Title
Microcystin Toxin and Associated Trophic Level Effects

Permalink
https://escholarship.org/uc/item/2f876902

Author
Gibble, Corinne Michele

Publication Date
2016

Peer reviewed|Thesis/dissertation
UNIVERSITY OF CALIFORNIA
SANTA CRUZ

MICROCYSTIN TOXIN AND ASSOCIATED TROPHIC LEVEL EFFECTS

A dissertation submitted in partial satisfaction
of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

OCEAN SCIENCES

by

Corinne M. Gibble

June 2016

The dissertation of Corinne M. Gibble is approved:

______________________________
Professor Raphael M. Kudela, Chair

______________________________
Professor Peter Raimondi

______________________________
Melissa Miller, DVM, MS, PhD

Tyrus Miller
Vice Provost and Dean of Graduate Studies
Copyright © by

Corinne M. Gibble

2016
# TABLE OF CONTENTS

List of Tables and Figures......................................................................................... vi

Abstract......................................................................................................................... viii

Acknowledgements......................................................................................................... x

**INTRODUCTION**....................................................................................................... 1

References....................................................................................................................... 8

**CHAPTER 1: DETECTION OF PERSISTENT MICROCYSTIN TOXINS**

**AT THE LAND-SEA INTERFACE IN MONTEREY BAY, CALIFORNIA**

........................................................................................................................................... 13

Abstract......................................................................................................................... 14

1. Introduction................................................................................................................. 14

2. Materials and Methods............................................................................................. 15

   2.1 Initial survey ............................................................................................................. 15

   2.2 Time-series ............................................................................................................. 15

   2.3 Toxin Analysis ........................................................................................................ 16

   2.4 Statistics ................................................................................................................ 16

3. Results....................................................................................................................... 16

   3.1 Survey results (2010–2011) .................................................................................... 16

   3.2 Time series (2011–2013)......................................................................................... 17

4. Discussion.................................................................................................................... 17

Acknowledgements......................................................................................................... 20

References....................................................................................................................... 20
CHAPTER 2: EVIDENCE OF FRESHWATER ALGAL TOXINS IN
MARINE SHELLFISH: IMPLICATIONS FOR HUMAN AND AQUATIC
HEALTH ................................................................. 22

Abstract .................................................................................................................. 22

1. Introduction .......................................................................................................... 23

2. Methods .............................................................................................................. 26

   2.1 Tank Experiments .......................................................................................... 26

   2.2 Field Experiments .......................................................................................... 30

   2.3 Commercial Oyster Tank Experiments ......................................................... 30

3. Results .................................................................................................................. 32

   3.1 Particulate Microcystin Trials - Low Microcystin ........................................ 32

   3.2 Particulate Microcystin Trials - High Microcystin ......................................... 32

   3.3 Dissolved Microcystin Trials ......................................................................... 33

   3.4 Field Experiments ......................................................................................... 33

   3.5 Commercial Oyster Tank Experiments ......................................................... 34

4. Discussion ............................................................................................................ 35

Acknowledgements ............................................................................................... 41

References .............................................................................................................. 41

Figures ................................................................................................................... 47

CHAPTER 3: THE USE OF BLOOD COLLECTION CARDS FOR
ASSESSING THE PRESENCE OF MICROCYSTIN IN MARINE AND
ESTUARINE BIRDS ................................................................................................. 51
List of Tables and Figures

CHAPTER 1: DETECTION OF PERSISTENT MICROCYSTIN TOXINS AT THE LAND-SEA INTERFACE IN MONTEREY BAY, CALIFORNIA

Figure 1: Map of Monterey Bay, California, USA. ................................................................. 15

Figure 2: Microcystin toxin (MCY) time series from SPATT samplers for two locations ................................................................................................................................. 17

Figure 3: Microcystin toxin from SPATT time series from August 2011 to August 2013 ................................................................................................................................. 19

Figure 4: Alkalinity versus microcystin toxin box plots ...................................................... 19

Table 1: Survey data for microcystin toxin from 21 locations in and around the Monterey Bay area in year one (2010–2011) ................................................................. 17

Table 2: Survey data for microcystin toxin and environmental variables from four watershed locations at the land–sea interface in Monterey Bay in years two and three (2011–2013) ........................................................................................................... 18

Tables 3: Results from the mixed effects model evaluating principal components, and temperature versus microcystin toxin ........................................................................ 19

CHAPTER 2: EVIDENCE OF FRESHWATER ALGAL TOXINS IN MARINE SHELLFISH: IMPLICATIONS FOR HUMAN AND AQUATIC HEALTH

Figure 1: M. californianus and particulate microcystin toxin in low concentrations experimental trials ........................................................................................................ 47

Figure 2: M. californianus and particulate microcystin toxin in high-concentration experimental trials ........................................................................................................ 47

Figure 3: M. californianus and dissolved microcystin toxin experimental trials. ...... 48
Figure 4: Environmental samples of mussels collected monthly from central San Francisco Bay for April to September 2015 ........................................ 49

Figure 5: Commercial oyster and microcystin toxin experimental trials ................... 50

CHAPTER 3: THE USE OF BLOOD COLLECTION CARDS FOR ASSESSING THE PRESENCE OF MICROCYSTIN IN MARINE AND ESTUARINE BIRDS

Figure 1: Map of Monterey Bay stranding locations .................................................. 66

Figure 2: Control blood concentrations versus spiked blood concentrations ........... 67

Table 1: Limit of detection for volume of blood tested and levels of detectable microcystin in blood samples ......................................................... 67

Table 2: Species specific detection of microcystin on binned birds ...................... 68
Abstract

MICROCYSTIN TOXIN AND ASSOCIATED TROPHIC LEVEL EFFECTS
by
Corinne M. Gibble

Microcystin toxin is a known environmental stressor with implications for ecosystem harm throughout many inland waterways in California. This purported freshwater toxin has recently emerged in the marine environment in Monterey Bay. Microcystin abundance and frequency, as well as environmental variables that drive toxin production in the Monterey Bay area were examined by deploying Solid Phase Adsorption Toxin Tracking (SPATT) samplers and evaluating water samples for 3 years. Microcystin was found in 15 of 21 monitored sites and was shown to be correlated with anthropogenic nutrient loading. Because it is relevant to identify an indicator organism to better monitor this toxin, uptake and release of microcystin toxin in California mussels (*Mytilus californianus*), and commercial oysters (*Crassostrea sp.*) were also examined. To provide environmental relevancy *Mytilus sp.* were also collected at 4 sites in San Francisco Bay for 6 months and analyzed for microcystin toxin. The results indicate that mussels purge microcystin toxin slowly, while oysters released toxin more quickly, however, both have the ability to retain toxin for 8 weeks post-exposure. To investigate potential effects to marine and estuarine birds at upper trophic levels the use of Whatman® FTA® blood sample collection cards were evaluated in tandem with competitive enzyme-linked immunosorbent assay (ELISA), and this method was shown to have utility for postmortem analysis and large die off events. Blood was also collected from birds admitted to rehabilitation in Monterey Bay between 2011-2015 and two groups of waterbirds tested positive for microcystin toxin. The
extensive manifestation of microcystin in the Monterey Bay area exhibits the need for better monitoring and management of this toxin. Management decisions made at the terrestrial level, may now impact freshwater, estuarine, and marine ecosystems, particularly given the demonstrated capacity for bio-accumulation in shellfish. The identification of a potential bio-indicator through this study, could be used to inform the current Mussel Watch Program or could aid in establishing an analogous monitoring program specific to this toxin. Because current data on the effects of toxic algae on waterbirds is meager, this study addresses this deficiency by identifying a new technique for detecting microcystin in upper trophic levels.
Acknowledgements

I am very pleased to thank the many people who have contributed to the work in this dissertation. First, my advisor Raphe Kudela has always been willing to listen to my ideas, support my goals, and push me to think and succeed. I would also like to thank my committee members (Pete Raimondi and Melissa Miller) for their consistent guidance, useful advice, and eager encouragement. I am also indebted to Kendra Hayashi for her willingness to teach a “bull in the china shop” how to perform sensitive laboratory work, and Rob Franks for his patience during my work using the Lachat. I’d also like to thank the past and present members of the Kudela Lab, especially Regina Radan and Misty Peacock, as well as the 4th floor Bird House Crew Office for laughs, advice, and kindness. I’m incredibly thankful to Rosemary Romero for being the better half of Operation Bay Mussel, and for her endless joy for science. To my mentors past and present, especially, David Hirth, Alan Strong, and Jim Harvey, who encouraged and inspired me all along the way.

I am also exceptionally grateful to my scientific and personal support network including: my Santa Cruz Family especially Tammy Bye and Ashley Neway who are always there for me; Erica Donnelly and Casey Clark, the former for her unwavering support, the latter for his ridiculous enthusiasm; Brian Hoover, and Colleen Young who can always be counted on to tell you you’re wrong. Most of all, I’d like to thank my family: Mom, Dad, Sarah, James and Grammy for their devoted and shining support of all of my endeavors; and my partner in crime, Aaron, for constant and steadfast encouragement, patience, and love.
The chapters of this dissertation include reprints of the following accepted and in preparation manuscripts:

**Chapter 1:** Gibble CM, Kudela RM. 2014. Persistent detection of microcystin toxins at the land-sea interface in Monterey Bay, California. *Harmful Algae*. 39: 146-153

I contributed to this chapter by performing three years of field sampling including deploying SPATT and collecting water samples. I also prepared and analyzed SPATT, whole water samples, ammonium, urea, nitrate, phosphate and silicate in the laboratory. I analyzed the data statistically and wrote the manuscript. Raphael Kudela supervised the method development, operated the liquid chromatography mass spectrometer (LCMS), and provided assistance with the writing of the manuscript.


I contributed to this chapter by performing all collection of organisms and water for experimental trials for both *Mytilus californianus* (dissolved and particulate microcystin) and commercial oysters (particulate microcystin). I also collected all of the environmental samples of *Mytilus sp.* at 4 locations in San Francisco Bay for six months. I prepared all samples in the lab, analyzed the data and wrote the manuscript. Melissa Peacock prepared and analyzed *Mytilus sp.* samples via LCMS and provided editorial support in writing the manuscript. Raphael Kudela supervised the method development, operated the LCMS, and provided assistance with the writing of the manuscript.

**Chapter 3:** Gibble CM, Hayashi K, Kudela RM. *Submitted*. The use of blood cards for assessing the presence of microcystin in marine and estuarine birds. *Journal of Wildlife Rehabilitation*.

I contributed to this chapter by collecting blood cards, running the ELISA method, evaporation method, and participating in method development. I also analyzed data and wrote the manuscript. Kendra Hayashi contributed by designing and developing the method, analyzing data, and provided editorial support in writing the manuscript. Raphael Kudela supervised the method development, and provided assistance with the writing of the manuscript.
INTRODUCTION

Harmful algal blooms (HABs) are a problem both nationally and internationally in both freshwater and marine ecosystems. The occurrence of blooms of this nature and associated subsequent toxic events may be exacerbated by environmental degradation coupled with a warming climate (Zehnder and Gorham 1960, Welker and Steinberg 2000, Guo 2007, Paerl and Huisman 2008, Davis et al. 2009, Kudela 2011). Microcystin, a toxin produced by a freshwater cyanobacteria *Microcystis aeruginosa*, has long been considered a problem in lakes, rivers and streams. Ashworth and Mason (1946) originally recognized generation of this toxin in American waters in the 1940’s. Today, *M. aeruginosa* blooms are common in freshwater lakes and rivers throughout North America as well as in international freshwater systems (Chen et al.1993, Lehman et al. 2005). Environmental variables like: high nutrient supply high light levels and warm temperatures, have the ability to influence formation of *M. aeruginosa* blooms and affect subsequent toxin production (Zehnder and Gorham 1960, Tsuji et al.1994, Jacoby et al. 2000, Welker and Steinburg 2000, Paerl and Huisman 2008, Davis et al. 2009, Paerl and Otten 2013). Both *M. aeruginosa* and microcystins are able to survive and persist in both saltwater and freshwater habitats (Robson and Hamilton 2003, Ross et al. 2006, Tonk et al. 2007, Miller et al. 2010), providing the potential for exposure to organisms in the marine environment.
High concentrations of microcystin have been shown to have negative effects including impairment and death in many trophic levels including zooplankton, invertebrates, fish and mammals (DeMott and Moxter 1991, Malbrouck and Kestemont 2006, Richardson et al. 2007, Miller et al. 2010). Although *M. aeruginosa* is a purportedly freshwater cyanobacterium, it was recently found in the near-shore marine environment in California, and ocean outflows of freshwater systems in Monterey Bay, CA tested positive for microcystin toxins. This occurrence of toxins at the land-sea interface was later proved to be a health hazard to local marine wildlife feeding at these coastal locations (Miller et al. 2010). Numerous sea otters (*Enhydra lutris*) were found dead in Monterey Bay, and during examination in necropsy and consequent biochemical testing, there was confirmation of the presence of toxin in the livers of twenty-one individuals. (Miller et al. 2010). Miller et al. (2010) also showed freshwater to marine transfer of microcystins in the areas where sea otters had been recovered, and additional experimentation in this study demonstrated the uptake of microcystin in popular prey items.

While the freshwater to saltwater transfer of these toxins to Monterey Bay was discovered by Miller et al. (2010), and is undoubtedly harmful to the ecosystem, potential population-level impacts of these biotoxins on upper trophic levels remain undetermined. Microcystin toxin has been shown to, not only persist, but biomagnify in the environment and in the food web, and the stability of microcystin allows it to accumulate in water (Sivonen and Jones 1999, van der Oost et al. 2003, Dionisio Pires et al. 2004, Kozłowsky-Suzuki et al. 2012, Poste and Ozersky 2013). Although
microcystin poisoning in marine mammals was confirmed, the true source of these toxins to Monterey Bay waters is unclear. The location where Miller et al. (2010) detected microcystin was identified as a major source for toxin production, but this source was only consistent with the stranding location of some of the stranded otters, which were distributed throughout Monterey Bay. This suggests that there may be, several sources of toxin, or perhaps more obscure sources of toxin to the coastal environment. Advanced monitoring of microcystin and associated water quality is needed to better determine sources of toxin, affected areas, and environmental drivers propelling this toxin into sensitive sanctuary waters.

To detect the presence of dissolved or particulate harmful algal bloom toxins, current water quality monitoring programs commonly use general water sampling processes. Some programs have also begun using SPATT (Solid Phase Adsorption Toxin Tracking), a fairly new technology to act as passive monitors of toxin abundance (MacKenzie et al. 2004, Kudela 2011). In addition to direct water quality monitoring, it is useful to determine a biomarker species to investigate the duration of toxin in the ecosystem to evaluate potential affects to human and wildlife consumers. Aquatic organisms are often used in management practices as biomarker indicator species and these data can be applied to water quality assessment and detection of HABs. Animals used as biomarker indicators can signify exposure to environmental substances such as algal toxins, and chemical pollutants such as dichloro-diphenyl-trichloroethane (DDT), Polychlorinated biphenyls (PCBs), and mercury and biomarkers have been measured in various invertebrates such as mollusks and
bivalves, making these organisms excellent beacons of change (Södergren et al. 1972, Tavares et al. 1988, Cossa 1989, de Lafontaine et al. 2000). Invertebrates have historically been used as sentinel species for assessing ocean ecosystem health (de Lafontaine et al. 2000, Shumway et al. 2003, Jessup et al. 2004, Kvitek and Bretz 2005). In addition to aiding managers in detecting toxin in the environment, biomarkers used in tandem with water quality sampling can provide an extended overview of the natural environment. Using invertebrates in this capacity provides increased information about potential pathways for trophic transfer during chronic exposure to low levels of toxins, as well as exposure during bloom conditions. They also provide temporal information about consumption safety; and additionally, because shellfish are generally widespread, and can also be removed from natural environments and transported to alternate locations, these animals can also provide information about the spatial boundaries of areas being affected by HAB toxins.

Marine bivalves, such as California mussels (*Mytilus californianus*), are particularly convenient in assessing accumulation of harmful algal bloom toxins in California. Mussels not only have a broad range throughout California, but they also are a common prey item for humans and wildlife alike, and have used in prior studies to demonstrate trophic transfer of algal toxins (Miller et al. 2010, Kvitek and Bretz, 2005). Furthermore, the ability of these organisms to accumulate toxin is elevated by certain life history traits that they possess. Shellfish are both filter feeders and detritivores; by nature, they consume large amounts of cyanobacteria and subsequently have the potential ingest high levels of toxin (Christoffersen 1996).
Many studies have experimentally demonstrated the accumulation of microcystin toxin in freshwater and saltwater invertebrates (Vasconcelos 1995, Williams et al. 1997, Amorim and Vasconcelos 1998, Dionisio Pires et al. 2004, Miller et al. 2010), and these studies have also linked invertebrate indicator organisms to affects in vertebrates that occupy upper trophic levels. However, trophic level interactions and vulnerability of organisms to toxins at different trophic levels has not been well defined (Turner and Tester 1997, Shumway et al. 2003, Kvitek and Bretz 2005, Smith and Haney 2006). If microcystin is transferred trophically, there may be detrimental impacts for intermediate and apex predators, as well as for humans.

As mentioned above, apex predators in Monterey Bay, CA, sea otters, have recently had adverse interactions with microcystin in the near-shore marine environment. In addition to marine mammals, other species of marine wildlife, such as marine birds, may be adversely affected by microcystin in this area. Because sea otters use the same nearshore environment as many estuarine and coastal marine birds, they may be serving as sentinels for as-yet-unrecognized impacts of microcystins on nearshore-feeding seabirds. Birds not only share habitat with sea otters, but also consume many of the same foods. Although marine HABs are a regular occurrence along the central California coast, potential temporal and spatial links with mortality events in seabirds have lagged far behind similar studies in sympatric marine mammals (Shumway et al. 2003). Additionally, investigations into possible effects of microcystin on marine and estuarine birds is currently unexplored.
Many marine birds, including Common Loons (Gavia immer), Pacific Loons (Gavia pacifica), Red-throated loons (Gavia stellata), Brown Pelicans (Pelecanus occidentalis), Brandt’s Cormorants (Phalacrocorax penicillatus), Western Grebes (Aechmophorus occidentalis), and Clark’s Grebes (Aechmophorus clarkii) all forage in the nearshore environment of Monterey Bay. The unique and varied habitats provided by the central coast of California serve an array of marine and estuarine bird species. These birds may be especially vulnerable to HABs that occur nearshore and overlap with critical feeding areas, and this may be especially true for HAB toxins that have the ability to concentrate in prey items. It is possible that these populations may have been heavily impacted in the past by HABs and/or Cyanobacterial Harmful Algal Blooms (CyanoHABs) in the past. However, to confirm potential population-level impacts of microcystin to marine or estuarine birds, systematic biochemical testing is needed.

Investigations into potential interactions between waterbirds and HAB toxins has been sparse. There is a desire, especially in wildlife rehabilitation settings, to address these interactions. Metrics that are used frequently to determine toxin loads in wildlife include postmortem analysis and biochemical testing using liquid chromatography mass spectrometry (LCMS). Testing during necropsy negates the chance to treat birds while still alive in a rehabilitation setting, and the latter makes testing for most facilities financially out of reach. In addition to being affordable, blood cards, are easy to use and easy to store, making them a potentially viable technique for use at wildlife rehabilitation centers.
There is a critical need for a more affordable diagnostic and monitoring methods for wildlife managers and wildlife rehabilitators to assess in the populations that they care for and manage. The joint use of blood collection cards and enzyme-linked immunosorbent assay (ELISA) to evaluate HAB toxin loads in wildlife may provide a cost-effective measure for scientists, managers and rehabilitators. Several earlier studies have used this metric to examine exposure of HAB toxins in marine and terrestrial mammals (Woofter et al. 2003, Maucher and Ramsdell 2005, Maucher et al. 2007, Schwacke et al. 2010, Twiner et al. 2011). However, this method has not been used previously to detect microcystin toxin loads in wildlife, nor has it been applied to birds. As a hepatotoxin, microcystin is capable of remaining in the blood stream of organisms after exposure (Runnegar et al. 1995), and therefore may be detectable in the blood of affected birds. Blood cards are easy to use, store, transport, and are inexpensive, giving them wide applicability for many different parties for detecting microcystin toxicity in birds.

The overall goal for my dissertation work was to examine three related issues that encompass a full ecosystem approach. My first objective was to determine the freshwater sources of microcystin toxin to the marine environment in Monterey Bay, CA. Along with this, I was interested in major affected watersheds and environmental drivers of microcystin toxin in these areas. These questions are addressed in Chapter 1. Because there is currently is no sentinel species to help monitor impacts of microcystin toxin to higher trophic levels, I also addressed the need for a relevant and
convenient indicator organism to be used for toxin monitoring and effective management practices in Chapter 2. In association with this, I was interested in determining the uptake and depuration of microcystin in shellfish, which would give scientists and managers a better temporal estimate when evaluating safety of consumption for both humans and wildlife consumers. These findings also provide information about potential trophic interactions of this toxin in the local near-shore food web. Due to concern regarding potential wildlife and human consumption of toxin that may currently be going unnoticed, and to provide environmental relevancy, I was also interested in examining shellfish in their natural nearshore environments for microcystin toxin. In Chapter 3, I extended this concept to more deeply investigate transfer of this toxin to upper trophic levels. Because microcystin toxin may be posing a yet unrecognized impact to wildlife consumers, I investigated the potential exposure of this toxin to marine and estuarine birds in Monterey Bay. My objectives for this chapter were two-fold. My first goal was to determine if blood cards can be used in tandem with market available ELISA kits. My second objective was to determine if marine and estuarine birds in the Monterey Bay area have come into direct contact with microcystin and have detectable levels of toxin in their blood. By combining these three bodies of work, I was able to capture a snapshot of the dynamics of microcystin toxin in Monterey Bay and adjacent watersheds, and the role of this toxin in the local food web.

References


CHAPTER 1: DETECTION OF PERSISTENT MICROCYSTIN TOXINS AT THE LAND-SEA INTERFACE IN MONTEREY BAY, CALIFORNIA

Corinne M. Gibble and Raphael M. Kudela (2014)


Reprinted with permission from Elsevier
Detection of persistent microcystin toxins at the land–sea interface in Monterey Bay, California

Corinne M. Gibble\*, Raphael M. Kudela

Oceans Sciences Department, 2556 High Street, University of California, Santa Cruz, CA 95064, USA

ABSTRACT

Blooms of toxin-producing Microcystis aeruginosa occur regularly in freshwater systems throughout California, but until recently potential impacts in the coastal ocean have been largely ignored. Twenty-five sites in and around Monterey Bay were surveyed for evidence of microcystin toxins (2010–2011) at the land–sea interface. Following this initial survey four major watersheds in the Monterey Bay area were surveyed (2011–2013) for microcystin concentration, nutrients, alkalinity and water temperature to identify potential environmental factors correlated with the abundance of microcystin at the land–sea interface. During the first year microcystin was detected in 13 of 21 sites. Data from years two and three were analyzed by principal components analysis and mixed effects model. Results indicated that coastal nutrient loading (silicate, phosphate, silicate, ammonium, urea) were statistically significant predictors of the microcystin concentrations in the watersheds with clear evidence for seasonality at some sites. Microcystin was frequently at highest concentration in the autumn; however, at some locations high levels of toxin were measured during spring. Because this toxin has the ability to biomagnify and persist within food webs, elevated levels within the watershed may decrease potential for health and survival of wildlife and humans exposed to freshwater and marine waters. The widespread occurrence of microcystin at low to moderate levels throughout the year and throughout the sampled watersheds demonstrates the potential for management. © 2014 The Authors. Published by Elsevier B.V. This is an open access article under the CC BY-NC-ND license (http://creativecommons.org/licenses/by-nc-nd/3.0/).

1. Introduction

Harmful algal blooms (HABs) are a global problem in both freshwater and marine ecosystems. The prevalence of HABs and subsequent toxic events may be intensified by a warming climate in tandem with increases in environmental degradation and eutrophication (Zehringer and Garst, 1999; Wolfe and Steinburg, 2000; Guo, 2007; Paerl and Huisman, 2008; Davis et al., 2009; Kudela, 2011). Production of the toxin microcystin by the cyanobacterium Microcystis aeruginosa, was originally recognized by Ashworth and Mason (1945) in American waters in the 1940s. M. aeruginosa blooms are now common in lakes and rivers throughout North America, including California (Chen et al., 1993; Lehman et al., 2005). M. aeruginosa bloom formation and consequent toxin generation increases with environmental variables such as: high nutrient supply, elevated light levels, and warm temperatures (Zehringer and Garst, 1999; Tsuji et al., 1994; Jacoby et al., 2008; Weiler and Steinburg, 2000; Paerl and Huisman, 2008; Davis et al., 2009; Paerl and Otten, 2013a, 2013b).

Recently toxins associated with the extensively freshwater cyanobacterium Microcystis aeruginosa have been detected in the near-shore marine ecosystem of central California, and have been confirmed as a danger to the health of sea otters feeding near ocean outflows of freshwater systems (Miller et al., 2010). M. aeruginosa is fairly salt-tolerant and microcystin toxins can be stable and environmentally persistent in both saltwater and freshwater habitats (Robison and Hamilton, 2003; Ross et al., 2006; Tont et al., 2007; Miller et al., 2010). In addition to direct toxic effects, exposure of aquatic organisms to elevated concentrations of microcystins may negatively affect all levels of the food web (Dempsey and Moster, 2001; Malbrouck and Kestemont, 2006; Richardson et al., 2007; Miller et al., 2010).

In 2007, numerous sea otters were found dead in Monterey Bay with signs of liver failure (Miller et al., 2010). Biochemical testing confirmed the presence of microcystin toxin with associated lesions in the livers of 21 otters. Because the occurrence of phytolipids derived toxins are a common phenomenon in Monterey Bay, the otters were evaluated for domoic acid, okadaic acid, nodularin, yessotoxin and anatoxin-A. Otters that were found
positive for microcystin toxin were negative for all other toxins in the tissues. A few of the microcystin positive oysters were also found to have low levels of domoic acid in the urine. However, this is a common finding during necropsy of stranded sea otters from this region, due to domoic acid being broadly dispersed in the sediments of Monterey Bay (Goldberg, 2003; Miller et al., 2010). Freshwater to marine transfer of microcystins was confirmed in areas where sea otters had been recovered, and uptake of microcystins by marine invertebrates and environmental persistence in seawater were demonstrated experimentally (Miller et al., 2010). At this time, potential population-level impacts of these biotoxins on otters and other coastal wildlife remains undetermined. The freshwater to marine transfer of microcystin to the Monterey Bay National Marine Sanctuary waters described by Miller et al. (2010) has the potential to cause major environmental harm. The stability of microcystin allows it to accumulate (van der Oost et al., 2003), and microcystin toxin has been shown to biomagnify and persist in the environment and the food web (Sivonen and Jones, 1999; Dionisi Pires et al., 2004; Kozlowsky-Suzuki et al., 2012; Posto and Ozeriski, 2013). Despite the confirmation of microcystin poisoning in marine mammals, the source of these toxins is unclear. Pismo Lake was identified as a “hotspot” for toxin production and subsequent transfer to the coastal ocean but this source was not consistent with the location of many of the otters (Miller et al., 2010), which were distributed throughout Monterey Bay, suggesting other, less obvious, sources of toxin to the coastal environment.

We took a wide ranging watershed-based approach to identify the potential pathways leading to microcystin contamination in coastal ecosystems in and around Monterey Bay, CA. Since initial surveys of other potential “hotspots” for toxin production were unsuccessful (Miller et al., 2010) via grab sampling, we deployed Solid Phase Absorptive Toxin Tracking (SPATT) samplers throughout the Monterey Bay area to provide a temporally integrated assessment of potential freshwater sources (Kudela, 2011). Because toxin frequency of occurrence, persistence, and associated environmental drivers may potentially be propelling this freshwater toxin into a sensitive and protected marine sanctuary, our overarching goals were to identify the freshwater sources of microcystin to the Monterey Bay ecosystem, and to identify the underlying environmental drivers influencing toxin production in this area.

2. Materials and Methods

2.1. Initial survey

We surveyed 21 freshwater, estuarine, and marine locations in and around the Monterey Bay area at the land–sea interface (June 2010–July 2011) for microcystin toxin presence and concentration (Fig. 1A). Sites included small and large rivers, estuaries, and nearshore marine locations traversing the six watersheds that surround Monterey Bay (Fig. 1A). Each site was sampled monthly using SPATT (Kudela, 2011). SPATT bags were constructed using 3 g Diatom® HP-20 resin (Sorboth Technologies Inc., Georgia, USA) placed between two 3 inch × 3 inch squares of 100 μM Nitex bolting cloth (Wildlife Supply Company, Product No. 24-C34), and secured in a Caron Westex 2.5 in. flat embroidery hoop (Caron International, Ontario, Canada). SPATT was activated by soaking each bag in 100% MeOH, for 4 h, and then rinsed with de-ionized water (Milli-Q), and stored in fresh Milli-Q until deployment (Macenszie et al., 2004; Lane et al., 2010). When deployed at the beginning of each month, SPATT bags were suspended below the surface of the water, and secured with twine to a stake near the edge of the water. This allowed each bag to be suspended in the water, while being weighed down by the ring so that it remained below the surface. Toxin concentration values are reported as nanogram toxin per gram resin SPATT toxin concentration levels are not directly comparable to grab sample values (ppb or μg/L), but previous studies suggest a rough correspondence of 10:1 for SPATT to grab samples (Kudela, 2011), i.e. 10 ng/g SPATT is equivalent to an average concentration of 1 ppb microcystin during SPATT deployment.

2.2. Time-series

In years two and three (August 2011–August 2013) sampling locations were reduced to four major affected watersheds in the Monterey Bay area: the Big Basin watershed, Pajaro River watershed, Salinas River watershed, and the Carmel River.

Fig. 1. Map of Monterey Bay, California, USA. (A) Sampling locations in year one (2010–2011) and sampling locations affected by microcystin toxin in year one. White symbols represent sites that were positive for microcystin, black symbols represent sites that were sampled but negative for microcystin toxin. (B) Sampling locations in year two (2011–2013). The watersheds, from north to south, are: Big Basin (dark blue), Pajaro River (red), Delia River (yellow), Salinas River (green), Carmel River (light blue). Ocean bathymetry is indicated with shading. Maps created using Ocean Data View (ODV) and Earlis Visual Information Solutions (ENVI).
watershed. These four sampling locations were determined by the initial year one survey to be highly impacted by microcystin toxicity. SPATT was deployed weekly at each site and whole water was collected and analyzed for temperature, ammonium, urea, nitrate, phosphate, silicate, and total toxin, with the whole water samples corresponding to the deployment and recovery dates for SPATT. With the exception of alkalinity, which was monitored only in year three (August 2012–August 2013), all other variables were measured in both years two and three. Temperature was monitored using Hobo Pendant® Temperature/Light Data Loggers (88-04-002-08; Onset Computer Corporation, Massachusetts, USA). When data loggers were unavailable for use due to theft or loss, field thermometers were employed in situ (−10 to 110 °C; Enviro-safe®; HB Instrument Company, Pennsylvania, USA). Ammonium, urea, nitrate, phosphate, and silicate samples were collected in the field, immediately filtered (0.7 μM GF/F filter), into 25 mL Falcon™ centrifuge tubes, and were stored frozen until processing. The average time until processing was less than one month. Ammonium was analyzed using the OPA method and RFU values were obtained via fluorometer (TD-700; Turner Designs, California, USA) as described by Holmes et al. (1995). Urea was analyzed using a Varian Cary 50 Bio UV/Visible Spectrophotometer (Varian Medical Systems, California, USA) following methods described by Mulvanny and Savidge (1992). Nitrate, phosphate, and silicate were analyzed using a Lachat QuickChem 8500 Flow Injection Analyst System and Omnim 3.0 software (Lachat Instruments, Hach Company, Colorado, USA). Alkalinity was determined using Total Alkalinity Test Strips, 0–240 mg/L (Hach Company, Colorado, USA) in the field. Whole water was also collected in the field.

2.3. Toxic analysis

Microcystin-LR, RR, YR, IA was analyzed by liquid chromatography/mass spectrometry (LCMS) with electrospray ionization (ESI) with selected ion monitoring (SIM) on an Agilent 6120i with a Phenomenex Kinetex 100 (2.10) C18 column. The method was adapted from Mekhiri et al., 2009 with minor modifications to account for the choice of column and LCMS/SIM instead of tandem mass spectrometry (Kudela, 2011). Briefly, a gradient-elution method was used with HPLC water (solvent A) and LCMS acetonitrile (solvent B), both acidified with 0.1% formic acid, as the mobile phase. The gradient was as described in Mekhiri et al. (2009), starting with 95:5 solvent A:B and ending with 25:75 at 19 min, held for 1 min, then followed by a 5 min equilibration at initial conditions prior to injection of the next sample. Samples were calibrated with standard curves (for each batch of samples) using pure standards (Fluka 31578 and Sigma–Aldrich M4194). Standards were run again at the end of the run for sample runs lasting more than 8 h.

Whole water was collected in the field, returned to the lab, and stored in 3 mL of 10% methanol. Samples were then sonicated using a sonic dismembrator (Model 100; Thermo Fisher Scientific, Massachusetts, USA) for 30 s at ~10 W, filtered (0.2 μm nylon syringe filter), and analyzed by direct injection of 50 μl onto the LCMS. SPATT samples were processed as described by Kudela (2011). Briefly, SPATT were recovered from the field and stored frozen until processing. The resin was transferred to a disposable chromatography column and sequentially extracted with 10, 20, 20 mL 50% methanol. Each extract (30 μl) was analyzed by LCMS and total toxin was determined by summing the individual extracts. Values are reported as μg/L (ppb) for whole water and ng/g resin for SPATT. The Minimum Detection Limit (MDL) was 0.05 ng/g for SPATT and 0.10 mg/mL (ppb) for whole water. Values <MDL were considered non-detect (zero) for statistical analysis. While it is possible that compound other than microcystin could be falsely identified, it would require the compounds to exhibit the same mass and retention time as the standards making false positives unlikely.

2.4. Statistics

Microcystin toxin presence, concentration, and persistence were evaluated at each sample location in year one, and for each watershed during years two and three. Data for both the discrete grab samples and the SPATT toxin concentrations were pooled for the analysis. The relationship between environmental variables (date, temperature, nitrate, phosphate, silicate, ammonium, urea) and microcystin were evaluated graphically and statistically. Because there was multicollinearity within the data from years two to three, a PCA was run to account for this, and the variables were grouped into components for further analysis (Zar, 1989; Quinn and Keough, 2002). A mixed effects model was chosen to account for autocorrelation caused by the seasonality component. Because of this autocorrelation the components (date and temperature) that comprised seasonality were removed from the PCA and added back into the model independently. When the model was run, components from the PCA (PC1 and PC2), and temperature were run against microcystin toxin. Date was added back into the model and was set as the random effect, with microcystin toxin concentration set as the fixed effect. This model is appropriate due to the nature of the flexibility it provides for correlated data (Quinn and Keough, 2002; Seltman, 2013). Variables used in the model were transformed via square root transformation to meet assumptions of normality, and u = 0.05. From the results of the model, both negative and positive relationships between environmental variables and microcystin concentration were examined, and statistical significance was obtained. PCA and mixed effect model statistical tests were conducted using Systat 13.1 (Systat Software Inc., Chicago, Illinois, USA). The relationship between microcystin concentration and alkalinity was investigated via simple bivariate correlation using IBM SPSS Statistics 21 (IBM Corporation, Armonk, New York, USA).

To investigate any statistical relationship between river discharge and toxin presence, microcystin toxin concentration was compared to river discharge data (USGS, 2014) for each of the four watersheds. Data for the comparison were obtained from the United States Geological Survey (USGS) National Water Information System Web Interface database, and river discharge data were reported in cubic feet per second. Data were analyzed via simple bivariate correlations using IBM SPSS Statistics 21 and the significance was set at p < 0.05. Cross correlation function analysis (CCF) was evaluated using Systat 13.1 for effectiveness of introducing temporal lags into the data.

3. Results


In year one, 15 out of 21 locations surveyed in the Monterey Bay area were positive for microcystin toxin concentration (Fig. 1A; Table 1). There were noticeably high levels of toxin in the autumn season, and at some sites, such as the Carmel River and Salinas River, there were also noticeable spring season peaks in toxin concentration (Fig. 2). From our first year of data, four watersheds (Big Basin, Pajaro River, Salinas River, Carmel River) were identified as persistently toxic; this directed our sampling in years two and three. Toxin concentration values varied from undetectable to 20 ng/g.
Table 1. Survey data for microcystin toxin from 21 locations in and around the Monterey Bay area in year one (2010-2011).

<table>
<thead>
<tr>
<th>Location</th>
<th>OBS</th>
<th>POS</th>
<th>Range</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Walsh Creek</td>
<td>8</td>
<td>1</td>
<td>0-1,880</td>
<td>0.138</td>
<td>0.699</td>
</tr>
<tr>
<td>Scott Creek</td>
<td>12</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>San Lorenzo River</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Santa Cruz Harbor</td>
<td>10</td>
<td>4</td>
<td>0-0.025</td>
<td>0.495</td>
<td>1.147</td>
</tr>
<tr>
<td>Twin Lakes State Beach</td>
<td>12</td>
<td>3</td>
<td>0-0.090</td>
<td>0.142</td>
<td>0.311</td>
</tr>
<tr>
<td>Sequoia Creek</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Pajaro River</td>
<td>13</td>
<td>1</td>
<td>0-2.930</td>
<td>0.225</td>
<td>0.813</td>
</tr>
<tr>
<td>Pajaro Lagoon</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Watsonville Slough</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Benett Slough</td>
<td>12</td>
<td>1</td>
<td>0-0.58</td>
<td>0.045</td>
<td>0.161</td>
</tr>
<tr>
<td>Moss Landing Harbor</td>
<td>11</td>
<td>3</td>
<td>0-0.097</td>
<td>0.387</td>
<td>2.315</td>
</tr>
<tr>
<td>Strawberry Pond</td>
<td>13</td>
<td>3</td>
<td>0-2.960</td>
<td>0.405</td>
<td>0.802</td>
</tr>
<tr>
<td>Moss Cibb</td>
<td>13</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Salinas River</td>
<td>13</td>
<td>3</td>
<td>0-4.700</td>
<td>0.472</td>
<td>1.298</td>
</tr>
<tr>
<td>Laguna Grande</td>
<td>13</td>
<td>1</td>
<td>0-0.155</td>
<td>0.013</td>
<td>0.046</td>
</tr>
<tr>
<td>Lake El Estero</td>
<td>12</td>
<td>1</td>
<td>0-0.414</td>
<td>0.382</td>
<td>0.115</td>
</tr>
<tr>
<td>Monterey Coast Guard Pier</td>
<td>11</td>
<td>2</td>
<td>0-0.190</td>
<td>0.067</td>
<td>2.345</td>
</tr>
<tr>
<td>Pebegrnem Wharf</td>
<td>10</td>
<td>2</td>
<td>0-5.382</td>
<td>0.500</td>
<td>1.548</td>
</tr>
<tr>
<td>Atollerm Creek</td>
<td>11</td>
<td>2</td>
<td>0-1.010</td>
<td>0.115</td>
<td>0.281</td>
</tr>
<tr>
<td>Carmel River</td>
<td>12</td>
<td>7</td>
<td>0-23.64</td>
<td>2.905</td>
<td>5.517</td>
</tr>
<tr>
<td>Big Sur River</td>
<td>11</td>
<td>1</td>
<td>0-0.1837</td>
<td>0.014</td>
<td>0.051</td>
</tr>
</tbody>
</table>

The number of observations where SPATT was deployed and also recovered, is represented by OBS, the number of months positive for microcystin toxin is measured in ng/l and is represented by POS. The range, mean, and standard deviation represented by SD, are also provided.

3.2. Time series (2011–2013)

In year two, all four watersheds (Fig. 1B) exhibited an increase in microcystin toxin presence compared to year one (Table 2). In year three, all watersheds again exhibited similar or increased occurrences of microcystin toxin. As seen in year one, high values of microcystin toxin concentrations were observed in both autumn and spring seasons (Fig. 3), but in years two and three this seasonal pattern was evident in all four watersheds.

The PCA produced three significant principal components (Table 3). Principal component one (PC1) was comprised of ammonium and urea (21.02% variance explained), principal component two (PC2) was comprised of temperature and date (19.57% variance explained), and principal component three (PC3) was comprised of nitrate, phosphate, and silicate (19.33% variance explained). All of the variables loaded positively with the exception of silicate. These PCA components were used in place of direct environmental variables to account for multicollinearity within the data. The seasonality component (date and temperature) produced an autocorrelation within the data. Seasonality was therefore removed and the PCA was re-run; two significant components were produced and grouped similarly. PC1 was comprised of ammonium and urea (29.36% variance explained), and PC2 was comprised of nitrate, phosphate, and silicate (27.25% variance explained). PC1, PC2 were then run in a mixed effects model against the presence and amount of microcystin toxin with date set as the random effect. The model showed that microcystin toxin concentration had a statistically significant relationship to all tested variables (p < 0.05; Table 3). Nitrate, phosphate, ammonium and urea were negatively associated with microcystin concentration within the model. Because silicate loaded negatively in the PCA, it was considered positively associated with microcystin in the model, while temperature was negatively associated with microcystin in the model (Table 3). The results of a CCF analysis indicated significant correlations between toxin concentration and individual environmental factors with temporal lags of three weeks. However, when the data were lagged, the overall model was greatly weakened. Microcystin toxin concentration remained significantly related to all tested variables (p < 0.05). For the reasons we present the statistical results without lags.

Bivariate correlations showed negative correlation with alkalinity and positive correlation with river flow. Alkalinity had a significant negative correlation (p < 0.05) with microcystin concentration (Fig. 4). River discharge and microcystin toxin concentration were significantly positively correlated for Big Basin, Pajaro River, and Carmel River watersheds (p < 0.05). Salinas River exhibited a weak, non-significant (p = 0.05) correlation. The results of CCF analysis indicated that a lag of four weeks may better align the data and increase the strength of the correlation. When the data were lagged all watersheds again were positively correlated; however, only the Pajaro River and Carmel River watersheds exhibited statistically significant correlations (p < 0.05).

4. Discussion

The results from this study show a serious condition at the nearshore interface in the Monterey Bay area, consistent with previous reports (Müller et al., 2010). In year one, approximately half of all tested locations were positive for microcystin toxins at some time during the year. We believe these toxins are being produced by
Table 2
Survey data for microcystin toxins and environmental variables from four watershed locations at the land-sea interface in Monterey Bay in years two and three (2011–2013).

<table>
<thead>
<tr>
<th>Location</th>
<th>Variable</th>
<th>Mean</th>
<th>SD</th>
</tr>
</thead>
<tbody>
<tr>
<td>Big Basin Watershed</td>
<td>Microcystin SPIRT</td>
<td>0.82</td>
<td>0.49</td>
</tr>
<tr>
<td></td>
<td>Microcystin water</td>
<td>0.12</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>0.07</td>
<td>0.56</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>0.13</td>
<td>0.98</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>0.30</td>
<td>11.24</td>
</tr>
<tr>
<td></td>
<td>Silicate</td>
<td>0.51</td>
<td>143.37</td>
</tr>
<tr>
<td></td>
<td>Alkalinity</td>
<td>0.10</td>
<td>117.28</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>0.21</td>
<td>13.53</td>
</tr>
<tr>
<td>Pajaro River Watershed</td>
<td>Microcystin SPIRT</td>
<td>0.83</td>
<td>0.59</td>
</tr>
<tr>
<td></td>
<td>Microcystin water</td>
<td>0.19</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>0.03</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>0.15</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>0.66</td>
<td>4.54</td>
</tr>
<tr>
<td></td>
<td>Silicate</td>
<td>10.37</td>
<td>129.38</td>
</tr>
<tr>
<td></td>
<td>Alkalinity</td>
<td>0.12</td>
<td>231.86</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>7.75</td>
<td>15.72</td>
</tr>
<tr>
<td>Salinas River Watershed</td>
<td>Microcystin SPIRT</td>
<td>0.72</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>Microcystin water</td>
<td>0.32</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>0.03</td>
<td>3.79</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>0.10</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Silicate</td>
<td>0.45</td>
<td>196.88</td>
</tr>
<tr>
<td></td>
<td>Alkalinity</td>
<td>0.18</td>
<td>211.77</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>5.10</td>
<td>15.04</td>
</tr>
<tr>
<td>Carmel River Watershed</td>
<td>Microcystin SPIRT</td>
<td>0.62</td>
<td>0.11</td>
</tr>
<tr>
<td></td>
<td>Microcystin water</td>
<td>0.03</td>
<td>0.04</td>
</tr>
<tr>
<td></td>
<td>Ammonium</td>
<td>0.29</td>
<td>0.08</td>
</tr>
<tr>
<td></td>
<td>Nitrate</td>
<td>0.10</td>
<td>0.02</td>
</tr>
<tr>
<td></td>
<td>Phosphate</td>
<td>0.11</td>
<td>0.03</td>
</tr>
<tr>
<td></td>
<td>Silicate</td>
<td>27.00</td>
<td>250.79</td>
</tr>
<tr>
<td></td>
<td>Alkalinity</td>
<td>40.24</td>
<td>131.15</td>
</tr>
<tr>
<td></td>
<td>Temperature</td>
<td>7.06</td>
<td>13.91</td>
</tr>
<tr>
<td></td>
<td>Microcystin</td>
<td>0.10</td>
<td>0.78</td>
</tr>
</tbody>
</table>

Microcystis aeruginosa in the nearshore freshwater environment and have the potential to be subsequently transported to the marine environment. Miller et al. (2010) found that cells lyed in seawater after 48 h; there is also possibility for some cells to be carried to the marine environment, lyse, and then release toxin. Monterey Bay is at high risk for this type of problem due to the nature of the surrounding land which is highly populated and widely used for agriculture. However, we believe this may be a phenomenon in other near-shore marine systems that have not been monitored for this particular toxin, and therefore, have gone unnoticed.

The use of SPIRT technology allowed us to access time integrative toxin survey data simultaneously at many different locations, thus providing more than a "snapshot" of information such as would be obtained with intensive surveying. While SPIRT was originally developed to mimic shellfish toxicity, its use has proven to be more beneficial and easy to use for toxin monitoring as compared to other popular monitoring methods like the use of shellfish testing, 24 hours plankton surveys, and whole water sampling (Mackenzie et al., 2004; Lane et al., 2010; Mackenzie, 2010; Kudela, 2011). The occurrence of two dominant peaks, in spring and autumn, indicate an unexpected seasonal pattern of microcystin toxin for all primary watersheds in the Monterey Bay area. It is widely accepted that bloom formation is largely driven by light and nutrient availability, and often water stagnation (Zehnder and Gorham, 1960; Webb and Walling, 1992; Tsuji et al., 1994; Jacoby et al., 2000; Wetker and Steinberg, 2000; Jeong et al., 2003; Parol and Orten, 2011). This often leads to a seasonal pattern, with optimal conditions for bloom formation occurring in summer and autumn seasons. (Reynolds et al., 1978; Parol, 1988; Lehnman et al., 2004; Meisander et al., 2009). This seasonal characteristic has similarly been identified in nearby waterways like San Francisco Estuary (Lehnman et al., 2005, 2005; Meisander et al., 2009). Additionally, Miller et al. (2010) reported that the Monterey Bay area generally experiences increases in microcystin presence and concentration in freshwater lakes and rivers during autumn. The patterns of microcystin presence and concentration observed during this study suggest that microcystins are likely present throughout the year. In years two and three microcystin toxin increased and remained elevated at all locations and the spring/autumn peaks persisted. While only three years in duration, these results suggest that microcystin production and subsequent transfer to the coastal environment has the potential to be a persistent issue in the Monterey Bay area. The statistical analysis exhibited a distinct delineation between variables. Ammonium, urea, nitrate, and phosphorus all exhibited a negative association with toxin in the model. We infer that the negative relationship was caused by biological drawdow of nutrients: toxin is produced by cells which are stimulated by the high nutrient levels, but toxin concentrations can become
uncoupled from cell growth and nutrient concentrations due to cell lysis and differences between cell toxin quota and cell growth. Given the likely disconnect between toxin production and detection (SPATT were deployed for one week to one month), there is consistency with nutrient enrichment leading to increased algal biomass, with subsequent toxin production. Low nutrient levels would then be correlated with elevated toxins due to the time lag. This theory could be tested by identifying lagged correlations between nutrients and toxins, but our toxin data are integrative (SPATT) while the nutrients were collected at the time of SPATT recovery (with a coarse time scale relative to nutrient dynamics), precluding such an analysis. The assertions we present is consistent with the relationship between microcystin concentrations and silicate concentrations identified in the model. Silicate was positively associated with microcystin. Silicate is not utilized by Microcystis aeruginosa and consequently remained in the environment while other nutrients were presumably biologically drawn down in the absence of diatom blooms. The link between Table 3: Results from the mixed-effects model evaluating principal components, and temperature versus microcystin toxin.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Estimate number</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>-0.1240</td>
<td>0.0520</td>
</tr>
<tr>
<td>Urea</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ammonium</td>
<td></td>
<td></td>
</tr>
<tr>
<td>PC2</td>
<td>-0.0100</td>
<td>0.0000</td>
</tr>
<tr>
<td>Nitrate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phosphate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Silicate</td>
<td>-0.0640</td>
<td>0.0028</td>
</tr>
<tr>
<td>Temperature</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The first principal component is represented by PC1 and contains the variables urea and ammonium. The second principal component is represented by PC2 and contains variables nitrate, phosphate, and silicate. The associated estimate numbers and p-values from the model are provided.

Fig. 3: Microcystin toxins from SPATT (ng/L) time series from August 2011 to August 2013. (A) Big Basin watershed; (B) Pajaro River watershed; (C) Salinas River watershed; (D) Carmel River watershed.

Fig. 4: Alkalinity versus microcystin toxin box plots. Data for all locations from August 2012 to August 2013 was pooled, zeros were removed and microcystin toxin data was square root transformed.
macronutrients, particularly ammonium and urea, and toxic concentrations, and the relatively high nutrient concentrations point to anthropogenic loading as a significant driver of toxic accumulation in these watersheds (Kudela et al., 2008), consistent with other studies (Parrish et al., 2001; Parrish, 2008; Schindler and Vallenprere, 2008; Wilkinson et al., 2011).

Temperature, unexpectedly, exhibited a negative association with microcystin toxin within the model. Several studies have demonstrated the link between elevated and increasing temperature and the frequency of toxic blooms (Butterwick et al., 2005; Reynolds, 2006; Parrish and Hussman, 2008, 2009). However, the phenomenon of a non-correlation relationship between microcystin toxin and temperature has been seen previously, where elevated toxin concentrations have been associated with a range of temperatures (van der Westhuizen and Elloff, 1982; Arnó and Wunderlin, 2005; Davis et al., 2009; Kudela, 2011). This may be indicative of a non-linear relationship between the two variables, possibly driven by different growth-temperature responses for different toxigenic cyanobacteria (Parrish and Otten, 2013a, 2013b).

The inverse relationship between alkalinity and microcystin was expected. This dynamic has been documented previously (Aboul et al., 2005); additionally, alkalinity has been correlated with shifts in algal groups when ammonium is available to provide the source of nitrogen (Brewer and Goldman, 1970). River flow had positive correlations with microcystin toxin before and after time lags. Before the data were lagged there were more significant positive correlations; after lags, all sites were positively correlated, but fewer were significant. Many studies have highlighted that stagnation of water is associated with increased cell density of Microcystis (Christian et al., 1986; Reynolds, 1992; Jeong et al., 2006; Lehman et al., 2008). Lehman et al. (2008) also found that Microcystis cell density was positively correlated with flow at some locations in the San Francisco Estuary. Cell density was highest during periods of lowest river flow, and toxin was potentially produced during subsequent stagnant, long retention-time periods. The weak positive correlations identified in this study suggest that while river flow has an effect on toxin concentration, it is not the dominant effect. Other contributors to toxin abundance with stronger relationships, such as nutrient loading, appear to have a greater influence on toxin levels.

Presence of microcystin toxins is often indicative of an unhealthy ecosystem (Miller et al., 2012). Within the Monterey Bay region, toxins are present and persistent in the four major watersheds flowing into the Monterey Bay National Marine Sanctuary. Despite the persistence of this toxin in California watersheds and the potential negative impacts to humans and wildlife, microcystins are not routinely monitored by federal, state, or local management agencies. Because this toxin has the capacity for accumulation, biomagnification, and persistence within food webs, elevated levels within the watershed may increase the possibility for morbidity and mortality of wildlife and humans in terrestrial, estuarine, and marine ecosystems. Additionally, there is increasing evidence to support chronic exposure to microcystins as a significant threat to wildlife and humans (Bury et al., 1995; Wiegand et al., 1995; Jacobs et al., 2004; de Figueiredo et al., 2004; Maibrunn and Kentemir, 2006; Wang et al., 2010). Thus, even the low but detectable levels identified in this study may be indicative of a potentially unhealthy ecosystem. It is possible that low levels of microcystins are endemic to California and therefore a natural component of the ecosystem. The lack of baseline studies makes this assertion difficult to test, but this study provides a reasonable baseline for assessing future changes in toxins within the Monterey Bay watersheds.

Cyanobacterial harmful algal bloom events are often intensified by anthropogenic activities such as discharge of sewage, as well as both urban and agricultural practices that cause nutrient rich runoff to flow into local watersheds (Zehnder and Gartham, 1980; Fogg, 1969; Reynolds, 1987; Parrish, 1988; Davis et al., 2009; Parrish et al., 2011). Because cyanobacteria have the capacity to thrive in water with both low and high nutrient concentrations, these organisms have the potential to outcompete other algal groups and dominate affected watersheds (Salomon and Huemppe, 2005). The combined effect of high growth response to nutrient input and the ability to outcompete other organisms creates the potential for microcystin toxic to overwhelm affected ecosystems.

The extensive manifestation of microcystin at low to moderate levels throughout the year and throughout all major watersheds in the Monterey Bay area exhibits the potential complication of managing environmental impacts, and ecosystem disruptions. Management agencies have long grappled with the problem of microcystin toxins in inland waters. However, the ubiquity of microcystin at the land-sea interface in the Monterey Bay area represents a new management obstacle. Decisions made at the terrestrial level in the proximity of the marine environment may now impact freshwater, estuarine, and marine ecosystems, particularly given the demonstrated capacity for bio-accumulation in commercially harvested shellfish (Miller et al., 2010). New management plans and implementations may now have to regard this freshwater epidemic as an expanding and pervasive problem.

Acknowledgments

We would like to thank Dr. Melissa Miller and the Marine Wildlife Veterinary Care and Research Center (MWVCR) for volunteer support for the first six months of sampling. We thank Kendra Negrey for methodological guidance, Rob Frank for laboratory support, and Dr. Petri Raimondi for statistical advice. This work was completed with salary and tuition assistance from the NOAA Nancy Foster Scholarship program (CMG/NOAA award number: NA10SEC4810031 and with partial support from the Central and Northern California Ocean Observing System (CeNCOOS), funded by NOAA, NOAA award number: NA11NOS0030303).

References


CHAPTER 2: EVIDENCE OF FRESHWATER ALGAL TOXINS IN MARINE SHELLFISH: IMPLICATIONS FOR HUMAN AND AQUATIC HEALTH

Corinne M. Gibble, Melissa B. Peacock, Raphael M. Kudela
Submitted for publication in *Harmful Algae*

Abstract

The occurrence of freshwater harmful algal bloom toxins impacting the coastal ocean is an emerging threat, and the potential for invertebrate prey items to concentrate toxin and cause harm to human and wildlife consumers is not yet fully recognized. We examined toxin uptake and release in marine mussels for both particulate and dissolved phases of the hepatotoxin microcystin, produced by the freshwater cyanobacterial genus *Microcystis*. We also extended our experimental investigation of particulate toxin to include oysters (*Crassostrea sp.*) grown commercially for aquaculture. California mussels (*Mytilus californianus*) and oysters were exposed to *Microcystis* and microcystin toxin for 24 hours at varying concentrations, and then were placed in constantly flowing seawater and sampled through time simulating riverine flushing events to the coastal ocean. Mussels exposed to particulate microcystin purged the toxin slowly, with toxin detectable for at least 8 weeks post-exposure and maximum toxin of 39.11 ng/g after exposure to 26.65 µg/L microcysts. Dissolved toxin was also taken up by California mussels, with maximum concentrations of 20.74 ng/g after exposure to 7.74 µg/L microcystin, but was purged more rapidly. Oysters also took up particulate toxin but purged it more quickly than mussels. Additionally, naturally occurring marine mussels collected from San Francisco Bay tested positive for high levels of microcystin toxin. These results suggest that ephemeral discharge of *Microcystis* or microcystin to estuaries and the coastal ocean accumulate in higher trophic levels for weeks to months following exposure.
1. Introduction

While Microcystis aeruginosa blooms and associated toxins have long been recognized as a problem for freshwater systems, recent studies have identified significant and severe impairment in coastal receiving waters (Miller et al. 2010, Gibble and Kudela 2014). Production of microcystin toxin is influenced by nutrient supply, light levels, and temperature, and although it is not regularly monitored in the marine environment, M. aeruginosa is somewhat tolerant of saltwater conditions and some microcystin toxins can be persistent in saline and freshwater ecosystems (Zehnder and Gorham 1960, Tsuji et al. 1994, Jacoby et al. 2000, Welker and Steinburg 2000, Robson and Hamilton 2003, Ross et al. 2006, Tonk et al. 2007, Paerl and Huisman 2008, Davis et al. 2009, Paerl and Otten 2013, Gibble and Kudela 2014). Microcystin is produced in the cells of *M. aeruginosa*, in the particulate or intracellular form. If the cell is lysed, the dissolved or extracellular form can be released to the environment (Ripley 2010). *M. aeruginosa* has been shown to lyse and release the dissolved form of microcystin toxin within 48 hours of contact with saline water (Miller et al. 2010), making both particulate and dissolved microcystin potential concerns in the nearshore marine environment. Microcystin is a known hepatotoxin and exposure to this toxin has impacted different marine trophic levels, including small planktonic invertebrates, fish, and large vertebrates (DeMott and Moxter 1991, Malbrouck and Kestemont 2006, Richardson et al. 2007, Miller et al. 2010). The emergence of this toxin as both stable in marine receiving waters and harmful for upper marine trophic levels, including apex predators and humans,
highlights the need for better understanding of trophic transfer to and effects on
humans and wildlife health alike.

Marine bivalves, such as California mussels (*Mytilus californianus*), are particularly
useful in assessing accumulation of toxins related to Harmful Algal Blooms (HABs)
because they are widespread, are important prey of birds and marine mammals, and
are also consumed by humans. Their unique life history traits both impact and
increase their toxin accumulation ability. Because they are very active filter feeders
and detritivores, these organisms have the ability to consume large quantities of
cyanobacteria, and concentrate their toxins (Christoffersen 1996). Several studies
have documented microcystin accumulation in freshwater and saltwater invertebrates
Pires et al. 2004, Kvitek and Bretz 2005, Miller et al. 2010). However, trophic level
interactions and vulnerability of organisms to toxins at different trophic levels has not
been well defined (Turner and Tester 1997, Lefebvre et al. 1999, Shumway et al.
2003, Kvitek and Bretz 2005, Smith and Haney 2006). If microcystin is transferred up
the food chain, there may be several detrimental impacts for intermediate and apex
predators, as well as for humans.

Microcystins have been recorded in the near-shore marine environment in the
Monterey Bay, CA area (Miller et al. 2010, Gibble and Kudela 2014), while San
Francisco Bay displays chronic impairment, including poor water quality,
eutrophication, and increases in harmful algal bloom activity (Cloern and Jassby 2012). This is especially true in the upper estuarine environment where microcystins have been found to impact the food web (Lehman et al. 2005, 2008, Baxa et al. 2010, Lehman et al. 2010). More recently evidence for widespread contamination of California’s watersheds by multiple toxins has been documented (Fetscher et al. 2015). Despite significant upstream concentrations and the demonstrated ability for these toxins to be transferred through the marine food web (Miller et al. 2010), there is no routine monitoring of marine invertebrates for freshwater toxins. Recent attention to expanding microcystin occurrence (Gibble and Kudela 2014, Fetscher et al. 2015) generates a need to help address this deficiency.

Aquaculture has been ongoing since the 1800’s in Marin County, CA but has been closed in San Francisco Bay since the middle of the 1900's due to poor water quality. Tomales Bay, located immediately north in Marin County, CA, continues to have a vibrant aquaculture business (Carlsen et al. 1996). Historically this bay has been known as a pristine ecosystem, but because it is downstream of three major tributaries (Lagunitas, Olema, and Walker Creeks), it also has the potential to be impacted by downstream transport of freshwater toxins, particularly given the known impacts within the San Francisco estuary (Fischer et al. 1996, Lehman et al. 2005, Ger et al. 2009, 2010 Lehman et al. 2010, 2013).
We investigated the rate of toxin accumulation and subsequent loss in the California mussel to determine the length of time it takes for mussels to clear toxin after a marine exposure event. This organism was chosen since it is both recreationally harvested for human consumption, and a common prey item for wildlife. In addition to laboratory experiments, mussels of the same genus (Bay mussel, *M. trossulus* and Mediterranean mussel, *M. galloprovincialis*) collected from San Francisco Bay were analyzed for microcystin LR, YR, RR, and LA to determine if microcystins are present in a tidally influenced estuarine food web. This work led us to examine the presence of microcystins in commercially raised oysters to compare rates and levels of toxin uptake and loss between a species highly used by wildlife and a species highly used by humans. Because there is no current monitoring for microcystin in either aquaculture raised shellfish in California or recreationally harvested shellfish, there is concern that contamination of oysters sold for human consumption and contaminated naturally-occurring mussels may be going unnoticed.

2. Methods

2.1 Tank Experiments

To investigate depuration of both particulate and dissolved microcystin by California mussels, three separate tank experiment trials were performed. The first trial involved particulate microcystin toxin in low concentrations (average of 5.6 µg/L per tank); the second trial involved particulate microcystin toxin in higher concentration (average of 26 µg/L per tank); the third trial involved dissolved microcystin toxin in moderate
concentration (average of 7.73 µg/L per tank). A naturally occurring bloom of *M. aeruginosa* was collected from Pinto Lake, in Watsonville, CA a well-known “hot spot” for microcystin toxin production in the Monterey Bay area. mixed with saline water (Instant Ocean, Spectrum Brand, Virginia) and administered to three separate tanks per trial. The total salinity in each tank was approximately 33-36ppt to mimic ocean conditions, and all tanks were placed in a water-table filled with constantly flowing fresh seawater to maintain ambient temperature and exposure to typical coastal seawater. For the dissolved toxin experiment the Pinto Lake water was filtered through a 0.2 µm capsule filter to remove particulates.

California mussels used for the tank experiment trials were collected from Davenport Landing Beach in Monterey Bay, California. Once collected, mussels were acclimatized in constantly flowing filtered seawater for 24 hours before the start of the trial. Three representative control mussels were sampled for microcystin toxin concentration via liquid chromatography mass spectrometry (LCMS) and the remaining mussels were divided into three 38 liter tanks. For each of 3 experimental trials, mussels were shocked with microcystin (as dissolved or particulate Pinto Lake water) in each tank for 24 hours. At the start of each trial, whole water and cell counts were collected. After the 24-hour emersion period mussels were transferred to constantly flowing filtered seawater and sampled through time. At each time point 3 individual mussels and one whole water sample were taken from each tank and were assessed for levels of microcystin in the laboratory. Mussels from each tank were
subsampled in intervals (24 h, 36 h, 48 h, 72 h, 96 h post initial exposure). After the 96 h time point, mussels in all tanks were sampled at weekly intervals.

In the laboratory, mussels were shucked and the entire mass of tissue was collected to simulate consumption. Mussels were homogenized, and body burden was evaluated via LCMS following the procedures adapted from and outlined in Vasconcelos (1995), Amorim and Vasconcelos (1998), Eriksson et al. (1998), and Mekebri et al. (2009). To address individual variation in toxin uptake, mussels from each tank and timepoint were homogenized using a BioHomogenizer (Model M133/1281-0, Biospec Products Inc., Oklahoma, USA) ~4g of homogenized mussel tissue was extracted using 20mL acid methanol, then sonicated for 30 seconds using a sonic dismembrator (Model 100; Thermo Fisher Scientific, Massachusetts, USA) at ~10 W. Samples were centrifuged for 10 minutes at 3400 rpm (Model IEC Centra CL2; Thermo Fisher Scientific, Massachusetts, USA) and then prepped for analysis using solid phase extraction as described by Mekebri et al. (2009)

Microcystin-LR, RR, YR, and LA in mussel tissue was analyzed by LCMS with electrospray ionization (ESI) and selected ion monitoring (SIM) on an Agilent 6130 with a Phenomenex Kinetix (100 × 2.10) C18 column. Whole water collected from the tanks at each time point were analyzed in the lab using 3 mL of whole water mixed with 3 mL 50% methanol. Samples were then sonicated using a sonic dismembrator (Model 100; Thermo Fisher Scientific, Massachusetts, USA) for 30 s at
~10 W, filtered (0.2 μm nylon syringe filter), and analyzed by direct injection of 50 μL onto the LCMS column, following Mekebri et al. (2009) but modified to account for use of SIM rather than tandem mass-spectrometry (Kudela 2011). A gradient-elution method was used with HPLC water (solvent A) and LCMS acetonitrile (solvent B), both acidified with 0.1% formic acid, as the mobile phase. The gradient was as described in Mekebri et al. (2009), starting with 95:5 solvent A:B and ending with 25:75 at 19 min, held for 1 min, then followed by a 5 min equilibration at initial conditions prior to injection of the next sample. Samples were calibrated with standard curves (for each batch of samples) using pure standards (Fluka 33578 and Sigma–Aldrich M4194). Standards were run again at the end of the run for sample runs lasting more than 8 h. For a subset of mussel samples, standard addition was used to verify peak identification. The Minimum Detection Limit (MDL) for particulate and dissolved toxins was 0.10 µg/L. For shellfish samples the MDL depends on the amount of mussel tissue analyzed and was 0.10 ng/g for 1 g tissue (sample sizes were typically 1-4 g).

Water samples collected for cell counts were preserved with 10 mL 25% glutaraldehyde to 40mL of sample (5% final concentration). Preserved samples were sonicated using a sonic dismembrator (Model 100; Thermo Fisher Scientific, Massachusetts, USA) at ~4 W for 1 minute to break up colonies. 1 mL was then filtered using a 0.2 μm black polycarbonate (PCTE) membrane filter (Poretics Corp, Livermore, CA). Filters were examined via epifluorescence microscopy (Zeiss
Axioskop, Zeiss Microscopy, Thornwood, NY) at a magnification of 400x and individual cells were counted.

2.2 Field Experiments

Bay mussels and Mediterranean mussels, an invasive species, are sympatric, inhabit the same region of San Francisco Bay, and are known to hybridize (McDonald and Koehn 1988, Sarver and Foltz 1993, Suchanek et al. 1997). Differentiation between species often requires the use of molecular methods. Because of this difficulty in identification, mussels collected in San Francisco Bay are conservatively identified as Bay/Mediterranean mussels. To relate our tank experiment results to naturally occurring assemblages, Bay/Mediterranean mussels were collected monthly from 4 locations in San Francisco Bay: Point Isabel, Point Potrero, Berkeley Marina, Alameda Island from April 2015-September 2015, and from a fifth location, Romberg-Tiburon Center from August 2015-September 2015 (Fig. 4). Mussels were prepared for LCMS analysis following the methods described above but were examined individually rather than pooled to further investigate individual variability.

2.3 Commercial Oyster Tank Experiments

To determine whether invertebrate species used for aquaculture in Tomales Bay were impacted by microcystin, we purchased oysters from a commercial aquaculture vendor for immediate testing. Additional oysters were purchased from the same vendor for use in tank experiments at a later date. The standard distribution protocol for the supplier includes placing oysters in a tank of fresh filtered flowing water for
five days prior to release for public purchase and consumption. Additionally, oysters were cultivated and harvested under 28500-28519.5 of the California State Health and Safety Code.

Oysters purchased for immediate testing underwent the standard 5-day distribution protocol from the supplier, and were processed directly thereafter following methods described above for mussels. Oysters purchased subsequently for tank experimentation, also underwent the standard 5-day distribution protocol from the supplier, and also were acclimated in constantly flowing filtered seawater for an additional 24 hours before experimentation. Three control oysters were collected and processed before the start of the trials, and the remaining oysters were separated equally into three tanks. *Microcystis* was again collected from Pinto Lake, CA mixed with saline water (Instant Ocean, Spectrum Brand, Virginia) and administered to three separate tanks with a concentration of 7.71 µg/L microcystin. Cell counts were collected and analyzed as described above. Oysters in all three tanks were exposed to microcystin for 24 hours, after which they were placed in a water table with constantly flowing filtered seawater for the remainder of the trial. Oysters were sampled and processed as described above.
3. Results

3.1 Particulate Microcystin Trials - Low Microcystin

Particulate trials were conducted by adding whole water with *Microcystis* from Pinto Lake, CA, a well-studied known ‘hotspot’ for toxin production (Miller et al. 2010) to seawater tanks. Cell count results indicated that seawater tanks contained an average of 1,815,640 cells/mL, with an average of 5.6 µg/L total microcystin during the 24-hour shock period. Following the 24-hour shock period, constantly flowing seawater did not contain microcystin (< minimum detection limit, MDL), and no mussel mortality was observed. Control mussels collected from nearby Monterey Bay and analyzed before the start of the trials were negative for microcystin (<MDL). Mussels used in the trials took up microcystin toxin during the 24-hour shock period (Fig.1), reaching tissue concentrations similar to the water they were placed in. Mussels retained toxin for longer than eight weeks after a single 24 h exposure. Toxin levels slowly decreased after the 24-hour exposure with a noticeable drop in toxin three weeks post exposure. At the end of the eight week trials, microcystin toxin was low, but still detectable in mussel tissue.

3.2 Particulate Microcystin Trials - High Microcystin

Cell count results indicated that average water per tank contained approximately 7,045,229 cells/ mL, and whole water samples indicated that tanks had an average of 26.65 µg/L total microcystin during the 24-hour shock period. Post shock period, constantly flowing seawater did not contain microcystin (<MDL), and no mussel
mortality was observed. Control mussels analyzed before high microcystin particulate trials were negative for microcystin (<MDL). Mussels again took up particulate microcystin toxin at levels similar to the water they were placed in (Fig. 2). Mussels again retained toxin for longer than eight weeks. However, toxin levels remained high for much longer. There was a prominent drop in toxin three to four weeks post exposure. At the end of the eight week trials, microcystin toxin was again low, but still detectable in mussel tissue.

3.3 Dissolved Microcystin Trials
Whole water samples taken before the start of the 24-hour shock period indicated an average of 7.74 µg/L total microcystin. Preceding the 24-hour shock period, constantly flowing seawater did not contain microcystin (<MDL), and no mussel mortality occurred. Control mussels analyzed preceding the 24-hour shock period were negative for microcystin (<MDL). Mussels took up dissolved toxin within 24 hours (Fig. 3), at levels similar to the water they were placed in during the 24-hour shock. Mussels in the trials immediately decreased in toxin at 36 hours and fell to levels below the MDL by 72 hours post exposure (36 hours after being removed from exposure; Fig. 3).

3.4 Field Experiments
Naturally occurring mussels were harvested from multiple sites in San Francisco Bay. We detected microcystin toxins in at least one individual every month except for August 2015 at Romberg-Tiburon Center. Naturally occurring mussels exhibited a
large range of toxin concentrations, as is evident in September for individual mussels collected from Berkeley Marina, where the toxins ranged from <MDL - 416.23 ng/g (n = 8). September was the most toxic month for all sites, except for the northernmost site, Point Potrero, which was most toxic in August (Fig. 4).

### 3.5 Commercial Oyster Tank Experiments

Oysters that were evaluated immediately upon purchase tested positive for low levels of microcystin (3.42 +/- 2.24 ng/g, n=6). However, a second batch of oysters purchased at a later date and used for tank experiments were negative for microcystin (<MDL). At the beginning of the experimental trials, cell count results indicated that average cells per tank were 1,068,023 cells/mL, and whole water samples indicated that tanks had an average of 7.71 µg/L total microcystin during the 24-hour shock period. Post shock period, constantly flowing seawater did not contain microcystin (<MDL), and no oyster mortality was observed. During experimentation, oysters took up microcystin toxin within 24 hours. In contrast to the mussels, toxin in the oysters was lower on average than concentrations in the shock treatment tanks (4.88 ng/g; Fig. 5). Oysters in the trials decreased in toxin content at 36 hours and maintained a somewhat steady level of toxin until 4 weeks post exposure when there was another decrease. Eight weeks post exposure, oyster samples still contained low but detectable levels of toxin.
4. Discussion

Toxic marine and freshwater HAB occurrence is a worldwide problem exacerbated by the deterioration of ecosystem health, eutrophication, and increasingly warmer climate due to human activities (Zehnder and Gorham 1960, Welker and Steinburg 2000, Guo 2007, Paerl and Huisman 2008, Davis et al. 2009, Paerl and Huisman 2009, Kudela 2011). Unfortunately, the coastal ocean, which is already affected by marine HAB occurrence is now also influenced by the transport of freshwater toxin to the marine environment, which ultimately impacts the marine food web. As a relevant example, Preece et al. (2015) deployed caged mussels (*M. trossulus*) in Puget Sound, WA to document transfer of microcystins from a nearby lake into marine waters, and also demonstrated transfer from freshwater to marine receiving waters. When in contact with seawater, *M. aeruginosa* lyses and releases microcystin toxin within 48 hours (Miller et al. 2010) making both particulate and dissolved microcystin potential concerns for shellfish consumption. Our results indicate that both forms of toxin are concentrated by shellfish and consequently, are a concern for human and wildlife health, and for the aquaculture and fishing industries. We have shown experimentally that California mussels bioaccumulate toxin quickly in both particulate and dissolved forms, at high and low levels of exposure, and they release toxin slowly, allowing for rapid detection in real time and conservative estimates for clearance of toxin. Further investigation by examination of shellfish species can ultimately illuminate temporal and spatial patterns and identify long-term trends in microcystin occurrence, which
may better aid in future monitoring practices. However, perhaps more importantly, this toxin could be posing a real and unrecognized threat to human health globally.

Particulate uptake of microcystin toxin by animal cells, and toxicology of microcystin toxin has been investigated in the past (Eriksson et al. 1990, Dawson 1998, Campos and Vasconcelos 2010). As a hepatotoxin, microcystin targets the liver cells specifically, where subsequent to hepatocellular uptake, it inhibits protein phosphatases by binding to enzymes, which later causes the failure of liver cells (Eriksson et al. 1990, Dawson 1998, Campos and Vasconcelos 2010). However, routes of ingestion of microcystin toxin have been less well defined. The currently accepted assumption is that the main mode for uptake of cyanobacterial toxins is through consumption of particulate forms (Yokoyama and Park 2003, White et al. 2006, Ibelings and Chorus 2007). Some studies speculate that dissolved toxin is somewhat inconsequential to organisms, citing that levels of dissolved toxin may not be high in the natural environment, or uptake of dissolved toxin may be unimportant (Ozawa et al. 2003, Ibelings and Chorus 2007). However, Karjalainen et al. (2003) showed that planktonic grazers can take up dissolved nodularin toxin, and our study shows the substantial uptake of dissolved microcystin toxin in marine mussels, which emphasizes the fact that both types of ingestion play a role in food web transport and bioaccumulation, and should be investigated further. Currently the mechanism for concentrating dissolved microcystin in shellfish remains unknown.
The fact that marine shellfish species take up and incorporate dissolved toxin into tissues may provide an even greater likelihood that prey species down stream of *Microcystis* bloom activity will encounter microcystin toxin at some time during the year. Because microcystin toxin is both stable and environmentally persistent, it remains in the environment for extended periods of time (Cousins et al. 1996, Robson and Hamilton 2003, Ross et al. 2006, Tonk et al. 2007). Dissolved microcystin may also be transported further and extend into more distant environments than particulate forms, which increases the possibility of exposure downstream of bloom formation and toxin production. There is a general assumption that because *Microcystis* lyses within 48 hours in the marine environment, the expected encounter and exposure rates of this toxin in saline water is minimal. However, our results strongly contradict this assumption, with demonstrated bioaccumulation of both particulate and dissolved toxins.

During field sampling of naturally occurring mussels, we found microcystin toxin in mussels at all sites, during all months except for August at the most oceanic site. This result was somewhat unexpected because field sampling occurred during a 6-month period when large blooms of *Microcystis* are less likely to occur (Lehman et al. 2005, 2008) and freshwater input is minimal (Cloern 1996). The variation in toxin load among tested mussels was substantial (<MDL - 416.23 ng/g) leading us to us to further elucidate the potential bias in examining individual organisms. Individuals were pooled during tank experimentation to deal with this bias. The differential
concentrations of toxin shown in Fig. 4 may have been caused by fluctuating concentrations of particulate and dissolved microcystin to different areas of the nearshore marine environment, or may have been caused by differential uptake and retention rates by individuals.

Because our sampling locations were in San Francisco Bay, a brackish environment, and *Microcystis* does not grow in salinities above 5 for this region (Lehman et al. 2005), it is likely that mussels encountered microcystin originating in the upper San Francisco Estuary. *Microcystis* laden freshwater runoff has been documented in both San Francisco Bay and Monterey Bay in the nearshore environment, and runoff is known to carry microcystin toxin to downstream areas (Lehman et al. 2005, Paerl and Huisman 2008, Miller et al. 2010, Gibble and Kudela 2014). Because of these factors, it is perhaps even more surprising that toxin was detected in mussels during a period of catastrophic drought. During dry seasons, there is a tendency for reduced toxin occurrence (Paerl and Huisman 2008, Miller et al. 2010, Gibble and Kudela 2014), because nutrients necessary to sustain blooms are often delivered to bodies of water containing *M. aeruginosa* through both groundwater and surface runoff from land (Paerl and Huisman 2008). Drought periods theoretically should make detecting microcystin in organisms that are downstream of *M. aeruginosa* colony and bloom formation less likely. However, Lehman et al. (2013) report that warm, dry conditions such as occurred during our sampling period, in a multi-year drought, could lead to westward (estuarine) expansion of *Microcystis*, leading to longer duration of blooms,
greater spatial extent, and more impacts to higher trophic levels in response to predicted climate change. Our findings suggest that downstream in the estuary, rain events following periods of higher bloom activity are likely to increase dispersal of toxin found in mussels collected in San Francisco Bay. Our results also suggest that ephemeral discharge to the coastal ocean could have the potential to carry toxin to higher trophic levels for weeks to months following exposure.

Our results also indicated that oysters used in aquaculture in the adjacent Tomales Bay are at risk for microcystin toxin exposure. Given the various agricultural practices and sources of nutrient loading upstream of Tomales Bay’s main tributaries, toxin produced higher in the watershed may be traveling from these freshwater sources (Lewis et al. 2005). Oysters purchased from a local commercial vendor and prepared for public consumption contained low but detectable levels of microcystin toxin. In addition, results of experimentation in this study also indicate that oysters, accumulated microcystins within 24 hours of contact, and exhibited low but detectable levels of microcystin toxin at 8 weeks post exposure. Shellfish aquaculture practices vary globally. While most counties have initiated extensive monitoring programs to prevent human illness from harmful algal blooms, many developing countries do not have this type of organization (Shumway 1990). Because it is a freshwater toxin, microcystin is not frequently monitored for in marine environments where aquaculture operations exist. It is even less likely that this toxin would be monitored for in countries where less advanced aquaculture practices are used.
Two other studies (Rita et al. 2014, Preece et al. 2015), documented presence of microcystins above the World Health Organization (WHO) Tolerable Daily Intake (TDI; 24 ng/g wet weight) in marine mussels. Here we documented levels routinely exceeding California Office of Environmental Health and Hazard Assessment (OEHHA; 10 ng/g wet weight for microcystins per week in seafood) and WHO guidelines. From our environmental mussel samples, between 8% and 16% of the individual mussels at each site exceeded the OEHHA guideline for weekly fish consumption, and in the month of September, four mussels from Berkeley Marina and Alameda Island had > 230 ng/g of microcystin toxins, with one mussel at 416.23 ng/g of toxin, more than 40-fold the suggested weekly intake for consumption of fish (Fig. 4).

The results of our study elucidate an extensive unrecognized problem for environmental health, wildlife and human health alike. Cyanobacterial blooms with toxin production can have debilitating effects on ecosystems, leading to eutrophication, light limitation of other species, reduction of food web efficiency, and mortality of species living in the environment (Jones 1987, Paerl and Ustach 1982, Havens 2008). Such impacts are detectable in both marine and freshwater environments in California at many different trophic levels (Miller et al. 2010). The implications of environmental degradation and trophic transfer of microcystin toxin is important not only for California, but also strongly suggest that communities reliant on aquaculture and wild-harvest mussels for sustenance worldwide are at risk. A
combined real-time monitoring process including water quality and biomarker species monitoring is recommended to control detrimental effects and to better understand the breadth of impacts caused by this suite of toxins. We advocate immediate use of bivalves for monitoring and management, especially for aquaculture practices where low levels of toxins were identified in commercially sold shellfish. The structure to implement these recommendations presently exists and auxiliary testing could easily compliment current monitoring practices.

Acknowledgements

We would like to thank Kendra Hayashi, Rosemary Romero, and Regina Radan for lab and field support, and Betsy Steele for facilities support. This work was completed with salary and tuition assistance from the NOAA Nancy Foster Scholarship program (CMG) NOAA award number: NA10SEC4810031. Collection of samples was completed under California Department of Fish and Wildlife Scientific Collecting Permit #12147, and Monterey Bay National Marine Sanctuary Permit #MBNMS-2012-005.

References


Figures

Figure 1. *M. californianus* and particulate microcystin toxin in low concentrations experimental trials. X-axis begins at 24 hours denoting removal from water containing microcystins.

Figure 2. *M. californianus* and particulate microcystin toxin in high-concentration experimental trials. X-axis begins at 24 hours denoting removal from water containing microcystins.
Figure 3. *M. californianus* and dissolved microcystin toxin experimental trials. X-axis begins at 24 hours denoting removal from water containing microcystins.
Figure 4. Environmental samples of mussels collected monthly from central San Francisco Bay for April to September 2015. Boxes are the 25th and 75th percentile, and whiskers indicate 1.5 IQR. Dots are statistical outliers, blue squares are the means, and the black dotted line indicates the OEHHA action level for fish at 10 ng/g. Bars and outliers are colored via the following scheme: levels of toxin < 5 ng/g are grey, levels of toxin between 5-10 ng/g are light red, and levels of toxin > 10 ng/g are bright red.
Figure 5. Commercial oyster and microcystin toxin experimental trials. X-axis begins at 24 hours denoting removal from water containing microcystins.
CHAPTER 3: THE USE OF BLOOD COLLECTION CARDS FOR ASSESSING THE PRESENCE OF MICROCYSTIN IN MARINE AND ESTUARINE BIRDS

Corinne M. Gibble, Kendra Hayashi, Raphael M. Kudela
Submitted for publication in the Journal of Wildlife Rehabilitation

Abstract

*Microcystis aeruginosa* blooms and production of associated toxin, microcystin, are a common occurrence in freshwater systems throughout California, and microcystins have recently been recognized in nearshore marine environments along the central coast of California. Because nearshore feeding birds may be especially vulnerable to harmful algal blooms (HABs), we investigated the use of Whatman® FTA® blood sample collection cards for the detection of microcystin in estuarine and marine birds admitted to wildlife rehabilitation facilities in Monterey, CA between 2011 and 2015. Blood cards were analyzed via competitive enzyme-linked immunosorbent assay (ELISA), and results indicated that a large volume of blood (0.5mL) was necessary to detect a realistic level of toxin (0.5ppb). This may not be obtainable from sick, dehydrated, or injured birds in rehabilitation, however this method was shown to have utility for postmortem analysis and large die-off events. Blood cards collected in this study were grouped by species, and two groups tested positive for toxin. Currently data on the effects of HABs on marine birds is lacking, and better detection techniques are needed.
1. Introduction

Harmful algal blooms (HABs) are naturally occurring phenomena in freshwater and marine environments, and are found worldwide. Some algae that produce these blooms have the ability to release powerful toxins to the environment. Recently, toxins (microcystins) associated with the blooms of a common freshwater cyanobacterium, *Microcystis aeruginosa*, were detected in the near-shore marine environment of central California in Monterey Bay, and have been confirmed to be entering these environments through freshwater outflows (Miller et al. 2010, Gibble and Kudela 2014). *M. aeruginosa* has been shown to tolerate salt water environments and microcystin toxins can persist in saltwater and freshwater habitats (Robson and Hamilton 2003, Ross et al. 2006, Tonk et al. 2007, Miller et al. 2010), thus marine and estuarine wildlife feeding near outflows are at high risk for exposure.

Microcystins are known hepatotoxins that have the ability to cause impairment at many levels of the food web. In addition to direct toxic effects, exposure of aquatic organisms to elevated concentrations of microcystins have been shown to negatively impact primary consumers like herbivorous zooplankton, secondary consumers such as fish and invertebrates, as well as upper trophic levels such as birds and mammals (DeMott and Moxter 1991, Malbrouck and Kestemont 2006, Richardson et al. 2007, Miller et al. 2010). In 2007, numerous sea otters were found dead in Monterey Bay with signs of liver failure and microcystin was found in outflow areas where sea otters had stranded (Miller et al. 2010). During necropsy investigations, liver tissue tested positive for microcystin toxin and hepatic lesions typical of cyanobacterial
hepatotoxins were also discovered. Miller et al. (2010) also successfully validated the uptake of microcystins in common prey items during experimental trials. However, at this time, potential population-level impacts of these biotoxins on otters and other coastal wildlife remain undetermined.

2. Methods

2.1 Blood Card and ELISA Compatibility Trials

To evaluate viability of our proposed method, Whatman™ FTA blood sample collection cards (Whatman; GE Healthcare Bio-sciences, Pittsburg, PA, USA) were used in conjunction with Abraxis Microcystins ELISA for Serum (Abraxis LLC; Warminster, PA, USA) kits. This particular ELISA assay has demonstrated satisfactory cross-reactivity with all cyanobacterial cyclic peptide toxin congeners, and has 100% cross-reactivity with microcystin-LR, the most common variant of microcystin (Abraxis 2016). Whole blood was collected from live marine birds at the Society for the Prevention of Cruelty to Animals for Monterey County (MSPCA) Wildlife Center, in Monterey, CA and International Bird Rescue (IBR) in Fairfield, CA. In the laboratory, blood was spiked with Calbiochem InSolution™ MCY-LR standard to a final concentration of 100ppb and applied to blood cards in 0.005mL applications. To provide a negative control, whole blood was also applied to blood cards in 0.005mL applications and was not spiked with MCY-LR standard. This volume was chosen because it is a reasonable amount of blood to be taken from a bird in rehabilitation. Blood on blood cards was extracted using methods adapted from
Maucher and Ramsdell (2005) and Maucher et al. (2007). Prior to analysis by ELISA, blood spots were cut from cards, and Reagent A (.25mL) provided in the ELISA kit was used to hydrate the blood spots. The spots were then sonicated using a sonic dismembrator (Model 100; Thermo Fisher Scientific, Massachusetts, USA) for 30s at ~10W and centrifuged at 3600 rpm for 10 minutes. The supernatant was used as the sample, and triplicate samples were analyzed by ELISA following the exact specifications provided by the manufacturer. Both 50% MeOH and MilliQ de-ionized water were run individually to assess cross reactivity. The limit of detection was calculated and evaluated using standard additions of the MCY-LR standard.

2.2 Evaporation Trials

To determine if blood cards could be pooled to examine multiple individuals at once, blood cards were also evaluated with the addition of an evaporation/concentration step prior to ELISA analysis. Blood was spiked at 50ppb and applied to blood cards. Individual blood spots were cut from the blood cards, sonicated for 30s at ~10W, and centrifuged at 3600 rpm for 10 minutes. The supernatant was evaporated in two different solutions using either 0.5mL 50% MeOH, or 0.5mL 100% Milli-Q de-ionized water, using a Caliper TurboVap II (Caliper Lifesciences, Hopkinton, MA) nitrogen evaporator at 40°C. After evaporation the dehydrated pellet was rehydrated using Reagent A, provided by the ELISA kit, and used as the sample. ELISA was performed with replication in triplicate.
2.3 Application of Methodology on Birds

Detection of microcystin toxin in marine and estuarine birds was achieved through a collaboration with the MSPCA Wildlife Center. Whole blood was collected from a subset of marine and estuarine birds admitted to the wildlife center between the summer of 2011 and the winter of 2015. These birds live-stranded in Monterey County, CA (Figure 1), and strand location was recorded for each individual. Blood was collected via Fisherbrand™ microhematocrit capillary tubes (Fisher Scientific; Thermo Fisher Scientific, Massachusetts, USA) and administered to blood sample collection cards. Blood cards were stored frozen (-20°C) until analysis. Blood card samples were later pooled by species group and by date to evaluate multiple individuals simultaneously, and to obtain the appropriate amount of blood needed for detection. A total of eight species groupings were created as follows: Common Murres [(*Uria aalge*; COMU) COMU 2011-2012, COMU 2013-2014, COMU 2015], Brandt’s Cormorants (*Phalacrocorax penicillatus*), Ducks [Surf Scoters (*Melanitta perspicillata*); Ruddy Ducks (*Oxyura jamaicensis*), American Coots (*Fulica americana*), Grebes [Western Grebes (*Aechmophorus occidentalis*); Clark’s Grebes (*Aechmophorus clarkii*); Eared Grebes (*Podiceps nigricollis*), Loons [Common Loons (*Gavia immer*); Pacific Loons (*Gavia immer*)]. After pooling blood card samples by the above groups, blood cards were processed following the evaporation procedure described above, using 50% MeOH evaporation solution exclusively.
3. Results

3.1 Blood Card and ELISA Compatibility Trials

There was clear delineation between spiked blood and control blood during the method validation trials. All spiked blood was well above non-detect levels at approximately 100 ppb for each replication. The control blood was below the limit of detection (Figure 2.). The lowest detectable concentration of microcystin using this method was calculated conservatively as 0.15 ppb. This was calculated based upon the lowest standard to provide us a conservative working point. For a conservative amount of blood (0.005 mL) to be taken from a live bird, the concentration of microcystin in the blood would have to be at least 31.2 ppb for detection. This is a very high level of toxin. To detect microcystin in bird blood at a realistic level (0.5 ppb), the blood sample would have to be at least a volume of 0.5 mL (Table 1). There was no cross reactivity detected between the ELISA kits and 50% MeOH or MilliQ alone, and these solutions were deemed appropriate for use in evaporation trials.

3.2 Evaporation Trials

Due to results from the compatibility trials, we determined that blood spots should be pooled to obtain the appropriate amount of blood. When blood spots were pooled, the evaporation technique was employed, and 50% MeOH was determined to be a superior extraction solution due to the short time period required to evaporate the samples completely. Pooling and evaporating blood cards was a successful means of
examining multiple individuals simultaneously, and 70% of spiked microcystin was recovered.

3.3 Application of Methodology on Birds

The results of our findings for species-specific detection of microcystin are summarized in Table 2. Only COMU had sufficient sample density to evaluate temporal trends; for all other groups, data include the years 2011-2015. Two groups of wild birds entering local rehabilitation facilities, tested positive for microcystin toxin presence in whole blood. The first group was Ducks (<2.56ppb), and the second group was COMUs 2015 (<1.06ppb). Both of these groups had a very low level of detection of microcystin; all other groups were non-detect. Of the bird groups that tested positive for microcystin, the most common areas of stranding were Del Monte Beach and Moss Landing State Beach. Sixty-three percent of 2015 COMUs were found at Moss Landing State Beach; 50% of the Duck group were found stranded at Del Monte Beach.

4. Discussion

Microcystin toxin has been implicated in the deaths of birds worldwide, and is an emerging issue in the nearshore coastal environment in California. Other studies have examined the internal effects of this toxin in birds during necropsy via biochemical testing in the organs (Konst et al. 1965, Chen et al. 2009), and in the feathers (Metcalf et al. 2006), and measured presence and absence through microscopy in gut contents (Matsunaga et al. 1999). However, no blood values have been recorded and the lower
limit for microcystin sensitivity for waterbirds remains unknown (Ibelings et al. 2005). Means for better investigating contact with and effects of microcystin on live birds remain largely undeveloped. Our results show that blood cards, while effective, have some limitations for routine use. They may be best for large birds, or when large amounts of blood are available, or if several blood cards are pooled to obtain the appropriate amount of blood. They can also be used for postmortem analysis, during necropsy, or retrospectively to investigate a large mortality event, as this method provides the means to screen many birds at once. While we did not test other animals, the same methods should be applicable to organisms for which more blood is available (e.g. dogs, cattle).

Because this method allows for collective testing, it has the potential to provide a cost effective evaluation of microcystin exposure in birds that may otherwise go unnoticed. Generally, attempts to detect toxin in live birds in rehabilitation can be cost-prohibitive. Outsourcing testing to labs often can cost several hundred dollars per sample. In contrast, blood collection cards are inexpensive, easy to use, easy to store and easy to transport, making them an excellent option for use in rehabilitation centers and researchers. Use of inexpensive metrics may become increasingly important if this toxin, formerly found exclusively in freshwater environments, continues to be a problem in the coastal and marine environment, as seen in Monterey Bay, California (Miller et al. 2010, Gibble and Kudela 2014).
Monterey Bay supports the largest density and biomass of seabirds in the entire California Current System (Briggs et al. 1987), and has a high abundance of birds inhabiting the land-sea interface, including threatened and endangered species, making this region especially important as critical habitat (Henkel 2004). The bay also forms the centerpiece of the Monterey Bay National Marine Sanctuary, and is considered the second most important area for sea ducks (*Melanitta* spp.) and other nearshore species that winter along the Pacific coast of the United States (Jessup et al. 2009). This region also serves as an important molting and foraging area for migratory birds, so local microcystin production may also impact wider-ranging species.

Our results reveal that at least two groups of birds in Monterey Bay had contact with microcystin toxin between 2011-2015. Other groups that were tested may also regularly be in contact with this toxin, but were not captured in our study due to sampling constraints. However, because we had so few detections, contact with microcystin toxin may not be a persistent problem in Monterey Bay. Birds encountering the toxin may be doing so through acute exposure. Alternatively, based on the fact that this study had a relatively small sample size of birds, the fact that it was detected at all, suggests that microcystin is quite possibly an issue in some way. Stranding location data taken during intake and admittance provided information about potential areas where birds may have encountered microcystin toxin, if exposure was acute. The MSPCA responds to strandings of marine and estuarine
birds for Monterey County only (Figure 1). The birds included in this study ranged from Carmel, CA to Moss Landing, CA, and birds from the group: 2015 COMUs, were most commonly stranded at Moss Landing State Beach, however they were also found at Asilomar State Park, Del Monte Beach, and Marina State Beach (Figure 1). Birds from the group: Ducks, were most commonly stranded at Del Monte Beach, but also found stranded near Salinas River State Beach and Carmel River State Beach. These strand locations are at or near areas known to be freshwater outflows of microcystin to the coastal ocean, and these locations were also near areas where stranded otters that were suspected of microcystin intoxication were found (Miller et al. 2010, Gibble and Kudela 2014), and making acute exposure a possibility.

The groups identified as positive for microcystin by this study are likely candidates for microcystin intoxication in Monterey Bay because of diet and foraging locations. Surf Scoters and Ruddy Ducks regularly consume invertebrate species (Siegfried 1973, Woodlin and Swanson 1989, Bordage and Savard 1995, Savard et al. 1998, Anderson et al. 2008), that are known to uptake microcystin toxin and depurate it slowly (Vasconcelos 1995, Williams et al.1997, Amorim and Vasconcelos 1999, Dionisio Pires et al. 2004, Kvitek and Bretz 2005, Miller et al. 2010, Gibble et al. 2016), and are found in estuarine and nearshore marine environments. These foraging habits provide the potential for a higher likelihood of contact, if microcystin is present in the area where prey species are being consumed. Common Murres commonly eat fish, but also consume invertebrates in some instances (Ainley et al. 1996). Both of
these prey types have been shown to take up microcystin toxin (Vasconcelos 1995, Dionisio Pires et al. 2004, Malbrouck and Kestemont 2006, Richardson 2007).

However, perhaps a more important factor for this group of birds, is the year in which they stranded. In 2015 the west coast of the US experienced unprecedented intensity, duration and frequency of harmful algal blooms. Pinto Lake in Watsonville, CA is known as a local area “hot spot” for microcystin toxin production, and has been implicated in microcystin production that caused the death of sea otters feeding at freshwater outflows in Monterey Bay (Miller et al. 2010). In 2015, Pinto Lake had numerous large blooms of microcystin producing *Microcystis aeruginosa* (Kudela unpublished data). Other freshwater sources of microcystin in the Monterey Bay area, may have experienced similar bloom conditions and toxin production found at Pinto Lake in 2015. If there were several blooms producing toxin, there would be a greater likelihood of trophic transfer through prey items to species in upper trophic levels, like Common Murres.

The magnitude and frequency of HABs have increased significantly in recent years (Kudela et al. 2005), and the interaction between HABs and birds has likely increased contemporaneously. Our findings suggest that some bird species were in contact with microcystin in Monterey Bay between 2011 and 2015. However, these results may not directly translate to mortality for these birds. Outcomes from this study can be used as a starting point to investigate the interaction between microcystin and species that tested positive. However, further testing and development of new tools and
techniques is required to gain a better understanding of the role this toxin may be playing in the morbidity and mortality of waterbirds. The scant data available on the effects of toxic algae on marine and estuarine birds coupled with the results of this study show the need for better surveillance and detection techniques for HABs, and the development of better tools to evaluate algal toxins in birds (Shumway et al. 2003). Following further investigation, a systematic approach should be taken to develop a monitoring program for HAB-related marine and estuarine bird mortality. This type of monitoring could provide better detection of toxic events and subsequent exposure incidents for marine and estuarine birds.

Acknowledgements

We would like to thank the MSPCA Wildlife Center, and International Bird Rescue with great appreciation to Jessica Shipman, Rosanna Leighton, Dr. Rebecca Duerr, and Michelle Bellizzi who made this work possible. This work was completed with salary and tuition assistance from the NOAA Nancy Foster Scholarship program (CMG) NOAA award number: NA10SEC481003

References


Konst H, McKercher PD, Gorham PR, Robertson A, Howell J. 1965. Symptoms and pathology produced by toxic Microcystis aeruginosa NRC-1 in laboratory and
domestic animals. Canadian Journal of Comparative Medicine and Veterinary Science 29(9):221-228.


Figures

Figure 1. Map of Monterey Bay stranding locations. Red lines denote boundaries of stranding locations for birds examined in this study. Strand locations are labeled as follows: 1. Moss Landing State Beach, 2. Salinas River State Beach, 3. Marina State Beach, 4. Del Monte Beach, 5. Carmel River State Beach, 6. Asilomar State Park. Black dots indicate live stranding locations for birds binned in the group: COMU 2015; white dots indicate live stranding locations for birds binned in the group: Ducks. Map used with permission by required attribution: Tracey Saxby, Kate Boicourt, Integration and Application Network, University of Maryland Center for Environmental Science (ian.umces.edu/imagelibrary/).
Figure 2. Control blood concentrations containing no microcystin versus spiked blood concentrations, which were spiked at 100ppb microcystin.

Table 1. Limit of detection for volume of blood tested and levels of detectable microcystin (MCY) in microcystin-spiked avian blood samples

<table>
<thead>
<tr>
<th>Volume of blood (mL)</th>
<th>Detectable MCY (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.005</td>
<td>31.2</td>
</tr>
<tr>
<td>0.01</td>
<td>15.6</td>
</tr>
<tr>
<td>0.05</td>
<td>3.12</td>
</tr>
<tr>
<td>0.1</td>
<td>1.56</td>
</tr>
<tr>
<td>0.5</td>
<td>0.312</td>
</tr>
</tbody>
</table>
Table 2. Group-specific ELISA detection of microcystin in pooled blood card samples from naturally-exposed, wild birds

<table>
<thead>
<tr>
<th>Species Bin</th>
<th>Years</th>
<th># Birds</th>
<th>MCY in blood (ppb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Common Murre</td>
<td>2011-2012</td>
<td>14</td>
<td>nd</td>
</tr>
<tr>
<td>Common Murre</td>
<td>2013-2014</td>
<td>18</td>
<td>nd</td>
</tr>
<tr>
<td>Common Murre</td>
<td>2015</td>
<td>16</td>
<td>&lt;1.06</td>
</tr>
<tr>
<td>Brandt's Cormorants</td>
<td>2011-2015</td>
<td>26</td>
<td>nd</td>
</tr>
<tr>
<td>American Coots</td>
<td>2011-2015</td>
<td>7</td>
<td>nd</td>
</tr>
<tr>
<td>Grebes</td>
<td>2011-2016</td>
<td>8</td>
<td>nd</td>
</tr>
<tr>
<td>Loons</td>
<td>2011-2017</td>
<td>26</td>
<td>nd</td>
</tr>
<tr>
<td>Ducks</td>
<td>2011-2018</td>
<td>7</td>
<td>&lt;2.56</td>
</tr>
</tbody>
</table>

*nd = non-detect, Grebes Group = Western Grebes, Clark's Grebes, Eared Grebes; Ducks Group = Surf Scoters, Ruddy Ducks; Loons Group = Common Loons, Pacific Loons
CONCLUSION

This dissertation greatly expands the knowledge base of research for microcystin toxin in the marine environment and associated trophic level interactions. My Chapter 1 research demonstrates the widespread issue of microcystin impacting all major watersheds in the vicinity of Monterey Bay area, and shows that low to moderate levels of microcystin were detectable in ocean outflows of streams and rivers into the Monterey Bay National Marine Sanctuary at all times of the year. Increased levels of toxin occurred, not only during the expected autumn season, but were present in spring as well. This supports the idea that blooms producing microcystin may be far more pervasive than we once imagined in Central California. Freshwater blooms and subsequent increases in environmental toxin presence, were also statistically correlated with nutrient loading and nutrient drawdown previous to increases in toxin. Nutrient loading is presumably from agricultural and anthropogenic runoff, which exacerbates the inherent complexity of managing this toxin across multiple environments. Ecosystem-based management decisions must now include terrestrial, freshwater, estuarine and marine ecosystems, to best regulate this toxin at the land-sea interface in Monterey Bay. Governmental and non-governmental agencies have been dealing with management problems relating to microcystin toxins in inland watersheds for many years. However, the pervasiveness of microcystin in near-shore estuarine and marine waters in the Monterey Bay area represents a new hurdle for management entities. New regulatory actions and management plans should implement and incorporate this new information, since the
pervasiveness of microcystin toxin is no longer a solely freshwater dilemma. It should now be acknowledged as an increasingly ubiquitous problem. Integrative management is particularly important now that the capacity for bioaccumulation and retention of microcystin in commercially and recreationally harvested shellfish was demonstrated in Chapter 2 of this dissertation.

In my second chapter, I examined the potential trophic transfer of this toxin in marine shellfish, and also focused on the need to establish a sentinel species for detection and monitoring in order to protect public health. Sentinel organism assessment can form the basis for monitoring bioaccumulation and trophic transfer, complementing traditional grab-sample monitoring by providing a time-integrated assessment that documents potential links to higher organisms. The National Status and Trends Mussel Watch Program has been ongoing since 1986 and is both successful and wide-ranging throughout the coastal and Great Lakes regions of the US. However, this program does not currently monitor for microcystin toxins in coastal waters. In Chapter 2, I show that inclusion of freshwater toxins would be straightforward and effective, capitalizing on the existing program’s effort.

*Mytilus californianus* is an excellent bioindicator species for monitoring microcystin in the nearshore environment in coastal California. California mussels uptake toxin quickly in both particulate and dissolved forms, at high and low levels of exposure, and they release toxin slowly, allowing for rapid detection in real time and conservative estimates for clearance of toxin. Use of this species can illuminate temporal and spatial patterns and identify long-term trends in microcystin occurrence.
and help protect public health from microcystin exposure through consumption of marine foods. In addition, this species is already being used in both the current National NOAA Mussel Watch Program, and California State Mussel Watch Program to monitor for other HAB toxins in the coastal ocean including paralytic shellfish toxins and domoic acid. The addition of testing for microcystin could be an easy and cost effective addendum to current protocols. Alternatively, the use of this species would aid in establishing an analogous monitoring program specific to this toxin. Gunther et al. (1999), showed that transplanting California mussels to other locations can be used as a viable tool for monitoring locations that are not normally inhabited by the mussel. The practicality of this type of application can further expand the use of this species as a biomarker.

My results also indicated that oysters used in aquaculture in adjacent Tomales Bay are at risk for microcystin toxin exposure. Aquaculture growers often harvest oysters and place them in fresh filtered water before they are available to the public. The length of time commercially raised oysters are kept in fresh filtered water varies by grower. However, it is recommended that shellfish remain in water for at least 48 hours when there is concern about transporting HAB toxins to other environments (Hégaret et al. 2008). Aquaculture operations are monitored for other HAB toxins in the State of California but are not currently required to monitor for microcystins. Results from Chapter 2 indicate that oysters accumulated microcystins within 24 hours of contact, and exhibited low but detectable levels of microcystin toxin at 8
weeks post exposure. This exposes a critical need for better monitoring of this toxin, especially in species harvested for human consumption.

While drinking water health advisory guidelines have recently been created by the Environmental Protection Agency (EPA) for microcystins in the US (USEPA 2015), there are currently no federal or California statewide seafood regulations pertaining to microcystin toxin specifically. The California Office of Environmental Health and Hazard Assessments (OEHHA) recently provided suggested action levels for consumption of fish and shellfish of 10 ng/g wet weight for microcystins per week (Butler et al. 2012), lower than the WHO Tolerable Daily Intake (TDI) of 24 µg/kg wet weight. Two other studies (Preece et al. 2015, Rita et al. 2014), documented presence of microcystins above the WHO TDI in marine mussels. Results documented levels routinely exceeding OEHHA guidelines with exposure to both dissolved and high levels of particulate microcystins, and exceeding WHO TDI with exposure to high levels of particulate microcystins.

Action levels provide some regulatory guidance. However, without frequent testing for microcystin toxin, typical consumers are likely unaware of daily ingestion rates. *Mytilus californianus* and *Mytilus trossulus/galloprovincialis* are collected recreationally for human consumption in San Francisco Bay year-round. This fishery monitors for both domoic acid and saxitoxin, with common quarantines in May-October, but with no equivalent monitoring for microcystins. The maximum bag limit is 10 lbs. of mussels in the shell, and typically many mussels are consumed per meal. My results suggest that recreational shellfish collectors have the potential to
reach the maximum level of ingestible toxin per week in one mussel, and potentially well over the maximum limit per week in one meal. The results of Chapter 2 elucidate an extensive unrecognized problem for environmental health, wildlife and human health alike. I address this potential for detriment to wildlife in Chapter 3.

Recently microcystin was recognized as a hazard for sea otters in Monterey Bay, CA. However, there has previously been no monitoring for this toxin in estuarine and marine birds that share the same prey species and habitat as sea otters. Monterey Bay supports a large number of estuarine and marine birds that use the near-shore environment. This region is essential and crucial habitat for threatened and endangered species including: Marbled Murrelets (*Brachyramphus marmoratus*) and Snowy Plovers (*Charadrius alexandrinus nivosus*; Henkel, 2004). Monterey Bay is also heavily used by migratory species, so detrimental impacts acquired here may implicate a broader range of species, rather than confined to only local and native waterbird species.

Real time detection of microcystin toxin in birds is especially important in a rehabilitation setting. Antemortem detection of microcystin intoxication can help direct care of affected birds; and rehabilitation centers could better identify unusual mortality events relating to contact with microcystin toxin, if birds could be tested in real-time during intake and exam. The results of Chapter 3 demonstrate that blood cards can be used in tandem with commercial ELISA kits to detect the presence of microcystin in the blood of pooled avian samples or single samples from large birds in rehabilitation or in the wild. This method did not work successfully for smaller, or
dehydrated birds, where the proper amount of blood is not obtainable. Results from Chapter 3 also demonstrate the utility of this method for use in evaluating large groups of potentially affected birds simultaneously, or for postmortem analysis during necropsy. Blood card samples can also be logged and stored for future analysis during large events of suspected microcystin intoxication. This may allow scientists and rehabilitation centers to determine cause of death or track patterns of contact with microcystin.

Chapter 3 results also confirm that marine and estuarine birds in Central California have been exposed to microcystin toxin. Presence of toxin in whole blood was detectable for two groups, encompassing three species. These results can be used as jumping off point for future evaluations, and these species: Surf Scoters (*Melanitta perspicillata*), Ruddy Ducks (*Oxyura jamaicensis*), and Common Murres (*Uria aalge*) should be investigated further to determine potential impacts. There is a critical need for a method that can detect the presence of algal toxins in birds of any size in a rehabilitation or wild setting. In general, there have been very few studies available that report or describe effects of algal biotoxins on waterbirds. Shumway et al. (2003) and this study, both highlight the need for better detection and monitoring practices to evaluate this potential problem. Without these metrics harmful algal bloom related morbidity and mortality in birds will presumably continue to go unnoticed.

This complete dissertation expands on current scientific knowledge on several different levels. It contributes new discoveries to the role of microcystin toxin in the
food web and in the near-shore environment in Monterey Bay, but also can be
extended globally. Because microcystins are rapidly becoming a worldwide problem,
this research contributes data that has the ability to influence wildlife conservation
and water management policy across the United States and in numerous other
countries.

References

Gunther AJ, Davis JA, Hardin DD, Gold J, Bell D, Crick JR, Scelfo GM, Sericano J,
Stephenson M (1999) Long-term bioaccumulation monitoring with transplanted

transport of harmful algae via relocation of bivalve mollusks. *Mar Ecol Prog Ser*

microcystin in Puget Sound, Washington, mussels (*Mytilus trossulus*). *Lake Reserve
Manage* 31(1):50-54.

Contamination in Sea Mussel Farms from the Italian Southern Adriatic Coast

Henkel LA (2004) Seasonal abundance of marine birds in nearshore waters of

sporadic victims or under-reported events? *Harmful Algae* 2:1-17.

Butler N, Carlisle J, Linville R (2012) Toxicological summary and suggested action
levels to reduce potential adverse health effects of six cyanotoxins. Office of
Environmental Health Hazard Assessment, California Environmental Protection

Health Advisory for the Cyanobacterial Microcystin Toxins. U.S. Environmental
Protection Agency Office of Water (4304T) Health and Ecological Criteria Division
Washington, DC 20460. 67pp.