Environmental Factors Associated with Toxic Cyanobacteria in Pinto Lake, a Coastal Lake in the Monterey Bay Area

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ENVIRONMENTAL FACTORS ASSOCIATED WITH TOXIC CYANOBACTERIA IN PINTO LAKE, A COASTAL LAKE IN THE MONTEREY BAY AREA

A Thesis
Presented to the
Faculty of the
Division of Science and Environmental Policy
California State University Monterey Bay

In Partial Fulfillment
of the Requirements for the Degree
Master of Science
in
Coastal and Watershed Science and Policy

by
Erin Rose Stanfield
Summer 2013
The Undersigned Faculty Committee Approves the

Thesis of Erin Rose Stanfield:

ENVIRONMENTAL FACTORS ASSOCIATED WITH TOXIC CYANOBACTERIA

IN PINTO LAKE, A COASTAL LAKE IN THE MONTEREY BAY AREA

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DEDICATION

This Master’s Thesis is dedicated to the memory of my father, Steven C. Stanfield who passed away August 14, 2008. His vibrant curiosity and life-long learning inspired me to explore my thesis research and make the world a better place through the lens of environmental science.
ABSTRACT

Environmental Factors Associated with Toxic Cyanobacteria in Pinto Lake, a Coastal Lake in the Monterey Bay Area
by
Erin Rose Stanfield
Master of Science in Coastal and Watershed Science and Policy
California State University Monterey Bay, 2013

Cyanobacterial harmful algal blooms (CHABs) dominate and disrupt aquatic ecosystems by virtue of their rapidly expanding biomass and the production of secondary metabolites, including potent toxins. Since 2007, toxin-producing CHABs have been documented in freshwater bodies draining into the Monterey Bay National Marine Sanctuary (MBNMS) located on the California Central Coast. In this study, we combined freshwater ecology and molecular biology approaches to characterize the abundance of potentially toxic cyanobacteria, intracellular microcystin levels, and presence of microcystin synthesis genes in association with environmental factors in Pinto Lake, located in Watsonville, CA, a freshwater body seasonally draining into the MBNMS. We observed potentially toxic cyanobacteria increasing with water temperature, thermocline depth and decreasing ammonium and nitrate with microcystin levels increasing with cyanobacteria abundance during the summer and autumn months of 2009 through 2011. Additionally, our results support an association between toxic *Microcystis* sp. abundance and abundance of *Aphanizomenon* sp. indicating a positive association between the presence of microcystin toxin genes, intracellular microcystin levels, and abundance of potentially toxic cyanobacteria in Pinto Lake.

Keywords: California, cyanobacteria, CHAB, Mediterranean climate, microcystin, shallow lake
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CHAPTER 1
ENVIRONMENTAL FACTORS RELATED FRESHWATER TOXIC CYANOBACTERIA BLOOMS: A LITERATURE REVIEW

Cyanobacteria are largely responsible for the development of the Earth’s plentiful oxygen and moderate climate (Olson and Pierson 1987; Blankenship 1992; Schopf 2000; Knoll 2003). At least two and a half billion years ago, cyanobacteria evolved photosynthesis as a metabolic pathway for harvesting solar radiation and synthesizing organic compounds with oxygen released as a byproduct (Des Marais 2000; Xiong et al. 2000). Cyanobacteria remain essential for current biogeochemical processes including atmospheric oxygen production and the recycling of carbon, nitrogen, sulfur and phosphorus (Falkowski 2008). As ancient organisms, cyanobacteria demonstrate adaption to a wide variety of ecosystems including hot springs and other geothermally heated waters (Stockner 1967), hyper-saline conditions (Krumbein et al. 1977; Ley et al. 2006), the coldest and most desiccated Antarctic deserts (Friedman and Ocampo 1976), frigid permanent snow fields and glaciers (Stomp 2007), in symbiosis with a variety of organisms (Rai et al. 2000) and in a variety of marine, freshwater and estuarine systems (Pinckney and Paerl 1998; Potts and Whitton 2000). Most plentiful in most aquatic systems, cyanobacteria form an important portion of phytoplankton assemblages (Schindler 1978; Fisher 1992)

HARMFUL ALGAL BLOOMS
The domination of the phytoplankton assemblage by a single or a few taxa, given a suite of environmental and ecological community factors, is the fundamental definition of
a harmful algal bloom (HAB) (HARRNES 2005; Carpenter 1998). HABs can disrupt ecosystems, alter food chains and release potent toxins (Pearl 1988). In freshwater systems, HABs are most often composed of cyanobacteria forming cyanobacterial harmful algal blooms (CHABs) (Anderson 2002). CHABs alter the physical and chemical conditions of freshwater ecosystems to their advantage through several mechanisms. By virtue of their rapidly expanding biomass and buoyant physiology, CHAB often occupy surface waters and physically block light from reaching other photosynthesizing organisms and lower levels (Horne 1972; Huisman 1999). The developing CHAB often increases the pH level beyond what is acceptable to other phytoplankton (Mogelhøj et al. 2006). Nitrogen-fixing cyanobacteria can contribute significant amounts of nitrogen into the ecosystem, contributing to eutrophication and supplementing the nitrogen cycle (Horne 1977; Horne 1979; Paerl 1988). The decomposition of CHAB biomass and the organic molecules produced can result in seasonal or longer term hypoxic conditions and fish kills (Diaz and Solow 1999; Lopez et al. 2008).

CHABs demonstrate several means towards bottom-up control of freshwater food webs (Shurin et al. 2006). By dominating primary productivity, CHABs can replace the diverse and more nutritious phytoplankton at the base of the food chain (Bernardi and Giussani 1990; Muller-Navarra et al. 2004). CHABs colonial forms and filamentous structures inhibit grazing by many zooplankton as the particles are often too massive or too gelatinous for filter feeding structures (Fulton and Paerl 1987; Gliwicz and Lampert 1990; DeMott et al. 2001). Prevalent mucilage and biofilms also reduce palpability, digestibility and assimilation of cyanobacteria by zooplankton and larger grazers (Porter 1975). CHABs may further inhibit grazing by releasing potent biochemicals (Haney et
al. 1994; Jungmann 1994 and 1995; Rohrlack et al. 1999 and 2001; Lurling 2002; Weigand 2002). These modes of CHAB grazing inhibition a positive feedback loop for continuing the CHAB formation and presence as zooplankton positively select for and reduce the biomass of otherwise competitive phytoplankton (Lehman 2005).

**Toxic CHABs**

The aspect of CHABs that has garnered the most attention from the media and world governments is the production and release of potent cyanotoxins by some CHAB species (Anderson 2000; Carmichael 2008). A variety of cyanotoxins are present in natural and anthropogenic water systems (Codd et al. 1989; Namikoshi and Rinehart 1996; EPA 2006; Falconer 1996; WHO 1999; Codd et al. 1999). Several freshwater cyanobacteria toxins pose serious risks of illness and death through ingestion of infected water or by incidental contact through recreational activity (Stewart 2006; Sandifer 2007). Food chain bioaccumulation and magnification may serve as a latent yet powerful cyanotoxin exposure vector (Vasconcelos et al. 2001; Pflugmacher et al. 2002; Magalheas et al. 2003; Lehman et al. 2008). Documented reports of cyanotoxin-related human fatalities (Reynolds 1999) and illnesses (Falconer 1996; Carmichael et al. 2001; Stewart et al. 2006) are dwarfed by cyanotoxin-related pet, livestock and wild animal illness and mortality (Yoo et al. 1995; Briand 2003; Stewart et al. 2007). There may be an increased risk for marine mammals from freshwater cyanobacteria that lyse and release toxins at freshwater-saline interfaces (Tonk et al. 2007; Lehman et al. 2008). Some cyanotoxins, including the ubiquitous microcystins, are also structurally stable, resistant to degradation in the environment and can persist weeks to months after release (Tsuji et al. 1994;). Cyanotoxins may serve as a mode of allelopathic competition against other phytoplankton...
Other important research has also demonstrated the deleterious effects of cyanotoxin-laced water used as crop irrigation (Crush et al. 2007; Peuthert et al. 2007).

Evidence of toxic cyanobacterial blooms persists in historical documents and anecdotal reference for over 500 years in Europe (Codd and Beattie 1991) in traditional practices of North American indigenous people (Prakash et al. 1971) and Australian aboriginal communities (Hayman 1992). The journal *Nature* published the first documented report of a CHAB in 1878 as a livestock-killing toxic algae event in South Australian lakes (Francis 1978). Paerl and Huisman (2009) reported increasingly frequent and intense CHABs globally over the last 150 years. With equally increasing media coverage of CHAB, world governments have prioritized funding and legislation to the study of CHAB with an eye to prevention, prediction, reduction and remediation (Anderson 2000; Carmichael 2008). Most CHAB research has been dedicated to studying the environmental causes and dynamics of CHAB formation and proliferation. The success in forming robust predictive models has been incomplete.

**CHAB Nutrient Drivers**

The role of anthropogenic and natural nutrient loading into freshwater systems is the most studied environmental factor associated with the CHABs. Because some land use types enhance runoff, nutrient concentrations can also be understood as a proxy for the effect of land use on the formation of CHAB (Jones et al. 1996; Jassby 2005; Buford 2007; Piehler 2008). While there is widespread agreement that nutrient inputs play a significant role in the formation and attenuation of CHABs, the nature of nutrient connection is debated. Smith (1983), in one of the earliest wide-scale CHAB studies,
described a strong association between CHAB formation and the ratio of epilimnic total nitrogen to total phosphorus (TN:TP) from a collection of 17 worldwide lakes. When TN:TP ratios fell below 29:1, he reported a strong association with CHAB formation. Likewise, blooms tend not to form in water bodies with higher ratios (Smith 1983). Trimbee and Prepas (1987) repeated Smith’s analysis using data collected from 16 Canadian lakes. Their results supported the low TN:TP theory, but only in the case of nitrogen-fixing CHABs. Across all cyanobacteria species, they found TP to be a more robust predictor of cyanobacteria biomass than TN:TP. Their results suggested the importance of cyanobacterial species composition in combination with nutrient inputs for the promotion of CHAB (Trimbee and Prepas 1987). McQueen and Lean (1987) studied the role of nutrient concentrations in the development of CHABs on a much smaller scale of Lake St. George, Ontario during five nonconsecutive summer bloom seasons throughout 13 years. They did also not find support for TN:TP as predicting CHAB presence (McQueen and Lean 1987). Instead, they identified a significant negative correlation between cyanobacteria and concentrations of nitrate (NO3), total inorganic nitrogen (TIN) and NO3:TP. Furthermore, they found that this relationship was even more significant with temperatures factored in.

Canfield et al. (1989) investigated Smith’s TN:TP theory with a larger dataset. Analyzing nutrient concentration and CHAB species concentration in a collection of 165 lakes throughout Florida. Canfield et al. did not identify the association between cyanobacterial bloom formation and TN:TP. In fact, they did not find any significant relationship nutrients levels and cyanobacteria presence or domination of the freshwater phytoplankton community. They only identified a positive correlation between
cyanobacterial biomass and total plankton biomass, with cyanobacteria became consistently dominant when total phytoplankton biomass exceeded 100 mg/L (Canfield et al. 1989).

Several subsequent studies described significant associations between nutrients and CHAB promotion. In a small scale study closely following the development of CHABs in Steilacoom Lake, Washington, Jacoby et al. 2000 described a significant association between high total phosphorus (TP) concentration and bloom formation. In their large-scale meta-analysis, Anderson et al. (2002) reported support for the association between phosphorus inputs and freshwater CHAB and between nitrogen inputs and marine and estuarine CHAB worldwide. Downing et al. (2001) also reviewed the incidence of cyanobacteria in nearly 100 lakes and found low N:P ratio to be less significant than total nitrogen (TN) and TP. Gianni et al. (2005) identified a positive relationship between cyanobacterial biomass and both TN concentration and TP concentration. In their regional study of the San Francisco Estuary, Lehman et al. (2005) also found TN and TP to both be associated with cyanobacterial biomass.

Most of the studies following Smith (1983) involved datasets of varying spatial and temporal scales with a complex of potential compounding factors. As such, meaningful comparison between their results is somewhat problematic (Morris et al. 2002). Two long-term studies offer another perspective on the associations between cyanobacteria and nutrients and other environmental factors. Havens et al. (2003) presented the results of a 28-year observational data set investigating the incidence of CHAB as a function of TP, TN and the ratio of TN:TP in a sub-tropical Floridian lake.
Over the length of the study, TN:TP decreased from 30:1 to 15:1 correlating with an increase in cyanobacterial biomass (Havens et al. 2003).

Based on results from a 37-year study, Shindler et al. 2008 reported that reduced nitrogen inputs had no effect on reducing the incidence of CHAB in a natural freshwater Canadian lake under controlled nutrient inputs. Researchers fertilized the lake with constant annual inputs of phosphorus while simultaneously reducing the nitrogen while monitoring CHAB presence and duration. Cyanobacterial biomass persisted and was dominated by nitrogen-fixing species for years after cessation of nutrient inputs (Schindler et al. 2008). These results underline the importance of identifying CHAB species composition for understanding nutrient impacts. Also, because Schindler et al. (2008) performed a manipulative experiment, their results have the potential to be more informative and demonstrate a higher level of inference.

The limiting nutrients for Chesapeake Bay’s estuarine phytoplankton biomass shift from P and Si limitation in spring to N in the summer (Fisher et al. 1992). Winter and spring precipitation maximizes freshwater runoff, driving the estuary towards phosphorous-controlled cyanobacterial biomass as in freshwater systems. Conversely, low summer freshwater runoff drives the estuary towards the typical nitrogen-correlated algal biomass of marine systems (Fisher et al. 1992). The seasonal controls on phytoplankton assemblages in the San Francisco Estuary (SFE) appear to differ from East Coast systems. Because of the relatively constant nutrient-rich conditions of the SFE, nutrient concentrations do not seem to limit cyanobacteria biomass independent of season. Instead, CHABs are often associated with summer low stream flow conditions, high water temperature and high photosynthetically active radiation (Lehman et al. 1996;
Lehman et al. 2008). Seasonal and climatic variation consistently play a significant role in the formation of CHABs through increased water temperature and PAR (McQueen and Lean 1987; Pearl 1988; Wasmund 1997; Huisman et al. 1999; Havens 2003; Kardinaal et al. 2007; Pearl et al. 2009)

**CHAB and Sediments**

The nutrient content of lake sediment has also been implicated with the promotion of CHABs. Hyerstrand et al. (1998) identified a significant association between benthic ammonium deposits and CHAB formation. Johnston and Jacoby (2003) also found nutrient rich sediments offered a sort of reservoir for cyanobacteria in low nutrient waters or extreme low flow conditions. When conditions improve, the cyanobacteria can migrate from the sediments into the water column and mobilize a pulse of nutrients with them (Johnston and Jacoby 2003). Izaguirre et al. (2007) identified sediment as a latent source of cyanobacteria and cyanotoxins in Southern California drinking water reservoirs. Periods of summer water column stratification often result in redox conditions in the hypolimnion that favor the release of sediment bound phosphate, ammonium and other nutrients. While usually restricted to the hypolimnion by the stratification’s resistance to lake mixing, this nutrient reservoir is available to the highly mobile and buoyant cyanobacteria (Wagner and Adrian 2009; O’Neil et al. 2012).

**Potential Global Climate Change Impacts**

While the explicit nutrient dynamics promoting cyanobacterial blooms may not be consistent worldwide, in almost every system there is a documented association between temperature and cyanobacteria biomass (Kosten et al. 2012) and the frequency and duration of CHABs is increasing worldwide (Paerl & Huisman, 2008 & 2009; Paerl and
Hyerstraand 2009). Cyanobacteria have a higher optimal temperature for growth (for most cyanobacteria, greater than 25°C) than eukaryotic algae (Robarts and Zohary 1987; Butterwick et al. 2004). As such, global climate change poses a significant risk for worldwide increases in the prevalence and intensity of CHABs.

Understanding the diversity of cyanobacteria community is important for appreciating the effect of water temperature on CHAB formation due to a variable response depending on taxa (O’Neil et al 2012). For example, species of both *Oscillatoria* and *Microcystis* have optimal growth above 25°C, *Microcystis* sp. is severely limited below 15°C, while *Oscillatoria* sp. often can persist below 10°C and even down to 4°C (Robarts & Zohary 1987). In contrast, *Aphanziomenon flow-aquae* has been document to grow above 35°C (Butterwik et al. 2004). Some cyanobacteria genera have higher growth rates for toxin producing species than non-toxic species under increased temperature conditions (Davis et al. 2009).

Horizontal and vertical mixing patterns (or lack thereof) can also contribute to the formation of CHABs (Watson et al. 1997; Huisman 2004; Dokulil 2006). Eutrophic or hypertrophic lakes (high nutrient and high primary productivity) often exhibit stratification with distinct thermal and dissolved oxygen concentration layers (Stralling 1966; Schindler 1978). Many taxa of cyanobacteria outcompete other phytoplankton in stratified lake conditions due to the superior buoyancy control and ease at moving through the thermocline and access otherwise unavailable habitat and resources (Wagner and Adrian 2009). The buoyancy control advantage demonstrated by many cyanobacteria may be further enhanced under conditions of climate change. As the surface water temperature increases, the density of the epilimnion water will correspondingly decrease.
and the less buoyant phytoplankton will increasingly sink and yield their habitat to cyanobacteria (Paerl and Huisman 2009).

**Cyanotoxin Drivers**

Another important area of CHAB research is the study of the factors associated with the potency, production and release of cyanotoxins as distinct from the development of CHABs. Many researchers perform controlled laboratory studies to examine the environmental parameters related to cyanotoxin production. Westhuszen et al. (1985) asserted a strong association between the growth phase of cyanobacterial cultures, temperature and toxin production. Watanabe and Oishi (1985) found that N deficiency could increase toxin concentration. In contrast to Watanabe and Oishi, Vezie et al. (2002) found higher N and P requirements for toxic over nontoxic strains. Rapala et al. (1998) and Tonk et al. (2005) both found that water temperature and light intensity can have species specific and toxin-variant specific effects on cyanotoxin production. In their meta-analysis of existing cyanotoxin research Sivonen and Jones (2000) report that while controlled laboratory studies can offer some insight into theoretical relationships and models, they have very limited applicability to the complex array of factors found in the natural systems. Zurawell et al. (2005) recommends a combination of detailed field study combined with controlled laboratory practices for the widest applicability and highest inference.

Several field-based studies suggested that CHAB toxicity is related to the similar environmental factors that favor the formation of CHAB. In their two-year study of a freshwater lake in Washington state, Jacoby et al. (2000) found that cyanotoxin concentration was positively associated with TP concentration, water column stability,
water temperature and decreased mixing. In contrast to Jacoby et al., Kotak et al. (2000) found cyanotoxin to increase with total cyanobacterial biomass according to ratio of TN:TP concentrations. Park et al. (1998a) found that some CHABs show considerable temporal variation in the concentration of cyanotoxins, growing more toxic as the bloom ages. In addition, temporal variability in toxicity may result from the waxing and waning of CHAB species and toxin strains with varying toxicity (Park et al. 1998b).

**CHAB Research Methodology**

**Challenges of Microscopy for CHAB Research**

Oh et al. (2001) asserted that in CHAB dominated by one or two toxic species, cyanobacteria biomass is a proxy for toxin concentration. The possibility of using phytoplankton biomass as an indirect approximation of microcystin and other cyanobacteria toxin concentration offers and exceptionally promising tool gauging potential CHAB toxicity. However, the crux of this method, the need to determine cyanobacterial species, highlights cyanobacterial genetics and diversity as another important area of CHAB research. Woese (1987) and Whitton et al. (2008) emphasized the need for molecular biology-based cyanobacteria taxa determination as an array of environmental factors can induce morphological changes on both short and long time scales, hindering effective morphological-based species identification. Researchers Yang et al. (2008) and Pan et al. (2008) also supported the notion for a molecular approach for identifying cyanobacteria taxa as morphological traits can be deceiving and imprecise. However, the efficacy and accuracy of molecular biology approaches depends on the completeness and extent of the known genome catalog. Several studies have concluded that more basic cyanobacteria molecular research is crucial to perform the emerging
molecular techniques that depend on genetic fingerprints for rapid and efficient identification and quantification of CHABs and cyanotoxin production (Hornder-Devine et al. 2003; Mankiewicz-Boczek et al. 2006; Ouellette et al. 2006; St. Amand et al. 2007). For the near future, CHAB researchers will continue to employ microscopy to assess morphology as an indicator of cyanobacteria diversity and by extension potential toxicity (Via-Ordorika et al. 2004; Lehman et al. 2008; Pan et al. 2008). Cell counts and density estimations also will be performed visually to double-check quantitative molecular approaches and as an inexpensive alternative in some regions (APHA 1998; Rogalus and Watzin 2008).

**Molecular CHAB Detection**

Neiland (2002) presented the rapid and highly sensitive polymerase chain reaction (PCR) to perform molecular characterization of cyanobacteria toxin genes from environmental samples. This approach has formed the background of much current CHAB research to characterizing cyanobacteria taxonomy phylogeny and as a proxy for toxin assay (Neiland 2002). However conventional PCR offers only the binary data on the presence or absence of specified gene sequences. Foulds et al. (2002) presented the next step in molecular approaches in the real-time, quantitative PCR (Q-PCR). Q-PCR detects specified gene sequence but has the added advantage of quantifying the number of copies of the gene sequence in an environmental sample. Rinta-Kanto et al. (2005) improved the q-PCR method using a combined approach targeting both cyanobacteria-specific 16S-rRNA genes and microcystin synthetase genes with a taq-man probe in *Microcystis aeruginosa* blooms in Lake Erie. In 2010, Baxa et al. used q-PCR to quantify *M. aeruginosa* in San Francisco Bay Estuary and found the ratio of toxic to non-toxic
cyanobacteria to be surprisingly variable and deserve more attention for assessing health-
risks from CHABs.

Rudi et al. (2000) described the computer-assisted DNA chip (micro array) that identifies all cyanobacteria that are present in a sample accurately and identify the toxin producers depending on the designed probe. The development of the microarray based on 16S rRNA genes for a few groups of cyanobacteria and Eubacteria. The environmental DNA was isolated, amplified by PCR, labeled and hybridized with the complementary probes on the membrane (Rudi et al. 2000).

In the 2008 article, “A Synopsis of Research Needs Identified at the Interagency, International Symposium on Cyanobacterial Harmful Algal Blooms Incidence of cyanobacterial harmful algal blooms (CHABs),” Hudnell et al. present many near-term CHAB research goals with emphasis on genetic-based field-ready tests to identify and quantify cells and toxins (Hudnell et al. 2008). Long-term goals include the creation and dissemination of CHAB occurrence and genetic information into accessible databases. They also assert the necessity of broad-based training to effectively perform emerging genetic-based analytic techniques. With a more extensive library of cyanobacteria genetic information, exploring environmental factors regulating toxin production at the genetic level can be a valuable tool for understanding the complex associations between environmental factors and CHAB formation and toxin production (Lopez 2008). The need for a broader and accessible cyanobacteria genetic dataset is further emphasized by Izaguirre et al. (2007). Researchers reported the discovery of previously unidentified and unusual microcystin-
producing benthic cyanobacteria from water reservoirs that supply drinking water to over
18 million people in southern California (Izaguirre 2007). This study also highlights the possibility of latent cyanobacteria toxicity and the risks of that toxicity entering the municipal water supplies. Broader understanding and accessibility of cyanobacteria genetic can assist with rapid assessment of water bodies for such latent toxic cyanobacteria. Until more complete genetic documentation is available for the mostly understudied cyanobacteria, genetic approaches will continue to be paired with researchers using microscopes (St. Amand et al. 2007)

**CHAB Sampling and Experimental Design**

While very precise genetics-based analyses and sophisticated computer-based statistics are in reach for many researchers studying CHAB, several essential questions regarding experimental design and effective methods are often overlooked. Consistent with a review on microbial biodiversity studies (Morris et al. 2002), Catherine et al. (2008) reported that the majority of phytoplankton and cyanobacteria surveys lack explicit focus on sample representativeness and unbiased sampling strategies (e.g. Ernst et al. 2001; Oh et al. 2001; Lehman et al. 2003; Rolland et al. 2005; Lehman et al. 2007; Rogalus and Watzin 2008). Catherine et al. also emphasized the use of cost-effective and rapid sampling techniques in tandem with geographic information system technology (GIS) and remote sensing data to design and implement unbiased sampling on regional-scale CHAB studies.

**Tiered Sampling Design**

Rogalus and Watzin (2008) presented the results of their examination of sampling methods for a tiered cyanobacteria monitoring protocol proposed by the World Health Organization (WHO) and adapted by Watzin et al. (2006). The *Alert Level Framework*
recommended by WHO, consists of a three-tiered monitoring system based on chlorophyll \(a\) concentration and cell density thresholds (Chorus and Bertram 1999). The adapted monitoring framework presented by Watzin et al. (2006) focused on a rapid phytoplankton screening method to efficiently estimate cell density using vertical plankton net tows and cyanobacteria colony size. Rogalus and Watzin evaluated the ability of this rapid screening protocol to capture the overall risk posed by cyanobacteria exposure through recreation use and found that this method regularly underestimated cell counts and toxicity levels and that the difference was inconsistent across cyanobacteria taxa (Rogalus and Watzin 2008). This discrepancy between the sampling methods is consistent with the results that Watzin et al. (2006) described at high \((>4000 \text{ cells·ml}^{-1})\) cyanobacteria cell densities.

CHAB Sampling Interval

The majority of CHAB research employs analysis on multiple temporal and spatial scales, often rendering the results difficult to compare (Anderson 2002; Carmichael 1999). In order to effectively track the environmental factors affecting CHAB formation and toxin production in the real conditions of the field, appropriate sampling design includes randomization and representativeness detailed by Catherine et al. (2008) and efficiency of practice as emphasized by Rogalus and Watzin (2008). The tradeoff between these two poles is obvious by the many sampling schemes employed. Znachor et al. (2006) and Kardinaal et al. (2007) recommend regular repeat sampling to best track the environmental conditions associated with the development of CHAB. This end of sampling spectrum can involve biweekly (Visser et al. 1996; Lehman et al. 2008; Rogalus and Watzin 2008; Kann 2005; Briand et al. 2008) weekly sampling (Ernst 2001).
and sub-weekly (Jacoby et al. 2000) sampling schedules that maximize sample size and put effort to controlling for temporal variability. At the other end of the spectrum are more wide-scale studies that employ snap-shot, single sampling events (Lehman et al. 2001; Lehman et al. 2004; Rogalus and Watson 2008).

**CHAB Research Gaps**

Using an array of available approaches, researchers have demonstrated moderate success with understanding the multitude of environmental factors influencing CHAB and cyanotoxin production, several authors suggest that these associations are only applicable in temperate lakes of moderate depth and dependable mixing regimes (Pearl et al. 1988; Anderson et al. 2002; Lehman et al. 2005; Lehman et al. 2008). CHAB prediction in the much more multivariate and complex mixing and flushing patterns of non-temperate climates, estuaries, and the interface with marine systems remains challenging and needing further research. Lehman et al. (2007) and Catherine et al. (2008) suggest that the essential nutrients for CHAB promotion depend on the local systems and on regional hydrologic network patterns. Catherine et al. (2008) also reported that most CHAB research effort has gone towards studying the dynamics of CHAB promotion, growth and decline, and a shortage of well-designed investigations of regional-scale CHAB spatial distribution and controlling factors. Such regional-scale studies have the potential to yield valuable information about cyanobacterial species richness, spatial distribution, the extent of potential toxicity and CHAB interactions environmental variables (Catherine et al. 2008).

This is the context for much needed research into diversity, distribution and potential toxicity of cyanobacterial communities within the context of the Monterey Bay
Region. The combination of this region’s long history intensive conventional agriculture and the Mediterranean climate’s extreme seasonality of winter rainfall and hot, sunny and drought-prone summers offer near ideal habitat for CHAB formation. Most of the published literature about cyanobacteria in California has been focused on San Francisco Estuary (Lehman et al. 1992-2010; Jassby 2005; Moisander et al. 2009) or on drinking water reservoirs in southern California (Izaguirre et al. 2004). Before more complex experiments to examine the controlling factors for CHAB in complex systems such as Mediterranean climate and estuaries, the basic research identifying the dominant species must be performed. For example, in the San Francisco estuary, *M. aeruginosa* is one of the most common species of freshwater cyanobacteria and it spends most of the year as a single-celled version with minimal toxicity or impact on the aquatic ecosystem (Lehman and Smith 2001). Seasonally, following the decline of diatoms and in step with warmer weather (Lehman 2000), *M. aeruginosa* cells fuse together forming the massive colonial version that dominates the phytoplankton community and presents an increasingly toxic threat.

Working towards understanding Monterey Bay Area cyanobacteria, in 2010 Miller et al. implicated microcystins in the deaths of over twenty marine mammals in the Monterey Bay National Marine Sanctuary (MBNMS) and attributed the cyanotoxins to CHABs in Pinto Lake, a lake several kilometers onshore. In 2011, Kudela used an absorptive resin to track microcystins at the Pinto Lake boat dock and found the toxin to correlate with cyanobacterial biomass and total dissolved nitrogen.
**PINTO LAKE, CALIFORNIA**

Pinto Lake is a 0.37 km$^2$ hyper-eutrophic lake located within the Pajaro River watershed in Santa Cruz County. The lake is bordered by two public parks and private lands. Land use in the lake’s 15 km$^2$ watershed is primarily agricultural and ranch land, with some suburban and rural residential areas and businesses including stables, kennels and a composting facility. A history of the lake’s trophic status and watershed land use changes, as derived from sediment core data, includes increases in productivity with sediment runoff following agricultural development of the watershed in the 19th century. The shift of the lake to a much more eutrophic system with frequent cyanobacterial blooms, however, does not appear to have begun until the second half of the 20th century (Boyle et al. 2011).

Pinto Lake exhibits seasonal CHABs with microcystin toxin levels measuring at an average of 183 ppb, during the summer-autumn bloom season, in 2007 through 2012. These toxin levels supersede the safe recreational exposure limit of 0.8 ppb established by the State of California (Cal EPA 2012). Health risks to park visitors and the community that are linked to water contact would be significantly reduced through eradication of the cyanobacteria and associated toxins. The lake poses human health risks from frequent CHABs which dominate the lake’s aquatic ecosystem.

Annually, over 50,000 people visit the Pinto Lake’s two parks enjoying boating, fishing and lakeside picnics and camping. Many visitors include local low income families with young children. A low-income housing project for agricultural workers is located on the lake’s western shore.

The 2010 documented 21 sea otter deaths in the MBNMS were linked potentially with Pinto Lake cyanobacteria and cyanotoxins (Miller et al. 2010). Pinto Lake drains
seasonally into the greater Pajaro River watershed and eventually into the MBNMS. Also in 2010, The State of California listed Pinto Lake on the California Impaired Water Bodies 303(d) list for CHABs. The City of Watsonville was awarded US EPA Clean Water Act section 319(h) grant to identify the environmental drivers of the blooms (temperature, nutrients, and/or sediments) and develop an implementation strategy to mitigate and restore Pinto Lake water quality based on the results of the water quality sampling data and modeling. The strategy was required to include a summary of Management Measures/Management Practices, an implementation sequence of actions needed to minimize and/or eliminate the cyanobacteria blooms, actions related to minimizing the loading of nutrients into the lake, treatments recommended for the nutrients in the lake itself, and/or any other action or treatment required at the lake water outflow.

The Pinto Lake 319 (h) project was designed to encompass an array of factors most commonly associated with CHABs. Monitoring was focused on the temporal variation in CHAB development across Pinto Lake in association with several environmental parameters. In particular, shallow areas with high nutrient loadings (from surface or ground water), redox shifts at the water-sediment boundary, and benthic nursery areas for cyanobacteria to maintain presence and their subsequent dominance later in the year. The project also included monitoring of potential nutrient sources to the lake including from the watershed through the surface water in Pinto Creek, from groundwater through groundwater monitoring wells and from the sediments via monitoring flux chambers.
In 2012 and 2013, the Watsonville public works manager, project collaborators with the Santa Cruz Resource Conservation District, Santa Cruz County Health staff and researchers met with a Pinto Lake Watershed community members to present the results of the 319(h) project. At this time, the beginnings of the community action group, “Friends of Pinto Lake” was also established.
CHAPTER 2
ENVIRONMENTAL FACTORS ASSOCIATED WITH TOXIC CYANOBACTERIA IN PINTO LAKE, A COASTAL LAKE IN THE MONTEREY BAY AREA

INTRODUCTION
Worldwide, freshwater bodies and near-shore coastal environments are increasingly threatened by cyanobacterial harmful algal blooms (CHABs) (Paerl et al. 2011; Smith 2003). Pinto Lake is a shallow, eutrophic freshwater lake that develops regular toxic CHABs and drains seasonally into the Monterey Bay National Marine Sanctuary (MBNMS) (Figure 1). Located offshore of California’s central coast, the federally-protected MBNMS supports one of the world’s most diverse marine ecosystems in a uniquely productive environment (NOAA 2011). In 2007, researchers detected Pinto Lake micocystins at 2.1 g · L\(^{-1}\) (Mekebri et al. 2009.), greater than six orders of magnitude above the current California State recreational contact guideline level of 0.8 µg·L\(^{-1}\) (CalEPA 2012) were documented in Pinto Lake (Mekebri et al. 2009). In 2010, deaths of endangered sea otters in the MBNMS were linked to microcystin poisoning with Pinto Lake being identified as a putative source of cyanobacteria and toxins (Miller et al. 2010).

Hepatotoxic microcystins, are potent eukaryotic protein phosphatase inhibitors (Rogaus and Watzin 2008; Schindler et al. 2008). Acute high dose microcystin ingestion can cause acute liver failure and death, with chronic lower-dose exposure linked to liver tumor development (Falconer 1991; Yoshizawa et al. 1990). Several cyanobacterial genera
synthesize microcystins, including *Anabaena*, *Microcystis*, *Oscillatoria*, *Planktothrix* and *Woronichinia*, among others (Falconer 1991; Rantala et al. 2006). More than 90 microcystin variants have been identified and are all produced by multifunctional enzyme complexes coded by the 55kb 10-gene microcystin synthesis gene cluster (Tillett et al. 2000). The stable monocyclic heptapeptide structure of microcystin confers a half-life in the environment of weeks to months after release and potentially facilitates transport to the near-shore marine environment (Jones and Orr 1994; Tsuji et al. 1994).

Specific environmental drivers of CHAB formation and toxicity remain system-specific, however, a variety of physical and biological environmental factors have been reported to promote the development, dominance, and toxicity of CHABs (Havens et al. 2003; Jacoby et al. 2000; Smith 1983). While CHABs and cyanotoxin levels can correlate with nitrogen and phosphorus levels, light intensity, water temperature, diversity of cyanobacteria community and growth phase, researchers have been unsuccessful at specifically accounting for the production or release of cyanotoxins (Dolman et al. 2012; Downing et al. 2001; Oh et al. 2001; Rinta-Kanto et al. 2009; Schindler et al. 2008).

In 2011, Kudela demonstrated correlations between Pinto Lake microcystin levels and environmental factors including chlorophyll *a* (as a proxy for biomass) and dissolved nutrients (Kudela 2011). In our study, we further investigate the CHAB abundance and diversity dynamics, with examinations of cyanobacterial community interactions and the molecular basis of CHAB toxicity in Pinto Lake. We also examine the impact of the Mediterranean climate and the limnology of a shallow, eutrophic lake on the promotion of CHABs. Our three-year study represents the most in-depth study of a freshwater CHAB in the Monterey Bay area to date.
MATERIALS AND METHODS

We collected surface water samples from Pinto Lake from June 2009 through June 2011 in the morning between 09:00 and 11:00 am when wind-induced mixing is lowest (Kromkamp and Mur 1984; Wallace et al. 2000). Sample collection was based on the Alert Levels Framework, a tiered sampling protocol with increasing sampling effort as the CHAB developed to capture CHAB development, peak and decline phases (Watzin et al. 2006). Samples were collected monthly during the winter and spring months, based on low seasonal cyanobacterial abundance. As cyanobacterial biomass increased, sampling frequency increased to biweekly in the late spring and weekly in the summer and autumn. Intensive sampling continued through the early winter when the first significant rains resulted in increased mixing of the water column and resulting cyanobacteria cell dilution.
At each sampling event, water samples were collected for microscopy, molecular analyses, intracellular microcystin quantification, nutrient levels, and chlorophyll $a$ concentration. All samples were collected in one liter dark polycarbonate or glass bottles and stored at ambient temperature during transportation.

At each sampling event, we measured in situ environmental parameters including water temperature, dissolved oxygen (DO), pH, and depth with a calibrated Hydrolab DSX water quality multiprobe. We estimated water clarity using a Secchi disk and measured photosynthetically active radiation (PAR) through the water column with an underwater PAR sensor. The PAR sensor was also used to estimate the depth of the photic zone and calculate the extinction coefficient. We defined photic zone as the depth of the water column where irradiance reaches 1% of surface irradiance (Kirk 1994). We obtained meteorological information, air temperature, precipitation and wind from a the proximal CIMIS weather station number 129 (CIMIS 2012).

Water samples for analyses of dissolved inorganic nutrients (ammonium, nitrate + nitrite and orthophosphate) and total dissolved nitrogen and organic carbon were filtered through 0.45µm pore membrane Millipore filters. Dissolved inorganic nutrient water samples were frozen at -20°C prior to analysis and total dissolved nitrogen and organic carbon samples were acidified and stored at 4°C. All samples were analyzed within the 28-day holding time (AWWA 2005).

Dissolved inorganic nutrients were analyzed colorimetrically with the Lachat Instruments QuikChem 8500 flow injection analyzer. We used the Shimadzu Combustion TOC Analyzer to analyze dissolved organic carbon and total nitrogen. We measured
chlorophyll $a$ by fluorescence with a Turner Designs fluorometer calibrated to calculated chlorophyll $a$ corrected for pheophytins following acetone extraction (AWWA 2005). All analyses included quality control samples of known concentration, laboratory blanks, field and laboratory replicates and matrix spikes to maintain precision and accuracy.

Samples destined for microscopy (for cyanobacterial enumeration and identification) were preserved with 2% Lugol’s iodine solution (AWWA 2005) in 50 ml centrifuge tubes and stored at 4°C. After concentration by passive sedimentation, we examined 1 mL of the sample concentrate in Sedgwick-Rafter counting cells on an Olympus IX51 inverted microscope at 100x magnification. We examined twelve fields of the counting chamber, identifying cyanobacteria to genus (Komarek 2003) and recording and measuring all observed cyanobacteria cells captured with the Olympus DP2-BSW microscope digital camera software. We estimated cyanobacterial cell concentrations based on the natural unit method (Rogalus and Watzin 2008). This natural unit method estimates abundance of the most common cyanobacteria colony forms based on natural unit categories for size and shape. To efficiently estimate the cell density of a sample, the number of natural units were counted per taxa and multiplied by the number of cells contained in that natural unit. To verify the accuracy of the natural unit method, we counted cells from a randomly selected 10% of the natural unit method samples.

We performed polymerase chain reaction (PCR) with established primer sets (Table 1) to amplify cyanobacteria-specific 16S-rRNA genes (Nubel et al. 2002) and the 320 bp fragment of the microcystin-synthetase gene $mcyB$ (Nonneman and Zimba 2002). We used the 16S-rRNA gene primers to screen for naturally-occurring PCR inhibitors in our environmental samples and to examine the diversity of Pinto Lake cyanobacteria.
PCR primers targeting the 16S-rRNA gene included a second reverse strand with degenerencies in the sequence for optimal binding to most cyanobacterial 16S-rRNA (Nubel et al. 2002). The mcyB primers were used to estimate the potential for microcystin production and understanding the diversity of cyanobacteria containing the gene. We paired each PCR with a positive control of purchased cyanobacteria and a negative control of molecular-grade water.

We extracted cyanobacterial genomic DNA with the Qiagen DNeasy Plant Kit with a modified protocol for cyanobacteria cells. In addition to the kits’ extraction steps, we performed three freeze-fracture cycles, two minutes of bead beating along with a proteinase K digestion for one hour. The resulting genomic DNA was maintained at -20°C. We performed separate PCR amplifications of a 450 bp 16S-rRNA gene fragment and a 320 bp mcyB gene fragment for each sample. Each PCR reaction (50μl total) contained a final concentration of 1X PCR buffer, 2 mmol·l$^{-1}$ MgCl$_2$ buffer, 800 μmol·l$^{-1}$ dMTPs, 0.4 μmol·l$^{-1}$ each of forward and reverse primers, 0.5 U Taq polymerase and 50–200 ng DNA template or 25μl 2x Brilliant PCR Master Mix containing, 0.4 μmol·l$^{-1}$ each of forward and reverse primers and 50 – 200 ng DNA template. We examined the PCR products with gel electrophoresis stained with 1 μL ethidium bromide (10 mg · mL$^{-1}$) and visualized the bands on a UVP bench top UV illuminator.

To confirm the identity of the PCR amplicons, we sequenced every fourth sample. These samples were sequenced at Sequetech Labs (Mountain View, CA) using single primer extension / dye terminator sequencing on an Applied Biosystems 3730xl DNA Analyzer with the same primers used to amplify the sequences (Table 1).
We quantified intracellular microcystin levels with an enzyme-linked immunosorbent assay (ELISA) from Envirologix. Samples destined for ELISA were gently homogenized and filtered onto Whatman 934-AH filters. We transferred the filters into 5ml glass vials wrapped in foil and stored the vials at -80°C prior to analysis. To extract the microcystins, we subjected the filters to a freeze-thaw cycle and then transferred the filters to 25ml glass centrifuge tubes containing a 50% solution of methanol solution (Fastner et al. 1998). To further promote cell lysis and toxin release, we disrupted the samples with the Fisher Scientific sonic dismembrator and ultrasonic converter (Carmichael and An 1999). To separate cell debris from the extract, we centrifuged the filters in methanol for 10 minutes at 2,200g followed by dilution to 5% methanol solution (Metcalf et al. 2000). We applied 20μl aliquots of the samples and microcystin standards to the ELISA microtiter plate with reagents according to manufacturer instructions. Samples that exceeded the highest concentration of the standard curve were diluted and reanalyzed. We monitored precision and accuracy with quality control samples, of known concentration, laboratory blanks and sample duplicates.

We evaluated relationships between environmental factors and total observed cyanobacteria abundance, abundance of cyanobacteria taxa, and intracellular microcystin levels using Pearson correlation with a significance level of 0.05. Some variables were logarithmically transformed to improve normality of the data. All distribution diagnostics and analyses were performed using the statistical language R (R Core Team 2012).
Table 1: Primers used in this study

<table>
<thead>
<tr>
<th>Target</th>
<th>Name</th>
<th>Sequence</th>
<th>Length</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cyano 16S-rRNA</td>
<td>Cyan395F</td>
<td>GGGGAATYTTCCGCAATGGG</td>
<td>450 bp</td>
<td>Nübel et al., 1997</td>
</tr>
<tr>
<td>Cyano 16S-rRNA</td>
<td>Cyan781Ra</td>
<td>GACTACTGGGGGTATCTAATCCC</td>
<td>ATT</td>
<td></td>
</tr>
<tr>
<td>Cyano 16S-rRNA</td>
<td>Cyan781Rb</td>
<td>GACTACAGGGGTATCTAATCCC</td>
<td>TTT</td>
<td></td>
</tr>
<tr>
<td>mcyB</td>
<td>meyB2959F</td>
<td>TGGGAAGATGTTCCTCCAGGTAT</td>
<td>320 bp</td>
<td>Nonneman and Zimba 2002</td>
</tr>
<tr>
<td>mcyB</td>
<td>meyB32789R</td>
<td>AGAGTGGAAACAATATGATAA</td>
<td>GCTAC</td>
<td></td>
</tr>
</tbody>
</table>

**RESULTS**

Planktonic cyanobacteria cell density was below detection limit in January through March 2010 and 2011 and increased to $10^6$ to $10^7$ cells·ml$^{-1}$ in the peak bloom periods. Likewise, chlorophyll $a$ concentrations peaked at nearly $10^4$ ug·L$^{-1}$ in November 2009, $10^3$ ug·L$^{-1}$ in the summers of 2010 and 2011. Throughout the study period, the observed CHABs were dominated by *Aphanizomenon* sp. while other major taxa included *Anabaena* sp. and *Microcystis* sp. (Figure 2). We also intermittently observed several members of Oscillatoriales and Chroococcales. Intracellular microcystin levels were at or below detection limit from December 2009 through June 2010 and December 2010 through June 2011. Microcystins peaked in October 2009 at 569 ug·L$^{-1}$, September 2010 at 25 ug·L$^{-1}$ and October 2011 at 360 ug·L$^{-1}$ (Figure 2).
Using the described primers (Table 1), we amplified the 450 bp fragment consistent with the expected cyanobacteria-specific 16S-rRNA gene product throughout the study (Figure 3a). We amplified the 320 bp fragment consistent with the mcyB PCR product in the summer and autumn of each study year (Figure 3b and table 2).
Figure 3: Electrophoresis gels of a selection of samples from each month of the study beginning with June 2009 (J) and continuing through December 2011 (D): (a) 450 bp PCR product consistent with cyanobacteria-specific 16S-rRNA gene fragment and (b) 320
We sequenced a subset (every 4\textsuperscript{th} sample) collected of the 16S-rRNA and \textit{mcyB} gene fragments. Of the 65 sequenced cyanobacteria 16S-rRNA gene fragments, 59 were 99\% sequence matches with \textit{Aphanizomenon flos-aquae} when searched using BLAST and the NCBI microbial genomic database (NCBI 2012). Likewise, the \textit{mcyB} gene products matched 99 – 100\% with published sequences of \textit{Microcystis aeruginosa}.

Throughout the sampling period, Pinto Lake nutrient and chlorophyll \textit{a} levels and water clarity (Table 3) were within the range described for hypereutrophic waterbodies (Carlson 1977; Nurnberg 1996). We observed a temporal pattern in the levels of nitrate and ammonium, with the highest concentrations corresponding to the winter and spring,
and the lowest levels in the summer and autumn (Figure 4). A seasonal pattern was not observed for levels of soluble reactive phosphorus in any years at the surface.

Table 3: Environmental parameter data summary

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Mean</th>
<th>S.D.</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air Temp (°C)</td>
<td>12.4</td>
<td>3.5</td>
<td>6.2 – 22.1</td>
</tr>
<tr>
<td>Ammonium (mg · L⁻¹)</td>
<td>0.57</td>
<td>0.91</td>
<td>0.02 – 5.92</td>
</tr>
<tr>
<td>Chlorophyll a (ug · L⁻¹)</td>
<td>137</td>
<td>150</td>
<td>1.27 – 596</td>
</tr>
<tr>
<td>Degree days</td>
<td>12.9</td>
<td>5.1</td>
<td>2.9 – 24.4</td>
</tr>
<tr>
<td>DO (mg · L⁻¹)</td>
<td>9.58</td>
<td>3.08</td>
<td>3.04 – 19.40</td>
</tr>
<tr>
<td>Extinction Coefficient</td>
<td>1.9</td>
<td>0.5</td>
<td>1.4 – 3.3</td>
</tr>
<tr>
<td>Log₁₀ Secchi depth (m)</td>
<td>2.0</td>
<td>0.2</td>
<td>1.6 – 2.6</td>
</tr>
<tr>
<td>N:P ratio</td>
<td>3.69</td>
<td>4.24</td>
<td>0.16 – 21.47</td>
</tr>
<tr>
<td>Nitrate (mg · L⁻¹)</td>
<td>0.09</td>
<td>0.13</td>
<td>0.05 – 0.65</td>
</tr>
<tr>
<td>NPOC (mg · L⁻¹)</td>
<td>16.3</td>
<td>11.4</td>
<td>8.2 – 74.9</td>
</tr>
<tr>
<td>pH</td>
<td>7.86</td>
<td>0.804</td>
<td>6.60 – 10.04</td>
</tr>
<tr>
<td>Phosphate (mg · L⁻¹)</td>
<td>0.265</td>
<td>0.276</td>
<td>0.0078 – 1.631</td>
</tr>
<tr>
<td>Photic zone depth (m)</td>
<td>2.4</td>
<td>1.1</td>
<td>1.1 – 4.5</td>
</tr>
<tr>
<td>Surface PAR (μmol photons · m² · s⁻¹)</td>
<td>2691</td>
<td>1368.7</td>
<td>149.5 – 6215.0</td>
</tr>
<tr>
<td>Thermocline depth (m)</td>
<td>2.7</td>
<td>1.9</td>
<td>0.0 – 5.0</td>
</tr>
<tr>
<td>Water temperature (°C)</td>
<td>19.68</td>
<td>3.77</td>
<td>10.46 – 24.88</td>
</tr>
<tr>
<td>Wind speed (average daily)(m · s⁻¹)</td>
<td>2.18</td>
<td>0.63</td>
<td>1.40 – 6.30</td>
</tr>
</tbody>
</table>
Figure 4: Pinto Lake mean monthly water quality data: Chlorophyll $a$ (ppb); Nitrate + nitrite (as N); Ammonium (as N); Orthophosphate (as P); N:P ratio; Non-purgeable organic carbon (NPOC); Temperature (°C)
A shallow lake, averaging 2 meters in depth during the dry season (summer and autumn), Pinto Lake maintained seasonal thermal stratification, maintaining a stable thermocline through the mid-summer. As the lake surface warmed in the late spring and early summer, a warm-water epilimnion developed, overlaying a cooler hypolimnion (Figure 5). The resulting difference in density between these layers prevented overall water column mixing. In correspondence with the thermal stratification and the development of the CHABs, the lake was also dissolved oxygen (DO) stratified. The surface waters were consistently supersaturated in DO (> 100%) due to CHAB photosynthesis, while levels in the hypolimnion fell to nearly zero. The depth of the thermocline gradually descended in the late summer and early autumn with consistent solar heating and no surface flow throughout the summer. By early autumn, nearly the entire water column warmed and the thermal stratification disappeared (Figure 5). As the thermocline descended, the DO through the water column remained very low except at the oxygen supersaturated surface.
Nine of the environmental parameters measured were significantly related to cyanobacterial abundance (Table 4). All taxa, besides *Anabaena*, were positively associated with degree day (as a proxy for season). All taxa aside from *Anabaena* were also negatively associated with dissolved nitrate, while *Anabaena* negatively correlated with ammonium (Table 4). The observed filaments of *Anabaena* sp. and *Aphanizomenon* sp. were both noted to contain heterocysts throughout the bloom periods. We found no significant relationship between any taxa abundance and dissolved surface phosphate.
Table 4: Pearson correlation coefficients (r), n and p-values for correlations with Pinto Lake cyanobacteria

<table>
<thead>
<tr>
<th></th>
<th>Log$_{10}$ Total observed Cyanobacteria (cells $\cdot$ L$^{-1}$)</th>
<th>Log$_{10}$ Aphanizomenon sp. (cells $\cdot$ L$^{-1}$)</th>
<th>Log$_{10}$ Anabaena sp. (cells $\cdot$ L$^{-1}$)</th>
<th>Log$_{10}$ Microcystis sp. (cells $\cdot$ L$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>r$_p$   n  P</td>
<td>r$_p$   n  P</td>
<td>r$_p$   n  P</td>
<td>r$_p$   n  P</td>
</tr>
<tr>
<td>Log$_{10}$ Ammonium (mg $\cdot$ L$^{-1}$)</td>
<td>-0.219  86  0.043</td>
<td>-0.252  87  0.020</td>
<td>-0.313  87  0.003</td>
<td>0.208  87  0.100</td>
</tr>
<tr>
<td>Chlorophyll a (µg $\cdot$ L$^{-1}$)</td>
<td>0.768  87  0.000</td>
<td>0.518  87  0.000</td>
<td>0.226  87  0.039</td>
<td>0.227  87  0.038</td>
</tr>
<tr>
<td>Degree days</td>
<td>0.288  87  0.019</td>
<td>0.596  87  0.000</td>
<td>-0.348  87  0.005</td>
<td>0.289  87  0.020</td>
</tr>
<tr>
<td>DO (mg·L$^{-1}$)</td>
<td>0.112  87  0.310</td>
<td>0.224  87  0.077</td>
<td>0.074  87  0.502</td>
<td>0.372  63  0.036</td>
</tr>
<tr>
<td>Extinction Coefficient</td>
<td>0.482  87  0.000</td>
<td>0.310  87  0.014</td>
<td>0.289  87  0.154</td>
<td>-0.167  87  0.195</td>
</tr>
<tr>
<td>N:P</td>
<td>0.016  85  0.695</td>
<td>-0.039  85  0.757</td>
<td>-0.373  85  0.008</td>
<td>0.182  85  0.151</td>
</tr>
<tr>
<td>Nitrate (mg·L$^{-1}$)</td>
<td>-0.525  87  0.000</td>
<td>-0.563  87  0.000</td>
<td>-0.121  87  0.262</td>
<td>-0.263  87  0.036</td>
</tr>
<tr>
<td>NPOC (mg·L$^{-1}$)</td>
<td>0.377  86  0.006</td>
<td>0.450  86  0.000</td>
<td>-0.193  86  0.077</td>
<td>0.246  86  0.050</td>
</tr>
<tr>
<td>pH</td>
<td>0.398  87  0.000</td>
<td>0.553  87  0.000</td>
<td>0.012  87  0.909</td>
<td>0.193  87  0.127</td>
</tr>
<tr>
<td>Phosphate (mg·L$^{-1}$)</td>
<td>0.064  87  0.560</td>
<td>0.168  64  0.186</td>
<td>0.048  87  0.709</td>
<td>-0.035  87  0.783</td>
</tr>
<tr>
<td>Photic zone (m)</td>
<td>-0.195  86  0.171</td>
<td>-0.306  86  0.029</td>
<td>-0.143  86  0.064</td>
<td>0.184  86  0.196</td>
</tr>
<tr>
<td>Log$_{10}$ Secchi depth</td>
<td>-0.127  87  0.238</td>
<td>-0.370  87  0.003</td>
<td>0.114  87  0.379</td>
<td>0.003  87  0.982</td>
</tr>
<tr>
<td>Surface PAR</td>
<td>0.149  84  0.613</td>
<td>-0.242  84  0.072</td>
<td>-0.088  84  0.560</td>
<td>0.370  84  0.075</td>
</tr>
<tr>
<td>Thermocline (m)</td>
<td>0.265  87  0.033</td>
<td>0.307  87  0.013</td>
<td>0.468  87  0.000</td>
<td>-0.071  87  0.574</td>
</tr>
<tr>
<td>Water temperature (˚C)</td>
<td>0.481  87  0.000</td>
<td>0.509  87  0.000</td>
<td>0.307  87  0.013</td>
<td>0.024  87  0.852</td>
</tr>
<tr>
<td>Wind speed (m·s$^{-1}$)</td>
<td>0.001  87  0.997</td>
<td>0.024  87  0.850</td>
<td>-0.046  87  0.719</td>
<td>-0.075  87  0.558</td>
</tr>
</tbody>
</table>

Microcystin levels correlated most strongly with the abundance of *Microcystis*, slightly less with *Aphanizomenon* and negatively with *Anabaena*. As with cyanobacterial abundance, microcystins also were associated with chlorophyll *a* and degree day (Table 5).
The most abundant taxa, *Aphanizomenon* was most strongly associated with the total observed cyanobacterial abundance, while *Anabaena* and *Microcystis* demonstrated weaker, but significant correlations with total cyanobacteria abundance. While
Microcystis and Aphanizomenon were positively correlated with each other, they both negatively correlated with Anabaena (Table 6).

### Table 6: Pearson correlation coefficients (rs), n and p-values for correlations among Pinto Lake cyanobacteria

<table>
<thead>
<tr>
<th></th>
<th>Log(_{10})Aphanizomenon sp. (cells · L(^{-1}))</th>
<th>Log(_{10})Anabaena sp. (cells · L(^{-1}))</th>
<th>Log(_{10})Microcystis sp. (cells · L(^{-1}))</th>
</tr>
</thead>
<tbody>
<tr>
<td>Log(_{10}) total observed cyanobacteria (cells · L(^{-1}))</td>
<td>0.75785 85 0.000</td>
<td>0.257 85 0.039</td>
<td>0.266 85 0.031</td>
</tr>
<tr>
<td>Log(_{10}) Aphanizomenon sp. (cells · L(^{-1}))</td>
<td>-0.099 85 0.430</td>
<td>0.272 85 0.028</td>
<td></td>
</tr>
<tr>
<td>Log(_{10}) Anabaena sp. (cells · L(^{-1}))</td>
<td>-0.159 85 0.205</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The cyanobacterial abundance strongly correlated with chlorophyll \(a\) (\(r=0.76, p < 0.01\)). While levels of chlorophyll \(a\) increased as cyanobacteria cell density increased, chlorophyll \(a\) was never below detection limits. The persistence of chlorophyll \(a\) reflected the presence of chlorophyll \(a\)-containing phytoplankton observed in the winter and spring.

**DISCUSSION**

Pinto Lake microcystins pose a documented health risk to the thousands of lake visitors who engage in recreational contact both through accidental direct contact or potentially through aerosolized toxins (Cheng et al, 2007). In 2009 through 2011, the levels of intracellular microcystins were consistently above the 0.8 μg · L\(^{-1}\) threshold recommended as safe for recreational exposure (CalEPA 2012). While lower than the
levels reported in 2007 (Mekebri et al. 2009) the 2009 through 2011 Pinto Lake microcystin data are more robust. In our study, samples were collected from throughout the lake and are more representative of toxin levels across the lake, as 2007 levels were taken from the concentrated scum accumulated at the boat dock.

The documented microcystins also pose risks to lake and downstream fauna. With the Mediterranean climate cessation of precipitation by late spring and summer-autumn drought, the level of the lake usually recedes and loses connectivity to the stream network during the period of peak toxicity. However, it is possible for stream connectivity to be reestablished with late season precipitation (as observed in June 2011). Early springtime CHABs, as observed in 2012 and 2013 (data not included), also pose an increased risk for downstream ecosystem and human health.

The observed correlations between the levels of intracellular microcystin and chlorophyll \(a\) and degree days (as a proxy for air and water temperature) correspond with CHABs in other Mediterranean-climate California water bodies (Kudela 2011; Mayers 2001). Microcystin levels were positively correlated with both *Microcystis* and *Aphanizomenon* and negatively with *Anabaena*. While both *Microcystis* and *Anabaena* have been documented to produce microcystin in other systems (Hisbergues et al. 2003), in Pinto Lake, the correlation data support *Microcystis* as the dominant toxin producer. We found no documented examples of *Aphanizomenon* producing microcystin or containing the microcystin synthesis gene cluster in the literature. Furthermore, our microcystin synthesis gene sequence data only matched with sequences of *M. aeruginosa*. The correlation between microcystin levels and *Aphanizomenon* could be a reflection of the nitrogen-fixing capacity of *Aphanizomenon* in association with non-
nitrogen fixing *Microcystis* (Beversdorf et al. 2013). Our current focus will also help us further understand the role of non-toxic cyanobacteria and environmental parameters in the promotion of toxic blooms.

The timing of seasonal changes in cyanobacterial abundance showed similar patterns across the sampling period. However, the magnitude of overall cyanobacteria abundance was significantly higher in 2010 than the other two years, while microcystin levels were higher in both 2009 and 2011 (Figure 2).

While we consistently detected intracellular microcystin from July through December concurrent with the observation of *Microcystis*, extracellular microcystins have been described throughout the year at much lower, but detectable, levels when collected via the solid phase adsorption toxin tracking (SPATT) media analyzed by LC-MS/MS (Kudela 2011). The resulting difference in detection during non-bloom periods is most likely due in part to methodology, but also could be a reflection of microcystins stable structure and resistance to degradation in natural systems (Kardinaal et al. 2007; Tsuji et al. 1994).

In all years, primary succession of CHABs was first dominated by nitrogen-fixing taxa with evident heterocysts (*Anabaena* and *Aphanizomenon*) followed by non-nitrogen fixing *Microcystis* The spring chlorophyll *a* levels, prior to the June cyanobacterial blooms, were the result of sequential blooms of various green algae followed by *Bacilliarophyta*, with *Aulacoseira* sp. in April and *Astroniella* sp. in May and June.

The strongest correlations observed were between *Aphanizomenon* and environmental factors, the most abundant cyanobacteria observed throughout the study
The factors most strongly associated with *Aphanizomenon* abundance were seasonal drivers of primary productivity (temperature, degree days). *Aphanizomenon* was negatively associated with nitrate in the surface waters (Table 4) and demonstrated at least on heterocyst in every filament observed. This correlation, consistent with another recent Pinto Lake study (Kudela 2011) is indicative of the nitrogen fixing capacity of *Aphanizomenon* and the advantage that nitrogen fixing cyanobacteria have when dissolved nitrogen is depleted (Smith 1990; Smith et al. 1990). We found no significant associations between cyanobacterial abundance and orthophosphate (soluble reactive phosphorus) which may be non-limiting in this system.

Though *Microcystis* sp. was not the most abundant cyanobacteria, it is the likely source of microcystin in the lake and rapidly increased from below detection to $10^4$ cells·ml$^{-1}$ in late summer and autumn. Similar to *Aphanizomenon*, *Microcystis* abundance also was positively associated with chlorophyll $a$ and degree days, and negatively so with surface nitrate levels (Table 4). *Microcystis* abundance also corresponded with DO, PAR and organic carbon and demonstrated the highest correlation with microcystins (Table 5). The positive association between *Microcystis* and *Aphanizomenon* abundances may also reflect the reliance of *Microcystis* cells on *Aphanizomenon* for fixed nitrogen (Agawin et al. 2007; Beversdorf et al. 2013).

We documented the microcystin synthesis gene *mcyB* in months when we detected intracellular microcystin and observed *Microcystis* microscopically (Figure 2, Table 2). Further, sequenced *mcyB* gene PCR products matched sequences for *M. aeruginosa* *mcyB* when analyzed by nucleotide BLAST (NCBI 2012). Together, the molecular biology and microscopy results support *Microcystis* as the dominant toxin-
producing cyanobacteria in the 2009-2010 study period. Detection of cyanobacterial 16S-rRNA gene products throughout the study period, including periods when planktonic cyanobacterial abundance was below detection via microscopy is emblematic of the higher sensitivity of the molecular assays. We have also documented (but not included in this study) the presence of benthic mat-forming cyanobacteria in the winter months.

For a more interdisciplinary approach to monitoring the abundance, diversity and potential toxicity of Pinto Lake CHABs, we combined cell counts and molecular biology data with a study of the Pinto Lake’s physiochemical and limnological factors driving CHAB proliferation. Pinto Lake’s hypereutrophic nutrient and chlorophyll \(\alpha\) levels were intensified by the shallow water column and the seasonal drought-driven Mediterranean climate of Central California (Alexrod 1973). Heating of the water column during summer resulted in the development of a persistent thermocline, with the warmer upper portion of the lake gradually increasing in depth as the overall water column temperature warmed (Figure 5). The DO stratification along the thermocline, with supersaturated surface DO and hypoxia near the interface with the lake sediments. The anoxic conditions near the sediments promote the release of phosphorus and reduced iron into the water column (Mortimer 1942; Sondergaard et al. 2003). We also observed the presence of bioturbation from non-native carp and chironomid larvae, both benthic hypoxia-tolerant fauna that can promote the release of ammonia from lake sediments, the preferred nitrogenous species for cyanobacterial primary productivity (Capone and Kiene 1988; Svensson 1997). These bioturbating species also increase the release of phosphorus from the benthos (Havens 1991).
The elevated nutrient levels near sediments were usually restricted in depth by thermocline-induced resistance to mixing. Seasonally-driven warming of the lake caused thermocline deepening, increasing the volume of epilimnion. These increases in epilimnion were associated with increased mixing, increasing the availability of nutrients found near sediments in the greater water column beyond the hypolimnion.

The drivers of cyanotoxins often also promote the development of CHABs, and toxicity may be closely linked to overall increases in cyanobacteria biomass. In this study we found that nearly the same environmental factors were associated with both microcystin levels and *Microcystis* cell density. Furthermore, while *Microcystis* abundance and microcystin levels correlated well in all years, *Microcystis* abundance remained within the same range across the years as microcystin levels were an order of magnitude lower in 2010 than other years. The difference in microcystin levels between these study years may reflect variability in the toxicity of the *Microcystis* strains present in response to environmental conditions (Kardinaal et al. 2007; Moisander et al. 2009; Rinta-Kanto et al. 2009). While this study has succeeded in describing the environmental conditions under which Pinto Lake CHABs form and microcystin levels increase, parsing out the specific factors driving cyanobacterial proliferation and toxin production will require further investigation of cyanobacterial gene expression and the composition and relationships among the microbial community.

We also intend to extend this research with PCR primers targeting *mcyE* that have been designed to elucidate taxonomy of toxin producers (Vaitomaa et al. 2003). Performing additional *mcy*-targeting PCRs will reduce the possibility of biased inferences on toxin-producing taxa and increase the coverage of detected toxin genes. We also plan
to examine other cyanotoxins, as there are many potent cyanotoxins commonly identified in freshwater systems associated with the CHAB-forming genera observed at Pinto Lake (Sivonen 1996; Codd et al. 1999). To better understand the diversity of cyanobacteria and the dynamics among cyanobacteria and with other organisms, we are planning a genomic approach to understanding the phytoplankton and microbial community. The genomic community study is particularly important in the context of understanding the impacts of the Mediterranean climate and Pinto Lake’s shallow water body on cyanobacterial community and interactions.

While Pinto Lake is just one of several waterbodies along the California coast, the consistent seasonal CHABs and toxin levels, small scale, connectivity to the marine environment and central California location provide a unique opportunity to study the environmental factors driving CHAB formation and toxin production in Mediterranean coastal freshwater lakes.

This study also provides a framework for future research and helps expand regional knowledge of the environmental drivers and potential risks to human and ecological health posed by CHABs and associated toxins. Pinto Lake also will prove to be an effective model lake for applying a genomic approach to understand the interplay between environment and microbial community in the production of toxic freshwater CHABs.
REFERENCES


Kann J. 2006. Microcystis aeruginosa occurrence in the Klamath River system of Southern Oregon and Northern California. Yurok Tribe Environmental and Fisheries Programs.


Mogelhoj M, Henriksen P, Lundholm N. 2006. High pH and not allelopathy may be responsible for negative effects of Nodularia spumigena on other algae. Aquatic Microbial Ecology 43: 43-45.


Moreno-Ostos, E, Cruz-Pizarro L, Basante a, George Dg. 2008. The spatial distribution of different phytoplankton functional groups in a Mediterranean reservoir. Aquatic Ecology, 42(115-128).


Pflugmacher S. 2002. Possible allelopathic effects of cyanotoxins, with reference to microcystin-LR in aquatic ecosystems. Environ Toxicology 17: 407-413.


