsorption Toxin Tracking (SPATT) technique which integrates exposure of dissolved toxins over longer time spans (Kudela 2011). While valuable to indicate a potential for exposure to cyanotoxins, the comparison of SPATT to existing guidelines for human and aquatic health is problematic because SPATT detected concentrations are not directly comparable to traditional, instantaneous grab samples. For example, in Clear Lake microcystin detected with SPATT (ng/g resin) was 5-115 times lower than grab samples (ng/L) taken the last day of the SPATT deployment during the height of a *Microcystis* bloom (Figure 4.5). Post bloom, microcystin detected with SPATT was either comparable to, or double, levels measured in grab samples (Figure 4.5). While microcystin was

detectable both with SPATT and with grab samples in Clear Lake, microcystin was detectable with SPATT in the Delta, at similar levels as in Clear Lake, but not with grab samples. In the former system *Microcystis* was very abundant and in the latter it was not. The above example illustrates that given longer equilibration times, SPATT becomes more senstitive than grab samples at lower concentrations of toxins. Although difficult to "translate" directly into effects on aquatic life (i.e. Echols *et al.* 2000), SPATT detection may be a very useful system for identifying regions at risk for harm to aquatic life from toxin exposure (Gibble and Kudela 2014).

4.2.4 Potential for CyanoHAB Risk to Delta Beneficial Uses

Characterization of the risk of cyanoHABs to Delta beneficial uses is generally poor. While no guidelines for toxicity of cyanotoxins to aquatic life have been established for California, total microcystin levels found in the Delta are within the range of potential impacts to aquatic health, as recently reviewed by the California Office of Environmental Health Hazards (OEHHA 2009). For example, microcystins are acutely toxic to fish at concentrations as low as a fraction of a microgram per liter (OEHHA 2009). Chronic exposures can also be problematic; embryos and larval fish appear to be very sensitive to chronic exposures to microcystins, resulting in oxidative stress, reduced growth, developmental defects, and lethality; exposures as low as 0.25 µg/L resulted in oxidative stress to zebrafish embryos (OEHHA 2009).

Consumption of prey items with body burdens of cyanotoxins can also be a potential pathway of impact. Lehman *et al.* (2010) traced increasing concentrations of microcystins from the water (25-50 ng L^{-1}) to zooplankton (0.4-1.5 µg g dry wt^{-1}) to striped bass muscle tissue (1-3.5 µg g dry wt^{-1}) at Central Delta Stations. These values are within the range of sublethal microcystin doses to fish (2.5 µg g dry wt^{-1}; OEHHA 2009). The striped bass caught at stations where *Microcystis* cells comprised 100% of the surface Chl *a* had tumor lesions in their liver tissue, consistent with the sublethal effects caused by microcystin-LR toxin (OEHHA 2009, Lehman *et al.* 2010). This is consistent with fish feeding studies which demonstrate that microcystin-LR spiked diets result in lesions of the liver (Deng *et al.* 2010; Acuna *et al.* 2012a,b). Zooplankton are also acutely sensitive to *Microcystis aeruginosa* cells; diets consisting of 50% toxigenic and non-toxigenic *Microcystis* strains result in 100% mortality in the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* (Ger *et al.* 2010). Interestingly, when fed diets containing only 10-25% *Microcystis* cells, both copepods demonstrate significantly greater survival on the toxigenic strain than the non-toxigenic strain, suggesting that bioactive compounds other than the microcystin toxin exert a greater adverse impact on the zooplankton (Ger *et al.* 2010). This is consistent with a number of the studies of the effect of cyanoHABs on zooplankton mentioned in Section 2.2.6.

Determination of risk to human health in the Delta is problematic because cyanoHABs monitoring has been focused on aquatic health (depth-integrated sampling) rather than human

health (via surface-scum sampling). With this caveat, toxin concentrations of 10-50 ng L^{-1} (Lehman *et al.* 2008) are 16-80 times lower than the Office of Environmental Health Hazard Assessment (OEHHA) Action Level for human health (Table 4.1), but the 2012 concentrations approaching 2000 ng L^{-1} in the Stockton shipping channel (Spier *et al.* 2013) exceed both the OEHHA Action level and the WHO guideline of 1000 ng L^{-1} (Table 4.1).

Table 4.1. Action levels developed by OEHHA (2009) for human health exposure to cyanotoxins compared with the WHO guidance level for microcystins and the EPA 10-day average exposure threshold.

Toxin	OEHHA Recreational Use (µg/L water)	OEHHA Consumption Level (ng/g fish)	WHO recreational Use (µg/L water)	EPA 10-day average (µg/L)
Microcystins	0.8	10	1.0	0.3
Cylindrospermopsin	4	70		
Anatoxin-a	90	5000		

4.2.5 Summary of Potential for Adverse Effects on Delta Beneficial Uses

A thorough characterization of the risks for adverse effects on Delta beneficial uses is hindered by the fact that cyanoHAB prevalence and toxin concentrations are currently not routinely monitored in the Delta; moreover, sampling has been focused on aquatic health and does not include sampling for human health risks. Determination of risk to human health is not possible at this time because surface scums are not currently being monitored. The current risk to Delta aquatic health is of concern and merits a more thorough investigation. This observation is based on total microcystin levels found in Delta fish tissues that are within the range of sublethal effects to fish as recently reviewed by the California Office of Environmental Health Hazards (OEHHA 2009). In addition, dissolved toxin concentrations (10- 50 ng L^{-1}) that are generally 16-80 times below the OEHHA action level, occasionally exceed both the OEHHA action level and the WHO guideline of 1000 ng L^{-1} in certain “hotspots” of the Delta. Whether or not these hotspots are expanding is currently not known and merits further investigation and monitoring.

5.0 Synthesis of Factors Influencing CyanoHABs presence and Toxin Production in the Delta

The charge of the cyanobacterial workgroup, as outlined in the Delta Nutrient Management Charter, is to "assess whether observed increases in the magnitude and frequency of cyanobacterial blooms in the Delta is the result of long-term changes in nutrient concentrations and whether management of nutrient loads can remedy the problems associated with cyanobacteria." The best way to characterize the relationship between the extent and frequency of bloom occurrence and nutrient concentrations is by regression analysis. Ideally, this type of analysis ought to be performed in multiple locations for longer time scales. Given that temperature, irradiance and water column clarity are such powerful triggers of blooms, stepwise multiple regression analysis to test the influence of several environmental indicators simultaneously on cyanoHAB cell densities would be even more useful in order to ascertain key triggers of the blooms in the Delta region.

While environmental indicators such as salinity, turbidity, temperature, total phytoplankton biomass (as Chl *a*), and phytoplankton species composition are monitored on a monthly basis by DWR, surface concentrations of phytoplankton, which requires special sampling, are not routinely monitored in this program. Therefore, the statistical analyses needed to answer the charge of the cyanobacterial working group cannot be performed at this time. Instead, this section focuses on summarizing factors known to favor cyanobacterial prevalence (from Section 2) and synthesizing available literature on the extent to which those factors may also be at play in the Delta.

5.1 Present and Future Factors associated with cyanoHAB prevalence in the Delta

5.1.1 Flow and mixing

Environmental and population drivers that promote growth of cyanoHABs in freshwater bodies around the world also play key roles in regulating growth of cyanoHABs in the Delta (Table 5.1). Chief among these is low flow. For example, Lehman *et al.* (2013) noted that increased abundance of *Microcystis* is associated with up to a 50% reduction in flow of water in the San Joaquin River. In 2004, *Microcystis* only appeared in the Central Delta when stream flow was 1-35 $m^3 s^{-1}$ (Lehman *et al.* 2008). In addition to direct effects of decreased flow such as increased stratification of the water column, changes in flow and mixing also impart indirect effects that may influence cyanobacterial growth. These include changes in turbulence, sediment resuspension (therefore turbidity), chemical consitutents, and water temperature to mention a few. Changes in these parameters typically cannot be separated from that of flow to determine their relative importance. For example, in the Delta, reduction in flow is accompanied by a 50% reduction in turbidity and volatile suspended solids. Decreased flow also leads to increased water temperatures. Conditions of decreased flow occur more predictably in dry years (Lehman *et al.*

2013). Within the summer season, reduced flows typically occur in the July-August time frame (Figure 5.1) and set the stage for the two factors necessary for bloom initiation, including increased water column temperature and water column clarity (decreased turbidity).
While decreased flow may increase the abundance of *Microcystis*, increasing rates of flow decrease its abundance because of the negative effects of water column mixing, such as light limitation, on its growth. Artificial mixing is even used as a strategy to mitigate blooms of harmful cyanobacteria in lakes and reservoirs (Reynolds *et al.* 1983, Burford and O'Donohue 2006). In the Delta, natural mixing rates may be sufficient to restrict the abundance of *Microcystis* to 10-15% of the total phytoplankton community.

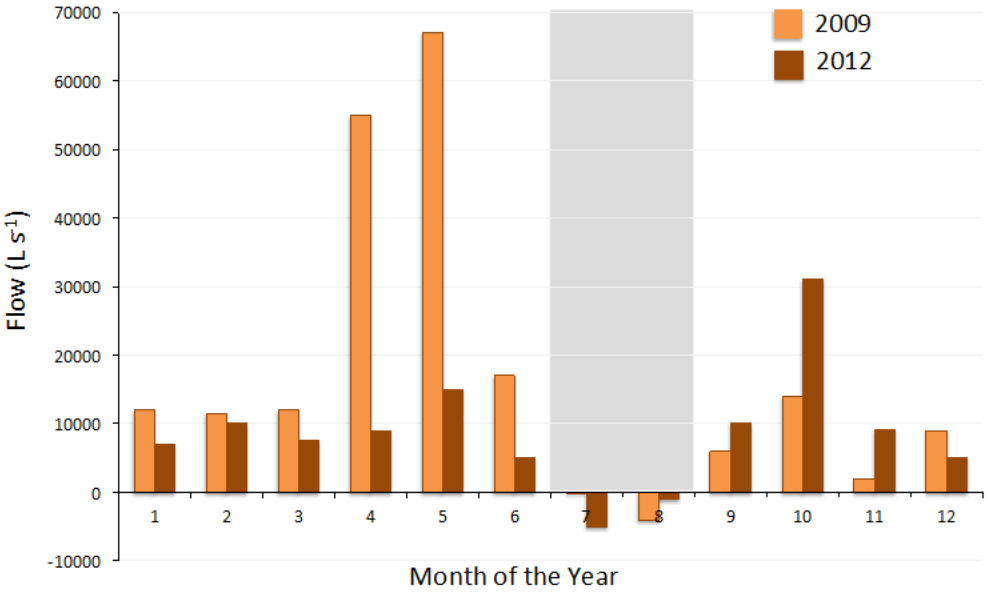

Figure 5.1. Variation in flow at Brandt Bridge in the Delta (years 2009 and 2012) illustrating the low- and reverse-flow window in July-August (shaded grey). Data and plot from Spier *et al.* 2013.

5.1.2 Temperature

Aside from the rate of water flow, water temperatures have increased globally over the last few decades as a result of global warming (Gille 2002, Hansen *et al.* 2005). In the Central Delta, a change from mainly negative deviations in the water temperature from the long-term mean to positive deviations occurred in 1999 (Figure 5.2). This local change in the water temperature may be part of the larger-scale global patterns and/or the Pacific Decadal Oscillation weather pattern which also changed sign in the same year (Cloern *et al.* 2007).

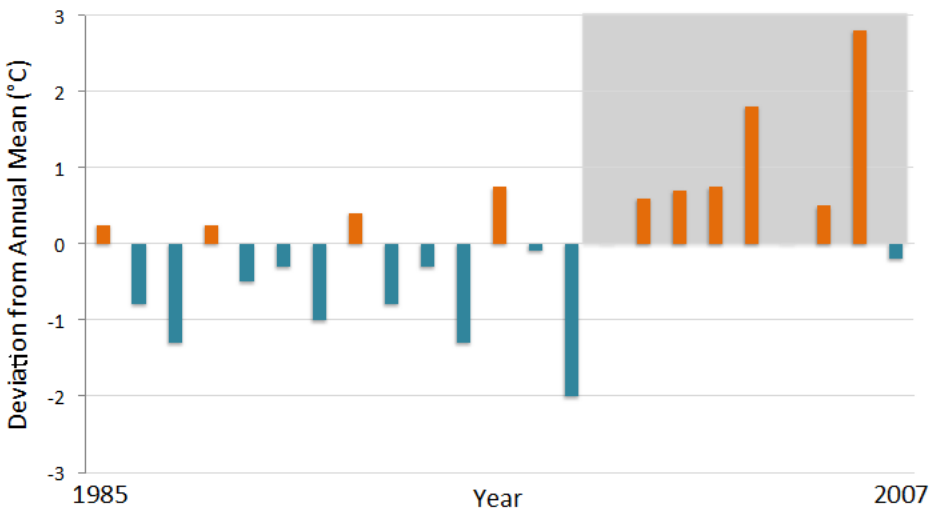

Figure 5.2. Deviation from the annual mean of maximum water temperatures at Stockton in the Central Delta. Grey shaded area indicates period from 1999 onwards with increased positive temperature deviations. Data from Brooks *et al.* 2011.

The interesting question with respect to changes in water temperatures is whether they are great enough to affect competition between cyanobacteria and other members of the phytoplankton community in the Central Delta. Presently, 40-75% of the phytoplankton community in the Delta is comprised of diatoms, followed by chlorophytes (15-30%), cyanobacteria (15-40%), cryptophytes (5-10%) and flagellates (0-10%), including dinoflagellates (Lehman 2007). In order for cyanoHAB species to to grow faster than diatoms and displace diatoms as the dominant member of the phytoplankton community, they would have to be able to accelerate their growth rates upto 2-3 fold. Alternatively, a scenario where the growth rate of diatoms would decrease and cyanobacteria would increase is necessary. Examining variation in growth rates with changes in environmental data, temperature appears the most likely candidate for bringing about such a change. Data from Figure 3.6 indicates that a doubling in cyanobacterial growth rates occurs with an increase in temperature from 20-27°C, whereas diatom growth rates decrease over the same temperature range. Therefore, a rise in temperature is a scenario under which cyanobacteria are able to outcompete diatoms.

This scenario is consistent with differences in temperature between a system, such as Clear Lake, where cyanoHABs dominate community composition, and the Delta. Comparing the 2011 environmental variables from Clear Lake and the Central Delta, two pre-bloom (June) differences become immediately clear. One is that the water temperature in Clear Lake is 7°C degrees warmer than the Delta (Figure 4.3). The other is that the Secchi disk depth is 2.6-fold greater in Clear Lake compared with the Delta (Figure 4.3). This difference in water clarity disappears in July when the *Microcystis* bloom takes off in Clear Lake, increasing Chl *a* 35-fold and decreasing the water clarity (Figure 4.3). Lehman *et al.* (2013) also predicted that the two

factors that potentially would make the greatest impact on accelerating the growth of *Microcystis*, and increase the frequency and duration of blooms in the Delta, would be increased water temperatures and increased water column clarity. The earlier in the growth season that these increases would occur the greater the window of opportunity for growth would become (see also Peeters *et al.* 2007).

5.1.3 Water Clarity

The Central Delta is highly turbid due to large amounts of sediments transported into the upper estuary via the Sacramento River as well as due to sediment resuspension. However, as more and more of the sediment load is being caught behind dams, sediment transport is on the decline and the upper estuary is becoming less turbid (Schoellhamer *et al.* 2012). Since 1975, turbidity at Stations D26 and D28 has declined by on average 2 and 4% per year, respectively (Jassby 2008). These average declines are accentuated by declines in turbidity of up to 50% during the low flow months (Lehman *et al.* 2013). If these present declines in turbidity in the Central Delta continue into the future, they may substantially promote growth of cyanoHAB species.

5.1.4 Nutrient Concentrations

If water temperatures did not increase above the summer-time average of 18-20°C, could there be a 2-fold acceleration in cyanobacterial growth rates with changes in N source, or with N:P ratio, at non-limiting nutrient concentrations that would enable them to outcompete diatoms and become dominant? To answer this question, we can 1) look to growth results from culture investigations and 2) investigate how nutrient ratios differ between a system that is overwhelmed by *Microcystis* (such as Clear Lake) compared with the Delta.

1) Culture investigations demonstrate that there is no significant, or consistent, change in growth rates with change in N source, or N:P ratios, at nutrient concentrations in excess of demand (Tilman *et al.* 1982, Tett *et al.* 1985, Reynolds 1999, Saker and Neilan 2001, Roelke *et al.* 2003, Sunda and Hardison 2007).
2) Comparing the ratios of dissolved N:P between the Delta and Clear Lake, 3.6±0.6 and 2.9±0.8, respectively, it's clear that these are essentially the same (Mioni *et al.* 2012). Nutrient ratios also do not vary from pre-bloom to bloom in the Delta, indicating that nutrients are in excess of phytoplankton demand for the entire summer season (Lehman *et al.* 2008, Mioni *et al.* 2012). Moreover, nutrient concentrations, or ratios, do not change sufficiently from year–to–year in order to explain year–to–year variation *Microcystis* biomass or occurrence. For example, since 1994 there has been no change in concentrations or ratios of nutrients in the Cental Delta (Appendix A).

Therefore, the initiation of *Microcystis* blooms around 1999 in the Delta was probably not associated with changes in nutrient concentrations or their ratios. However, as with all

phytoplankton blooms, once initiated, cyanoHABs cannot persist without an ample supply of nutrients. It is important to keep in mind that while nutrient reduction may not limit the onset or frequency of bloom occurrence, it will limit bloom duration, intensity and possibly also geographical extent. If, in the future, nutrient concentrations were to decrease to the point where they start to limit phytoplankton biomass, then the magnitude of the nutrient pool, as well as seasonal changes in the magnitude, would impact cyanoHAB concentration, distribution and bloom duration.

Interestingly, the long-term record for station D26 demonstrates that a decline in Chl *a* and corresponding increases in nitrogen concentrations (NH_4^+ and NO_3^-) and N:P ratios occurred in the period from 1985-1994 (Appendix A). Jassby (2008) reported similar changes in Chl *a* (decrease) and nitrogen (increase) at Central Delta Stations D16 and D28 between the years 1985 and 1994. Van Nieuwenhuyse (2007) hypothesized that the changes in N:P ratios and Chl *a* were driven by a decrease in phosphorus loadings to the Sacramento River that occurred in 1994; however the step change in P loading that year does not explain the gradual decrease in Chl *a* that started prior to 1994 (Appendix A).

Gradual decreases in Chl *a* concentrations may have been brought about by relative changes in flow and benthic grazing, leading to a new and lower Chl *a* equlibrium by the mid-1990's (Lucas and Thompson 2012). According to Lucas and Thompson (2012) the areas of the Delta where benthic grazing typically overwhelms phytoplankton growth rates are the same as those where *Microcystis* tends to bloom (Figure 4.1; Lehman *et al.* 2005). Because *Microcystis* floats at the very surface, it may avoid being grazed by clams in contrast with other phytoplankton that are distributed throughout the water column. It's important to bear in mind that large-scale (temporal and spatial) variation in environmental factors such as flow and grazing by clams may have a more profound impact on phytoplankton standing stocks, and competition among different phytoplankton taxa, compared with many of the autecological adaptations discussed in this review.

5.2 Summary

In the review of the global literature on factors influencing cyanobacterial blooms and toxin production, five principal drivers emerged as important determinants:

1) Water temperatures above 19°C
2) High irradiance and water clarity
3) Availability of N and P in non-limiting amounts; scientific consensus is lacking on the importance of N:P ratios and nutrient forms (e.g. ammonium) as a driver for cyanoHABs
4) Long residence times and stratified water column
5) Low salinity (<10 ppt) waters

Comprehensive understanding of the role of nutrients vis-à-vis other environmental factors in influencing cyanoHAB presence in the Delta is severely hampered by the lack of a routine monitoring program. The DWR monitoring program currently measures many of the environmental factors of interest, except cyanobacterial abundance and toxin concentration, which require a different approach than that used in standard phytoplankton monitoring. Drawing on the five factors influencing cyanoHABs, we can conclude the following:

- Because of the large effects of temperature and irradiance on accelerating, and decelerating, the growth rates of cyanoHABs, these two factors appear to exert key roles in the regulation of the onset of blooms. Cyanobacteria require temperatures above 20°C for growth rates to be competitive with eukaryotic phytoplankton taxa, and above 25°C for growth rates to be competitive with diatoms (Table 5.1). In addition, they require relatively high irradiance to grow at maximal growth rates. This is in contrast with diatoms that are able to keep near-maximal growth rates at irradiances limiting to cyanoHABs in the Delta, e.g., 50 µmol phot m^{-2} s^{-1} (Table 5.1).
- It appears that N and P are available in non-limiting amounts in the Delta; moreover concentrations, or ratios, do not change sufficiently from year-to-year to explain year-to-year variation in Microcystis biomass or occurrence. Therefore, the initiation of *Microcystis* blooms and other cyanoHABs are probably not associated with changes in nutrient concentrations or their ratios in the Delta. However, as with all phytoplankton blooms, once initiated, cyanoHABs cannot persist without an ample supply of nutrients. As long as temperatures, flow rates and irradiance remain favorable for growth, the size of the nutrient pool will determine the magnitude and extent of cyanoHAB blooms.
- Salinity is controlling the oceanward extent of cyanobacterial blooms in the Delta, but salinity gradients do not explain the spatial distribution of cyanoHABs in the Delta (Table 5.1). Notably, salinity regime is not a barrier to toxin transport, as cyanotoxins have been detected in San Francisco Bay.
- Higher flows, turbidity and lower temperatures during most of the year are likely restricting cyanobacterial blooms to the July-August time period.

Climate change and anthropogenic activity associated with land use changes have the potential to alter cyanoHAB prevalence in the future. Climate change will likely result in warmer temperatures and increased drought, the latter of which could result in reduced flows, increased residence time and water column stability leading to higher light availability in the Delta. Both higher temperatures and reduced flows would presumably result in a greater prevalence of cyanoHABs. It’s noteworthy that phytoplankton biomass and primary productivity are depressed relative to available nutrients in the Delta, so it’s unclear what the effect of modifying nutrient loads will have on frequency and intensity of cyanoHAB occurrence in the future.

Table 5.1. Summary of general physiological drivers of cyanobacterial growth, how they are manifested in population growth and competition with diatoms, and how they compare with environmental drivers observed to be operating in the Delta.

Physiological Driver	Population Driver	Observations in the Delta
Growth significantly slower below 20°C, and greater above 25°C, compared with eukaryotic phytoplankton taxa	Requires temperatures above 25°C for growth rates to be competitive with diatoms	Not observed at temperatures <19°C
Cyanobacteria have greater cellular N:P ratios than diatoms due to two light harvesting systems and peptide toxin production	At non-limiting nutrient concentrations, changes in ratios of nitrogen substrates or N:P does not affect competition among species or taxa	Nutrient concentrations, nitrogen speciation, and dissolved N:P ratios have not changed in the Delta over the last 25 years
Production of bioactive peptide compounds (toxic and non-toxic) results in high N demand of cells	Toxin production per cell is greatest at maximal growth rates; linked with external N concentrations and decrease at N limiting conditions; cyanoHABs do not secrete toxin	Inorganic N and P concentrations are at non-limiting concentrations for growth and toxin production; Variation in toxin produced per cell or in number of toxigenic vs non-toxigenic strains is not related to any specific environmental condition
Inefficient photosynthesis, low alpha; efficient at dissipating excess light energy via high concentration of carotenoid pigments in photosystems (*Microcystis*, *Anabaena* and *Aphanizomenon*)	CyanoHABs (*Microcystis*, *Anabaena* and *Aphanizomenon*) require high irradiance to grow; diatoms able to keep near-maximal growth rates at irradiances limiting to cyanoHABs (e.g. 50 µmol phot m^{-2} s^{-1})	High rate of water flow and mixing most of the growing season restricting blooms to low-flow periods (July-August), when turbidity is < 50 NTU, flow is <30 m^3 s^{-1} and irradiance > 50 µmol phot m^{-2} s^{-1} (Central Delta 2004-2008)
Growth optimal at salinities <10 ppt for most cyanoHAB species	CyanoHABs generally restricted to freshwater habitats and estuaries with salinities <10 ppt (Baltic Sea, San Francisco Delta, North Carolina)	Does not proliferate outside the Delta in the Sacramento River (freshwater) or Suisun Bay (mesohaline) suggesting that the primary agent restricting its spread is not salinity

6.0 RECOMMENDATIONS

The goal of this review is to synthesize available information to provide insight into cyanobacterial bloom occurrence in the Delta. The review has three major objectives:

1) Provide a basic review of biological and ecological factors that influence the prevalence of cyanobacteria and the production of cyanotoxins;
2) Summarize observations of cyanobacterial blooms and associated toxins in the Delta;
3) Synthesize literature to provide an understanding of what ecological factors, including nutrients, may be at play in promoting cyanobacterial blooms in the Delta.

This review found that the lack of a routine monitoring of cyanoHAB occurrence in the Delta greatly hindered our ability to summarize, with confidence, the status and trends of cyanoHABs in the Delta (Objective 2), and to what extent nutrients versus other factors were controlling their occurrence (Objective 3). Given this finding, our recommendations are focused on two principal actions:

1) Strengthening routine monitoring; and
2) Development and use of an ecosystem model, coupled with routine monitoring and special studies, to 1) understand controls on primary productivity and phytoplankton assemblage in the Delta and 2) test hypotheses regarding factors promoting or curtailing growth of cyanobacteria.

R1: Implement Routine Monitoring of CyanoHABs

DWR is currently conducting a monitoring program that routinely samples many of the variables of interest known to influence cyanoHABs. Comprehensive cyanoHAB monitoring should be added as a component to this program to fully evaluate risk to human and aquatic health as well as better understand linkages to factors that may be promoting or maintainting blooms.
To begin, a work plan should be developed which specifically scopes the needed changes in the program to comprehensively monitor cyanoHABs. Monitoring should include enumeration of major cyanobacterial species (e.g. *Microcystis*, *Aphanizomenon* and *Anabaena*). Sampling of toxins should include water column concentrations as well as mussel tissue concentrations or other important taxa that represent sentinels for bioaccumulation in the food web. Analyses of toxin concentrations should be expanded to include the six major cyanotoxins of concern identified in the OEHHA guidance in year 1 then adjusted based on the most commonly encountered toxins thereafter. In addition, selective sampling for analysis of concentrations of herbicides and fungicides commonly encountered in the Delta should be considered. The workplan should also consider monitoring needed to develop and calibrate an ecosystem model to further investigate controls on primary productivity and phytoplankton assemblage (see R2 below).

After an initial period of 3-5 years, the monitoring data should be used to comprehensively report on the status and trends of cyanoHABs and the factors that favor bloom occurrence in the Delta.

R2: Develop an Ecosystem Model of Phytoplankton Primary Productivity and HAB Occurrences to further Inform Future Risk and Hypotheses on Factors Controlling CyanoHABs

The Delta is at an advantage with respect to management of cyanoHABs in that naturally occurring high rates of flow and turbulence act to keep cyanobacteria in check. Despite this, future increases in temperature and residence time associated with climate change, increasing the degree and duration of stratification events, may substantially degrade the effectiveness of the Delta's breaking mechanism and increase the risk of cyanoHAB occurrences. Because nutrients are not currently limiting cyanobacterial blooms, it is critical that an improved understanding is gained of the factors that are controlling phytoplankton primary productivity in the Delta, since a relaxation of those factors followed by increased growth of phytoplankton could lead to increased risk of cyanoHABs.

To inform management actions moving into the future, an ecosystem model of phytoplankton primary productivity and HAB occurrences should be developed. This model should have the capability to provide information on primary productivity and biomass as well as planktonic food quality and transfer of carbon to higher trophic levels. Moreover, such a model could be used to assess the relative importance of environmental factors such as benthic grazing, flow, water column stability, temperature, to mention a few, at various times and locations in the Delta, on cyanobacterial growth. To step into model development, four steps should be taken: 1) examine existing models already available to determine suitability for this task, 2) utilize existing data from the Central Delta to explore, to the extent possible, the relationships between Chl *a*, phytoplankton composition, climate variables and other factors at stations where cyanoHABs are known to occur (e.g. D26, D28 and turning basin in the Stockton Shipping Channel). 3) Develop hypotheses regarding the environmental conditions in those areas that promote cyanoHABs. In addition, develop hypotheses regarding conditions needed to curtail cyanoHABs; including the effect of reducing nutrient loads on the entire phytoplankton community (including cyanobacteria) and on the transfer of carbon to higher trophic levels. These hypotheses can subsequently be tested through model development as well as potential future scenarios, and 4) a work plan should be developed that lays out the modeling strategy, model data requirements, and implementation strategy.

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APPENDIX A

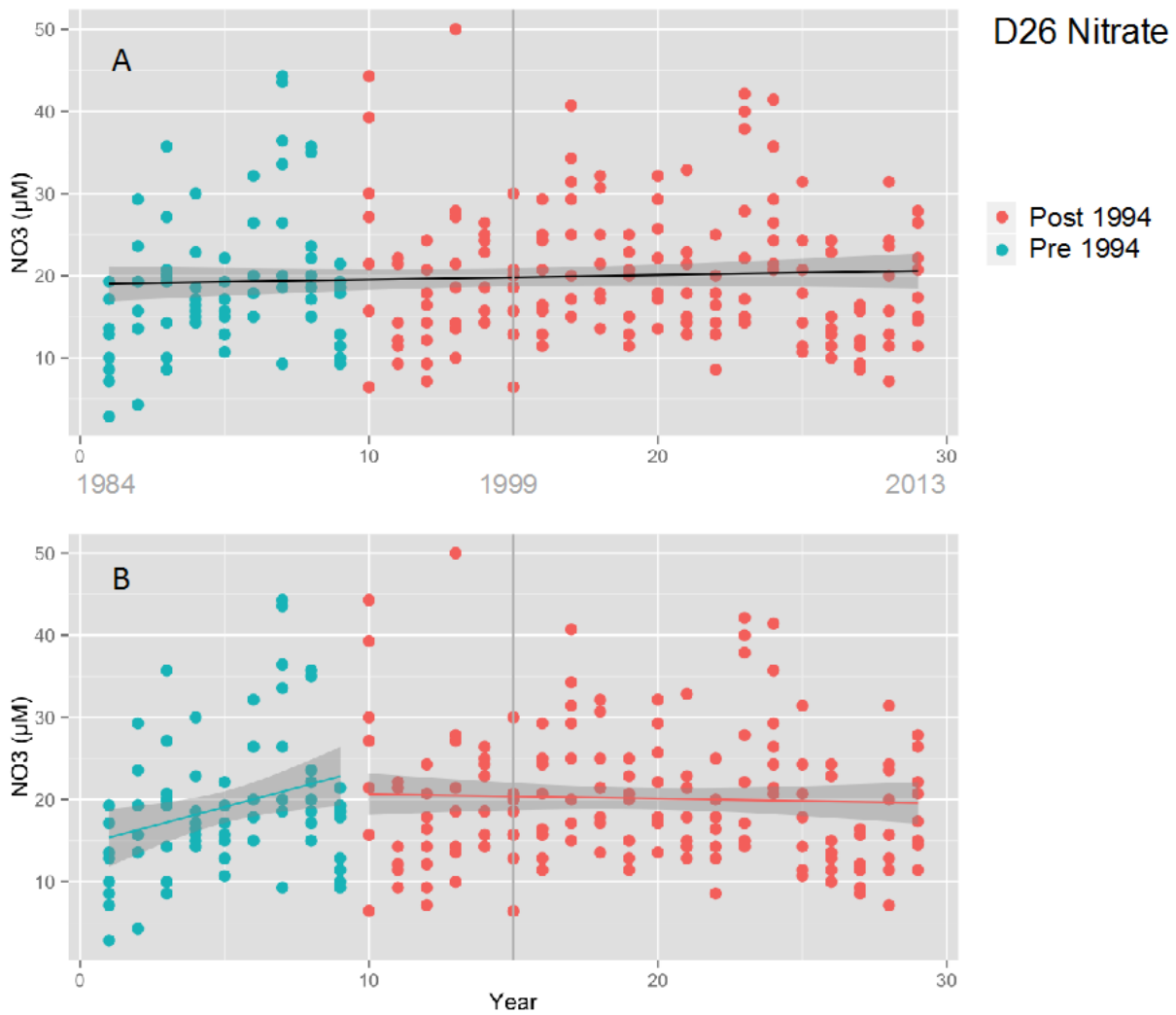

Figure A-1. Changes in the concentration of nitrate (NO_3^-) over time (1985-2013) at station D26 in the Delta. Green filled circles denote period before 1994 and red filed circles denote the period after 1994. Vertical grey line denotes the year 1999 when Microcystis started occurring. A) Regression of NO_3^- versus time for the period 1985-2013 (black line) with 95% confidence interval in grey. B) Regression of NO_3^- versus time for the period 1985-1994 (green line) and the period 1994-2013 (red line). Slopes significantly different from zero in bold in regression table:

Nitrate	1985-2013	1985-1994	1994-2013
Slope	0.09066	**1.374**	-0.02962
Probability	0.226	0.00149	0.832
multi- R^2	0.00424	0.09127	0.0001988

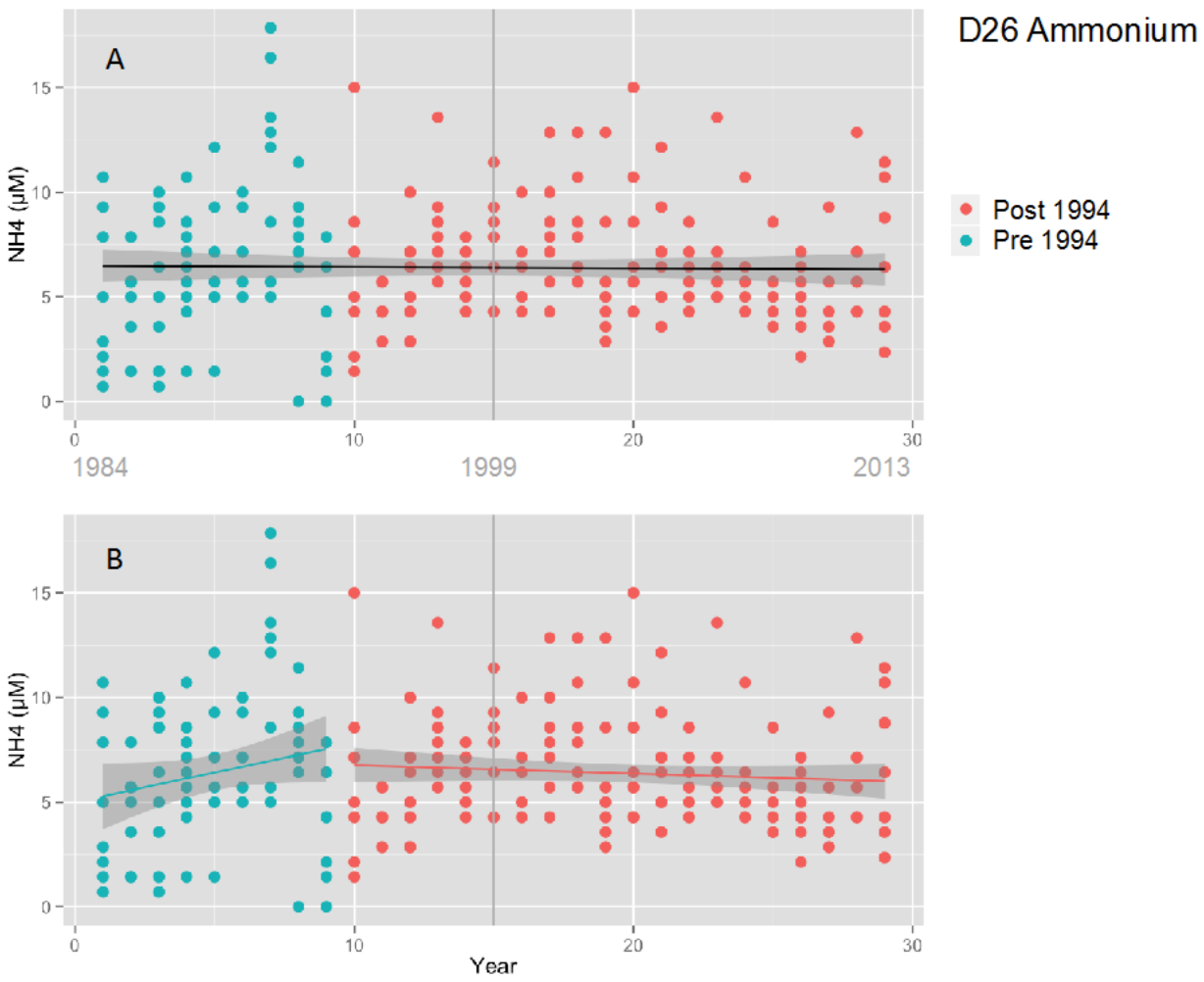

Figure A-2. Changes in the concentration of ammonium (NH_4^{+-}) over time (1985-2013) at station D26 in the Delta. Green filled circles denote period before 1994 and red filed circles denote the period after 1994. Vertical grey line denotes the year 1999 when Microcystis started occurring. A) Regression of NH_4^+ versus time for the period 1985-2013 (black line) with 95% confidence interval in grey. B) Regression of NH_4^+ versus time for the period 1985-1994 (green line) and the period 1994-2013 (red line). Slopes significantly different from zero in bold in regression table:

Ammonium	1985-2013	1985-1994	1994-2013
Slope	-0.038	**0.3801**	-0.03525
Probability	0.108	0.023	0.358
multi- R^2	0.007448	0.04779	0.00374

Figure A-3. Changes in the concentration of phosphate (PO_4^{3-}) over time (1985-2013) at station D26 in the Delta. Green filled circles denote period before 1994 and red filed circles denote the period after 1994. Vertical grey line denotes the year 1999 when Microcystis started occurring. A) Regression of PO_4^{3-} versus time for the period 1985-2013 (black line) with 95% confidence interval in grey. B) Regression of PO_4^{3-} versus time for the period 1985-1994 (green line) and the period 1994-2013 (red line). Slopes significantly different from zero in bold in regression table:

Phosphate	1985-2013	1985-1994	1994-2013
Slope	**-0.048906**	0.03673	-0.008772
Probability	2.00E-16	0.263	0.157
multi- R^2	0.2594	0.01183	0.008855

Figure A-4. Changes in the N:P ratio (mol:mol) over time (1985-2013) at station D26 in the Delta. Green filled circles denote period before 1994 and red filed circles denote the period after 1994. Vertical grey line denotes the year 1999 when Microcystis started occurring. A) Regression of N:P ratio versus time for the period 1985-2013 (black line) with 95% confidence interval in grey. B) Regression of N:P ratio versus time for the period 1985-1994 (green line) and the period 1994-2013 (red line). Slopes significantly different from zero in bold in regression table:

N:P Ratio	1985-2013	1985-1994	1994-2013
Slope	**0.3726**	**0.6236**	0.02932
Probability	3.79E-16	0.000572	0.736
multi- R^2	0.1747	0.1064	0.0005047

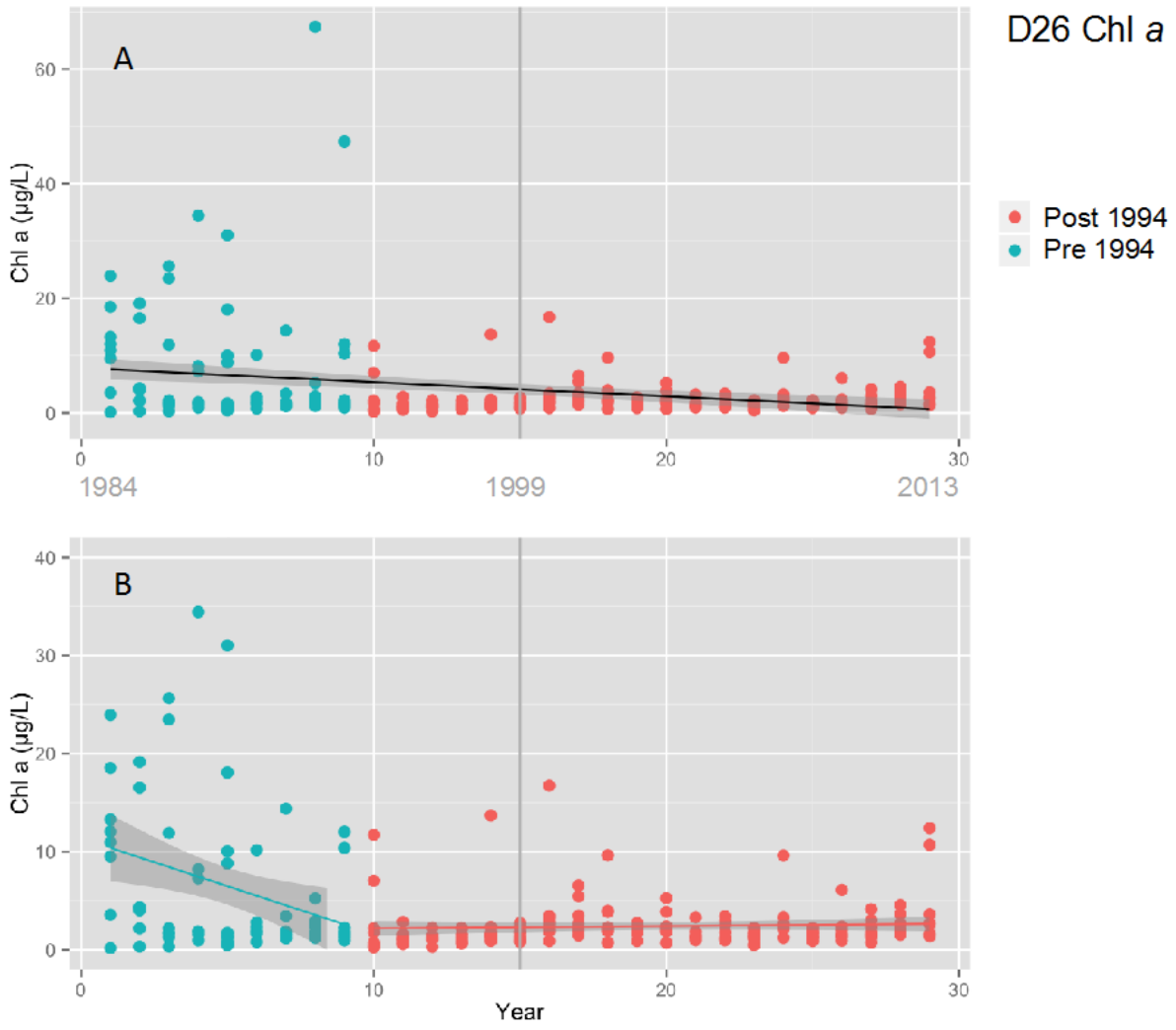

Figure A-5. Changes in the concentration of Chlorophyll a (Chl a) over time (1985-2013) at station D26 in the Delta. Green filled circles denote period before 1994 and red filed circles denote the period after 1994. Vertical grey line denotes the year 1999 when Microcystis started occurring. A) Regression of Chl a versus time for the period 1985-2013 (black line) with 95% confidence interval in grey. B) Regression of Chl a versus time for the period 1985-1994 (green line) with two of the high values from 1994 removed, and the period 1994-2013 (red line). Slopes significantly different from zero in bold in regression table:

Chl *a*	**1985-2013**	**1985-1994**	**1994-2013**
Slope	**-0.1676**	-0.7386	0.03936
Probability	2.87E-05	0.00759	0.1148
multi- R^2	0.05143	0.07266	0.01116

APPENDIX B

Comments from the Scientific Working Group and responses from the authors.

Author	Page	Comment	Response
Anonymous	iii	Under Finding #3, second bullet, regarding ratios of Nand P in Delta: I'm reading this to mean ratios of total N and total P (including various forms of each). I don't know that enough research has been done to determine if the ratios of the different forms can be an important driver.	Ratios of N:P are important drivers when one nutrient is in limiting supply and slows the growth rate down. Ratios of different forms of the same nutrient are important if a certain form produces a lower growth rate than the other; research on this topic is discussed under section 3.2.3 p24.
Foe	11	Under section 2.2.5, first paragraph, last sentence: Add something like this to last sentence on page 11, "it was deduced that *under nutrient limiting conditions*phytoplankton would become …"	Done
Foe	19	On pages 19, 22, and 38 you note that nutrient concentrations are one factor constraining the accumulation of cyanoHAB biomass. Can you estimate either from information from the delta or other waterbodies what range of N and P concentrations would be needed to limit cyanoHAB biomass and toxin levels below a low or moderate probability of human and wildlife health effects? Presumably there are a number of complicating factors including the fact that cyanoHABs co occur with blooms of other algal species which would also pull down nutrient levels. I understand that your estimate is likely to be fairly gross. Would it be possible to refine the range through a series of laboratory and/or field experiments? Could this be considered an information gap? Maybe discuss this somewhere around page 37?	I tried to do this in the original version where based on measurements of microcystin toxin that was harmful to aquatic life (0.8 µg/L) I calculated the amount of Microcystis-associated surface Chl a needed to produce that amount (7 µg/L). Because the science group did not like this estimation I've removed it from the paper. However, using 7 µg/L surface Chl a as a rough estimate, you would need greater or equal to 7 moles N/L to sustain such a level; this is not discussed in the current version
Foe	29	Second paragraph: You might note that Ger *et al.*, 2010 found that both toxin producing and non-toxin producing strains of Microcystis reduced the survival of both Eurytemora affinis and Pseudodiaptomus forbesi in 10 day lab bioassays. This suggests that the presence of other microcystis metabolites also contribute to overall toxicity.	A new section (4.2.4) on p39 entitled "Potential for CyanoHAB Risk to Delta Beneficial Uses" has been where the Ger (2010) paper and additional papers mentioned by Peggy Lehman are discussed
Foe	32	Under section 4.2.3, second paragraph: Brannan Island is located inside the legal boundary of the delta.	This sentence has been changed to read "Sacramento River" instead
Foe	35	Under section 4.2.4 under potential adverse effects on Delta beneficial uses: What can be concluded about the potential toxicity of cyanoHABs to aquatic organisms including zooplankton and larval fish in the Delta? Presumably there is the possibility of both direct and indirect effects. See Ger et al 2010 for an example of direct toxicity and Acuna et al (2012) and Deng et al (2010) for examples of bioaccumulation related effects. Peggy gave citations for all these papers. If uncertainty exists about the extent of	These effects and papers are discussed in a new section (4.2.4) on p39 entitled "Potential for CyanoHAB Risk to Delta Beneficial Uses". I think uncertainty exists regarding 1) whether the organisms reflect concentrations that are in the water column or 2) they bioaccumulate the toxin 3) what affects the zooplankton - toxic or non-toxic cells

		potential toxicity, then should this be listed as an information gap? What information is most important to collect first?	
Foe	38	Figure 5.2 shows nutrient trends at station D26 in the delta between 1994 and 2014. The conclusion is that nutrients concentrations are not changing. Longer term nutrient analysis suggest otherwise. Nutrient concentrations, N speciation, and dissolved N:P ratios have changed in the delta over the last 40 years. More DIN, more NH4, less SRP and an increase in the N:P ratio (Jassby 2008; Glibert, 2010[3] ; Van Nieuwenhuyse, 2007[4]) [3] Reviews in Fishery Science, 18:211-232 [4] Canadian journal of fisheries and aquatic science 64:1529-1542	I reanalyzed the nutrient data going back to 1985. My new interpretation is in section 5.1.4 on p43. I included the Van Nieuwenhuyse and Jassby citations. Appendix A provides plots of NO3, NH4, PO4, N:P, and Chl a from station D26. I demonstrate that one can draw different conclusions from these data depending on whether they are broken into separate time periods or analyzed as one long time course.
Foe	39	Around page 39. You note that cyanoHAB growth rates are a positive function of water clarity. The Delta has become clearer. The delivery of suspended sediment from the Sacramento River to the Delta has decreased by about half during the period between 1957 and 2001 (Wright and Schoellhamer (2004)[1] and this has resulted in a statistically significant -2 to -6 percent decrease per year in SPM between 1975 and 2005 (Jassby, 2008)[2]. Of course, it is uncertain whether the trend will continue. Might this increase in clarity also increase the frequency and magnitude of cyano blooms in Delta and make other factors like nutrients more important? [1] San Francisco Estuary and Watershed Science, 2004 volume 2, issue 2 [2] San Francisco Estuary and Watershed Science, 2006 volume 6, issue 1	This is true and I've added a new section (5.1.3) entitled Water Clarity (p 43) where this additional information is discussed.
Joab	ii	Second paragraph, second sentence. Add "the" between "by" and "Water Board".	Done
Joab	ii	Under Finding #2, item 1), change "e,g." to e.g.,"	Removed
Joab	1	Under section 1.1, first sentence. Add "in" between "found" and "Northern California".	Done
Joab	1	Last paragraph, first sentence regarding the commissioning of literature reviews: Actually we only commissioned two white papers (to date) on cyano	Changed to "two"

		and macrophytes. We are working on commissioning the third.	
Joab	4	Under section 2.1, first paragraph, fourth sentence. In sentence, "Cyanobacteria also produce and array…" Change "and" to "an".	Done
Joab	5	In Table 2.1, under the Nostocales (Group 4), is Cylindrospermum the correct name?	It is the correct name; however, I could just as easily have mentioned Cylindrospermopsin which is a more recognizable species.
Joab	6	Second paragraph, second sentence. You identify Group 5 as having toxic cyanoHAB-forming cyanobacteria: Don't you mean Group 4 based on the species identified in Table 2.1? Also, which group is Planktothrix in? I did not see them identified in the table - can they be added?	I did mean Group 4; it's been changed. I've also indicated in the text which subgroup Planktothrix belongs to
Joab	8	Under Ammonium transport section, third paragraph. Change "alterate" to "alternate".	Done
Joab	8	Under Nitrate transport and reduction section, last sentence regarding nitrate uptake: What concentrations of ammonia are relevant? Are these concentrations in the cells or the water column?	External; sentence changed to reflect this
Joab	9	First paragraph, first sentence: Carbon fixation seems to be very important in the nutrient uptake process. What controls carbon fixation? Is there someway to reduce their carbon fixation?	Irradiance controls CO2 fixation; this has been mentioned
Joab	9	Fourth paragraph, last sentence. Remove "have" between "their genomes" and "demonstrates".	Done
Joab	10	Under Nitrogen fixation, second paragraph, last sentence relating to n2 fixation under iron-limiting conditions: What is the iron-limiting condition? Do we know?	Where iron is not enough to support cell division
Joab	10	Under nitrogen fixation, last paragraph, seventh sentence. Correct the spelling of "heterocyst".	Done
Joab	11	First paragraph: What are the conditions for N starvation?	When N concentration is not enough to support cell division of available biomass
Joab	19	In Figure 3.1, step 6 states to add grazers: Are their cyanobacteria grazing fish and zooplankton?	This figure was very busy and included many processes not discussed in the White Paper; I've substituted a new and simpler figure
Joab	38	Under section 5.2, first paragraph, first sentence: This citation is now 8 years old. Is there any recent information to suggest if these percentages have changed significantly?	Not that I'm aware
Joab	39	First paragraph: Correct the spelling of "cyanHABs" to "cyanoHABs". Do global search in document to check spelling of cyanoHAB.	Done
Joab	39	Second full paragraph: In sentence, "In Clear Lake, Both N and P…" delete capital B and make lowercase.	Sentence changed

Joab	41	In Table 5.1, Observations in the Delta "temperatures above 25° C rarely occur." - Temperatures in the San Joaquin River near Stockton have over the past 3 years (2012-2014) reached over 25°C from June through October, most likely due to this persistent drought and overall increase in temperature.	Sentence has been removed
Kudela	31	Figure 4.2. I think this is an issue with Peggy's original figure, because I remember seeing it before, but the chlorophyll units don't make much sense. 0.1 ng/L is barely detectable under the best of circumstances.	Y-axis corrected to µg/L
Kudela	N/A	The toxin table is very thorough, but it might be worth pointing out that, based on available information, Central California seems to be dominated by microcystins. We have all of those genera present but we don't very often see saxitoxins or anatoxin-a. Admittedly we don't look that often either, but we have tested some samples from Clear Lake, SF Bay, and Pinto Lake. We very rarely get low levels of STX, and one low hit for anatoxin-a in Clear Lake. We did see low levels of anatoxin-a in Lake Chabot also, and if you go further north, anatoxin-a becomes dominant in the Eel River basin. This supports Mine's decision to focus on microcystins in the report, but the implication of that section is that we could see a wide variety of toxins, and we usually don't.	This has been pointed out in the first paragraph of section 4.2.3
Kudela	N/A	Temperature. While I completely agree with Mine's summary, bear in mind that we do see toxin at low temperatures (this is documented in Kudela 2012 and Gibble and Kudela 2014). We were not tracking species, but it seems likely that it's related to a shift in composition to more cold-tolerant species such as Planktothrix. We tend to get two peaks of toxicity—one at lower biomass and cooler temperatures, and the second (larger) when Microcystis is dominant.	I was not aware of the Gibble Kudela paper; would like to add appropriate discussion
Kudela	N/A	Marine toxins. I'm not sure I completely believe it but there is a recent article (which I can't find right now—looking for it) that documents presence of microcystins in marine waters, from marine cyanobacteria.	Noted

Kudela	N/A	I'd be very supportive of developing an ecosystem model, but for CHABs in particular you probably need a fairly complex model that can parameterize both end-members (riverine and marine). A good hydrodynamic model would be a great place to start. I'm not sure how easy or difficult it would be to add a biological model on top of that, or whether you'd need two models, etc. It's probably my own bias but I would start with assembling all the available data and run statistical analyses on that (Peggy's done quite a bit of this already) to see what variables emerge as most important. Cecile Mioni has been attempting that with the Bay/Delta data and it's been interesting, in that there are no clear physical drivers related to cell abundance or toxicity. She looked at all the usual ones, temperature, salinity, nutrients, etc. suggesting that either there's not enough data (a real possibility) or that it's not a simple relationship. That of course leads back to the need for more monitoring and modeling.	Noted
Mussen	iii	Under Finding #4, third sentence regarding increased nutrient loading: With continued regluatory controls on nutrient loads into the system, we should not necessarily expect nutrient loading to increase substantially in the future.	This has been removed
Mussen	1	Under section 1.1, in fourth sentence "The Delta is widely recognized as in "crisis" because of competing demands…" Add "human effects on the environment and" between "because of" and "competing".	Done
Mussen	4	Last paragraph, second sentence. Add "in local communities" between "irrigation of farms" and "as well as". Plus, remove the words "drinking water to" after the words "as well as".	Sentence has been revised
Mussen	7	Under Carbon Fixation, fifth sentence. Add "near" between "concentrate CO2" and "its vicinity".	Sentence has been revised
Mussen	28	Under section 4.1 Ecosystem Services, second paragraph, third sentence: Change "Striped Bass" to "juvenile-Striped Bass".	Done
Mussen	29	First paragraph, fourth sentence: "At high densities…(Paerl 2004, Paerl and Fulton 2006)" is a repeat from text in the paragraph above on page 28.	Noted; the repeat text has been removed
Mussen	29	First paragraph, sixth sentence "At dense concentrations…" - If low nutrient concentrations can be used to limit the magnitude of future cyanoHAB blooms, the effects of lower nutrient concentrations must also be considered for all other plant and algae species growing in the system (this is especially important for the period followin onset of a future cyanoHAB blooms where nutrients in the area would be fully depleted).	Noted; this point has been brought up in the recommendations section (6.0) in conjunction with hypotheses development

Mussen	38	Under section 5.2, second paragraph, first sentence refering to growth of cyanoHABs versus diatoms: Without nutrient limitation, growth rates may not determine which phytoplanktong species is dominant in the system. Other factors such as light availability, buoyancy, temperature, salinity and grazing pressure may determine the dominant species.	This sentence, presently in section (5.1.4) has been revised to clarify point
Mussen	40	Under second bullet, third sentence concerning blooms not persisting without ample supply of nutrients: Once a bloom consumes the available nutrients, would nutrient remineralization be able to sustain some lower concentration of cyanoHABs presence throughout the remainder of the growth season? Could cyanoHABs persist at harmful levels in this manner?	I think typically not; harmful levels require a certain level of biomass to be sustained
Mussen	40	Under second bullet, third sentence: Add "flow rates," between "temperatures," and "and irridiance".	Done
Mussen	40	Under second bullet, third sentence: Remove "s" from word "remains".	Done
Mussen	40	Last paragraph, fourth sentence starting with "Increase nutrient loading…": Please see my comment above on increased nutrient loading.	This has been removed
Mussen	42	Under R1, second paragraph discussing enumeration of cell counts: What about the inclusion of "and average biomass?"	Controversy regarding how it is to be measured; could be discussed under recommendations
Mussen	43	Under R2, first paragraph, second sentence: Replace "higher chlorophyll a" with "increased phytoplankton growth in the Delta".	Done
Mussen	43	Last paragraph, first sentence concerning informing management actions: It is also important to model expected nutrient levels with levels of reduced loading. The time required for a reduction and the amount of nutrient regeneration in a system can be highly variable.	Section expanded in order to note this point
Mussen	43	Last paragraph, first sentence. Add "s" to "action" making it "actions".	Done
Mussen	43	Last paragraph, second sentence regarding modeling primary productivity and biomass: CyanoHAB growth rates under ideal conditions (which may be used as the basis for a model design) can be quite different from their growth rates at near-limiting nutrient conditions. Do we know what low nutrient concentrations (thresholds) would be necessary to prevent the overgrowth of different cyanoHABs? How would other plants and algae in the system be affected by low nutrient concentrations? With limited nutrients, can we predict which phytoplankton species would be dominant in the system, and how the dominant species may change with climatic factors such as temperature, flow, and turbidity, or with differing grazing rates?	Section expanded in order to note this point

Orr	iii	Under #3, first bullet - During the last meeting lower temperatures (18°C) were discussed. Are there references for the blooms at lower temperatures in the delta?	None that I'm aware of
Orr	28	For the last sentence on page 28 under section 4.1. Ecosystem Services, "CyanoHABs also can cause night-time dissolved oxygen depletion via bacterial decomposition and respiration of dense blooms which results in fish kills and loss of benthic fauna (Paerl 2004, Paerl and Fulton 2006) - Does this occur in the Delta or is flow mixing sufficient to prevent the issue?	This is an example of an adverse effect noted in other systems
Orr	29	In the second paragraph, the sentences starting with "At low concentrations…(Lehman *et al.* 2010)" are already in the preceding paragraph. Consider removing.	This has been removed
Orr	29	Regarding the thrid sentence at the top of the page, "However, even at low concentrations, toxins released (upon death and cell lysis, or by grazing) can bioaccumulate in higher trophic levels (Lehman *et al.* 2010) - There is some disagreement on this topic in the literature. Based on the Lehman paper alone it seems unclear whether the toxins bioaccumulate or simply occur in tissue at concentrations that are not greater than the surrounding environment. In other systems it depends on the particular toxin and species in question. I recommend removing the "even at low concentrations" to make a more conservative statement. Another option would be to state they have been observed in higher trophic levels in the delta and leave the bioaccumulation to be addressed in recommendations or further research.	This sentence has been modified
Orr	32	Under section 4.2.3, last sentence in first paragraph "Using the relationship 115 ng microcystin µg surface Chl a^{-1} (Figure 4.4), *Microcystis*-associated surface Chl a concentration of 7 µg L^{-1} (sampled using a horizontal net tow) would produce enough microcystin (800 ng L^{-1}) to reach the OEHHA Action Level, and constitute an action level for the Delta." I am concerned with the concept of using Chl a to determine actions levels. While Chl a and microcystin levels are related the correltation is not linear and does not take other cyanotoxins into account. Whether or not chl a correlates with other toxins would be an interesting question.	This can be discussed further; to be on the safe side I removed Figure 4.4 and the calculation of a surface Chl a level that could potentially constitute an action level

Orr	36	Under section 5.1, last half of paragraph relating to flow and turbidity - Is there data to suggest that increased turbidity reduces risk of HABs in the delta that is independent of flow rate or temperature? HABs are common in other water bodies with high turbidity. The observation the HABs are controlled by turbidity may be an artifact of higher flows and lower temps. In low flows and turbid water could buoyancy regulating species stay near the surface to receive the necessary light intensity?	Yes, I do think that the effect of turbidity cannot be separated from the effect of flows in the Delta; whether turbidity alone has the same effect is not clear. I have revised this statement to reflect that the two covary
Orr	42	Under R1, second paragraph discussing monitoring - Consider not listing species. If the plan is long term the species of concern may change or expand.	Adaptive management strategies should take care of that; the species are listed as an example
Orr	42	Under R1, last sentence in first paragraph, correct the misspelling of "calibrate".	Done
Orr	N/A	The introductory sections have a broad perspective regarding toxigenic algal species. However, the discussion of factors influencing cyanobacterial blooms appears to focus on microcystins as a model for all blooms. I think the dicussion of other species should be increased.	The literature is heavily tilted towards microcystins therefore the white paper as well. However, Kudela noted in his comments that cyanobacterial toxins other than microcystins are almost not detected in the Delta; a statement to this effect has been added in the first paragraph of section 4.2.3
Orr	N/A	I am concerned about how tubidity is discussed. If data is available I recommend discussing it separately from flow and temperature. If turbidity related data is not available avoid general assumptions regarding its influence on blooms.	I have repeated previously published statements regarding turbidity and Microcystis in the Delta; the assumptions in the published work are stated. A new section (5.1.3) on water clarity in the Delta has been added.
Orr	N/A	It was unclear to me what the end goal of the monitoring program is. If a clearer question(s) can be developed I encourage adding a more specific monitoring plan.	To be discussed at the next meeting
Orr	N/A	I heard some monitoring questions from the group and am interested in how common these questions are among the group. I suspect there will be some disagreement about the hypothesized answers but the questions seemed shared. (See 4 questions below)	Noted
Orr	N/A	1. When and where do we reach the required surface temperatures for a bloom? (microcystis exclusively?) a. What is the appropriate depth to measure temperature?	Noted
Orr	N/A	2. Do nutrient limited conditions occur during blooms in the delta? Presumed not to. a. Does this occur in some areas but not others? b. Are we close enough for this to occur in near future? c. Is this question species or nitrogen source dependent in a non-limited system?	Noted
Orr	N/A	3. Spatially where are both temperature and nutrients high and do we need more spatial resolution?	Noted

Orr	N/A	4. Is chlorophyll a the right parameter to be measuring? a. Does it correlate with microcystin concentrations?	Noted
Taberski	iii	Delete "already exists" under the section R1, first sentence.	Done
Taberski	1	Add "of" under section 1.1, 4th sentence "…Delta is widely recognized as in "crisis" because *of* competing demands…"	Done
Taberksi	1	Delete "d" in word "declined" under section 1.1, last sentence "…including the continued declined of …"	Done
Taberski	22	The paragraph under sub-section "Confounding factors:" is not clear, particularly the last sentence is confusing.	This sentence has been revised
Taberski	29	In the 5th sentence at the top of the page, insert a space in the word "watercolumn".	Done
Taberski	32	In table 4.1, I think you should also include the OEHHA thresholds.	Table below has OEHHA thresholds
Taberski	39	Under the last paragraph for section 5.2, the last sentence "…nutrients are unlikely to play a role in the onset or frequency of bloom occurrence in the Delta." - I agree. Nutrient concentrations would play a role, though, in the magnitude (concentration) and duration of a bloom. If nutrients were lower, they would be depleted more quickly and the bloom would crash. This was stated in the Summary bullet #2. That clarification should be added to this paragraph.	This has been added
Taberski	40	Under the second bullet, in the third sentence, correct the misspelling of "initiated".	Done
Taberski	40	In the last paragraph, in the second sentence, put a space in the word "watercolumn".	Done
Taberksi	40	In the last paragraph, in the third sentence, change the sentence to read as "Both *higher* temperatures and reduced …"	Changed
Taberksi	42	Under R1, first sentence, delete the wording "already exists".	Done
Taberski	N/A	A section should be added on risk to aquatic life.	Done
Taberski	N/A	Historical data should be analyzed based on driving factors to evaluate risk (areas with high temperatures/low turbidity/long residence time)	Example analysis of nutrient concentrations at station D26 performed; included in Appendix A
Taberski	N/A	Recommended monitoring should be based on specific management questions related to status and trends, hotspots, risks to humans, animals and aquatic life, and directing management actions.	Noted
Taberski	N/A	Monitoring information should be collected on processes and projections needed for modeling cyanoHABs and directing management actions. The SF Bay RMP's management questions could be used as a model for developig management questions for cyanoHABs. The RMP's management questions are:	Noted

Taberski	N/A	1. Are chemical concentrations in the Estuary at levels of potential concern and are associated impacts likely? a. Which chemicals have the potential to impact humans and aquatic life and should be monitored? b. What potential for impacts on humand and aquatic life exists due to contaminants in the Estuary ecosystem? c. What are appropriate guidelines for protection of beneficial uses? d. What contaminants are responsible for observed toxic responses?	Noted
Taberski	N/A	2. What are the concentrations and masses of contaminants in the Estuary and its segments? a. Do spatial patterns and long-terms trends indicate particular regions of concern?	Noted
Taberski	N/A	3. What are the sources, pathways, loadings, and processes leading to contaminant-related impacts in the Estuary? a. Which sources, pathways, and processes contribute most to impacts? b. What are the best opportunities for management intervention for the most important contaminant sources, pathways, and processes? c. What are the effects of management actions on loads from the most important sources, pathways, and processes?	Noted
Taberski	N/A	4. Have the concentrations, masses, and associated impacts of contaminants in the Estuary increased or decreased? A. What are the effects of management actions on the concentrations and mass of contaminants in the Estuary? B. What are the effects of management actions on the potential for adverse impacts of humans and aquatic life due to Bay contamination?	Noted
Taberski	N/A	5. What are the projected concentrations, masses, and associated impacts of contaminants in the Estuary? A. What patterns of exposire are forecast for major segments of the Estuary under various management scenarios? B. Which contaminants are predicted to increase and potentially cause impacts in the Estuary?	Noted
Thompson	ii	You only have four, not five, major findings identified in the Executive Summary section	Corrected
Thompson	iii	Under Finding #3, first bullet, second sentence relating to temperature for growth: Should we specifiy the time frame over which the temperature is measured? e.g., instantaneous, daily average, daily max or min. This will matter more when we get to modeling phytoplankton dynamics.	Save for the modeling

Thompson	19	Under section 3, first sentence: Correct spelling of word "prompted" by adding a "p" between "m" and "t".	Done
Thompson	20	Under section 3.1, in sentence "Indeed, recent decades has witnessed…" Replace word "has" with "have".	Done
Thompson	20	Under section 3.2.1, first paragraph, reference Edmondson and Lehman 1981 was not included in the reference section.	Done
Thompson	21	Under Cellular N:P compostion section: Reference Mills *et al.* was not included in the reference section and date missing in citation.	Corrected; citation added
Thompson	22	Under Confounding Factors, third sentence: Should we introduce the concept that there may be time lags between nutrient uptake and increased biomass, such that a correlation between two variables at a given point in time may not imply causality?	Good idea; sentence added under confounding factors on page 23 of revised manuscript.
Thompson	22	Under Confounding Factors, third sentence discussing parameters: Is there a diagram from a paper or textbook that we could borrow and reference, that shows the patterns of these variables over time before, during and after a bloom? (e.g., temperature, nutrient concentration, nutrient uptake rate, phytoplankton biomass). Something to show phytoplankton biomass peaking as nutrients draw down.	I found one diagram that showed a dinoflagellate peaking as nutrients were drawn down but nothing for cyanobacteria; after looking for the same pattern for cyanobacteria for half day I gave up
Thompson	27	Last paragraph under section 3.6 on stratification and residence time: Suggest adding a brief discussion of the potential role of ferrous iron. See Molot *et al.* 2014. A novel model for cyanobacteria bloom formation: the critical role of anoxia and ferrous iron. Freshwater Biology 59:1323-1340. The article mainly deals with lakes but there is a section on page 1330 that mentions shallow, nearshore regions of lakes, including harbors, inshore areas of Lake Erie, and embayments of Georgian Bay (Lake Huron). [**Text from Introduction shown on next line**.]	The potential role of toxins acting as siderophores and aiding cyanobacteria with iron uptake providing an advantage in competition with eukaryotes is discussed in a new expanded paragraph on p. 19 and the Molot *et al.* citation has been added to this section.

Thompson	27	**Here's some text from the Introduction**: "We cannot predict with any certainty when a cyanobacteria bloom will begin once temperatures are warm enough to support growth or the duration of a bloom except through empirical observations from previous years. Nor do we know why the problem is worsening in some mesotrophic systems." "Clearly, the predictive state of cyanobacteria science is unsatisfactory. This dissatisfaction may have contributed to the recent debate challenging he supremacy of the P paradigm in eutrophication management. Wurtsbaugh, Lewis, Paerl, and their colleagues argue that N plays a major role alongside P in promoting cyanobacteria blooms and that both N and P should be controlled (refs). This argument has been vigorously challenged in return by Schindler and his colleagues who claim that controlling N to control cyanobacteria will not work because N-fixation by cyanobacteria will compensate to a large extent for induced N shortages (refs). The outcome of this on-going debate can be expected to influence the direction of billions of dollars in public expenditures to remedy nutrient loading." "Our purpose here is to present a novel model that does not supplant the important roles of P and N as major macronutrients, but instead weaves additional ideas into older ones to create a novel and more comprehensive conceptual framework with much more explanatory power that spans the range of conditions where cyanobacteria blooms have been observed."	Noted
Thompson	28	Under section 4.1 Ecosystem Services, second paragraph, Reference Sommer *et al.* 1997 not included in reference section.	Citation added
Thompson	30	Figure 4.1 - Can we get a higher resolution version of this map? It was blurry in the original Word version, prior to becoming a Google doc.	Will investigate
Thompson	36	Under section 5.0, first paragraph, last sentence: Should we specify that the variables may need to be time-lagged in order for the correlations to be apparent?	I actually prefer to be vague in case entirely different statistics are needed
Thompson	38	Under section 5.2, first paragraph, second sentence referring to Microcystis and Aphanizomenon becoming more common: Is the reference for this statement the Lehman 2007 paper? I think it would be worth referencing it again at the end of this sentence, or adding an additional reference as necessary.	This is based on Lehman's 2008 paper and the Mioni *et al.* 2012 report; thesecitations have been added
Thompson	38	Under section 5.2, second paragraph, second and fourth sentence refering to Figure 2: I think this is now [Figure] 3.3. Check Figure number.	Corrected: now figure 3.6

Thompson	39	Second full paragraph, reference to Figure 4.5: This information is not shown in this figure. Check your Figure number.	Correct, the reference to this figure has been deleted
Thompson	39	Second full paragraph, last sentence related to culture investigations: It would strengthen the point to reference (re-reference) some key papers here.	Done
Thompson	41	In Table 5.1, Observations in the Delta "when turbidity is <50 NTU, flow is <30 m^3s^{-1} and irradiance >50 µmol phot $m^{-2}s^{-1}$": Please briefly state where in the Delta this was measured, and over what spatial and temporal scale.	Done
Ward	N/A	**Comment 1:** Of the five questions the Work Group is tasked with answering, the first is to determine whether the principal physical and biological factors promoting cyanobacteria blooms and toxin production in the Delta have been identified. My reading of the current work in this area leads me to conclude that these factors have not yet been adequately characterized. More importantly, the critical task of accurately gauging the relative weight of various factors that are known to influence/control the formation of toxigenic (or other) blooms still seems beyond our capability at present, whether in the Delta or in other waterbodies for which some relevant data is available. These deficiencies are particularly problematic for the development of a model that has practical utility. The field work and laboratory studies on Delta water quality and Delta species involved with the Pelagic Organism Decline that were cited in the draft white paper and/or distributed to the Work Group are largely “Microcystis-centric” and “microcystin-centric”. There is, in my view, a very large risk in attributing (1) all significant microcystin production to Microcystis in the Delta, and; (2) focusing on microcystin(s) to the exclusion of the effects of other possible toxigenic genera and other cyanotoxins. Dr. Berg’s draft white paper duly notes the existence of many other toxigenic genera and other cyanotoxins, but it seems the Delta-specific research on these possibilities may not yet be available for review.	Noted; Please see new comment under section 4.2.3 on toxin data available from Central California demonstrating that very few detections of toxins other than microcystins have been made in the Delta

Ward	N/A	**Comment 1 continued:** This is not a trivial point: for example, various Aphanizomenon strains can produce saxitoxin, microcystin(s), cylindrospermopsin, BMAA, and anatoxin-a (Paerl & Otten, 2013), and Lehman *et al.* have noted the presence of this genus in the estuary, bay and/or Delta. Though it is quite possible that I have overlooked Delta-specific studies on Aphanizomenon strains which examined the possibility that one or more of these toxins is present, if it is true that these studies have not been conducted yet, it would be ill-advised to presume that microcystin(s) are some sort of "model" toxin that can be regarded as a generic equivalent of all of the others in a subsequent modeling exercise, especially given their chemical and toxicological heterogeneity. Similarly, the diazotrophic cyanobacteria such as Aphanizomenon may respond rather differently to "nutrient limitation" (of nitrogen) than the non-diazotrophic genera such as Microcystis. If both genera produce microcystins, then microcystin production per se may continue in a water body as nitrogen becomes more limiting for Microcystis. Comparisons of diazotrophic cyanobacteria with non-nitrogen fixing cyanobacteria to nitrogen-limited conditions tend to show the following pattern: diazotrophs (e.g., Aphanizomenon) tend to produce toxins such as microcystin under nitrogen-limited conditions, whereas non-nitrogen fixers such as Microcystis and Planktothrix increase toxin production under non-limiting conditions.	Not necessarily; please see Dolman 2012 citation for patterns of abundance of various species and toxin production in over 100 lakes in Germany under different N:P scenarios described in "Meta analyses of Lake Studies" on page 24.
Ward	N/A	**Comment 1 continued (references):** Holland, A., Kinnear, S. Interpreting the possible ecological role(s) of cyanotoxins: compounds for competitive advantage and/or physiological aide? Marine Drugs 2013, 11(7), 2239-2258 http://www.mdpi.com/1660-3397/11/7/2239 Paerl, H. Otten, T. Harmful Cyanobacterial Blooms: Causes, Consequences, and Controls. Microbial Ecology 2013 May;65(4):995-1010 http://www.unc.edu/ims/paerllab/research/cyanohabs/me2013.pdf Leao, P. *et al.* The chemical ecology of cyanobacteria. Natural Products Reports, 2012 Mar;29(3):372-91 http://www.ncbi.nlm.nih.gov/pmc/articles/PMC4161925/pdf/nihms-599340.pdf	
Ward	N/A	**Comment 2:** Given my time limitations for reviewing more recent work on how/whether nutrient management can reduce the magnitude and frequency of cyanobacteria blooms and toxin formation, I was unable to conduct the review I had originally anticipated on this question.	Noted

Ward	N/A	**Comment 3:** I believe the draft white paper correctly examines and compares the relative significance of various factors in controlling the growth and development of toxigenic blooms based on the limited data now available on this subject that is “Delta-specific”. However, as stated in answer to Question 1 (above), I also believe the factors considered, while appropriate, are nevertheless an incomplete list. At our meeting I mentioned the apparent role of competition for iron as a factor in bloom formation and dominance in freshwater ecosystems, and provided a citation for this. Other factors which should be considered include the differences in sensitivity to herbicides between cyanobacteria and other phytoplankton that are being reported in studies conducted elsewhere, and the role of allelopathy in bloom formation, dominance, and senescence. Allelopathy is also discussed in references provided in answer to Question 1. For pesticides – in this case, I focused on herbicides – please refer to references provided below.	Allelopathy was dicussed in the original version of the White paper under "Potential Functions of toxin production" on page 18. Two new references have been added to the previous references on allelophathy in this section.
Ward	N/A	**Comment 3 continued (references):** The USGS maintains an online geo-referenced database which charts the most commonly-used pesticides in CA as they have continued to change in recent years that is current through 2012: http://water.usgs.gov/nawqa/pnsp/usage/maps/compound_listing.php Lurling, M., Roessink, I.On the way to cyanobacterial blooms: Impact of the herbicide metribuzin on the competition between a green alga (Scenedesmus) and a cyanobacterium (Microcystis). Chemosphere, 2006, 65:4, 618-626. Peterson, H. *et al.* Toxicity of hexazinone and diquat to green algae, diatoms, cyanobacteria and duckweed. Aquatic Toxicology, 1997, 39(2), 111-134. Arunakumara, K. *et al.* Metabolism and degradation of glyphosate in aquatic cyanobacteria: a review African Journal of Microbiology Research, 2013 Vol. 7(32), pp. 4084-4090. http://www.academicjournals.org/article/article1380269900_Arunakumara%20et%20al.pdf	The potentially important influence of herbicides and fungicides on the prevalence of cyanobacteria vis-à-vis other phytoplankton is discussed in a new Section 3.7 on p. 31 and again under Section 4.2.1 p 33. Because concentrations of herbicides in the Delta have been demonstrated to be quite high, a recommendation has been added that selective sampling for herbicides and pesticides be instituted in the Delta.
Ward		**Comment 4**: In answer to this question, please see the additional references supplied in answer to Questions (1) and (3).	A citation by Holland and Kinnear (2013) has been added on the benefits of toxin production under iron limiting conditions as mentioned in previous comments.

Ward	N/A	**Comment 5:** Overall, I agree with the draft recommendation put forward regarding monitoring of CyanoHABs (Recommendation 1), but would place more emphasis on monitoring for more immediate threats to public health e.g., intakes for drinking water treatment plants either within the bloom-prone areas of the Delta. The waterboard's drinking water program staff has informed me that some public water supply systems are struggling to successfully contend with this issue elsewhere in California, and this may also be a recurrent problem for smaller communities in the Delta. With perennially limited resources, public health protection should be given the highest priority, followed closely by protection of beneficial uses such as threatened/endangered species already impacted by the Pelagic Organism Decline, and a (seasonal?) surveillance program for areas of the Bay/Delta which experience periods of frequent and prolonged recreational uses water-contact uses, fishing, etc. With respect to Recommendation 2, I am unclear as to what the model being described is intended to accomplish: will it, if properly deployed, facilitate successful toxigenic bloom "forecasting"? Will use of whatever model results from this development process be of assistance, say, to managers of local public water supplies whose intakes are situated in the Delta? Having worked on this issue for ten years, I am concerned that our scarce resources are not being directed at immediate (& often seasonally recurrent) cyanotoxin hazards, and that local public health officials and water system managers have too few resources to respond effectively, and in a timely manner, when these episodes occur.	Noted

Ward	N/A	**Comment 5 continued:** As an example, last year the public water supply system for 400,000 people in the greater Toledo area were shut down, causing a public emergency and immediate potable water shortage for the entire population, when a microcystin-producing Microcystis bloom swamped the treatment plant's capacity to remove it in the "finished" drinking water. The National Guard was called-up to help deliver potable to this large urban population, and the problem did not abate for several days. Prior to this episode, NOAA had been doing quite a bit of modeling, bloom-forecasting, and other scientific investigations on these recurrent toxigenic blooms on western portion of Lake Erie where Toledo area residents obtain their public water supplies. The NOAA investigations remain on-going, and no doubt have provided much useful information on the role of various environmental factors in bloom formation: their "mission", however, is not to protect specific public water supplies from catastrophic events such as this episode. http://www.washingtonpost.com/news/post-nation/wp/2014/08/04/toledos-water-ban-and-the-sensitivity-of-our-drinking-systems/	Noted

APPENDIX C

Comments from the Stakeholder and Technical Advisory Group (STAG) members and responses from the authors.

Author	Page	Comment	Response
Lee	N/A	Overall Comment: The findings expressed in the draft white papers are consistent with our many years of experience investigating nutrient-related water quality, our findings in investigating Delta nutrient impacts and control of excessive aquatic plants, as well as with the findings expressed in presentations made at the CWEMF Delta Nutrient Modeling Workhop discussed below.	Noted
Lee	N/A	There remains little ability to quantitatively and comparatively describe the role of nutrients (N and P) in controlling the excess fertilization of the Delta waters.	Noted
Lee	N/A	There is considerable misinformation in the professional arena on the relative roles of N and P concentrations and loads, and the ratios of N to P in affecting water quality in the Delta; some of the information presented on nutrient/water quality issues is biased toward preconceived positions.	Noted
Lee	N/A	Based on the results of the US and international OECD eutrophication study and our follow on studies of more than 600 waterbodies worldwide (lakes, reservoirs, estuarine systems) the planktonic chlorophyll levels in the Central Delta are well-below those that would be expected based on the phosphorus loads to the Delta.	Noted
Lee	N/A	There is a lack of understanding of the quantitative relationship between nutrient loads and fish production in the Delta.	Noted
Lee	N/A	The Delta Stewardship Council's timetable for developing Delta nutrient water quality objectives by January 1, 2016, and to adopt and begin implementation of nutrient objectives, either narrative or numeric as appropriate, in the Delta by January 1, 2018 is unrealistically short.	Noted
Lee	N/A	There is need for substantial well-funded, focused, and intelligently guided research on Delta nutrient water quality issues over at least a 10-yr period in order to develop the information needed to generate a technically sound and cost-effective nutrient management strategy for the Delta.	Noted
Lee	N/A	As discussed in our writings, some of which are noted below, it will be especially dfficult to develop technically valid and cost-effective nutrient control programs for excessive growths of macrophytes in the Delta.	Noted

Mioni	3	#2: pH may also be important (I see some correlations and I think Raphe mentioned a report). I believe some cyanobacteria can be more competitive when pH increases due to CO2 concentrating mechanism. I think Alex Parker did some research on the Delta pH... Also, the residence time may be affected by the pumping station located near the EMP Old River D28 station (a station with typically high Microcystis abundance).	Noted
Mioni	13	last paragraph: Please talk to Anke Mueller-Solger. I believe Microcystis was there before 2000 but was simply not monitored as closely or did not cause such bloom.	Noted
Mioni	16	Carbon fixation: I would include a few reference to the cyanobacteria carbon concentrating mechanism.	Noted
Mioni	16	Table 2.3: Microcystin LD50 varies depending on the variant	Noted
Mioni	20	typo "preceding"	Noted
Mioni	21	N:P ratio: I would cite Hans Paerl as well. I believe he has shown (in Lake Taihu?) that the N:P ratios were not so fixed for cyanobacteria.	Noted
Mioni	29	Salinity: I think Pia Moissander did phylogenetic studies in the SFBD and has shown that there were two types of Microcystis, one of those was associated with higher salinity.	Noted
Mioni	31	I agree that absolute concentrations of nutrients is more relevant than N:P ratios with regards to cyanobacteria. I believe Hans Paerl also demonstrated this (Nature paper? I can't recall the exact source).	Noted
Mioni	37	last paragraph: typo "water column"	Noted
Mioni	39	Old River stn (D28) usually has the highest abundance based on my monitoring. Antioch also has a high abundance of Microcystis. Pia Moisander's paper show that there may be two different strains (different requirements?) between antioch and other stations. It varies between years at other stations (see attached examples but please do not use as this is for the paper I am writing...)	Noted

Mioni	40	It really depends on the year. Aphanizomenon was very sporadic before 2011 and I focused on enumerating Microcystis which was the dominant cyanoHAB. But in 2011, Aphanizomenon was pretty significant. The tricky part here is that the Aphanizomenon cells are much larger than Microcystis so even if Aphanizomenon doesn't reach the cell density of Microcystis, it doesn't mean they are not dominating the bloom (e.g. 2011, it would clog my filters pretty quickly at some stations)... In 2012, Microcystis abundance was higher than in 2011 but Apha was still pretty abundant. I think that the "bloom" classification based on cell density should be revised to take into account the biovolume... Cell counts can be misleading.	Noted
Mioni	44	There is definitely variations explained by the method but there are also variations due to heterogeneity, patchiness and temporal variation. In Clear lake, while on station (within maybe 30min or less), we could see the scum moving very quickly with the wind. Also, the two net samples mostly applies to colonial forms of Microcystis although it occurs also as single cells and microcolonies. Another bias is the cell count. Prior to do my cell counts, I was homogenizing the samples by dislocating the colonies physically (based on prior research and comparison). I suspect that not dislocating the colonies prior to do the cell count may result in bias as the person enumerating the cells may not be able to count accurately as colonies can be more 3D than 2D (I hope it makes sense)... Although there is a bias in all methods, I do not think I ever collected samples in the same time than Peggy and at the same location. Thus, the comparison is a little puzzling to me. We never did intercomparison of the cell enumeration from the same samples. It would be more relevant to compare methods for the toxicology work since we did intercomparison of methods for the same samples.	Noted
Mioni	48	"colonial Microcystis have been more common", see my comments regarding the bias of tow net sampling versus grad raw water samples...	Noted

Mioni	4 & 35	#3 and page 35, temperature: Lenny Grimaldo generated a logistic model based on my CALFED data (see attached) which shows that Microcystis bloom probability raises to 50% when surface water temperature reaches 25C. Also, I suspect there is a minimum temperature that would need to be sustained for several days if not week for a bloom to initiate.	Noted
Mioni	42-43	I think the SWAMP report could be cited, especially for the SPATT results.	Noted
Mioni	Fig 4.5	Figure 4.5: the axis are not labelled and I have trouble understanding this figure.	Noted
Mioni	48	I could not find the figure 2 mentioned here…	Noted