Exhibit A: Attachment 1 – Project Narrative

THE ROLE OF MICROCYSTIS BLOOMS IN THE DELTA FOOD WEB: A FUNCTIONAL APPROACH

1. Project Purpose

We propose an interdisciplinary, collaborative investigation of blooms of the toxic, colonial cyanobacterium *Microcystis aeruginosa* (herein referred to as *Microcystis*) that have occurred in recent years in the upper San Francisco Estuary (SFE). We will investigate how blooms develop, what controls toxin production, and how these blooms affect the pelagic food web of the SFE, particularly the Sacramento-San Joaquin Delta. These blooms may be impairing pelagic food webs, and have been implicated in the decline in abundance of several estuarine fish species (Pelagic Organism Decline, Baxter et al. 2008).

Identification of the Problem

The Pelagic Organism Decline (POD) occurred around 2002 (Sommer et al. 2007, Thomson et al. 2010), roughly coincident with an apparent increase in frequency and intensity of *Microcystis* blooms centered in the freshwater Delta (Lehman et al. 2005). Several potential mechanisms may link these two temporal trends (Lehman et al. 2008). Although direct toxicity to fish is unlikely at observed toxin concentrations, indirect effects on fish may arise through the planktonic food web. Specifically, larvae and juveniles of POD species feed mainly on copepods, and copepod survival may be impaired by blooms (Ger et al. 2010a). Thus, a thorough investigation of the mechanisms forming these blooms and their effects on the pelagic food web would quantify the link between blooms and fish and help to identify management strategies.

Our proposed research builds on previous work on *Microcystis* in the Delta. The spatial and temporal scales of blooms have been identified along with their environmental covariates (Lehman et al. 2005, 2008). Analyses of toxin distributions have shown potential direct and indirect effects on fish (Lehman et al. 2008). *These largely correlative results pave the way for a mechanistic analysis of the conditions that distinguish bloom periods and locations from non-bloom periods and locations, and that result in production of toxins.*

Current understanding of *Microcystis* blooms in the Delta and globally indicates that bloom formation, dynamics, and toxicity are complex. Concentrations of total microcystin toxin and *Microcystis* cell numbers are not strongly correlated in the Delta (Lehman et al. 2008, Baxa et al. 2010, Mioni et al in prep). Different strains of *Microcystis* vary in their ability to produce toxins but cannot be distinguished by microscopy (Moisander et al. 2009). Preliminary research in the Delta and elsewhere also indicates that toxicity may not be due solely to *Microcystis,* but may also arise through the association of *Microcystis* with an unidentified filamentous cyanobacterium (Mioni et al in prep). *These findings point to a need for a deeper and more comprehensive understanding of Microcystis-dominated blooms and toxin production.*

Microcystis reduces survival of copepods in laboratory cultures even at concentrations below those observed in the field (Ger et al. 2010a, b). If this proves true also in field populations of copepods, the outcome would be a strong negative effect on the food supply of POD fish species. *These laboratory*

results indicate the immediate need for a study of the effects of Microcystis and its toxins on the feeding, survival, and population dynamics of copepods in the field.

Project Goal and Objectives

The goal of this project is to determine the environmental conditions leading to *Microcystis* blooms, their toxicity and their impact on the pelagic food web of the Delta.

- Objective 1. Determine the biotic and abiotic factors controlling *Microcystis* bloom formation and toxin production in the Delta.
- Objective 2: Determine the role that *Microcystis* strains and microbial associations play in resulting toxicity associated with blooms.
- Objective 3: Determine the role of *Microcystis* in the Delta pelagic food web and its effect on the POD through zooplankton grazing.

2. Background and Conceptual Model

Harmful algal blooms (HABs) have become a significant nuisance in freshwaters, estuaries, and coastal waters worldwide (Paerl 1988, Paerl et al. 2001). Blooms of cyanobacteria (CHABs) including *Microcystis* species have been on the increase in freshwaters globally, particularly in warm, stratified, eutrophic waters of long residence time (Paerl et al. 2001). All HABs can alter nutrient and biogeochemical cycles and deplete oxygen, but the most troublesome attribute of CHABs is the production of cyanotoxins that can impair food web function and fishery production as well as human uses (Paerl et al. 2001). Climate change, particularly warming and droughts, may favor further increases in the prevalence of CHABs (Paerl and Huisman 2008, 2009).

Microcystis is a colony-forming, non-N_2 fixing cyanobacterium and one of the most common species of cyanobacteria worldwide, dominating in eutrophic and hypereutrophic aquatic ecosystems (Christoffersen 1996). Several strains of *Microcystis* produce hepatotoxins including microcystins and microviridins (Dawson 1998, Rohrlack et al. 2003). The life cycle of *Microcystis* includes overwintering resting stages that remain dormant in sediments, with a shift to pelagic forms triggered by increases in water temperature and irradiance or a change in dissolved oxygen concentration (Stahl-Delbanco et al.2003). High nutrient concentrations combined with low N:P ratios can favor maximum recruitment of resting cysts (Stahl-Delbanco et al, 2003). While benthic colonies are near neutral buoyancy, photosynthesis prompts positive lift (Reynolds 1981). *Microcystis* colonies tend to concentrate at the surface, especially in turbid waters, due to vertical migrations and the ability to regulate buoyancy (Reynolds 1981).

Likely drivers of *Microcystis* blooms and resulting impacts on estuarine food webs are discussed in the following sections and summarized in Fig. 1. Our conceptual model contrasts non-bloom (left) with bloom (right) conditions to illustrate the factors that might facilitate the transition to a bloom, and the influences that the bloom might have on the pelagic food web. The diagram also depicts the topics to be addressed in the proposed research.

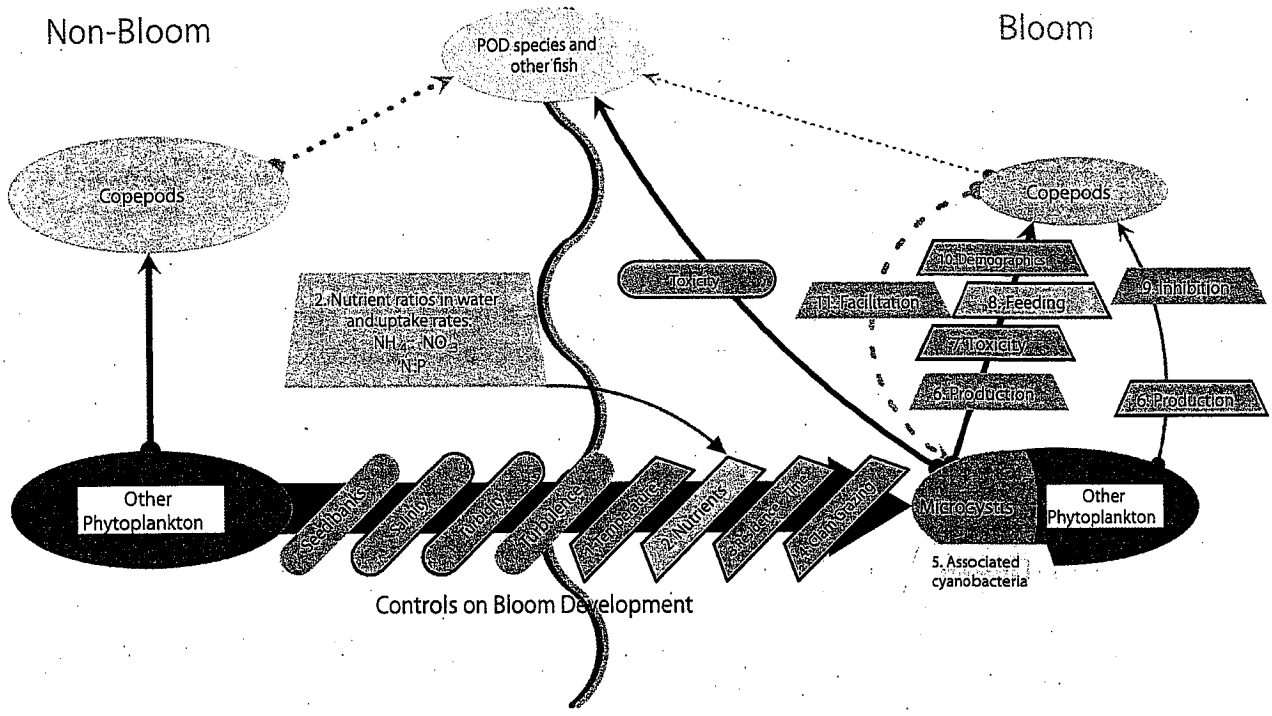

Figure 1: Simplified conceptual model of controls on development of *Microcystis* blooms and food web consequences in the Delta. Left side of wavy line: non-bloom conditions with high phytoplankton biomass supporting high biomass of copepods. Right side: bloom conditions including some potential food web effects. Heavy blue arrow indicates bloom development; small boxes indicate controls on bloom development or influences on the food web. Trapezoids indicate topics addressed in the proposed work plan, and ovals indicate topics reserved for subsequent investigations or not requiring investigation. Lines around the shapes indicate topics that have been addressed to some extent in previous studies. Food web effects may include either feeding on or inhibition of feeding by *Microcystis*, with consequent reduction in reproductive rate. Copepod grazing may also reduce other phytoplankton and facilitate *Microcystis*.

Controls of Bloom Development: Nutrients

CHABs are symptomatic of anthropogenic nutrient over-enrichment (Reynolds 1987, Paerl 1988, Paerl et al. 2001) (Fig 1 #2). Traditionally, phosphorus (P) input reductions have been recommended to control CHABs, because P limitation is widespread in freshwaters and some CHABs can satisfy their nitrogen (N) requirements by fixing atmospheric N_2 (Schindler 1971). However, systems undergoing eutrophication are increasingly plagued with non-N_2-fixing CHABs, including *Microcystis* spp., that are N and P co-limited or even N limited. Many of these systems are experiencing accelerating N loads, even as P loading is being controlled. Therefore both N *and* P input constraints are likely needed for long-term control of eutrophication and CHABs in such systems (Paerl 2009).

Several studies have investigated the importance of varying N and P concentrations on the growth of toxic and nontoxic strains of *Microcystis,* microcystin production, and competition with other phytoplankton (Yoshida et al. 2007, Downing et al. 2005, Marinho et al. 2007). Competition studies showed that *Microcystis* had a higher uptake capacity for N than a freshwater diatom and another

cyanobacterium (Fujimoto et al. 1997, Marinho et al. 2007), which may help explain why *Microcystis* thrives in high N:P waters (Xie et al. 2003, Marinho et al. 2007). At low nutrient concentrations, nontoxic strains had an advantage in N uptake capacity but in high nutrient environments toxic strains outpaced nontoxic strains (Vezie et al. 2002). Field studies corroborate laboratory findings, indicating that nitrate loading may be a significant promoter of toxic strains within *Microcystis* blooms (Yoshida et al. 2007). Finally, microcystin content in cells also appears to be modulated by N uptake (Downing et al. 2005).

N uptake estimates for *Microcystis* using ammonium (NH_4), nitrate (NO_3), and urea in Lake Kasungaura, Japan showed that *Microcystis* can use all three N substrates for growth but capacities for uptake of each N species differed at sub-saturating and saturating concentrations. Maximum uptake rates of, and affinity for N by *Microcystis* were high compared with some other phytoplankton taxa, which may impart a significant competitive advantage during conditions of N-limited growth (Takamura et al. 1987).

The inhibition of NO_3 uptake by the presence of NH_4 has been reported for a broad spectrum of phytoplankton species (Dortch 1990) and while it is generally accepted, it is not universal. We are aware of only one study that addressed NH_4 inhibition of NO_3 uptake for *Microcystis* (McLachlan & Gothan 1962 as cited in Dortch 1990), which did not show inhibition. A lack of inhibition of NO_3 uptake by NH_4 could provide a competitive advantage to *Microcystis* over other phytoplankton in high NH_4: high DIN environments such as the SFE.

Controls on Bloom Development: Climate

Climatic changes including warming, increased vertical stratification, salinization, and intensification of storms and droughts may play additional, interactive roles in modulating CHAB frequency, intensity, geographic distribution, and duration (Paerl and Huisman 2009) (Fig 1 #1). This has complicated management strategies for control because CHABs are capable of taking advantage of unprecedented levels of nutrient enrichment *and* regional and global climatic change, including warming and altered hydrology. In particular, reduced freshwater discharge increases water residence time in estuaries, which can favor CHABs because their growth rates are generally slower than their eukaryotic phytoplankton competitors (Paerl and Huisman 2009).

Hydrologic manipulation can suppress cyanobacterial blooms. Two approaches have been used. Artificial mixing is often aimed at decreasing water column stratification and enhancing vertical mixing of the phytoplankton, thereby preventing the formation of surface blooms of buoyant cyanobacteria (Paerl et al. 2001, Paerl and Huisman 2009) (Fig. 1 #3). Horizontal flushing, by increasing the water flow through lakes or estuaries, reduces water residence time, thus providing less time for the development of cyanobacterial blooms (Paerl and Huisman 2009).

Warming will lead to longer periods of elevated water temperatures worldwide (Fig. 1 #1). This trend favors expanding phytoplankton bloom periods, particularly dominance by CHABs (Paerl and Huisman 2007;2009) A recent example is the proliferating toxic *Microcystis* spp. blooms that caused a drinking water supply crisis at Lake Taihu, China (Qin et al. 2010). Here, blooms have increased in magnitude due to increased N and P loading, and duration most likely due to a multi-decadal warming trend. Summer droughts appear to be increasing in intensity and duration, possibly another symptom of global

warming (IPCC 2007). This, combined with increased use of freshwater for irrigation, has led to rising salinities around the world. Increased salinization a serious threat to freshwater supplies; it also has major impacts on freshwater plankton composition and possibly CHAB potentials. One impact of salinization is increased vertical density stratification, which would benefit buoyant, scum-forming CHABs. In addition, some species of common CHAB genera such as *Anabaena*, *Microcystis,* and *Nodularia* are more salt tolerant than their eukaryotic freshwater algal counterparts (c.f., Horne and Galat 1985, Moisander et al. 2002). For example, the growth rate of toxic strains of *Microcystis* remains unaffected by salinities ranging from 0 g L^{-1} up to 10 g L^{-1} (Tonk et al. 2006). Temporary salinity fluctuations of up to 15-20 g L^{-1} may still allow survival of *Microcystis* populations, but causes salt stress leading to leakage of cells and excretion of intracellular microcystin. Likewise, *Anabaena aphanizominoides* can withstand salt levels up to 15 g L^{-1}, while *Anabaenopsis* spp. and toxic *Nodularia spumigena* tolerate salinities ranging from 0 g L^{-1} to more than 20 g L^{-1} (Horne and Galat 1985; Moisander et al. 2002, Mazur-Marzec et al. 2005). Laboratory experiments indicate that the nodularin (toxin) content of *Nodularia* correlates positively with salinity. The high salt tolerance of these CHABs is reflected as expanding blooms in brackish waters, including the Baltic Sea, the Caspian Sea, Patos Lagoon Estuary, Brazil, the Swan River Estuary, Australia, Lake Ponchartrain, Louisiana, and the SFE (Paerl and Huisman 2008).

Toxin Production and Microcystis Strains

Like many other cyanobacteria, *Microcystis* produces several secondary metabolites with toxic properties (Fig 1 #7). The most studied toxins produced by *Microcystis* (also by *Anabaena* and *Oscillatoria*) are called microcystins. These are cyclic heptapeptides classified as hepatotoxins, with over 89+ forms that vary in toxicity (Watanabe et al. 1996, Zurawell et al. 2005). As an endotoxin, microcystin remains in the cell until rupture or death, when it enters the water column in its dissolved form, at which point it degrades relatively rapidly, typically taking days to weeks, depending on biodegradation and binding (Perez et al. 2003). However, *Microcystis* toxicity can also come from less identified non-microcystin secondary metabolites (Lurling 2003, Rohrlack et al. 2004, Wilson et al. 2006). Examples include lipopolysaccharides, which reduce detoxification efficiency, microviridin, which reduces protease activity, as well as various unidentified toxins (Kurmayer and Juttner 1999, Rohrlack et al. 2004, Wiegand and Pflugmacher 2005). Though the significance of non-microcystin metabolites has been emerging, the relative toxicity of microcystin versus non-microcystin metabolites to zooplankton remains unresolved (Kurmayer and Juttner 1999, Lurling 2003, Rohrlack et al. 2004). The negative effects of *Microcystis* on the copepods of the Delta occur mainly via non-microcystin toxins (Ger et al. 2010a). Nevertheless, microcystins have been the most practical and universal measure for *Microcystis* toxicity and is therefore the focus here. The production of microcystin variants differ among cyanobacteria strains and also depend on the intracellular availability of different amino acids and therefore the degree of nitrogen availability.

Traditionally, *Microcystis* taxonomy has been based on microscopic observation. However, due to the high degree of phenotypic plasticity exhibited in natural assemblages it is difficult to accurately and consistently identify species of *Microcystis* on microscopic observation alone (Otsuka el al. 2000), requiring a phylogenetic approach for identifying species and strains (Fig. 2). However, a more important question to ask about a given strain of *Microcystis* may be: is it toxic? In the past toxicity has been determined by either direct measurements of microcystins, or by assessing a strain's potential toxicity by PCR amplification of a portion of the toxin genes, *mcyB* for example. Microcystin

production is dependent on 10 genes (*mcyA*-J), spanning ~55 kbp. (Neilan et al. 1999) The problem with only amplifying one or a couple genes is that it does not provide a definitive answer to whether or not a strain is toxic. If a partial deletion is present in any of the *mcy* genes, for example due to genetic recombination or lateral gene transfer, then the assembly of the microcystin peptide is halted and the strain is rendered non-toxic. For this reason, a *Microcystis*-specific long PCR assay was developed which allows for the complete amplification and visualization of all 10 *mcy* genes simultaneously on a standard agarose gel (Fig. 3).

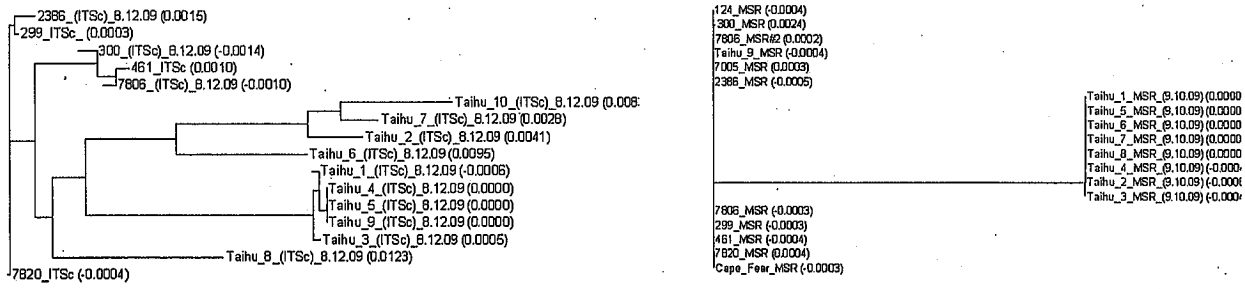

Figure 2: Phylogenetic resolution based on two different (L: 16S-ITS, R: MSR) sets of 16S rRNA oligonucleotides.

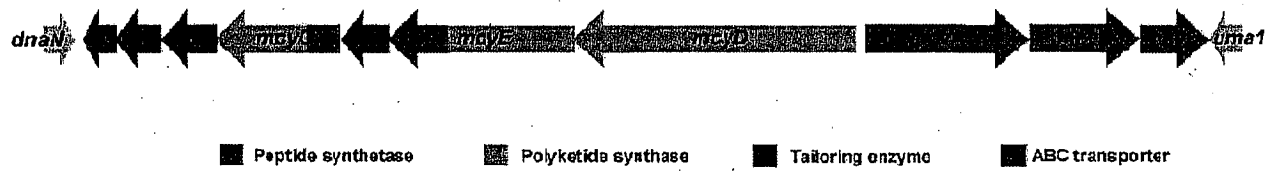

Figure 3: Diagram of long PCR primer coverage

Toxin Production and Microbial Associations (Consortia) with Microcystis

Compiled literature evidence suggests that the success of cyanobacteria is a result of complex and synergistic environmental factors rather than a single dominant variable (Lehman et al. 2008, 2010, Tan et al. 2008). However, microbial associations or consortia are of potentially great importance in regulating population dynamics are therefore potentially important for the formation and development of *Microcystis* blooms (Paerl and Pinckney 1996, Shi et al. 2009) (Fig. 1 #5, Fig. 4).

In planktonic environments, microbial consortia are widespread and play an important role for production and nutrient transformation processes. In lakes and rivers, highly specific associations between cyanobacteria and heterotrophic bacteria are very common (Paerl, 1976, Pearl and Gallucci, 1985; Paerl et al. 1988, Paerl and Pinckney 1996, Salomon et al. 2003, Shi et al. 2009). Similarly, cyanobacteria are frequently associated with algae such as diatoms (De Yoe et al. 1992, Paerl and Pinckney 1996). The exchanges of N between N_2-fixing cyanobacteria and heterotrophic bacteria are well documented (Paerl 1976, Pearl and Gallucci 1985, Paerl et al. 1988; Paerl and Pinckney 1996). Also, exchange of P has been observed between *Microcystis* and the associated bacterium *Pseudomonas* sp. (Jiang et al. 2007). Various studies have reported the diversity of the microbial flora associated with

the mucilage of *Microcystis* colonies (Brunberg 1999; Shi et al. 2009) and the stimulatory and inhibitory effects of this flora on *Microcystis* growth in culture (Salomon et al. 2003,Shi et al. 2009). Some *Microcystis* strains are difficult to grow axenically, and some freshwater cyanobacteria with bacteria added usually grow faster than axenic strains (Paerl and Pinckney 1996).

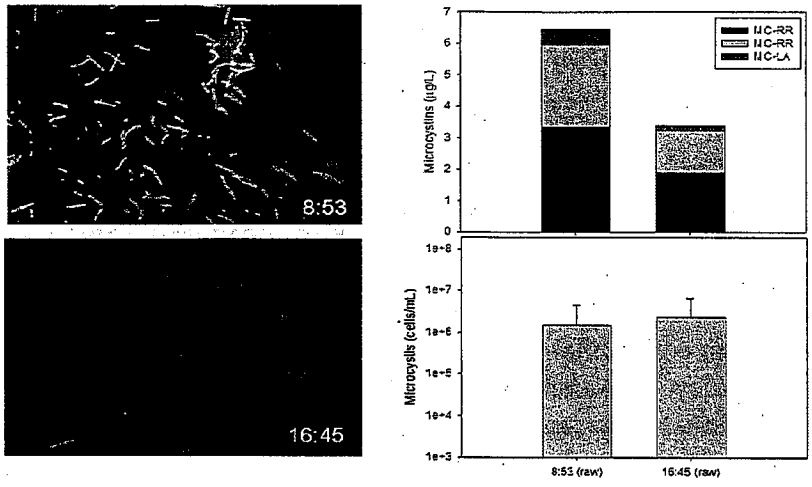

Figure 4: Left panel, *Microcystis* cells (background) and unknown associated filamentous cyanobacteria (Mioni et al. in prep) Samples collected near the Antioch Bridge (August 2009) in the morning (upper left) and late afternoon (lower left). Microcystin toxins concentrations were twice as high in the morning as compared to the afternoon (upper right). The decrease in toxin concentration was not explained by the *Microcystis* cell density (lower right), however, the associated filamentous cyanobacteria were more abundant in the morning compared to late afternoon (left). Toxins concentrations were measured using LC-MSMS (by Fish and Game, Rancho Cordova).

These microbial associations may also reflect consortial exchange of metabolites and growth factors (see Fig. 1 #5; Paerl and Pinckney 1996). Associated bacteria may supply specific growth factors (e.g. vitamins, chelators) that may play an important role in the development of a bloom (Paerl and Pinckney 1996). Cyanobacteria and eukaryotic phytoplankton excrete a variety of organic compounds (amino acids, peptides, alkaloids, carbohydrate, and lipopolysaccharides) that show chemotactic properties and support the growth of epiphytic microbes (Paerl and Pinckney 1996). Numerous bacteria are associated with the mucilage of *Microcystis* colonies (Brunberg 1999; Shi et al. 2009), some of which are able to metabolize microcystin (Maruyama et al. 2003) and therefore control the toxicity of the bloom. Microbial consortia thus play important roles in controlling *Microcystis* growth and in the ecophysiology of microcystin production.

Microcystis Food Web Effects

Cyanobacterial blooms can impair fish production either directly or through the food web (Fig. 1). Direct impacts on fish may arise through ingestion of toxic colonies or through uptake of dissolved toxins directly from the water. However, we focus instead on effects of cyanobacterial blooms occurring through their interactions with the food web. Most estuarine fish consume zooplankton during early life stages. Additionally, dissolved microcystins have not been recorded in the SFE, possibly due to rapid degradation, and are not expected to present a major risk. Thus, the degree of food web impact hinges on whether production of zooplankton is impaired by blooms.

Zooplankton grazing could be impaired by cyanobacterial blooms through several mechanisms, including direct toxicity or nutritional inadequacy of the bloom organism, toxicity of the water through

released toxins, or interference with feeding on nutritionally superior food sources (Kirk and Gilbert 1992, Smith and Gilbert 1995, Ghadouani 2003, DeMott and Tessier 2002, Fig. 1 #7,8,9). Mechanistic evidence from laboratory studies shows that *Microcystis* typically suppresses feeding in large zooplankton because of its colonial morphology, toxic properties, and nutritional deficiency (Wilson et al 2006, Lampert 1987, Christoffersen 1996, DeMott and Moxter 1991). Ingestion of *Microcystis* can vary considerably among zooplankton species and depends on their tolerance to ingested toxins, the feeding mode (including ability to avoid toxic food), and the availability of alternative food sources (DeMott and Moxter 1991, Kurmayer and Juttner 1999, Engstrom et al. 2000). Furthermore, preferential grazing by zooplankton or bivalves (through size selection) on more nutritional algae may favor smaller, slower-growing forms and foster cyanobacterial blooms through elimination of faster-growing algal competitors (Wang et al. 2010).

Copepods feed selectively on a wide variety of food particles including phytoplankton, protozoans, detritus, and larger prey including other copepods (Nejstgaard et al. 2008, DeMott and Moxter 1991, Kumar 2003, Koski et al. 1999). Mechanisms behind food selection depend heavily on the copepod species. These include copepod responses to biochemical and mechanical cues as well as the mode of feeding, i.e. raptorial versus suspension feeding, in conjunction with the size, shape, and behavior of the food organisms (Gasparani and Castelt 1997, Bouley and Kimmerer 2006). Some copepods can detect and avoid ingesting harmful food particles including toxic cyanobacteria (DeMott and Moxter 1991, Kleppel 1993, Koski et al. 1999).

Most information on zooplankton grazing on *Microcystis* is based on laboratory studies, mainly using the cladoceran *Daphnia* spp. as the test organism. Only a small fraction is based on copepods (reviewed in Wilson et al. 2006), yet copepods dominate the zooplankton of many estuarine and freshwater systems. Reduced survival of the copepods *Eurytemora affinis* and *Pseudodiaptomus forbesi* in the laboratory was due to toxicity of ingested *Microcystis* cells rather than nutritional inadequacy (Ger et al. 2010a).

Field evidence for *Microcystis* effects on zooplankton is less clear than laboratory results, because feeding interactions are difficult to observe in the field and species-specific differences causes contradictory effects (Kumar 2003, Ghaouani et al. 2003, Kirk and Gilbert 1992).*Microcystis* blooms can shift zooplankton community composition towards smaller species, or to those able to avoid it or to graze on it directly (Lampert 1987, Chorus and Bartam 1999, Nadini 2003, Graneli and Turner 2006).

Food limitation in calanoid copepods usually manifests first as reduced egg production (Fig. 1 #10). Both egg production rate and egg hatching success are sensitive to toxins. Thus overall reproductive rate (egg production times proportion hatching) is a good indicator of any influence of *Microcystis* on feeding. For juvenile copepods, development rate is moderately sensitive to food and proportion molting is sensitive to toxicity. These mechanisms together imply reduced survival during blooms through poor feeding or toxic impairment.

The effects of grazing by clams or copepods on *Microcystis* on bloom development depend on the degree and the mode of selectivity (Fig. 1 #11). In the short term, nonselective feeding may result in mortality of *Microcystis*, favoring more rapidly growing species. In the long term, toxicity (Ger et al. 2010a, b) or unsuitability as food (Rohrlack et al. 1999, Kumar 2003) could reduce the abundance of nonselective feeders, allowing bloom formation. If feeding is selective for other, more palatable algae,

the result may also be to stimulate formation of *Microcystis* blooms (Vanderploeg et al. 2001, Wang et al. 2010).

Microcystis in the Sacramento-San Joaquin Delta

Microcystis was first documented in the SFE in 1999 and has become widely distributed among freshwater to brackish reaches of the estuary (Lehman et al., 2005, 2008). While the distribution of Microcystis in the SFE includes the upper estuary (i.e. Suisun Bay) identified "hotspots" of *Microcystis* abundance is centered in the central Delta in the San Joaquin River (Fig. 5A).

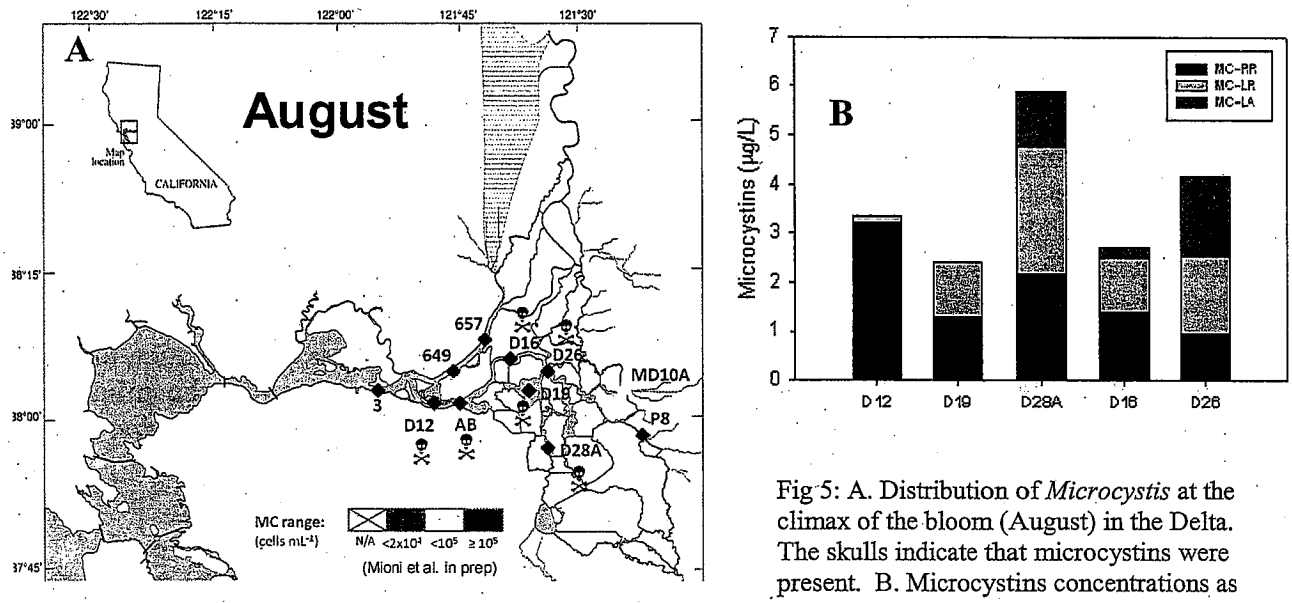

Fig 5: A. Distribution of *Microcystis* at the climax of the bloom (August) in the Delta. The skulls indicate that microcystins were present. B. Microcystins concentrations as determined by LC-MSMS. Mioni et al. in prep.

Long residence time appears to be an important precursor for *Microcystis* bloom development (Paerl et al. 2001). Residence time in subregions of the Delta vary from days to months depending on location and flow conditions (Kimmerer and Nobriga 2008). This suggests a basis for the strong spatial variability in bloom formation previously reported (Lehman et al. 2008).

Because inorganic nutrients are generally abundant in the SFE (Hager & Schemel 1996) nutrient availability has been largely overlooked as a driver of phytoplankton dynamics in the SFE. Recent experimental evidence as well as analysis of long-term trends of nutrients and phytoplankton suggests a role for nutrients in determining phytoplankton abundance (Van Nieuwenhuyse et al. 2008), frequency and timing of phytoplankton blooms (Wilkerson et al. 2006; Dugdale et al. 2007) and species community shifts (Dugdale et al. 2007; Glibert 2010) in the SFE. Substantial N loading to the Delta (and specifically increased NH_4 concentrations) has been hypothesized to sustain *Microcystis* growth in the ecosystem (Meyer et al. 2009).

While the overall toxicity of the Delta population has not been documented, compiled literature suggest that up to 11 microcystin variants contribute to the spatial and temporal variations in total microcystins in the delta (Lehman et al, 2004, 2008, 2010; Mioni et al. in prep). Microcystin-LR comprises the greatest percent of the total microcystins at most stations (Lehman et al. 2004, 2008, 2010, Mioni et al. in prep). The highest toxin concentrations is usually observed in the surface layer of the western and Central Delta stations (Antioch and Old River), which is characterized by lake-like flooded islands, including agricultural channels diverting water to storage reservoirs and the State Water project (Lehman et al. 2004, Lehman et al. 2010, Mioni et al. in prep). The toxicity has been increasing from the 0 - 81 ng/L range during the 2004 and 2005 blooms (Lehman et al. 2008, 2010) to the 0 - 10.81 ug/L range during 2008 and 2009 blooms (Baxa et al. 2010, Mioni et al. in prep)."

Preliminary results suggest that *Microcystis* forms microbial consortia with other microorganisms in the Delta (cyanobacteria, diatoms, and heterotrophic bacteria). More specifically, one unidentified filamentous cyanobacterium strain is found associated with *Microcystis* colonies when microcystin concentrations are elevated (nearly two-fold, Fig. 2). This epiphytic filamentous cyanobacterium is usually absent or uncommon when the microcystin concentrations are low (Mioni et al. in prep). Therefore, this cyanobacterium could possibly influence the production of toxins, including microcystins, and possibly unidentified toxins implicated in copepod mortality (Ger et al. 2010a) and kidney lesions in fish (S. Acuña, pers. com). The influence of this biological interaction on the success of *Microcystis* should be part of the comprehensive view of blooms in the Delta.

Microcystis has relatively slow growth rates, so blooms result more from biomass accumulation than rapid growth (Lehman 2008). Low total primary production in the Delta may exacerbate the influence of *Microcystsi* during summer and fall when it is abundant (Fig 1 #6). However, the contribution of *Microcystis* to total primary production appears to be small. Primary production estimates for phytoplankton of the size range of *Microcystis* colonies (>75 μm) ranged from 19 to 141 ng C m^2 h^{-1} using PI curves for whole water determined by the light/dark bottle oxygen technique (Lehman et al 2008). Assuming a 13-hr day-length in August, corresponding daily rates would be 0.2 to 1.8 μg C m^2 d^{-1}. In contrast, Jassby et al. (2002) reported mean gross primary production rates of 132,000 to 201,000 μg C m^2 d^{-1} for the Central Delta and Lidstrom (2008) reported 155,000 μg C m^2 d^{-1} in the western Delta during summer where *Microcystis* was not found (Fig. 1 #6).

Bivalve grazing is a dominant control on phytoplankton biomass accumulation in many parts of the SFE, including freshwater to brackish regions, and could reduce abundance of *Microcystis* cells. However, *Microcystis* colonies are probably too large to be ingested, so bivalves likely eject them as pseudofeces (Vanderploeg et al. 2001). Subsequent growth and survival of these cells depends on how tightly bound they are in the mucous matrix of the pseudofeces; if cells are released quickly, bivalve grazing could stimulate bloom formation by consuming some algae and allowing unconsumed *Microcystis* to continue growing. Thus, the effect of clam grazing may be negative early in the season before colonies are formed, and positive later when colonies form, partly because of rejection and partly because the colonies float and are not vulnerable to bivalve grazing. Clams in the Delta had low concentrations of microcystins in their tissues during the 2004 bloom, suggesting either rejection of colonies or rapid removal of toxins from clam tissue (Lehman et al. 2008).

If *Microcystis* is affecting the pelagic food web, the most likely mechanism for effects is through copepods, notably *Pseudodiaptomus forbesi.* This copepod is the most abundant calanoid in the freshwater-brackish habitat of *Microcystis*, and is important food for early life stages of nearly all fishes

in the Delta, as well as juveniles of some and all stages of delta smelt. Previous experiments have demonstrated an effect of *Microcystis* on *P. forbesi*, apparently through toxicity not related to microcystin (Ger et al. 2010a). The cyclopoid copepod *Limnoithona tetraspina* is much more abundant in brackish water than is *P. forbesi*, but is much less fed upon by fish because of its small size and cryptic behavior (S. Slater, CDFG, and L. Sullivan, SFSU, pers. comms. to W. Kimmerer). Furthermore, *L. tetraspina* feeds only on motile cells such as ciliates (Bouley and Kimmerer 2006, Gifford et al. 2007) and therefore is unlikely to consume *Microcystis*.

Thus, we are interested in the mechanisms by which *Microcystis* could impair *P. forbesi* (Fig. 1). The most likely mode of impairment is direct toxicity from the ingestion of the cells themselves, combined with a partial or complete inability of the copepod to reject or avoid *Microcystis*; this mode is supported by laboratory results (Ger et al. 2010). Second, *Microcystis* could be fed upon but be inadequately nutritious. Third, the copepod may reject *Microcystis* cells, but the time spent rejecting particles could impose a penalty in feeding. Fourth, the copepod could reject *Microcystis* or avoid colonies without penalty, but its feeding on other foods may be impaired, such as if the copepod avoids a part of the water column where *Microcystis* is abundant. Finally, there may be no effect in the field.

Critical Unknowns Related to Microcystis in the Delta (Table 1)

- Controls on bloom development: Water temperature, salinity tolerance, and water column stratification have been shown to contribute to *Microcystis* success in the Delta. Changes in nutrient (and specifically N) loading, leading to increases in NH_4 concentration, have been hypothesized to have shifted Delta phytoplankton towards cyanobacteria (Meyer et al. 2009; Glibert 2010). The role that nutrients play (including N speciation and increased NH_4 loading, as well as N:P ratio) remain largely speculative and unexplored for the Delta.
- *Microcystis* and toxin production: What proportion of cells are capable of producing toxin at any given time? How do these proportions vary in regards to time, space and environmental and nutrient factors? Although it is well documented that the *Microcystis* bloom in the Delta is toxic, the mechanism controlling the toxicity, especially biological associations are not well characterized. Previous work has focused on microcystin toxin but *Microcystis* can produce other cyanotoxins (e.g. microviridin). Recent work (Acuna, pers. com.) suggest that fish populations in the Delta are affected by other toxins than microcystin.
- Food web effects: Laboratory-cultured copepods survived poorly when provided a diet including cultured *Microcystis* (Ger et al. 2010a), and field-collected copepods and clams contained measurable microcystin concentrations (Lehman et al. 2008). However, the influence of *Microcystis* on copepod populations in the field has not been determined, nor has the precise mechanism for low survival. The importance of clam grazing in the development of blooms is also unknown.

3. Approach and Scope of Work

We propose a collaborative field and laboratory investigation into seasonal *Microcystis* dynamics and associated food web interactions in the Delta (Fig. 5A), focusing on the processes underlying observed patterns of abundance and distribution.

The proposed research will elevate our quantitative understanding of *Microcystis* bloom dynamics and food web impacts, and provide the missing rates and parameters necessary for development of

ecosystem-level models of these processes. To achieve this, we will answer questions and test hypotheses (Table 1) to achieve our two overall objectives. Key elements of our research plan are:

- To develop an understanding of *Microcystis* bloom development, including the nutrients used for growth and rates of primary production, and the roles of residence time and bivalve grazing (Objective 1).
- To characterize the types of *Microcystis* that occur in the Delta, including the strains' ability to form different toxins, and the role of associations with other cyanobacteria (Objective 2).
- To determine the impact of blooms on zooplankton demographics, assessing the link between blooms and the Delta food web (Objective 3).

The project is composed of five interrelated tasks:

Task 1: Project management and synthesis (Parker)
Task 2: Nutrients, primary production, and nitrogen uptake (Parker and Wilkerson)
Task 3: Microscopic characterization of blooms and microbial consortia, and toxin characterization (Mioni and Kudela)
Task 4: Molecular characterization of *Microcystis* blooms (Paerl)
Task 5: Food web interactions (Kimmerer and Ger)

The proposed 3-year work plan comprises 2 years of field and laboratory work and 1 year of synthesis. Our previous experience in such interdisciplinary, multi-investigator projects indicates the importance of the synthesis phase. Each project component, mapped to the tasks above, will be responsible for publishing at least one paper on results of the field and laboratory studies. However, all will contribute to the synthesis which will result in a paper presenting our best assessment of the roles and impacts of *Microcystis* blooms.

The first two years of research will center on a series of field surveys contrasting locations dominated by *Microcystis* with locations where *Microcystis* is not abundant, during pre-bloom, bloom, and post-bloom periods (Fig. 3). Stations will be characterized by investigating phytoplankton community structure (including *Microcystis* abundance and diversity), autotrophic C and N rate processes, toxin concentrations, and zooplankton. Additional experimental manipulations will test hypotheses about the underlying mechanisms that drive the observed *in situ* spatial and temporal patterns (Table 1). Each of the collaborators will participate in the field surveys, promoting full integration of each of the tasks and providing a spatially and temporally cohesive dataset to characterize *Microcystis* effects in the Delta.

We plan six two-day research cruises between early June and October of years 1 and 2. Because of interannual variation in the onset of *Microcystis* abundance in the SFE, we will coordinate with established monitoring programs (i.e. USGS, IEP, DWR) to determine the start of the field season in order to capture the full seasonal progression of *Microcystis* bloom formation. Cruises will be spaced at a 3-week interval (to avoid potential bias associated with spring/neap tidal cycles) with a higher sampling frequency during the time of peak *Microcystis* biomass. We will use RTC's 38-ft research vessel *Questuary,* which is outfitted with rosette/CTD and underway surface data acquisition for temperature, salinity and fluorescence. Samples will be taken from 6 stations at 3 depths: surface, sub-surface, and near-bottom. Surface samples are essential for capturing the peak biomass of floating colonies of *Microcystis*. Stations have been selected to complement rather than replicate existing monitoring programs by state agencies.

Three pairs of stations will be visited, with one of each pair in high *Microcystis* patch (if one is found). Each station pair represents two sites that are similar in terms of residence time and hydrology, have close geographic proximity, but contrast in terms of *Microcystis* abundance. Provisional station pairs within the Delta (region depicted in Fig. 5A) include the following locations but may be modified based on *Microcystis* distribution determined by members of our project team in summer-fall of 2010:

1) Franks Tract (relatively low *Microcystis* and has bivalves) vs. Mildred Island (high *Microcystis* abundance and no bivalves). This station pair represents the shallow water habitat of the Central Delta, where residence time is relatively high and hydrology is more similar to a lake than compared to a river.
2) Rio Vista (low *Microcystis*) vs. Antioch (high *Microcystis*). This station pair represents the large river channel habitat of the Western Delta region of the SFE. Both areas are dominated by high flows. Rio Vista represents Sacramento River water and Antioch represents San Joaquin water
3) Mokolumne River (relatively low *Microcystis*) and Old River (high *Microcystis*). This station pair represents Central Delta's small channel habitats.

At each station the water column will be profiled (temperature, salinity, fluorescence, PAR, transmittance) using a Seabird SBE-19 plus CTD equipped with a Wetlabs fluorometer, Licor 4□ PAR sensor, and a D &A OBS turbidity sensor. A Seabird Electronics SBE-32 rosette mounted with six 6-L Niskin bottles and fitted with a Seabird SBE-19 plus CTD will be deployed to collect vertical profiles of temperature and salinity and collect nutrient samples at depth. Other sampling is described under individual tasks below.

Questions Addressed	Example Hypotheses	Tasks
Obj. 1 Determine the environmental factors most likely to control *Microcystis (M)* bloom formation and toxin production in the Delta.		
What are optimal physical and biological conditions for *M.* growth in Delta?	*M.* blooms proliferate and persist at temps >20C, long residence times, high N:P ratio, low grazing.	2, 3, 4
Does *M.* grow better using NO_3 or NH_4 or organic N (e.g. urea) as a nutrient source?	*M.* dominated communities show higher NH_4 uptake than NO_3	2
Does the interaction or ratio of inorganic (and organic) nutrients favor *M.* bloom formation?	*M.* dominated communities favored with high N:P *M.*-dominated communities do not exhibit NH_4 suppression of NO_3 uptake	2
How do environmental factors affect *M.* toxicity?	*M.* toxicity is greater when *M.* is actively growing	2, 3, 4,5
Obj. 2 Determine the role that *Microcystis* strains and microbial associations play in determining toxicity associated with blooms		
Does the associated microbial flora favor *M.* growth and/or toxin production?	Toxicity is higher at sites dominated by associated filamentous cyanobacteria	3
How does the diversity of *M.* influence toxin concentrations and overall toxicity?	Different strains of *M.* will result in a diversity of toxins forms.	3,4
Objective 3. Determine the role of *Microcystis* in the pelagic Delta food web and its impact on the POD via zooplankton grazing		
How do primary production and N uptake vary between *M.*. and non *M.*-dominated blooms?	Primary production and nitrogen uptake rates are lower in blooms dominated by *M.*	1
How much *M.* do copepods ingest during the bloom?	*P. forbesi* guts contain more *M.* during peak of bloom	4, 5
How does *M.* grazing and toxicity of *M.* affect copepod survival and reproduction?	Ingestion of *M.* by *P. forbesi* increases mortality and reproductive loss	5
How do copepod grazing and behavior respond to blooms and toxins	During blooms and with high toxin concentrations, *P. forbesi* avoids ingesting *M.*	5
Do copepods avoid areas of high *M.* biomass?	Abundance of all life stages is higher in low-biomass areas	5
Does grazing by clams affect *M.* bloom formation?	*Corbicula* grazing reduces *M.* during pre-bloom periods	5

Table 1: Questions and example hypotheses addressed under the three objectives of the proposed project.

Task 1: Project Management

The lead PI (Parker) will be responsible for gathering information and developing semiannual progress reports, for liaison with contracting personnel, and for ensuring coordination among tasks. Each task leader will be responsible for ensuring the information is passed in a timely manner. Coordination of research tasks will be facilitated by semiannual participant meetings either at the RTC campus (prior to the start of the field season) or via conference call (or video conference call – e.g skype). Data sharing

between project tasks will be facilitated through a private ftp site (hosted by RTC/SFSU) with files made available to all PIs. In addition, input and output files will be provided to the IEP data web page no later than 1 year after completion of the manuscripts. The leader for each component will be responsible for ensuring that the component meets its goals and results in at least one manuscript submitted to a peer reviewed journal as well as the development of a synthesis paper combining the results of all components, in addition to the summary report to Delta Science Program (see deliverables table).

Project synthesis is an essential part of the project, and fostering synthesis is an essential element of project management. Year 3 of the project will be dedicated to synthesis of component (task) elements and will lead to insights not available from any one component. The lead PI will host and facilitate a project workshop at RTC during the beginning of Year 3 to initiate the synthesis paper. Additional time will be made to bring project members together with representatives of SFE resource management agencies to share project findings. We will also propose a special session on Microcystis for the Delta Science Program Biennial Meeting in Sacramento.

Task 2: Nutrients, Primary Production, and Nitrogen Uptake (Parker and Wilkerson)

Field Measurements of Nutrients, Size-fractionated Chlorophyll-a, Primary Production and Nitrogen Uptake

This part of Task 2 will be carried out to determine the depth-integrated primary productivity (CO_2 uptake) in *Microcystis*-stations to compare with non-*Microcystis* stations to evaluate the base of the food web under these contrasting conditions. In addition, autotrophic uptake of NO_3, NH_4 and dissolved organic nitrogen (urea) will be measured to establish whether rates of uptake are greater for different forms of N in *Microcystis* dominated communities (e.g. is there relatively higher NH_4 uptake rates in water with *Microcystis* compared to without *Microcystis*). Any relationship with ambient nutrient concentrations or ratios will also be considered.

Water will be collected at three depths for extracted size fractionated (>0.7-μm, >5-μm, >75-μm) chlorophyll-a measurements, size spectra of fluorescent particles (by flow cytometer) and inorganic nutrient concentrations (NO_3, NO_2, NH_4, PO_4, $Si(OH)_4$) (Wilkerson et al. 2006). Bulk dissolved organic matter (DOC and DON) will be assessed by high temperature combustion (Sharp et al. 2004) as well as urea concentrations will be measured (Revilla et al. 2006). Particulate organic carbon and PON will be assessed in the >0.7μm size fraction. Water from the depths at each vertical profile will be incubated for 24-h with $H^{13}CO_3$ (for primary productivity rates) and either $^{15}NH_4$, $^{15}NO_3$ or ^{15}N-urea (to evaluate uptake of different forms of N) (Parker 2005). These samples will then be incubated at simulated in situ irradiances using 100%, 50%, 25% and 5% shading of surface irradiance.

Tracking the nutrient concentrations and distributions as the blooms develop during the tri-weekly sampling along with uptake rates should allow the assessment of whether NH_4 inhibition of NO_3 uptake (Dortch1990, Dugdale et al. 2007) occurs in *Microcystis* -dominated communities in the SFE and how N:P ratios influence the occurrence of *Microcystis*.

Experimental Simulation of Bloom Progression and Bioassays of Microcystis-dominated Communities

Understanding whether different nutrient forms (especially reduced forms of N) or nutrient ratios play a role in determining *Microcystis* success is essential for future predictive modeling of *Microcystis* blooms. In the first year, water will be collected from each of the *Microcystis*-dominated sites for mesocosm studies to understand the nutrient interactions involved in the growth of different strains of *Microcystis* and toxin production. Our previous experiments using mesocosms in Suisun and Central San Francisco Bay and the Sacramento River revealed a predicable pattern of nutrient use and autotrophic biomass accumulation for eukaryotic phytoplankton dominated communities. Typically when water from the SFE is enclosed and relieved of light limitation by incubation at 50% of ambient irradiance, eukaryotic phytoplankton first use NH_4, reducing its concentrations to below inhibitory levels for NO_3 uptake. Once NO_3 inhibition has been relieved, rapid NO_3 uptake occurs along with chla synthesis and CO_2 uptake, producing a phytoplankton bloom. The full sequence for complete nutrient use occurs within 5 to 7 days and is extended in time with an increase in initial NH_4 concentration. All of our mesocosm experiments in the SFE have resulted in a diatom-dominated community regardless of the initial population. It is currently unknown whether the nutrient and chla sequence observed previously will hold in a *Microcystis* dominated community or if the interaction between NH_4 and NO_3 is an important determinant of *Microcystis* blooms. It is also currently unknown if, under these conditions, *Microcystis* will outcompete diatoms or as previously observed, mesocosms will favor diatoms.

Paerl and colleagues have conducted mesocosm bioassay experiments in a variety of field studies with colonial cyanobacteria including in the NH_4-dominated waters of the Neuse River Estuary, NC and Lake Taihu, China and will contribute to the design and interpretation of these mesocosm experiments. During 3 cruises each year, 20-L mesocosms will be filled at a pair of stations with and without the presence of *Microcystis* in order to capture the sequence of nutrient use and chlorophyll-a accumulation in these contrasting environments. Cubitainers will be held at 50% surface light in cooled incubators at RTC (Dugdale et al. 2007) and sampled daily for the same parameters that are proposed for the field station descriptions (see above). Mesocosms will be sampled for 5 to 10 days based on nutrient drawdown with at the start and end of the mesocosm experiments water will be sampled for phytoplankton enumeration, *Microcystis* species identification and toxin production. By measuring the physiological changes (uptake and photosynthetic rates) of the *Microcystis*-dominated samples as the potential bloom might progress, this approach offers a mechanistic way to interpret the field data collected during the spring-fall.

In the second year, nutrient bioassays will be conducted to further explore whether the growth and toxicity and dominance of certain *Microcystis* strains might be linked to nutrient availability and the form of N and the N:P ratio. Bioassays will explore saturation uptake kinetics for NO_3, NH_4, and urea and varying N:P ratios. Bioassays will involve small bottles (280-ml) incubations in which ambient water chemistry will be manipulated.

Task 3: Phytoplankton Microscopy and Toxin Characterization and Assessment of Microbial Consortia Associated with *Microcystis* Colonies (Mioni and Kudela)

Algal Identification and Toxin Measurements

We will collect surface water samples for algal identification and enumerations at the 6 sampling locations. We will perform algal identification and enumeration using epifluorescence microscopy (*Microcystis* cells) and inverted microscopy (all phytoplankton).

Samples will be analyzed to measure the concentration or microcystin toxins and to identify the presence of isomers and congeners. *Microcystis* can also produce other toxins (e.g. microviridin J) that have not yet been monitored in the Delta. Recent reports from toxicology analysis on Threadfin shad kidney suggest that Microcystis produces other toxic compounds in addition to microcystins that can affect the POD (Shawn Acuña, pers. com.). Samples for toxins will be analyzed using an Agilent 1200 liquid chromatograph (LC) connected to a 6130 quadrupole MS, using Selected Ion Monitoring (SIM) following a modification of the protocol reported by Mekebri et al. (2009). The following microcystin ions (m/z) will be monitored: 519.8 RR; 105.6 YR, 995.7 LR, 981.7 demethyl-LR, 910.6 LA, are monitored using $[M+H]^+$ in SIM mode. Full scan will also be collected over the range 100-1100 Da. The SIM windows were established for microcystins using the daughter ions, which are the Adda fragments of m/z 135.2 and m/z 213 produced by the transition of the protonated parent ions. We have previously run laboratory intercalibrations with an LC/MS/MS system operated by the California Water Pollution Control Lab, and our (LC/MS) results are comparable (with LC/MS/MS). The estimated method detection limits and reporting limits are 0.02 μg/L for MC. Samples will be prepared by sonication with 10% methanol of filtered biomass, and direct injection of filtered whole water, thereby providing particulate, dissolved, and total MC. For MC analyses, data are reported relative to calibration standards. For microviridin, no standards are commercially available, so we will follow published protocols for LC/MS analysis of these compounds and will report values based on identification of compounds based on mass and soft-ion fractionation of daughter ions.

Microbial Consortia Structure and Function

The proposed work involves isolating and culturing the phototrophic microbial consortia attached to *Microcystis*, establishing if they produce toxins and using a molecular approach to identify them. *Microcystis* colonies and associated flora will be collected in the field and washed to remove free living cells as described in Shi et al. (2009). Several isolation strategies will be tested, including manipulation of culture medium (e.g. addition of cyclohexamide to prevent the growth of eukaryotes or N depletion), agar overlay technique and serial dilution technique (for review, Acreman, 1994). Once pure cultures are obtained, we will determine if some of these associated phototrophs can produce cyanotoxin (microcystin and microviridin) themselves. We will specifically focus on the unknown filamentous cyanobacteria associated to *Microcystis* colonies in the Delta. We propose also to identify the epiphytic strains with a special focus on the unknown filamentous cyanobacterium using a molecular based approach. We will amplify cyanobacterial-specific 16S rRNA genes using the cyanobacterial primers CYA359F (Nübel et al. 1997) and CYA781R (Nübel et al. 1997) (in collaboration with Kudela and Paerl).

Task 4: Molecular Characterization of *Microcystis* Blooms (Paerl)

Microcystis strains from the SFE will be characterized molecularly based on 16S ribosomal RNA fingerprinting. Our lab group has acquired and tested various16S rRNA oligonucleotides on a suite of *Microcystis* containing environmental isolates. Based on these analyses a set of oligonucleotides first described by Janse (2004), has been shown to provide the highest resolution in genotyping closely

related strains of *Microcystis* (Fig.2). In general, rRNA is highly conserved within a group and it is therefore useful for verifying the presence of a particular genus of bacteria (*Microcystis* for example), but increased resolution can be obtained by sequencing a portion of the highly variable internal transcribed spacer (ITS) region of 16S rRNA. Using this approach, different strains within the same species can be differentiated.

Whole cell PCR will be used to amplify the 16S rRNA of each morphotype of *Microcystis* present in the estuary. PCR products will be separated on an agarose gel, excised and purified, ligated into a TOPO TA vector (Invitrogen) and transformed into TOP10 *E. coli* then sent for sequencing. Sequences will be analyzed and aligned using the computer programs Sequencher, VectorNTI and Mr. Bayes. Phylogenetic trees will be constructed and compared using the ClustalW algorithm which employs the neighbor-joining method and also by Bayesian inference. Regarding toxicity, each morphotype of *Microcystis* will have its entire *mcy* gene cassette responsible for microcystin production amplified. To accomplish this, a *Microcystis* -specific long PCR assay has been designed and tested which allows for the amplification of all ten genes and tailoring regions (~63 kb) in five PCR reactions (Fig. 3). Thus, each morphotype of *Microcystis* will be distinguished phylogenetically based on 16S rRNA and functionally based on whether its *mcy* cassette is complete, contains a partial deletion (and therefore is inactivated), or is totally absent. Once this preliminary information is known, samples collected throughout the SFE will be analyzed by real time quantitative PCR (qPCR) which will quantify the number of toxic and non-toxic cells of *Microcystis* along a spatial/temporal gradient.

Task 5: Food web Interactions (Kimmerer and Ger)
Residence Time

An index of residence time was previously determined for various Delta locations using the DSM2 particle tracking model (see Fig. 8 in Kimmerer and Nobriga 2008). These results will be used to index residence time as a function of flow conditions.

Clam Grazing Experiments

Grazing by clams could delay or reduce *Microcystis* blooms, especially before the blooms when cells are vertically mixed. We will measure grazing rates of *Corbicula fluminea* and *Corbula amurensis* on *Microcystis* and observe behavioral responses to various concentrations of *Microcystis*. Laboratory experiments will use natural water and added *Microcystis*, including cultured cells and colonies collected in the field (Ger et al. 2010). Grazing impact will be estimated using clam biomass data (IEP monitoring).

Zooplankton Responses

We will use a combination of field sampling and laboratory experiments to measure grazing, reproductive, development, and mortality rates of the calanoid copepod *Pseudodiaptomus forbesi* (Table 1). All measurements will be related to levels of *Microcystis* and toxins in the source water (Tasks 3 and 4).

Samples will be taken using water bottles, buckets and nets. Samples will be preserved for quantitative analysis, diluted into insulated buckets for experiments, or narcotized in carbonated water and stored on

ice for measurement of gut contents (Gannon and Gannon 1975). Preserved samples will be counted and copepods identified to species and life stage. Mortality will be calculated using a vertical life-table approach (Kimmerer and McKinnon 1987, Aksnes and Ohman 1996) using known stage development times augmented by molt rate measurements (below). The ratio of eggs to females in preserved samples and previously measured egg development time will be used to calculate egg production rate (Edmondson 1971, Gould and Kimmerer in press). Egg hatching success and juvenile development time will be determined by incubation of field-collected copepods. Ovigerous females will be isolated under a microscope and incubated in source water until eggs hatch or the egg hatching time has passed. Juvenile copepods will be sorted by life stage and incubated to determine molting rate (Gould and Kimmerer in press).

Feeding rates on *Microcystis* and total phytoplankton in the field will be determined as the ratio of gut contents (as carbon) to gut clearance rate. For total phytoplankton these measurements will be made using gut fluorescence (Mackas and Bohrer 1976, Slaughter et al. 2006), with a conversion to carbon using a suitable C:Chl ratio (Kimmerer et al. in prep.). Although this method has its critics (e.g., Tirelli and Mayzaud 1998), it is quick and its precision roughly matches that of the qPCR approach below. Gut fluorescence will be determined using a Turner Designs Model 10 fluorometer on batches of 20 copepods. Gut clearance rate will be determined on several occasions by collecting copepods in the field, placing them in filtered surface water, subsampling them over time and analyzing gut fluorescence as above (Irigoien 1998).

Feeding on *Microcystis* will be determined using qPCR on 16S rDNA and the *mcy* gene cluster (Oberholster et al. 2006, Ouellette and Wilhelm 2003). A PCR assay will be developed using primers (available from the Paerl Lab) on cultured axenic *Microcystis* to detect the presence of different strains based on the microcystin transcribing genes *mcy*B and *mcy*D (Ouelette et al. 2006). Fragments of the 16S rDNA gene region will be used to design specific SFE *Microcystis* primers for use in qPCR. Gene copy numbers of cyanobacteria and *Microcystis* specific target genes will be used to estimate the abundance of total cyanobacteria and various *Microcystis* strains in water samples and copepod guts (Rinta-Kanto et al. 2005, Ouellette and Wilhelm 2003, Baxa et al. 2010). Probes will be designed following the specifications of Applied BioSciences (Foster City, CA), and specificity will be tested on axenic *Microcystis* strains before the probes are used for field samples.

The gene copy number of the molecular biomarkers (16S rDNA and *mcy* genes) inside copepod guts will be converted into ingestion rates on *Microcystis* and other cyanobacteria using feeding experiments with axenic strains (Ger et al. 2010b). The calibration will be done by determining the gut clearance time for *Microcystis* and comparing the feeding rate estimated from qPCR with that determined by the disappearance of food. The same conversion will then be used to estimate *Microcystis* ingestion in the field. This is also the only way to quantify any strain specific differences in copepod grazing on *Microcystis* (MC+ vs. MC-). The ratio of ingested *Microcystis* cells to chlorophyll-containing cells will be used to assess how ingestion (or avoidance) of *Microcystis* affects demographic processes (reproduction, development, and survival).

Feeding and survival experiments will be used to extend earlier results (Ger et al. 2010a, b) to field conditions. One set of feeding experiments will contrast selected high- and low-*Microcystis* locations

with copepods from each location incubated in water from the same location and from the other locations in a replicated factorial design. This will determine both short and long-term effects on feeding and survival. In addition, field-collected *Microcystis* will be given to cultured copepods to determine the effect of large colonies on ingestion, survival and reproduction under controlled conditions (Ger et al. 2010a). Feeding experiments will be conducted using groups of ~100 adult female copepods in 4-L beakers incubated for 2 hours after a 5-hour period of starvation to allow gut evacuation. Feeding will be determined as above. Survival experiments will be set up like feeding experiments and survival over 1 week will be determined (Ger et al. 2010a).

Because we view synthesis of each of the tasks above as critical to the success of the project, we have proposed to use Year 3 to develop a synthetic manuscript integrating results from each of the components (tasks) (see task 1, Project Management). The goal of the synthetic paper is to provide the mechanistic understanding to guide development of predictive models of *Microcystis* bloom development in the Delta. Additional manuscripts (at least one paper per task), including collaborative papers, will be developed between tasks (groups) as outlined in Table 1. We will propose a special session on *Microcystis* at the Delta Science Program biennial conference and will submit at least one abstract per task for the conference.

We will hold a mini workshop at the beginning of Year 3 for SFE resource managers to discuss major project findings for use in management decisions. Additionally, this project will fund three graduate students for MS degrees at SFSU and PhD at UNC-CH. We have not provided for formal public outreach. However, SFSU and RTC are strongly committed to providing outreach and other non-traditional educational opportunities to the community. This commitment is embodied in the Education Coordinator at RTC. Opportunities are frequent for RTC scientists to engage in public speaking, lecturing to teachers or community organizations, and other such activities. We will work with the Educational Coordinator to seek such opportunities as our research unfolds, and present results of our research whenever possible.