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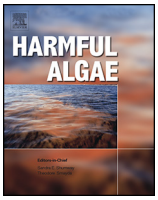

Wadeable streams as widespread sources of benthic cyanotoxins in California, USA

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ABSTRACT

Lentic water bodies and large rivers have long been recognized as being susceptible, under certain conditions, to toxin-producing ("toxigenic") planktonic cyanobacterial blooms. Although benthic cyanobacteria commonly inhabit wadeable (i.e., shallow) streams, little has been published on the potential for cyanotoxin (e.g., microcystin) production in this water body type. Recent research in Monterey Bay, California, USA has linked inland-derived microcystins to numerous sea otter mortalities in the marine environment, a finding that illustrates the negative effects cyanotoxins can have on ecosystem services, even far downstream from their origin, due to fluvial transport. For the present study, surveys of >1200 wadeable stream segments were conducted throughout California during the spring and summer of 2007 through 2013, and revealed a high occurrence of potentially toxigenic benthic cyanobacteria. In addition, benthic microcystins were detected in one-third of sites, where tested ($N = 368$), based primarily on one-time sampling, from 2011 to 2013 (mean concentration was 46 $\mu g/m^2$ of stream-bottom). Sites where microcystins were detected spanned a variety of surrounding land-use types, from open space (i.e., undeveloped land) to heavily urbanized/agricultural. Lyngbyatoxin ($n = 14$), saxitoxins ($n = 99$), and anatoxin-*a* ($n = 33$) were also measured, at subsets of sites, and were also detected, albeit at lower rates than microcystins. Results of this study provide strong evidence that wadeable streams could be significant sources of cyanotoxin inputs to receiving waters, a finding that has implications for the management of drinking water, wildlife, and recreational resources, within both the streams themselves and in downstream rivers, lentic water bodies, and the ocean.

1. Introduction

Cyanobacteria are photosynthetic prokaryotes that inhabit a wide variety of aquatic environments (Whitton, 2012). Many are capable of producing toxins ("cyanotoxins"), which can cause illness, and sometimes death, in humans, livestock, pets, and wildlife (Edwards et al., 1992; Van Halderen et al., 1995; Mez et al., 1997; Pouria et al., 1998; Backer et al., 2008; Stewart et al., 2008; Wood et al., 2010a,b; Backer et al., 2013). Although cyanotoxins are naturally occurring and cyanobacteria have existed for billions of years (Summons et al., 1999; Schopf, 2000), toxic blooms have become an increasing problem in lentic water bodies (O'Neil et al., 2012; Paerl and Otten, 2013) as well as large rivers (Quiblier et al., 2013; Hudon et al., 2014; Wood et al., 2014), with this proliferation attributed to a variety of anthropogenic factors (Paerl and Huisman, 2008; O'Neil et al., 2012).

For the purposes of this study, "wadeable" is defined as a stream segment that can be sampled by field crews wearing chest waders (i.e., estimated as measuring <1 m at its deepest). An important distinction of wadeable streams relative to other fresh water body types is that while the algal communities of lakes, ponds, lagoons, and large rivers are often dominated by phytoplankton, the major component of algal biomass in streams is typically benthic (Bellinger and Sigee, 2010). These communities can occur as

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microalgae within the "biofilm" coating on stream substrata. They also comprise macroalgae that are attached to stream substrata or that have detached and floated to the water surface, as well as filamentous forms loosely entrained in aquatic vegetation or occurring as diffuse masses in slow-moving water. All of these communities may contain species that produce cyanotoxins.

Despite the fact that cyanobacteria are known to inhabit streams (Ward et al., 1985; Becker, 1990; Dudley and D'Antonio, 1991), little has been published on these systems as sites for cyanotoxin production. Some exceptions include investigations in Spain (Aboal et al., 2002, 2005), which revealed microcystins in algal mats growing in shallow streams within calcareous catchments. Various studies on cyanobacterial mats (e.g., *Phormidium*) and the toxins they produce have also been conducted in New Zealand rivers (Heath et al., 2010; Harland et al., 2013). However, little work has been published for North American streams.

Microcystins, the most commonly occurring cyanotoxins (Sivonen and Jones, 1999; Rantala et al., 2004), have a half-life of several weeks under typical ambient conditions (Lahti et al., 1997). The stability of these and certain other cyanotoxins (e.g., nodularins; Twist and Codd, 1997) means they do not readily degrade during transit from the site of production, and thus may affect other locations, even far from their origin. For example, since 2007, at least 30 state/federally listed southern sea otters have died from microcystin intoxication in and around the Monterey Bay National Marine Sanctuary in California, USA (Miller et al., 2010; M. Miller, pers. comm.). Pinto Lake, a eutrophic water body that experiences frequent cyanobacterial blooms and drains to Monterey Bay via a 15-km segment of the Pájaro River, is believed to be a source of the toxin (Miller et al., 2010). Microcystin-laden water from the river, as well as from other tributaries to the Bay (Gibble and Kudela, 2014), flows to the coast, where the toxin can be biomagnified by bivalves or other prey items, and ultimately consumed by otters. The sea otter deaths illustrate the effect that cyanotoxins produced in a freshwater environment can have on biota (including marine species) downstream, and underscores an important role for fluvial systems as conduits that can transport intact toxins from inland waters to downstream marine environments.

This knowledge has prompted the following questions: (1) How abundant are potentially toxigenic benthic cyanobacteria in California wadeable streams? and (2) Are anthropogenic factors likely to influence the prevalence of these cyanobacteria, and/or cyanotoxin concentrations, in these systems? To begin addressing these questions, this paper presents the geospatial distribution of potentially toxigenic benthic cyanobacteria based on samples composited across 150-m-long stream segments that span a variety of surrounding land-use types throughout California. In addition, the frequency of detection of multiple cyanotoxins is reported, with an emphasis on microcystins. To the authors' knowledge, this is the first large-scale study to examine cyanotoxin concentrations in the wadeable stream benthic environment, accompanied by information on species-level cyanobacterial community composition.

2. Materials and methods

2.1. Study area

California's stream network is approximately 280,000 km long and drains a large (424,000 km^2), diverse landscape. There are temperate rainforests in the northwest and deserts in the northeast and southeast, but the majority of the state has a semi-arid, Mediterranean climate (Omernik, 1987). California's geology is complex, with recently uplifted and poorly consolidated marine sediments in the Coast Ranges, alluvium in its broad internal valleys, granitic batholiths along the eastern border, and recent volcanic lithology in the northern mountains. The native landscapes of some regions of the state have been nearly completely converted to agricultural or urban land uses (e.g., the Central Valley, the San Francisco Bay area, and the South Coast; Sleeter et al., 2011).

2.2. Sampling scope and site selection

Algal community composition samples were collected via stream monitoring surveys during the spring–summer of 2007 to 2013, and cyanotoxin samples were collected from 2011 to 2013. The target population for the surveys was perennial and non-perennial wadeable streams in California. The grand mean of depths across the sites sampled in the surveys was 12 cm (median = 10). The grand mean of wetted widths (i.e., the distance between the sides of the channel at the point where stream substrata are no longer surrounded by surface water) was 5.7 m (median = 4.1).

For the community composition data, 1565 sampling events occurred at 1279 unique sites (see maps in Results). For the toxin data, which largely correspond to a subset of the sites with community-composition data, 413 samples were analyzed for total microcystins across 368 stream sites. A subset of these were also analyzed for a select group of other cyanotoxins, including saxitoxins, anatoxin-*a*, lyngbyatoxin, nodularin, and cylindrospermopsin.

The majority of sampling sites were selected "probabilistically" (Stevens and Olsen, 2004), such that results (e.g., microcystin concentrations) from the surveys could be extrapolated to statewide estimates. The probability surveys were designed according to the methods described in Stevens and Olsen (2004), using the "SPSurvey" package (Kincaid and Olsen, 2008) in the R language and environment for statistical computing (version 2.15.1; R Core Team, 2012). SPSurvey employs an objective sampling-site-selection technique called "Generalized Random Tessellation Stratified" (GRTS; Stevens and Olsen, 2004). The GRTS procedure results in a list of randomly selected, spatially balanced sampling sites, such that the resulting dataset can be used to generate regional condition estimates (e.g., in terms of microcystin concentrations) with known confidence limits.

2.3. Sample and data collection

A "multi-habitat method" method (Fetscher et al., 2009) was employed to identify and quantify benthic algae from 150-m-long stream segments (hereafter referred to as sampling "sites"). "Composite" samples were collected by isolating benthic specimens from a known surface area over a variety of stream substrata in proportion to their relative abundances in the stream, and combining them. A fresh, "qualitative" sample was also collected by gathering an intact sample of all macroalgal types observed within the sampling site. By providing intact, unfixed specimens, the qualitative samples (1) aided laboratory identification of specimens in the quantitative sample that may have been fragmented in the course of collection (Fetscher et al., 2009; Stancheva et al., 2012), (2) were used, as needed, for isolation and culturing of specimens of interest, and (3) facilitated an assessment of overall macroalgal diversity in the stream segment sampled.

In addition to collecting benthic algae, percent areal cover of microalgae was recorded according to the methods of Fetscher et al. (2009). Microalgal cover was assessed based on the presence/thickness of the often slimy biofilm coating on stream substrata, the abundance of which was recorded by binning the coating into thickness categories (including zero thickness, for apparent absence of a biofilm) at 5 objectively determined points along

each of 11 equidistantly distributed transects down the length of the stream segment (for a total of 105 points).

2.4. Taxonomic analyses of stream cyanobacterial samples

Macroalgae were processed separately from the microscopic algal fraction of each composite sample to allow proper qualitative and quantitative identification and enumeration of the soft-bodied (i.e., non-diatom) algae in the sample, including cyanobacteria (Stancheva et al., 2012). To distinguish between fractions, the Sheath and Cole (1992) definition of "macroalgae" was adopted. Specifically, "macroalgae" include large (macroscopic) specimens that are filamentous, colonial, tuft-forming, crustose, tissue-like, or coenocytic algae or cyanobacteria that have forms recognizable with the naked eye. Example genera include *Nostoc*, *Rivularia*, *Cladophora*, *Oedogonium*, *Rhizoclonium*, *Batrachospermum*, *Lemanea*, *Spirogyra*, *Zygnema*, *Mougeotia*, and *Vaucheria*. See Sheath and Cole (1992) for definitions of forms.

Rather than homogenizing the entire original sample and using counting chambers (Lowe and Laliberte, 1996; Stevenson and Bahls, 1999), both algal fractions were processed separately to allow identification to the lowest possible taxonomic level (generally species), which was possible due to the high-quality preservation of macroalgal vegetative and reproductive structures, and the even distribution of microalgae on a standard microscope slide. Biovolumes measured in the laboratory were transformed into volume per area of stream bottom sampled ($\mu m^3\ cm^{-2}$).

In addition to collecting the biovolume information for each recorded specimen, up to 100 epiphytes on the macroalgae were enumerated, and taxa present in the qualitative sample recorded. Identifications were based on the cyanobacteria taxonomic concept and nomenclature of Komarek and Anagnostidis (1999), Komarek and Anagnostidis (2005) and Komárek (2013). Refer to Appendix A for a more in-depth discussion of taxonomic standards employed in this analysis.

2.5. Laboratory analysis of chlorophyll a samples

To estimate the amount of algal biomass in sampling sites, aliquots were drawn from the composite sample, filtered on to Whatman™ GF/F (i.e., glass-fiber) filters, which have a nominal pore size of 0.7 μm, stored frozen (−20 °C), and analyzed for chlorophyll *a* content using EPA method 445.0. Chlorophyll *a* concentrations measured in the laboratory were transformed into mass per area of stream bottom sampled ($mg\ m^{-2}$).

2.6. Laboratory analyses of cyanotoxin samples

At a subset of sites (19 in 2011, 98 in 2012, and 251 in 2013), aliquots were also drawn from the composite samples for the determination of cell-bound cyanotoxin concentrations. A known volume of composite sample was filtered on to Whatman™ GF/F filters and stored frozen (−20 °C) until analysis. Samples collected in 2011 were analyzed for four microcystin congeners (MCY-LA, MCY-LR, MCY-RR, MCY-YR) and anatoxin-*a* by liquid chromatography–mass spectrometry (LC–MS). Those collected in 2012 were analyzed for the same four congeners either by LC–MS (to yield results for each congener separately), or (for the four in aggregate) by enzyme-linked immunosorbent assay (ELISA). In addition, a subset of the samples were analyzed for saxitoxins (by ELISA) and/or for lyngbyatoxin, anatoxin-*a*, cylindrospermopsin, and nodularin (by LC–MS). All 2013 samples were analyzed for the four microcystin congeners by ELISA. Note that, hereafter, "total microcystins" refers to the combined values for the four microcystin congeners listed above, whether they are the ELISA results or summed results from the LC–MS analyses.

Microcystins were analyzed by ELISA using the Envirologix QuantiPlate™ kit (Envirologix, Portland, ME; Cat. No. EP 022; as described in Kudela, 2011). The BIOO Scientific MaxSignal™ Saxitoxin (PSP) test kit (BIOO Scientific Corp., Austin, TX; Cat. No. 1034) was used for saxitoxin analysis. Prior to analysis, the sample-containing filters were extracted in 3 mL of Milli-Q™ water, sonicated for 30 seconds to ensure cell disruption, and centrifuged for 10 min at 2147 g (as described in Seubert et al., 2014). The extract was then analyzed according to the manufacturer's instructions for both toxins.

For the samples analyzed by LC–MS, electrospray ionization (ESI) with selected ion monitoring (SIM) on an Agilent 6130 Phenomenex Kinetix™ C18 column was employed. This method was adapted from Mekebri et al. (2009) with minor modifications, to account for the choice of column, and LC–MS/SIM instead of tandem mass spectrometry (Kudela, 2011).

2.7. Generating regional estimates of the prevalence of toxigenic taxa and microcystin concentrations

To estimate the frequency of occurrence of potentially toxigenic cyanobacterial taxa, as well as microcystin concentrations, across California streams, data from the probability-survey sampling sites were used to generate descriptive statistics for data distributions and cumulative distribution functions (Kincaid and Olsen, 2008). A cumulative distribution function depicts the estimated probability distribution of a given measured value (e.g., benthic microcystin concentrations) relative to the cumulative proportion of the geographic unit of interest (e.g., percent of stream kilometers in the state). Estimates were calculated using the Horvitz–Thompson estimator (1952), which is a weighted average of sample values, where weights are adjusted according to the spatial relationship among sites. Confidence intervals were based on local neighborhood variance estimators (Stevens and Olsen, 2003), which assumes that samples located close together tend to be more alike than samples that are far apart. Estimates were generated using the "SPSurvey" package (Kincaid and Olsen, 2008) in R. All graphics presented here were prepared with the R package, "ggplot2" (Wickham, 2009).

2.8. Exploring land-use relationships

Potential anthropogenic influences on (1) the distribution of toxigenic taxa and (2) levels of benthic microcystin production were explored by looking at relationships with the proportion of coarse-resolution land-use types ("agricultural" vs. "developed" vs. undeveloped "open space") within three buffers of varying radii (10 km, 5 km, and 500 m) centered on each sampling site. Note that in this context, "open space" does not necessarily connote "pristine". In addition, Mantel tests (Mantel, 1967) were used to assess spatial autocorrelation of results among sites. This information is useful for generating hypotheses about potential anthropogenic vs. natural drivers of benthic microcystin production.

In preparation for analysis, microcystin concentration data were log-transformed and latitude/longitude were converted to Equidistant, Cylindric Map Projection coordinates using the "SPSurvey" package (Kincaid and Olsen, 2008) in R. Euclidean distance matrices were then calculated for each variable using the "ecodist" package (Goslee and Urban, 2007) and Mantel tests were performed using the "ade4" package (Dray and Dufour, 2007).

2.9. Identifying candidate microcystin producers in California streams

Indicator species analysis (Dufrêne and Legendre, 1997) was used to inform inferences about potential microcystin-producing

taxa in the sampling sites. The analysis was carried out using PC-ORD v6 software (McCune and Grace, 2002) on genus presence-absence data. Significance levels for each genus' group membership assignment were generated via Monte Carlo methods.

3. Results

3.1. Geospatial distribution of toxigenic cyanobacteria

Twenty-two cyanobacterial genera reported in the literature as possessing toxin-producing members (hereafter referred to as "toxigenic genera"), including 20 species known to possess toxin-producing strains (i.e., "toxigenic species"), were observed across the sampling sites during spring-summer monitoring surveys conducted from 2007 to 2013 (Table 1). *Leptolyngbya, Phormidium, Nostoc*, and *Anabaena* were the most frequently encountered toxigenic genera, and tended to exhibit some of the highest estimated biovolumes (among the toxigenic taxa) across sites (Table 2). Furthermore, three of the six potentially toxigenic species with the highest "prevalence index" (i.e., the product of the number of sites where the species was observed and its mean biovolume across sites) were *Nostoc* species (Appendix A).

Ninety-three percent of sites supported one or more toxigenic taxa during at least one sampling event. Of these, 25% supported species-level taxa known to be capable of cyanotoxin production. The subset of toxigenic taxa that are specifically microcystin-producers were nearly as common: Ninety-two percent of sites supported toxigenic genera, and of these, 16% supported toxigenic species. Toxigenic taxa were found throughout the state (Fig. 1). Most sites where such taxa were recorded supported one or two such genera, but some had as many as eight in a single sampling event. No spatial autocorrelation in the distribution of toxigenic taxa was evident: Mantel's r was 0.017 ($p = 0.18$) for cyanotoxin-producing taxa in general, and 0.020 ($p = 0.12$) for microcystin producers.

Based on the probability surveys, 90% of stream kilometers statewide are estimated to support toxigenic (Table 1) genera (with a 95% confidence interval of 81–99%), and 23% are estimated to support toxigenic species (confidence interval: 9–36%). Fourteen percent of stream kilometers are estimated to support microcystin-producing species (confidence interval: 0–27%).

3.2. Patterns of cyanotoxin detection

Of the 368 sites sampled for total-microcystins analysis, 33% tested positive during at least one sampling event. Overall, the distribution of microcystin concentrations in stream benthos was highly skewed toward the low end, with the median falling below detection limits, the 75th percentile at 7 $\mu g\ m^{-2}$, and a mean of 46 $\mu g\ m^{-2}$. Although uncommon, high concentrations were observed in a few sites, with the maximum exceeding the mean by >10-fold, at 4767 $\mu g\ m^{-2}$.

Samples from a subset of 35 stream sites were tested for four individual microcystin (MCY) congeners over the course of 2011–2012. The most commonly encountered was MCY–LA, which was detected at 43% of the sites and exhibited a maximum concentration of 75.4 $\mu g\ m^{-2}$. The next most common (MCY-LR) was detected at 37% of sites, with a maximum concentration of 2.5 $\mu g\ m^{-2}$, followed by MCY-YR and MCY-RR, both of which were detected at 6% of sites and had maximum concentrations of 3.1 and 2.5 $\mu g\ m^{-2}$, respectively.

Based on the probability surveys, the percentage of stream kilometers statewide that are estimated to harbor microcystins at some point during spring-summer is 34% (confidence interval: 7–60%). Based on the cumulative distribution function (Fig. 2), approximately 30% of California wadeable stream kilometers are estimated to harbor >25 $\mu g\ m^{-2}$ of benthic microcystins, and approximately 5% harbor >300 $\mu g\ m^{-2}$.

After microcystins, the class of cyanotoxin most frequently detected in stream benthos was lyngbyatoxin, which was present at 21% of sites ($N = 14$ samples collected; maximum concentration = 7.2 $\mu g\ m^{-2}$). Saxitoxin and anatoxin-*a* were detected much less frequently, at 7% ($N = 99$) and 3% ($N = 33$) of sites, respectively. The maximum observed concentration was 0.2 $\mu g\ m^{-2}$ for saxitoxin and 12.1 $\mu g\ m^{-2}$ for anatoxin-*a* (representing the sole instance of detection of that toxin). Tests for cylindrospermopsin and nodularin were conducted for a total of 14 stream sites, but neither was detected.

No spatial bias to the location of microcystin detections was readily apparent. The only possible exception was northwestern California, where relatively few samples were positive for the toxin (Fig. 3). Particularly high concentrations were observed in montane streams, especially in the Sierra Nevada (the highest-concentration in the data set overall was detected in the Lake Tahoe Basin) and parts of southern California. Weak spatial autocorrelation among sampling sites in terms of microcystin concentrations was evident (Mantel's $r = 0.08$; $p = 0.02$).

3.3. Relationship of microcystin concentrations to coarse-resolution anthropogenic influences

No significant relationships were observed between land use surrounding the sampling sites and the frequency of microcystin detection (Fig. 4), and there was no evidence of spatial autocorrelation in the tendency to produce microcystins (Mantel's $r = 0.004$; $p = 0.39$). However, where present, higher concentrations of the toxin tended to be associated with sampling sites in an undeveloped, open-space (as opposed to urbanized/agricultural) setting, regardless of the radius of the buffer around the site (Fig. 5).

The greatest concentrations of microcystins occurred in high-elevation streams with high microalgal cover at the time of sampling. The possibility that the high concentrations of toxin in these streams were simply a by-product of overall higher algal biomass was eliminated by regressing percent microalgal cover on chlorophyll *a* concentration, and plotting the residuals against elevation (Fig. 6). Even with the effect of overall algal biomass removed, higher microalgal cover was still associated with higher microcystin concentrations, among the higher-elevation sites.

The possibility that high-elevation/high-microalgal-cover conditions select for cyanobacterial taxa that can produce high levels of microcystins was explored via indicator species analysis (Dufrêne and Legendre, 1997). Several cyanobacterial genera, including *Nostoc* and *Phormidium*, were significantly associated with high-elevation/high-microalgal-cover stream sites (Table 3), thus providing further evidence that they could be microcystin producers in California.

4. Discussion

4.1. Prevalence and distribution of toxigenic taxa and cyanotoxins

Study results indicate that potentially toxigenic benthic cyanobacteria inhabit the majority of California wadeable streams, and are widely distributed throughout the state. Microcystins were commonly detected within the stream benthos (during the spring-summer timeframe), and while the other cyanotoxins measured were not detected as frequently (and were not sampled as comprehensively), the potential for

Table 1
Toxigenic cyanobacterial genera, and (where applicable) species within those genera, that were recorded in California wadeable streams ($N = 1279$ unique sites), along with toxins they can produce, according to the literature. Except where noted, only the results from literature for which chemical analyses were conducted on isolated cyanobacterial strains in culture conditions are included. Superscripts/bold-font indicate how species match with the toxins that they can produce and the literature source. *Note*: Morphological descriptions and photomicrographs for the cyanobacterial species from California streams are available from Stancheva et al. (2014).

Genus	% of sites where genus recorded	Species	Anatoxin-*a*	Aplysiatoxin	β-Methylamino alanine	Cylindrospermopsin	Debromoaplysiatoxin	Lyngbyatoxin	Microcystins	Neosaxitoxins	Nodularins	Pahayokolide	Saxitoxins	References
Anabaena	17		X			X			X	X			X	Vezie et al., 1998; Mohamed et al., 2006; Spoof et al., 2006
Anabaenopsis	<1								X					Lanaras and Cook, 1994
Arthrospira	1								X					Ballot et al., 2005
Coelomoron	1	*C. pusillum*[a]							**X**[a]					**Dos et al., 2005**[a‡]
Cylindrospermum	3	*C. stagnale*[a]	X						X				**X**[a]	**Sivonen et al., 1989a,b**[a]; Pandey and Tiwari, 2010; Borges et al., 2015
Dolichospermum	1	*D. flosaquae*[a], *D. planctonicum*[b]	**X**[a,b]						**X**[a]					**Sivonen et al., 1989a,b**[a]; Harada et al., 1991; **Bruno et al., 1994**[b]
Geitlerinema	6	*G. splendidum*[a], *G. amphibium*[b], *G. lemmermannii*[b]							**X**[a]				**X**[b]	**Aboal et al., 2005**[a]; Myers et al., 2007; **Borges et al., 2015**[b]
Gloeotrichia	1								X					Carey et al., 2007
Hapalosiphon	1	*H. hibernicus*[a]							**X**[a]					**Prinsep et al., 1992**[a]
Leptolyngbya	78								X					Mohamed et al., 2006
Lyngbya	3	*L. wollei*[a,b]		X		**X**[b]	X	X				X	**X**[a]	Onodera et al., 1997; **Yin et al., 1997**[a]; Berry et al., 2004; Dos et al., 2005; **Seifert et al., 2007**[b]; Harr et al., 2008
Microcystis	1								X					Botes et al., 1982
Nodularia	2	*N. spumigena*[a]									**X**[a]			**Sivonen et al., 1989a,b**[a]
Nostoc	33	*N. carneum*[a]							**X**[a]					Sivonen et al., 1992; **Mohamed et al., 2006**[a]
Oscillatoria	4	*O. tenuis*[a]	X			X			**X**[a]					Sivonen et al., 1989a,b; **Luukkainen et al., 1993**[a]; Brittain et al., 2000; Mazmouz et al., 2010
Phormidium	38	*P. formosum*[a], *P. uncinatum*[b], *P. autumnale*[c]	**X**[a,c]		X	X			X				**X**[b]	**Skulberg et al., 1992**[a‡‡], Mez et al., 1997; Gugger et al., 2005; Mohamed et al., 2006; Izaguirre et al., 2007; **Harland et al., 2014**[b], **Harland et al., 2013**[c]; **Borges et al., 2015**[b]
Rivularia	4	*R. biasolettiana*[a], *R. haematites*[a]							**X**[a]					**Aboal et al., 2005**[a]
Schizothrix	2			X										Sivonen and Jones, 1999
Scytonema	2	*S. crispum*[a]											**X**[a]	**Smith et al., 2011**[a]
Tolypothrix	12	*T. distorta*[a]							**X**[a]					**Aboal et al., 2005**[a]
Trichormus	1	*T. variabilis*[a]							**X**[a]					**Mohamed et al., 2006**[a]
Tychonema	10			X										Shams et al., 2015

‡ Extracts tested positive for toxicity, and toxin exhibited similarity to microcystin, but the exact chemical nature of the toxin was not conclusively determined.
‡‡ Specimen was not cultured.

Table 2
Frequency of occurrence and biovolumes of the microcystin-producing genera (based on Table 1) encountered in benthic samples from California wadeable streams. "Rank" refers to decreasing order of a "prevalence index", which is the product of the number of sites where the genus was observed and its mean biovolume across sites. Genera in bold exhibited the highest prevalence indices.

Genus	Rank (of 17 genera total)	# of sites where observed	Mean biovolume across sites ($\mu m^3\,cm^{-2}$)	Median biovolume across sites ($\mu m^3\,cm^{-2}$)	Maximum biovolume across sites ($\mu m^3\,cm^{-2}$)
Anabaena	**3**	**218**	**4.54E+09**	**2.89E+05**	**8.37E+11**
Anabaenopsis	16	1	4.06E+05	4.06E+05	4.06E+05
Arthrospira	15	10	1.17E+07	2.69E+03	1.17E+08
Coelomoron	17	11	3.05E+04	1.65E+04	9.01E+04
Cylindrospermum	8	38	4.25E+08	5.77E+06	3.24E+09
Dolichospermum	13	16	4.40E+07	5.87E+05	4.66E+08
Geitlerinema	10	73	6.38E+07	1.19E+05	1.87E+09
Gloeotrichia	9	10	1.06E+09	3.32E+08	3.84E+09
Hapalosiphon	12	16	1.22E+08	4.14E+06	1.42E+09
Leptolyngbya	**2**	**995**	**1.13E+09**	**7.36E+04**	**1.08E+12**
Microcystis	14	16	3.11E+07	2.68E+04	4.62E+08
Nostoc	**1**	**426**	**4.29E+09**	**2.89E+07**	**5.33E+11**
Oscillatoria	11	50	3.94E+07	8.02E+05	9.82E+08
Phormidium	**4**	**483**	**1.88E+08**	**4.58E+05**	**1.75E+10**
Rivularia	5	50	1.65E+09	1.25E+07	2.72E+10
Tolypothrix	6	152	2.96E+08	1.81E+07	7.62E+09
Trichormus	7	13	2.11E+09	1.48E+08	1.98E+10

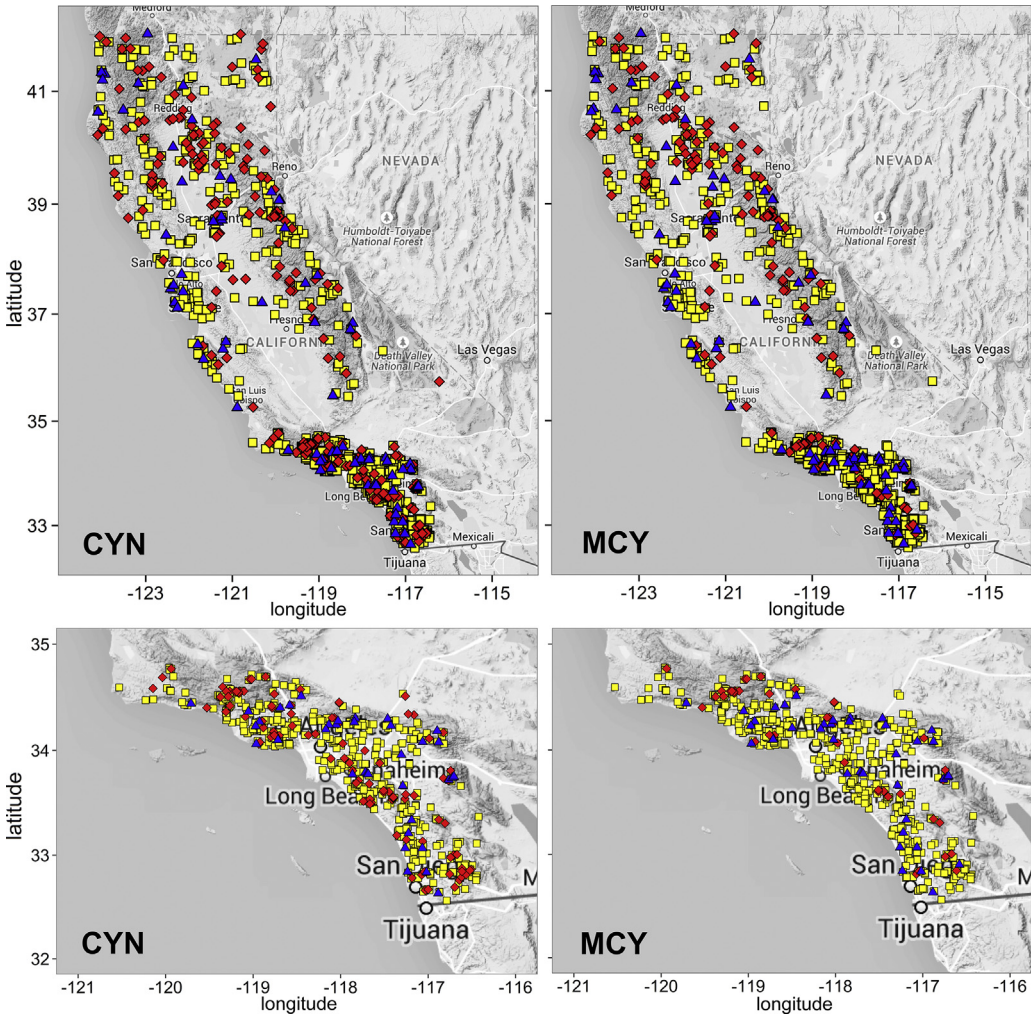

Fig. 1. Benthic algae sampling sites where taxa capable of producing cyanotoxins (CYN) in general (panels on left), or microcystins (MCY) specifically (panels on right), were observed during spring-summer monitoring surveys from 2007 to 2013. Blue triangles correspond to sites where no toxigenic taxa, based on current knowledge (Table 1), were observed; yellow squares correspond to sites where toxigenic genera (but not species) were observed, and red diamonds correspond to sites where toxigenic species were observed. Top panels correspond to the state as a whole, and bottom panels are zoomed-in on southern California, where the data density is highest. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

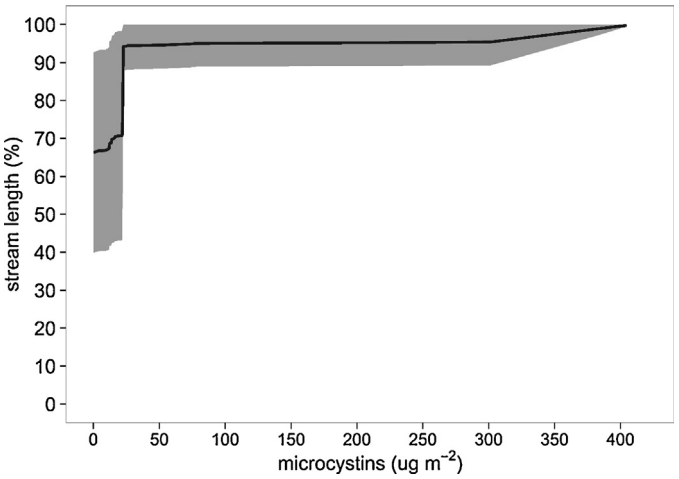

Fig. 2. Statewide cumulative distribution function for benthic microcystin concentrations during the spring-summer timeframe. The graph shows the estimated probability distribution of toxin concentrations relative to the cumulative proportion of length of California wadeable streams. The gray highlighted area delineates the 95% confidence interval for the estimate. Note: the x-axis is truncated at 400 μg m^{-2} to aid visualization.

these (and additional) toxins is high based on the results of the community-composition analysis. These findings challenge the conventional wisdom that only lentic water bodies and large rivers are susceptible to cyanotoxin-related impacts, and indicate that the risk of cyanotoxin export to downstream ecosystems is greater than previously thought.

While data are not available to identify conclusively which species are producing toxins, results of the study suggest some particularly common benthic cyanobacterial genera that have been shown elsewhere (reviewed by Quiblier et al., 2013) to include toxigenic species. For example, *Nostoc* and *Phormidium* were each recorded in >1/3 of stream sites surveyed, and indicator species analysis suggested that these genera are significantly associated with the types of sites (based on high elevation and high microalgal cover) where microcystin production was greatest in the study's data set (Fig. 6). Because *Nostoc* and mats of *Phormidium/Oscillatoria/Lyngbya* are relatively straightforward genera to identify macroscopically in the field, information on their tendency to produce toxins in streams could eventually help managers determine whether a toxic event might be underway or poised to occur. However, future,

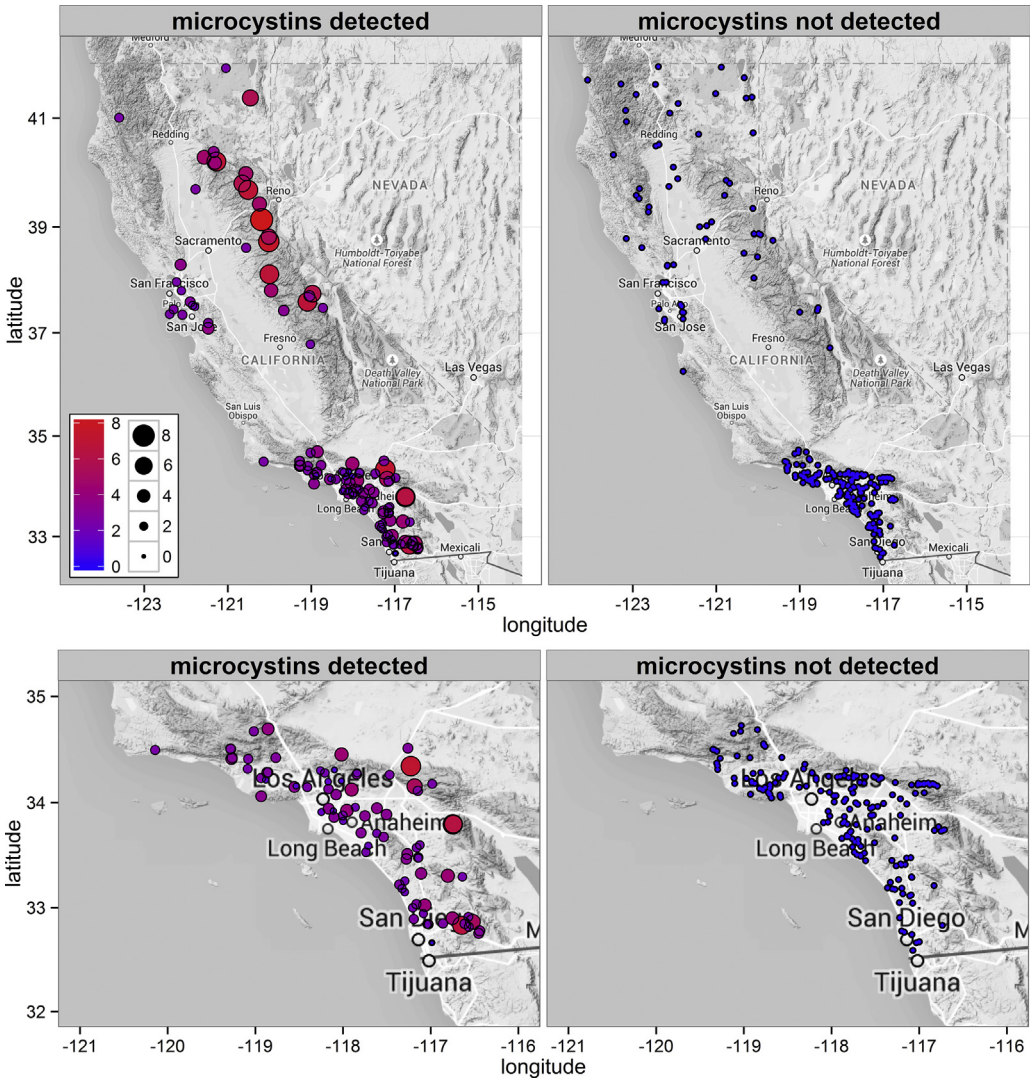

Fig. 3. Benthic algae sampling sites where microcystins were assessed during spring-summer of 2011 to 2013 (N = 368 sites sampled). Top panels correspond to the state as a whole, and bottom panels are zoomed-in on southern California, where the data density is highest. Panels on the left show all sites where microcystins were detected; icon size and shading indicate relative concentrations (μg m^{-2}) on a natural-log (ln) scale, with larger/red corresponding to higher values (see legend). Panels on the right show all sites where microcystins were tested for, but not detected. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

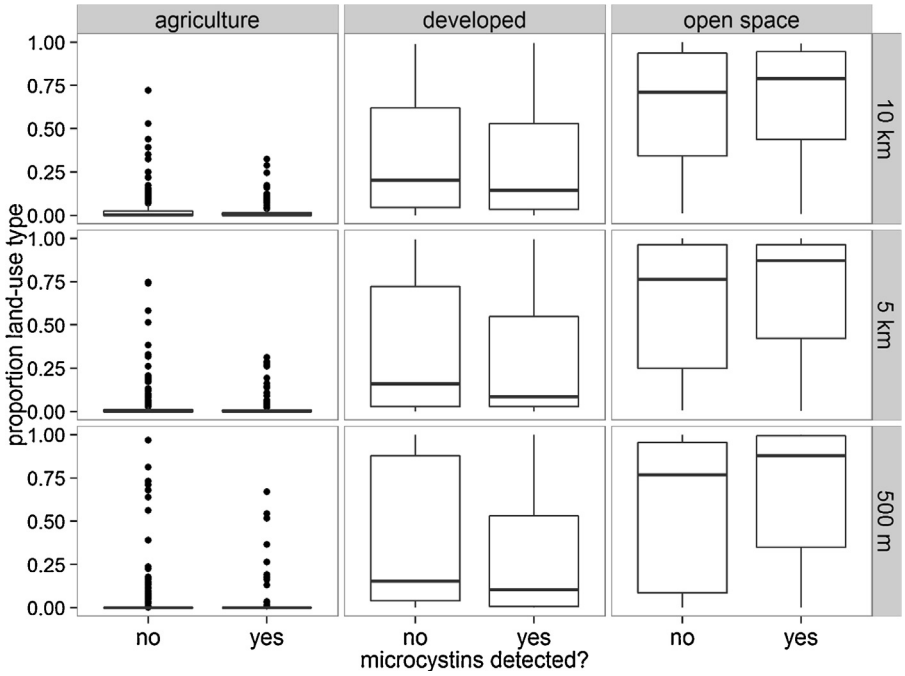

Fig. 4. Relationship between land uses (proportion of agricultural land use, urban development, or undeveloped open space) surrounding the sampling site, and whether or not benthic microcystins were detected at that site. All nine panels show data from the same (full) dataset, but depicted in different ways. Proportions of land-use types are shown at three scales (i.e., within 10 km, 5 km, and 500 m radii of buffer around sampling sites) and add up to 100, for each site, within each scale. Boxplots within each land-use category are stratified by whether or not microcystins were detected at the site in question.

more definitive, steps to identifying toxin producers in California will need to involve isolating likely specimens from the field and analyzing axenic tissues (i.e., those free of other contaminating organisms) individually, rather than in aggregate (as was done with the samples for this study).

4.2. Potential impacts of cyanotoxins within streams

Cyanotoxins could exert a variety of impacts within the local stream environment, in ways that have ramifications for monitoring and management of stream health. For example, they could be the cause of at least some instances of positive results from laboratory toxicity assays conducted as a part of monitoring surveys. Support for this phenomenon comes from studies showing toxic effects of cyanotoxin-containing extracts on invertebrates, such as cladocerans (Sotero-Santos et al., 2006, 2008; Okumura et al., 2007), which are often used as test organisms in water-column toxicity assays. Further support for this possibility comes from the finding, in southern California coastal watersheds, that sublethal toxicity (in terms of depressed reproduction) was more extensive in streams within open-space settings (33%) than those in agricultural (30%) or urban (19%) settings (Mazor, 2015). Because undeveloped catchments are less

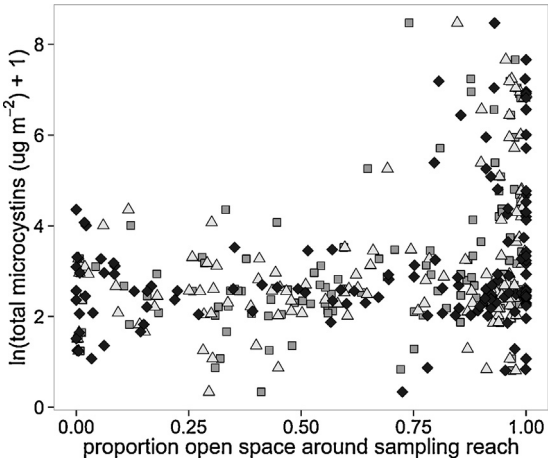

Fig. 5. Relationship between concentration of benthic microcystins and the proportion of land surrounding the sampling site that is open space (i.e., undeveloped). Data are shown for three spatial scales (buffer radii) around sampling sites: 10 km (dark gray squares), 5 km (light gray triangles), and 500 m (black diamonds). Non-detect samples are excluded.

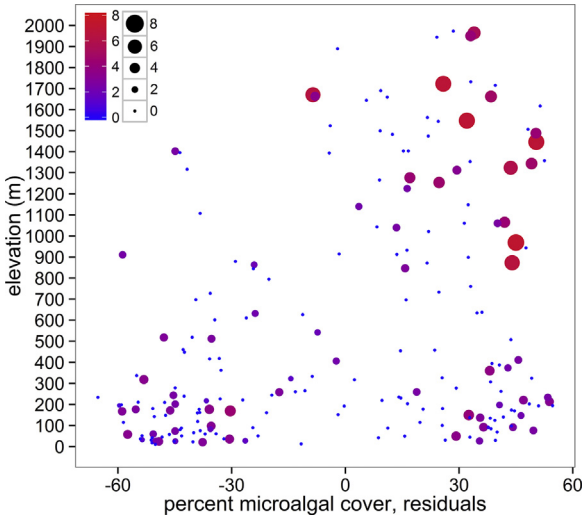

Fig. 6. Microcystin concentration as a function of elevation and the percent cover of microalgae, presented as residuals after the effect of chlorophyll *a* concentration was removed. Icon size and shading indicate relative concentrations (μg m^{-2}) of microcystins, on a natural-log (ln) scale, with larger/red corresponding to higher values (see legend). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)

Table 3
Results of indicator species analysis showing which genera were significantly associated with high-elevation sites (>700 m) supporting high microalgal cover (>70%) at the time of assessment. Indicator values can range from 0 to 100, with higher values corresponding to a stronger association between taxon and class of site. Shown are all genera with significant indicator values >10. Cyanobacterial genera in the list (shown in bold/italics) may be considered strong candidates for producing microcystins in California wadeable streams, based on their tendency to inhabit the high-elevation sites with high microalgal cover at the time of assessment (i.e., the type of site where microcystin concentrations were particularly high in the study data set; Fig. 6). Genera are listed in order of decreasing indicator value.

Genus	Indicator value	*p*
Chamaesiphon	42.3	0.0002
Nostoc	35.5	0.0002
Calothrix	28.9	0.0002
Homoeothrix	28.5	0.0002
Zygnema	26.7	0.0002
Phormidium	26.1	0.0020
Aphanocapsa	22.8	0.0354
"chantransia" stage (Rhodophyta)	22.1	0.0008
Tolypothrix	20.6	0.0002
Aphanothece	19.2	0.0216
Tribonema	17.5	0.0018
Ulothrix	12.6	0.0002
Microspora	11.2	0.0002
Closterium	11.0	0.0058

likely to harbor anthropogenically derived toxins, the likelihood of naturally occurring toxins (such as cyanotoxins) triggering positive bioassay results is worth examining.

Gaining a better understanding of cyanotoxin effects on macroinvertebrate communities in California streams could also prove useful for understanding otherwise unexplainable causes of low biomonitoring index scores, and for determining whether any impacts on these communities are the result of natural phenomena, or whether they are exacerbated by human activities, and potentially responsive to corrective management actions. Aboal et al. (2002) found adverse effects of microcystins on stream benthic macroinvertebrates, and suggested that cyanobacterial biomass and/or pigments be measured to provide a context for interpreting index scores.

Numerous studies have demonstrated accumulation and biomagnification of cyanotoxins in aquatic food webs: specifically, in freshwater and saltwater mussels (Williams et al., 1997; Amorim and Vasconcelos, 1999; Miller et al., 2010), farmed crustaceans (Vasconcelos et al., 2001; Zimba et al., 2006), corals (Richardson et al., 2007), fish (Malbrouk and Kestemont, 2006), and crabs (Miller et al., 2010). Wood et al. (2012a) confirmed that benthic cyanotoxins can enter freshwater food webs by showing that nodularins had been incorporated into crayfish hepatopancreatic tissue after feeding on ^{13}C-labeled cyanobacterial mats in lake-based field experiments. The effects, and propensity for bioaccumulation, of cyanotoxins in stream benthic macroinvertebrates have not been as well studied, and should be a focus of future research (Quiblier et al., 2013).

4.3. Potential for cyanotoxin loading to receiving waters

Coastal watersheds in many parts of California are mountainous, with a sizable proportion of waterways in the form of dense, low-order stream networks. The approximately 280,000 km of streams in California represents a potentially vast base for cyanotoxin production. Moreover, the general chemical stability of some cyanotoxins (Rapala et al., 1993; Jones et al., 1995; Tsuji et al., 1995; Twist and Codd, 1997; Lahti et al., 2001; Rapala et al., 2005) means that toxins produced in streams may not only have undesirable effects locally, but could also be exported, intact, to receiving waters, either in the form of pieces of cyanobacterial growths that detach from the benthos and are transported downstream, or in dissolved form released from lysed cells (Wood et al., 2011).

It will be important to understand the significance of cyanotoxin inputs from wadeable streams to drinking water reservoirs, recreational lakes, and coastal lagoons and estuaries that support wildlife. For example, are cyanotoxin concentrations in receiving waters meaningfully increased by contributions from tributaries? The answer to this question could influence whether it is deemed sufficient to rely on the appearance of in situ planktonic blooms for forecasting the likelihood of an impending toxic event, or whether additional monitoring of benthic cyanobacterial blooms in the contributing watershed are warranted. Future studies in streams should be directed toward determining (1) the typical concentrations (both cell-bound and dissolved) of toxins of benthic origin, (2) toxin fate and transport, (3) whether benthic blooms and toxin production have increased as a result of human activities, (4) the appropriate reference values to protect human and wildlife health, and (5) what concentrations may be of concern for listing purposes and other management actions (e.g., Wood and Williamson, 2012).

4.4. Potential drivers of benthic cyanotoxin production in streams

Cyanotoxins were detected in California streams within both developed and undeveloped landscape settings, and concentrations were overall substantially higher in the latter. Thus it is difficult to ascertain, based on available data, whether any anthropogenic factors may promote toxic events in these systems, in contrast to the mounting evidence that human influences have exacerbated toxigenic planktonic blooms in lentic water bodies (Paerl and Huisman, 2008; Paerl and Paul, 2011; Paerl et al., 2011). It is possible that the patterns observed in the concentration of stream benthic microcystins could more be a function of what species are selected for by specific environments than site-specific drivers boosting cyanotoxin production per se. For example, *Nostoc*, a nitrogen fixer, is a likely candidate for producing microcystins in California streams, and it tends to flourish in oligotrophic, minimally disturbed systems (Stancheva et al., 2013; Fetscher et al., 2014); it may be that *Nostoc* is inherently a more prolific microcystin producer than other benthic cyanobacterial taxa (that inhabit other types of streams). This scenario could help explain the observation of greater microcystin concentrations in largely undeveloped catchments.

It is also worth noting that, if the high microalgal cover in certain sites is a symptom of low scour, the relationship between low scour and high microcystin production (Fig. 6) suggests that some of the key taxa that produce microcystins may not be well adapted to high-flow conditions. Indeed, *Phormidium* is a benthic genus that has been observed to proliferate in lower-flow sites of rivers in New Zealand (Heath et al., 2011), presumably due to its weak physical connection with the streambed; thus reduced flows due to natural climate cycles, climate change, and/or hydromodification could potentially select for toxin-producing cyanobacteria in lotic water bodies. If there is a relationship with flow/scour, it should be explored, because climate change and future water management activities have the potential to alter streamflow patterns.

With respect to establishing a connection between human activities and cyanotoxin production in streams, an important consideration is that the toxin results presented here were based mostly on one-time grab samples during a restricted index period, which could miss toxic events due to the ephemeral and episodic nature of toxin production. This, in conjunction with the inherent patchiness of algae within stream benthos (Sheath et al., 1986), as

well as the patchiness of toxin concentrations even within individual cyanobacterial mats (Wood et al., 2010a,b, 2012b), suggests that the results presented may underestimate the true prevalence of cyanotoxins in California streams. Because of difficulties in drawing concrete conclusions about drivers of toxin production based on the opportunistically collected data available to date, further, more focused, efforts should be made to understand the potential for anthropogenic factors influencing toxin production in streams, as this has implications for what can be expected to be achievable via management actions. Issues that should be explored include: (1) temporal/seasonal variability in toxin production (i.e., when to sample, and whether there is a need for multiple samples per year), (2) the necessary level of sampling effort (i.e., to account for spatial variability and patchiness of toxigenic benthic cyanobacteria), and (3) factors that may trigger or exacerbate toxin production.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.hal.2015.09.002.

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