Benthic cyanobacterial proliferations drive anatoxin production throughout the Klamath River watershed, California, USA

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Abstract: Blooms of toxin-producing cyanobacteria are an enduring public health threat in lakes and rivers. In addition to more commonly studied planktonic taxa in lakes, attached cyanobacteria covering riverbeds and lake littoral zones can produce anatoxins, potent neurotoxins of growing concern. However, relative to planktonic blooms, the geographical and temporal extent and ecology of anatoxin-producing benthic cyanobacteria are poorly documented. To increase understanding of the distribution of these cyanobacteria and their relationships with physicochemical variables, we surveyed sites throughout the Klamath River watershed in Northern California, USA, for anatoxins from benthic mats. We used visual surveys, composite mat samples, water samples, and samples of transported coarse particulate organic matter (CPOM) to quantify benthic cyanobacteria and anatoxin extent. Benthic anatoxins were widespread, adding to a growing body of evidence that anatoxins from benthic cyanobacteria may be more common than previously recognized. Anatoxin concentrations were highest in benthic mats compared with water column and CPOM samples. Anatoxin detection frequency, as indicated by anatoxin synthetase genes, was high in both transported CPOM and benthic mats (74 and 86%, respectively), and transported CPOM anatoxin concentrations reflected benthic mat anatoxin concentrations and cover. Relationships between observed taxa, toxin concentrations, and genetic source tracking indicated that *Microcoleus* was the dominant anatoxin producer. Clear, low-nutrient tributaries supported anatoxin concentrations as high as, and in some cases higher than, mainstem sites that had higher nutrients. Weak, negative relationships among water quality parameters and anatoxins suggest that some aspects of the tributary streams not captured in this analysis promote the proliferation of benthic *Microcoleus* and associated anatoxin production. Monitoring benthic mats, including the use of nets targeting sloughed benthic material, can inform public health notifications and document changes in the proliferation of benthic cyanobacteria and associated cyanotoxin production in rivers.

Key words: anatoxins, benthic cyanobacteria, cyanotoxins, *Microcoleus*, periphyton, large rivers

Blooms of toxin-producing cyanobacteria are an enduring public health threat in lakes and rivers (Testai et al. 2016, Rosero-López et al. 2022). When ingested, cyanobacterial toxins can cause illness and death to humans, livestock, pets, and wildlife (Wood 2016, Chorus and Welker 2021). Our understanding of the drivers and impacts of toxin-producing cyanobacterial blooms has relied largely on research of planktonic blooms in lakes (Carey et al. 2012, Graham et al. 2020, Wood et al. 2020), but cyanobacteria also proliferate as attached forms on river and lake bottoms (Quiblier et al. 2013,

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Fetscher et al. 2015, Wood et al. 2020). Although cyanobacteria are a natural component of algal biofilms, proliferations of cyanobacteria-dominated mats and associated toxins appear to be more widespread than expected (Fetscher et al. 2015, McAllister et al. 2016, Bouma-Gregson et al. 2018, Rosero-López et al. 2022, Bauer et al. 2023). Benthic taxa have different distributions, toxin-production dynamics, and habitat requirements than planktonic cyanobacteria, yet there are far fewer studies of attached cyanobacteria than of their planktonic counterparts (Scott and Marcarelli 2012, Wood et al. 2020). This lack of knowledge inhibits predicting when and where these benthic blooms pose a risk to public health and the extent to which benthic proliferations may be increasing.

Anatoxins (including anatoxin-a, homoanatoxin-a, dihydroanatoxin-a, dihydrohomoanatoxin-a, and other derivatives) are potent neurotoxins commonly associated with freshwater cyanobacterial blooms. Increasingly, dog deaths following visits to rivers and lakes have been attributed to anatoxin exposure (Puschner et al. 2008, Backer et al. 2013, Quiblier et al. 2013, Hobbs et al. 2021). Anatoxins are fast-acting postsynaptic nicotinic agonists that bind to acetylcholine receptors, overstimulating muscles and causing convulsions, paralysis, and death from respiratory failure (Christensen and Khan 2020, Colas et al. 2021). Anatoxins break down quickly in UV light (Kaminski et al. 2013), making ingestion of cyanobacteria cells that contain anatoxins a larger exposure risk compared with ingestion of extracellular toxins. In plankton-dominated waters, strains of *Aphanizomenon* and *Dolichospermum* (formerly classified as *Anabaena*) are common anatoxin producers (Christensen and Khan 2020), but more distantly related benthic taxa also produce anatoxins. Genetic techniques and lab cultures increasingly identify *Phormidium*/*Microcoleus* as the primary benthic anatoxin producers in rivers, highlighting how the basic natural history of anatoxin production from benthic sources differs from planktonic blooms (Wood et al. 2012, 2020, Bouma-Gregson et al. 2019, Kelly et al. 2019, Conklin et al. 2020, Junier et al. 2023).

Drivers of benthic cyanobacterial proliferations likely relate to the specific taxa dominating the community and to their benthic growth forms. High nutrients, warm temperatures, and stagnant water cause planktonic cyanobacterial blooms (Paerl and Huisman 2008, Romo et al. 2013, Huisman et al. 2018), but drivers of benthic proliferations may be less generalizable. In contrast with planktonic cyanobacteria, benthic cyanobacteria may proliferate under a wide range of discharges and flow velocities (Heath et al. 2015, Robichon et al. 2023). Increased nutrient concentrations can promote cyanotoxin cover (Heath et al. 2015, 2016, Wood et al. 2017a), decrease toxin quotas (Heath et al. 2014, 2016), or cause minimal discernible effects (Gaget et al. 2020, Robichon et al. 2023), resulting in equivocal effects of nutrients on anatoxin from benthic sources.

The state of the knowledge surrounding benthic cyanobacteria occurrence, toxin-production dynamics, and ecology is not sufficiently advanced to address the public health concerns stemming from benthic cyanotoxins, in part because of difficulties associated with documenting benthic cyanobacteria. High spatial and temporal heterogeneity in the distribution of algae on stream beds makes quantifying benthic cyanobacterial proliferations and toxin dynamics difficult (Ledger et al. 2008). Even within patches that appear homogeneous in the field, toxin-production within small, adjacent patches can vary considerably because of the co-occurrence of toxic and nontoxic genotypes (Wood et al. 2010, 2012). Collecting composite samples from many mat patches represents toxin risk better than single point samples (Wood et al. 2020), but representative composite samples are often impractical to collect in deep and swift streams. Grab samples from the water column, although more homogeneous than benthic mat samples, may not be very useful for quantifying toxins from benthic taxa because these water column samples underrepresent the biomass of benthic algae (and, thus, intracellular toxins) by orders of magnitude (Tekwani et al. 2013) and because of the rapid degradation of extracellular anatoxins from UV light (Kaminski et al. 2013).

A potential solution that takes advantage of the homogeneity of water column sampling while targeting benthic mats is to sample transported sloughed algal material in large volumes of water as it flows downstream. This approach allows for integration of mats from many habitats and may represent reach-scale toxins from benthic cyanobacteria. Clumps of sloughed cyanobacteria can stay in suspension, presenting an exposure risk to downstream swimmers by increasing the likelihood that mat material with high intracellular cyanotoxin concentrations will be ingested (Bouma-Gregson et al. 2017, Wood 2017, Chorus et al. 2021). Using nets to capture coarse particulate organic material (CPOM) enables estimating the flux of coarse organic C in rivers (Perry and Perry 1991, Minshall et al. 1992, Tockner et al. 1999, Ng 2012), but these nets have not previously been used to quantify cyanotoxins (but see Carpenter and Wise 2023).

The Klamath River in Northern California, USA, is a nonwadeable, eutrophic river that can carry cyanobacteria toxins (Jacoby and Kann 2007, Genzoli and Kann 2016). Microcystin, a commonly documented cyanotoxin in the Klamath River, is sourced from planktonic blooms in upstream reservoirs (Otten et al. 2015, Howard et al. 2023), but the source of anatoxins is unknown. As in many rivers and lakes, water column grab samples have been used to monitor cyanotoxins in the Klamath River to align with water quality thresholds and established protocols for large rivers (USEPA 2019, Graham et al. 2020). However, observations of benthic, mat-forming cyanobacteria (LG, personal observation) provide evidence that benthic taxa may be primarily responsible for anatoxin production. In this study, we aimed to better understand the extent, sources, and drivers of anatoxin production throughout the Klamath River

watershed. Specifically, we asked: 1) How does anatoxin vary across environmental media (i.e., water, benthic mats, CPOM), geographically across the watershed, and through time? 2) What taxa are responsible for anatoxin production? and 3) What are the potential drivers of variation in anatoxin production across the Klamath River watershed? We expected that 1) anatoxin would be concentrated in benthic mats and CPOM throughout the summer, 2) that benthic taxa would be responsible for anatoxin production, and 3) that anatoxins and benthic cyanobacterial mats would be more prolific when nutrients and water temperature were high and water clarity was low.

METHODS

To address our research questions, we surveyed benthic cyanobacteria at 15 sites in the Klamath River and adjacent tributaries during summer 2021. Over 3 mo, we sampled cyanobacterial mats and transported CPOM and collected water column samples to compare anatoxin concentrations in different environmental media over the course of the summer. We used microscopy, real-time quantitative polymerase chain reaction (qPCR), and amplicon sequencing to identify anatoxin-producing cyanobacteria throughout the watershed. Finally, we used correlation analysis and regularization methods to explore associations between anatoxin concentrations and water quality variables across sites.

Study site

The Klamath River is 423 km long with a 40,600-km^2 watershed that spans South-Central Oregon and Northwestern California (Fig. 1). The upper watershed supports heavy livestock and row crop agriculture, resulting in water withdrawals for irrigation and increased nutrient runoff above already high background levels (Snyder and Morace 1997, Walker and Kann 2022). The nutrient-rich waters pass through a series of hydroelectric dams and reservoirs that increase water residence time and harbor planktonic blooms of *Microcystis* (Genzoli and Kann 2016, Oliver et al.

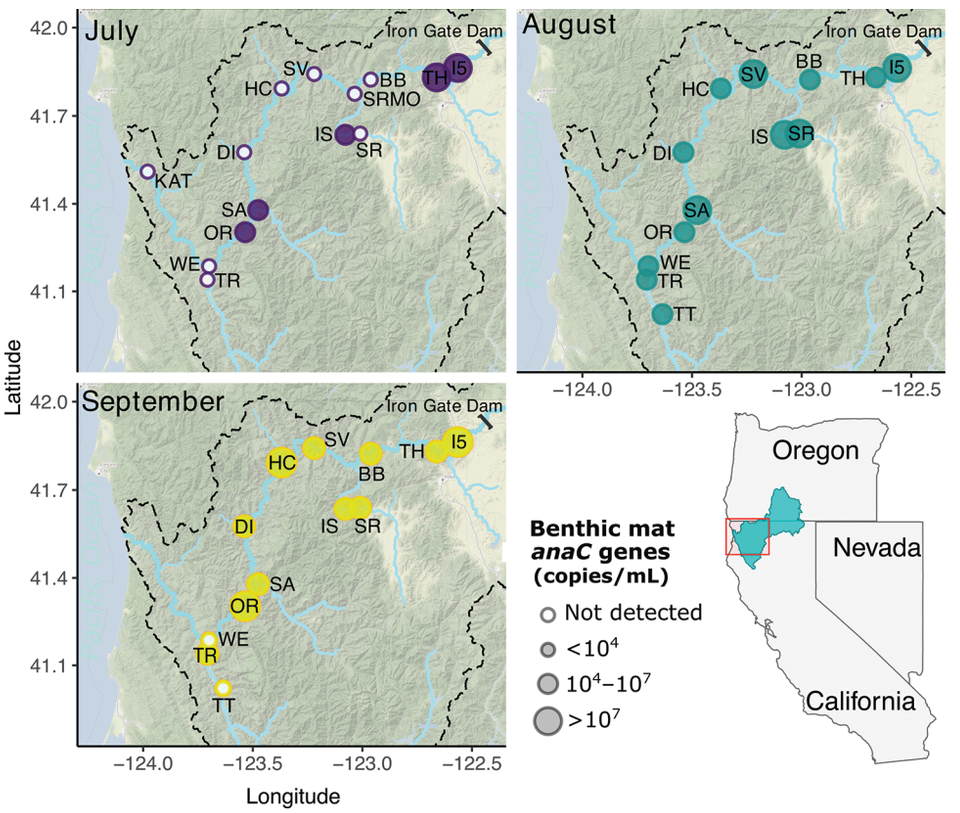

Figure 1. Sampling sites in the lower Klamath River watershed in Northern California, USA. Inset map shows the location of the Klamath River watershed in blue, with the red rectangle delineating the lower watershed shown in map panels above and to the left. Circles show locations where benthic cyanobacterial mats were sampled each month. Open (white) circles indicate no conspicuous cyanobacterial mats were found or that observed mats were below the detection limit for *anaC* genes. Point sizes indicate *anaC* gene concentrations (copies/mL). Colors for each month correspond with Fig. 4. Site codes represent the following sites: BB = Brown Bear, DI = Dillon Creek, HC = Happy Camp, I5 = Interstate 5 Bridge, IS = Indian Scotty, KAT = Klamath at Terwer, OR = Orleans, SA = Salmon River, SR = Scott River, SRMO = Scott River Mouth, SV = Seiad Valley, TH = Tree of Heaven, TR = Trinity River, TT = Trinity at Tish Tang, WE = Weitchpec.

2016) that spill into the Klamath River from an epilimnetic reservoir release, prompting public health warnings for microcystin toxin that extend downstream for 306 km to the Pacific Ocean (Otten et al. 2015, Howard et al. 2023). Occasional detections of anatoxins from river and reservoir grab samples have shown that anatoxins are present but rare in the Klamath River water column (Otten 2017, Genzoli and Kann 2020). Because microcystin in the river derives from the upstream reservoirs (Otten et al. 2015, Howard et al. 2023), it was assumed that anatoxins were also sourced from upstream reservoirs until more recent examination (Otten 2017).

Below the hydroelectric reservoirs, the Klamath River flows freely south and west through semiconfined canyons, with vegetation transitioning from oak savanna to dry pine forest, mixed conifers, and coastal redwoods. Wet winters and dry summers, along with mountain snowmelt, cause high winter and spring flows and stable, low summer baseflows. Flow magnitude increases downstream of Iron Gate Dam (Fig. 1) because of tributary inputs, whereas nutrient concentrations decrease because of a combination of dilution by low-nutrient tributaries and biological uptake (Asarian et al. 2009). Most primary production and respiration in the Klamath River and its tributaries occur in the benthic zone (Genzoli and Hall 2016), where the combination of relatively high water clarity and short water residence time promotes growth of attached algae and aquatic plants. Steep river slopes and confined river channels create swift and deep conditions where only portions of the mainstem river are wadeable.

Field methods

During summer 2021, we surveyed 15 sites in the Klamath River watershed below Iron Gate Dam, including sites in 4 tributaries: the Scott, Salmon, and Trinity rivers and Dillon Creek. We chose sites on the mainstem that were evenly spaced, to the extent possible, while prioritizing public access sites where the Karuk, Yurok, Hoopa, and Quartz Valley tribes monitor water quality. We sampled ≤13 of the 15 sites monthly in July, August, and September 2021 (Table S1).

At all sites, we conducted semiquantitative area searches of cyanobacterial mat cover in wadeable river margins and collected benthic mat samples. We performed visual surveys of conspicuous cyanobacterial mats by first walking along the shore so as not to disturb benthic mats, then by wading upstream in shallow river margins for ~15 min at each site. Conspicuous mats were those that we macroscopically identified as cyanobacteria-dominated benthic mats in the field (Fig. 2A–D). We estimated the cover of conspicuous mats on a scale of 0 to 5, where 0 indicated no conspicuous mats, 1 indicated few isolated patches totaling <0.1 m^2, 2 indicated cover between 0.1 and 0.4 m^2, 3 indicated 0.4 to 1 m^2, 4 indicated 1 m^2 to 20% total cover, and 5 indicated >20% cover in the search area. At each site, we collected cyanobacterial mat subsamples by hand from throughout the survey area and composited them in a 60-mL amber glass jar. We filled jars 40% with mat material and 40% with water and left 20% head space such that mat slurry samples from each site contained ½ water and ½ cyanobacterial mats.

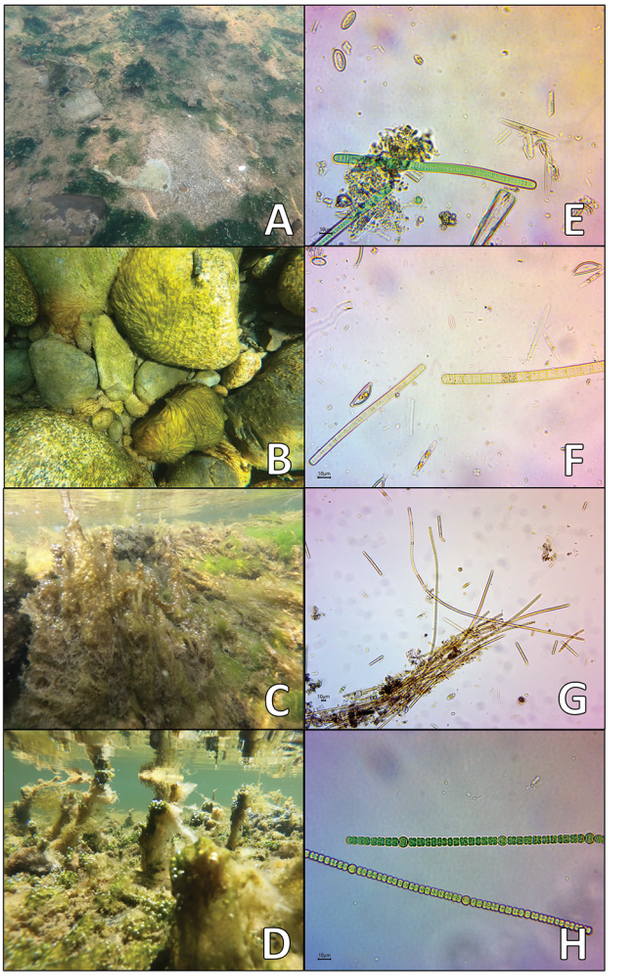

Figure 2. Field images of conspicuous cyanobacterial mats (A–D) and paired microscopic images (E–H) typical in the Klamath River and tributaries in Northern California, USA in summer 2021. *Microcoleus*-dominated cyanobacterial mats had diverse field appearances (A–C), whereas *Anabaena*-dominated mats had more consistent field appearances (D). Samples were collected from the following sites and dates: A and E from Tree of Heaven on 10 September 2021, B and F from Trinity River on 4 August 2021, C and G from Trinity at Tish Tang on 3 August 2021, and D and H from Orleans on 4 July 2021.

At a subset of 8 to 10 sites/mo, we collected additional anatoxin samples from both the water column and CPOM. Using 250-mL amber glass jars rinsed 3× in river water, we collected water column grab samples from the thalweg at locations >1 m deep by submersing a jar 0.5 m and bringing the jar upward to collect an integrated sample to the

water surface. We sampled CPOM by setting a drift net with a 0.30 × 0.45-m opening and 363-μm Nitex mesh in the current, securing the net with stakes so that it was submerged with the top at the water surface. We measured velocity at 3 points in front of the net using a FlowTracker Handheld acoustic doppler velocimeter (ADV®; SonTek/YSI, San Diego, California) and recorded net deployment time. We repeated net deployments 3× at each site, moving the net to a new location each time and allowing the dolphin bucket to fill with enough CPOM to partition for both anatoxin and CPOM analysis (total time of 3 deployments ranged from 11–89 min). We composited the net-captured CPOM from the 3 deployments into a single jar. All samples were immediately moved into a dark cooler on ice in the field.

Lab methods

CPOM processing We processed transported CPOM samples for analysis of ash-free dry mass (AFDM) and anatoxin indicators by homogenizing samples with scissors or by light blending with an immersion blender. We thoroughly rinsed all equipment with clean tap water between samples. We partitioned 40 mL of CPOM slurry for anatoxins and genetic analysis and processed ≥10% of the remaining volume of the sample for AFDM by drying then combusting the sample slurry at 500°C for 4 h. Samples were analyzed for indicators of anatoxins and taxonomy at Bend Genetics Laboratory in Sacramento, California, as described below.

Microscopy For all samples, we identified the 3 most abundant cyanobacteria taxa in each sample (water column, benthic mat, and transported CPOM) through qualitative microscopy conducted at 400× magnification. We identified the dominant and subdominant taxa and up to 1 additional taxon, if present, in the sample and photographed dominant and subdominant taxa for later reference. Because of difficulty in distinguishing between *Phormidium* and *Microcoleus*, along with work in nearby rivers that argue *Phormidium* observed in the Eel and Russian rivers (nearby Northern California rivers) should be classified as *Microcoleus*, we refer to all the strains of *Phormidium/Microcoleus* as *Microcoleus* (Bouma-Gregson et al. 2019, Conklin et al. 2020).

Anatoxins by enzyme-linked immunosorbent assay For transported CPOM samples and composite mat samples (except for July mat samples when funding was limited), we processed samples for total anatoxins by enzyme-linked immunosorbent assay (ELISA). Prior to ELISA analysis, we subjected all samples to 3 cycles of freezing–thawing to lyse the cells. We syringe filtered (0.45-μm glass fiber filter) the samples, then analyzed them for total anatoxins with a commercial kit (ABRAXIS® Anatoxin-a ELISA Plate Kit PN520060; Gold Standard Diagnostics, Davis, California). Any samples exceeding the highest concentration standard (5 μg/L) were diluted and reanalyzed until the value was within the standard curve. We used 4-parameter logistic regression interpolation to estimate toxin concentrations by relating absorbance from each environmental sample to the standard curve.

DNA extraction For DNA analysis, water samples were first concentrated by vacuum filtration onto 25-mm GF/C filters (1.0-μm pore size). We subsampled composite mat and transported CPOM samples by adding 1 mL of slurry to a microcentrifuge tube and centrifuging for 1 min at 12,000 *g* to pellet the material. We then decanted the supernatant and extracted the pellet in the same manner as the GF/C filters. We used a DNeasy® Powerlyzer® PowerSoil® DNA extraction kit (Qiagen®, Hilden, Germany) to extract total genomic DNA from filters or pellets per the manufacturer's instructions. The extracted DNA was stored frozen at −20°C until further analysis (a few days for real-time qPCR and ~6 mo for amplicon sequencing).

Real-time qPCR We used qPCR to quantify potential anatoxin-producing cyanobacteria in all samples. A conserved region of the anatoxin-producing cyanobacteria (*anaC*) gene (present for production of all anatoxin congeners) was targeted using the following oligonucleotides, which are being reported on for the 1st time in this study: *anaC-F* (5′-TCC CAATARCCTGTCATCAA-3′), *anaC-R* (5′-ATGAATTGG TAGYTAARGGTGARGT-3′), and *anaC-Probe* (5′-[Cy5]-CGCACRCAAAGCTCACCCA-[BHQ2]-3′). Each qPCR reaction consisted of 10 μL of 2× Luna® Universal Probe (New England BioLabs, Ipswich, Massachusetts), 2 μL of DNA template, 500 nM each of forward and reverse primers, 200 nM fluorescent probe, and 6 μL of sterile, nuclease-free water. We analyzed all samples with a CFX Opus Real-Time PCR system (Bio-Rad Laboratories, Hercules, California) with the following cycling conditions: initial denaturation at 95°C for 3 min, followed by 40 cycles at 95°C for 20 s and 60°C for 40 s. Each batch of qPCR reactions included serial dilutions of positive control DNA (gBlocks™ Gene Fragments; Integrated DNA Technologies, Coralville, Iowa) ranging from 10^7 to 100 gene copies/reaction. We interpolated the absolute concentration of target DNA in each sample from the standard curves.

Amplicon sequencing For amplicon sequencing, we selected 62 samples that had *anaC* gene copies >1000/mL, and we included 3 controls derived from cultures of *Anabaena* (strain WA102), *Microcoleus* (strain PTSR1), and *Microcystis* (strain UTEX2385). The *Anabaena* and *Microcoleus* controls were positive controls, whereas the *Microcystis* was included as a nontoxic control. We constructed 2 different sequencing libraries that targeted total cyanobacteria (16S rRNA) and total *anaC*. We amplified the DNA from each sample with PCR (22 cycles) in duplicate reactions consisting of: 10 μL 5-Prime 2.5× Taq Master Mix (Qiagen), 100 nM of the 16S or *anaC* forward and reverse

primers, 1 μL DNA/reaction, and sterile nuclease-free water to a final reaction volume of 25 μL. We used cyanobacteria-specific primers (359F and 784R, Nübel et al. 1997; *anaC-gen-F* and *anaC-gen-R*, Rantala-Ylinen et al. 2011; Table S2). The Cy16S primers span 425 bp, and the *anaC* primers span 366 bp. Both primers sets were amended to contain Illumina adapter sequences (colored blue; Illumina®, San Diego, California) to allow for sample indexing and multiplex sequencing.

After the 1st round of PCR, we pooled amplicons from each set of duplicate PCR reactions together and used KAPA Pure Beads (Roche, Basel, Switzerland) to purify them. We then subjected the purified amplicons to an additional 8 cycles of PCR to ligate Illumina index primers onto the amplicons. We purified the PCR products a 2nd time as before. We used a Nextera XT library prep kit (v2; sets A and B; Illumina) to prepare each sample with 1 ng of the purified amplicons, then we used a Sequal Prep™ Plate (Thermo Fisher Scientific, Waltham, Massachusetts) to normalize the purified amplicons to 25 nM for each sample, then used a single flow cell of an Illumina MiSEQ sequencer (2 × 300 bp) to pool and sequence samples.

Bioinformatics We used the QIIME 2™ suite (Bolyen et al. 2019) to analyze the 16S rDNA sequences separately from the *anaC* sequences. Only the forward (R1) reads (300 bp) were analyzed because of their overall higher quality. We used Cutadapt (version 3.5; Martin 2011) to remove primer sequences from the raw reads and then processed them with only Phred scores >25 retained as a quality assurance procedure. We used DADA2 (version 2021.4.0; Callahan et al. 2016) to denoise the data and to remove chimeric sequences. We used the Silva v138 reference database (https://www.arb-silva.de/documentation/release-138/) to assign taxonomy to the 16S rDNA sequences and related the *anaC* sequences to a custom curated database consisting of anatoxin-producing representative sequences corresponding to *Anabaena*/*Dolichospermum*, *Cuspidothrix*, *Cylindrospermum*, *Phormidium*/*Microcoleus*, and *Oscillatoria* (Appendix S1). After quality control and assurance, the 16S sequencing effort generated a mean of 56,849 reads/sample (median = 55,835, minimum = 23, maximum = 181,293), and the *anaC* sequencing effort produced a mean of 101,955 reads/sample (median = 119,069, minimum = 116, maximum = 222,384). Rarefaction curves for both gene targets indicated that a sequencing depth of 10,000 reads was adequate to resolve >95% of taxa (amplicon sequence variants). We deposited all sequencing reads into the National Center for Biotechnology Information Short Read Archive under submission SUB14002607.

Water quality and discharge data

We used data on physical, chemical, and biological water quality parameters from tribal water quality departments. These tribal water quality departments maintain YSI multiparameter sondes (Yellow Springs Instruments, Yellow Springs, Ohio) and collect water quality samples twice each month across the basin. Where available, we used water temperature data from these probes. In the Scott River, we used water temperature data from the Quartz Valley Indian Tribe, at Seiad Valley, Orleans, and Salmon rivers, we used data from the Karuk Tribe, and at Weitchpec, Trinity, and Klamath at Terwer, we used data from the Yurok Tribe. At 4 sites without sondes (Interstate 5-Bridge, Tree of Heaven, Brown Bear, Happy Camp), we deployed miniDOT® loggers (Precision Measurement Engineering, Vista, California) to record water temperature. We obtained alkalinity, nutrient, water clarity indicators, and algal pigment data (total N, $NO_3^- + NO_2^-$, NH_4^+, total P, soluble reactive P [SRP], dissolved organic C [DOC], turbidity, total suspended solids, phaeophytin *a*, and chlorophyll *a*) from Karuk and Yurok water quality programs, and we paired survey sites with grab sample data collected adjacent to the sites.

We assigned daily mean discharge to each sample location and date with data from nearby United States Geological Survey gauges on the Klamath River and major tributaries (Table S1). For tributary sites, we used direct measurements from gauges upstream from the sites that did not have substantial gains between the gauges and sample sites. For Klamath River sites not adjacent to gauged discharge locations, we used a flow model that assigned gains between gauges to tributaries based on their proportional watershed area (Perry et al. 2019). We then estimated daily mean discharge at each site based on upstream gauges plus additional contributions of ungauged tributaries between gauging locations and survey sites.

Analyses

Variation in anatoxin across environmental media, space, and time We calculated concentrations and loads of total anatoxins and organic material in transported CPOM. We scaled CPOM AFDM to water column concentration as mg AFDM/L by dividing the total AFDM of the field sample by the volume of water that passed through the net, which we calculated from the net opening size, time of deployments, and mean water velocity in front of the net. We similarly scaled CPOM anatoxin concentrations as ng/L from the transported CPOM samples by dividing the sample concentration by the volume of water that passed through the net during deployments. We also calculated anatoxin concentrations in the transported CPOM as ng anatoxins/g AFDM as an indicator of the relative abundance of anatoxin producers in the photoautotrophic assemblage. Finally, we calculated instantaneous anatoxin load as water column anatoxin concentration × discharge.

We compared total anatoxin concentrations in attached cyanobacterial mat samples, transported mat material, and water samples to examine the extent that different sampling

mediums reflected reach-scale conditions in the benthos. For sample types where we measured both *anaC* gene copies and anatoxin concentrations, we related *anaC* gene copies to anatoxin concentrations with major axis regression using package *lmodel2* (version 1.7-3; Legendre 2018) in R (version 2023.12.1+402; R Project for Statistical Computing, Vienna Austria) on the unscaled samples with positive detections of *anaC* gene copies and anatoxins. We tested for an effect of sample type by including sample type as a grouping variable in the model. We compared environmentally scaled concentrations of *anaC* gene copies among benthic mats, transported CPOM, and water to inform exposure risk to different mediums. We used linear regression to relate benthic mat toxins and extent of benthic mat cover to toxins in transported CPOM to assess the degree to which sampling isolated, accessible locations reflected reach-scale anatoxins. We compared Akaike information criterion scores from simple and multiple linear regression models of transported CPOM *anaC* gene copies as predicted by 1) benthic mat *anaC* gene copies, 2) benthic mat cover score (0–5), and 3) benthic mat *anaC* gene copies + benthic mat cover score (0–5). Anatoxins and *anaC* genes were log transformed in all analyses to increase symmetry of the residuals.

We graphically assessed patterns of anatoxins through space and time by using net-captured transported CPOM samples to compare anatoxins in tributary and mainstem sites longitudinally and by month. We considered patterns in anatoxin concentrations in the water, within the transported CPOM, anatoxin load, and transported CPOM as AFDM.

Identification of anatoxin-producing taxa To identify benthic cyanobacteria taxa responsible for anatoxin production, we compared the ELISA results with microscopy assessments of the dominant and subdominant taxa present in each sample, in conjunction with estimates of *anaC* gene abundance and taxonomic assessments of cyanobacterial 16S and *anaC* genes. We graphically compared dominant taxa, grouped to family, in each of the benthic mat samples and transported CPOM samples with *anaC* gene copies to explore patterns between dominant taxa and anatoxins. To examine *Microcoleus* specifically, we graphically compared qualitative microscopy results of *Microcoleus* dominance (dominant, subdominant, present, not detected) with *anaC* gene copies.

Relationships between anatoxins and water quality We explored relationships between anatoxins and physical, chemical, and biological water quality parameters to identify potential covariates with benthic anatoxin concentrations. We used the *zoo* package (version 4.2.2; Zeileis and Grothendieck 2005) in R to interpolate concentrations of nutrients and other water quality constituents on the day of toxin sampling based on linear interpolations of twice monthly water quality samples. We calculated Pearson's correlation coefficients between log anatoxin concentrations in transported CPOM and water quality parameters. For parameters that were concentrations and ratios, we performed correlation analysis on log-transformed data. For the 2 temperature metrics (7-d rolling mean of daily minimum and daily maximum water temperature), we performed correlation analysis on untransformed data.

We used regularization methods to examine multivariate covariates with anatoxin concentrations. We used the sum of single effects model (Wang et al. 2020), which is useful when the number of potential parameters is high relative to the number of measurements and when predictors covary. We included the 14 variables (total N, NO_3^- + NO_2^-, NH_4^+, total P, SRP, N:P (molar ratio), DOC, turbidity, total suspended solids, maximum daily water temperature, mean daily water temperature, phaeophytin *a*, chlorophyll *a*, and alkalinity) from the water quality dataset. We also included location (i.e., tributary or mainstem) as a dummy variable (0, 1) to test the influence of site type. We centered and standardized the log of all data in the model except for temperature and location variables, which were not logged. We used the *susieR* package (version 0.12.35; Wang et al. 2020) in R, which uses iterative Bayesian stepwise selection to fit the model.

RESULTS

Anatoxin indicators across environmental mediums, sites, and time

Variation between anatoxin concentrations and anaC genes Anatoxin concentrations positively covaried with *anaC* gene copies in benthic mats and transported CPOM samples (Fig. 3). The fitted major axis regression model of anatoxins by *anaC* gene copies, grouped by sample type (benthic mat or transported CPOM), resulted in a shared-slope, variable-intercept model (shared slope = 1.22 [1.04, 1.42]). In this model, transported CPOM samples are predicted to have higher anatoxin concentrations at a given concentration of *anaC* gene copies than benthic mat samples (intercept = −7.0 [−8.3, −5.7] and −6.0 [−7.2, −4.8] for benthic mat and transported CPOM, respectively; $p < 0.0001$; Fig. 3). The r^2-value was higher for benthic mat samples (0.80) than for transported CPOM samples (0.72).

Anatoxin variation among sample media Anatoxin concentrations varied strongly by the medium sampled when anatoxins in these media were scaled to the mean concentration in the river. Field-identified attached cyanobacterial benthic mats contained the highest indicators of anatoxins, followed by water grab samples and transported CPOM (Fig. 4A). The mean of *anaC* gene copies in benthic mats was 8.7×10^7 copies/mL compared with a mean of 950 copies/mL in water samples and 0.01 copies/mL in CPOM. Although unscaled benthic mat and transported CPOM

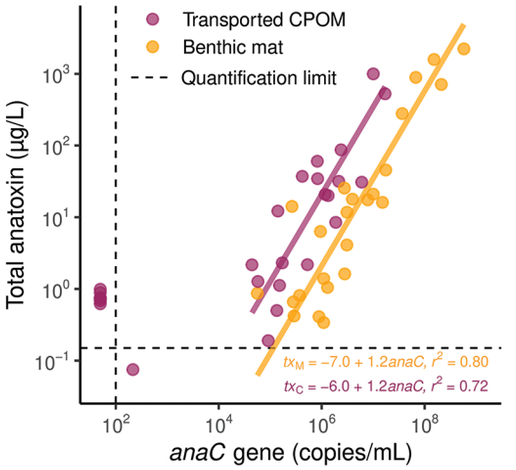

Figure 3. Total anatoxins positively related to *anaC* gene copies in benthic mats (orange points) and transported coarse particulate organic material (CPOM; pink points) in the Klamath River and tributaries in Northern California, USA, in summer 2021. Regression lines show the major axis regression of positive detections of *anaC* genes and total anatoxins by enzyme-linked immunosorbent assay (ELISA) for benthic mats (tx_M) and CPOM (tx_C). Samples below the laboratory quantification limit for either variable (points to the left and below dashed lines) are not included in the regression. Sample concentrations are raw samples (i.e., not scaled to environment concentrations).

samples had similar anatoxin concentrations (Fig. 3), the large volume of water passing through the nets relative to the mass of CPOM captured resulted in low reach-scale anatoxin concentrations within transported CPOM. Within sample media, *anaC* gene copies varied by month, but this temporal variation was smaller than the variation among sample mediums (Fig. 4A).

Seasonal variation in anatoxins Anatoxin concentrations were lower in July than in August and September. Median anatoxin concentration in transported CPOM samples were 10× lower in July (median = 0.006 ng/L) than in August and September (medians = 0.09 and 0.12 ng/L, respectively; Fig. 4A). Benthic mat *anaC* gene copies were higher in August than in September (median = 2.0×10^7 and 6.2×10^6 *anaC* copies/mL, respectively), but both of the late summer samples were substantially higher than the July benthic mat samples (median = 1.2×10^6 *anaC* copies/mL). Similar to mat samples, water column samples had lower anatoxin concentrations in July, peaked in August, and declined in September (median = 50, 1300, and 120 *anaC* copies/mL, respectively; Fig. 4A).

Detection frequencies of toxin indicators among sample media

Despite strong variation in environmental concentrations among sample materials, the detection frequency of

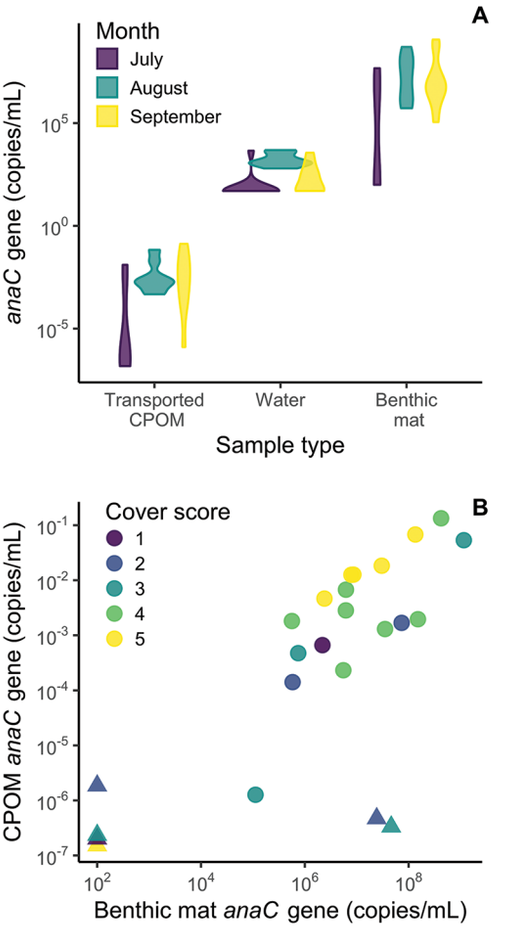

Figure 4. *anaC* gene copies, a proxy for anatoxin concentrations, were highest in benthic mats, followed by water column samples (water), and were lowest in transported coarse particulate organic material (CPOM) when scaled to mean concentration in river water in the lower Klamath River watershed in Northern California, USA. Anatoxins varied from month to month but less than the variation in environmental medium sampled (A). Despite differences in magnitude, transported CPOM *anaC* gene copies reflected concentrations in benthic mats and mat cover at survey locations, shown as a semiquantitative cover score, with 1 being the lowest cover and 5 the highest (B). Triangles in panel B indicate when *anaC* gene copies in transported CPOM samples were below detection limits.

toxin indicators was high in all sample media. We detected *anaC* genes in 15 of 31 water samples (48%), 20 of 27 transported CPOM samples (74%), and 31 of 36 benthic mat samples (86%). For the 26 benthic mat samples and 27 transported CPOM samples where all samples were processed for anatoxins by ELISA, anatoxins were detected in all the benthic mat samples and 26 of 27 transported CPOM samples (Table S3).

Not only were anatoxin detection frequencies similar between transported CPOM and benthic mat samples, but anatoxin concentrations in transported CPOM reflected benthic

mat sample concentrations, with the extent of mat cover further improving the relationship between benthic and CPOM anatoxin relationships (Fig. 4B). Variation in anatoxins in mats (A_{mat}) explained variation in transported CPOM (A_{cpom}), with Akaike information criterion scores reduced slightly (2.8 points) when the semiquantitative mat cover score (M) was added to the model (final model: $\log(A_{cpom}) = -20.5 + 0.6\log(A_{mat}) + 1.1M$, $R^2 = 0.53$, $p < 0.0001$).

Spatial and temporal anatoxin variation Anatoxin concentrations varied among sites and throughout the summer. Transported CPOM, representing average conditions at a site (as opposed to the benthic mat samples that were more patchily distributed), showed that tributaries and sites lower on the mainstem of the Klamath River had generally higher anatoxin concentrations than upriver sites (Fig. 5B, C). In contrast, CPOM AFDM concentrations were generally lower in tributaries and sites lower on the mainstem (Fig. 5A). Despite opposing patterns of anatoxins and CPOM AFDM, anatoxin:CPOM ratios more closely reflected patterns in anatoxin concentrations because CPOM varied less (range = 0.0022–0.31 mg/L) than anatoxin concentrations (range = 0.00044–6.7 ng/L) among sites. Patterns in anatoxin load in transported CPOM were similar to the patterns in anatoxin concentrations in the mainstem. Although CPOM-associated anatoxin concentrations in the tributaries were among the highest sampled, CPOM anatoxin loads were similar to mainstem sites because of lower summer discharge in the tributaries (Fig. 5D).

Timing of peak anatoxins depended on location. At downriver mainstem sites, anatoxin concentrations and loads peaked in September, whereas at tributary sites, August samples had higher anatoxin concentrations (Fig. 5B, C). At upriver mainstem sites, variation in time was less pronounced because of lower anatoxin concentrations and loads at these sites.

Despite similar levels of CPOM-associated anatoxins at tributary sites and some mainstem sites, samples with notably high anatoxins came from tributary sites. Of the 6 benthic mat samples with the highest anatoxin concentrations and *anaC* gene copies, 2 were in the mainstem and 4 were in tributaries, including the Salmon, Trinity, and Scott rivers.

Cyanobacteria taxa and anatoxin production

Microcoleus was the most common cyanobacteria taxa identified in our study, occurring in 26 of the 34 mat samples and dominating in 13 of those samples. *Anabaena/Dolichospermum* was the 2nd-most dominant taxa, followed by *Microcystis*, which commonly occurred in the transported CPOM samples (Fig. 6A). Less common taxa included *Oscillatoria*, *Cylindrospermum*, *Nostoc*, *Geitlerinema*, and *Tolypothrix*.

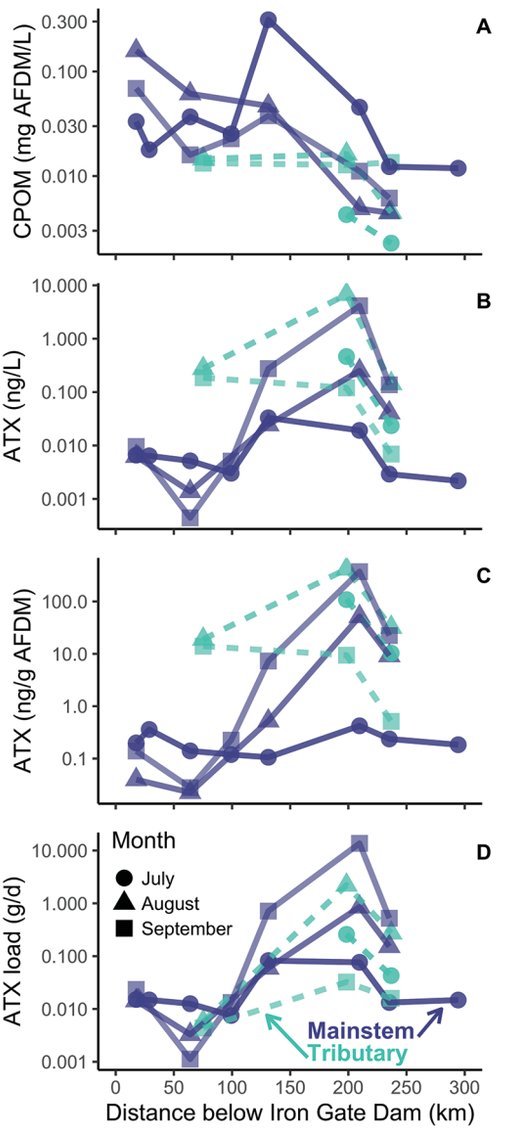

Figure 5. Longitudinal trends from transported coarse particulate organic matter (CPOM) as ash-free dry mass (AFDM) (A), anatoxins (ATX) associated with CPOM (scaled to water column concentrations) (B), anatoxins associated with CPOM as a ratio of CPOM AFDM (C), and daily anatoxin load (D) in the lower Klamath River watershed in Northern California, USA. Mainstem Klamath sites (purple, solid lines) are shown from upriver to downriver, and tributaries (blue, dashed lines) are plotted at the location of their confluence with the Klamath River. Shape indicates sample month.

The dominance of *Microcoleus* was associated with higher anatoxin concentrations. Highest *anaC* gene copies and anatoxin concentrations were found in samples where Oscillatoriales taxa, which include *Microcoleus*, were identified as the dominant cyanobacteria, followed by *Geitlerinema* and Nostocales taxa, which includes *Anabaena* (Fig. 6B). The amount of *Microcoleus* in a sample (if it was dominant,

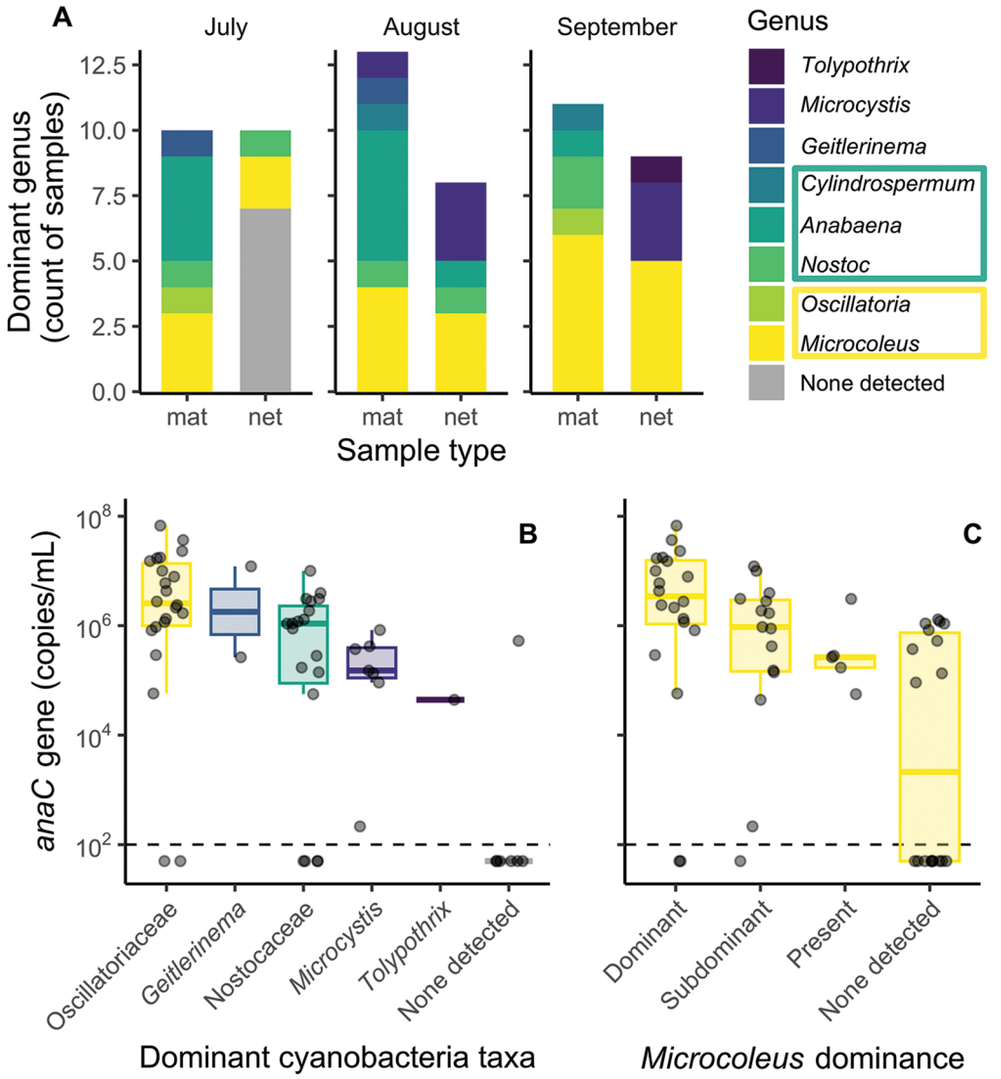

Figure 6. Benthic cyanobacteria taxa present in the lower Klamath River watershed in Northern California, USA. *Microcoleus* was most often the dominant genus across samples for both coarse particulate organic matter (CPOM) and mat samples throughout the summer as identified by microscopy (A). Cyanobacteria were found in all CPOM and mat samples except in July, shown in gray. Boxes around genera names in the legend show families in common. Anatoxins (indicated by *anaC* gene copies) were highest in samples where Oscillatoriales (*Microcoleus*, *Oscillatoria*) were dominant, followed by *Geitlerinemia*, and Nostocales (*Nostoc*, *Cylindrospermum*, *Anabaena*) (B). Highest *anaC* gene concentrations occurred when *Microcoleus* was dominant or subdominant in the sample (C). Plots include results from unscaled transported CPOM and unscaled benthic mat samples. Colors in panels B and C correspond to taxa in panel A. In panels B and C dashed lines show the quantification limit for *anaC* genes, boxes extend from the 25th to 75th percentiles of the data, the median is indicated by the mid-box horizontal line, and whiskers extend to 1.5× the interquartile range.

subdominant, present, or not detected) also corresponded to anatoxin concentrations in the transported CPOM and mat samples (Fig. 6C).

The vast majority of *anaC* sequences were most closely related to *Microcoleus/Phormidium/Tychonema* sequences in GenBank, which indicates that 1 or multiple members within Oscillatoriales were primarily responsible for anatoxin production in the Klamath River and tributaries. However, there were 2 exceptions to this pattern. One came from a transported CPOM sample from the Trinity River that contained 17% of a Nostocaceae-like *anaC* sequence; however, this sample returned very few reads (n = 116) after quality assurance, suggesting this may be a spurious result. The other sample contained 0.3% of an unidentified *anaC* sequence from the Scott River, which came from a more deeply sequenced sample consisting of 92,949 sequencing reads, 417 of which were of a novel *anaC* sequence more closely related to Nostocales than Oscillatoriales.

Environmental covariates of anatoxins

Variation in anatoxin concentrations correlated only weakly with water column characteristics. Correlations with material concentrations were negative, such that higher anatoxins occurred when nutrients, algal pigments, and suspended sediments were lower. The strongest correlations occurred between anatoxin concentrations and total N, total P, and DOC (r = –0.61, –0.50, and –0.60, respectively; Fig. 7). Correlations between anatoxin and water temperature and between anatoxin and N:P ratios were very weak.

Differences between tributaries and the mainstem Klamath partially drove the weak, negative correlations between anatoxins and water column constituents. The sum of single effects regularization method identified location (tributary vs mainstem) as a predictor of anatoxin concentrations (Fig. 8). For most water quality constituents, values were lower at tributary sites and higher at Klamath River sites, yet anatoxin concentrations were higher in tributaries (Fig. 7). In addition to location, the sum of single effects linear regression model identified 4 other variables in the credible set: SRP, total P, DOC, and total N. We did not include SRP in the simple correlation analysis because it is highly correlated with total P both in our dataset and generally in the Klamath River (Asarian et al. 2010, Oliver et al. 2014), but the other 3 variables identified in this model had the strongest correlation coefficients in the simple correlation analysis.

DISCUSSION

Our data showing widespread prevalence of anatoxins throughout the middle and lower Klamath River watershed add to a growing body of evidence that anatoxins from benthic cyanobacteria may be more common than previously thought (McAllister et al. 2016, Bouma-Gregson et al. 2018, Fastner et al. 2023). Sites with higher water quality, including clear, low-nutrient tributaries, supported anatoxin concentrations as high as, and in some cases higher than, the high-nutrient mainstem of the Klamath River. Relationships between observed taxa and anatoxin concentrations, and genetic source tracking, indicated that *Microcoleus* was likely the dominant anatoxin producer in the Klamath River and tributaries in summer 2021. Weak, negative relationships between water quality parameters and anatoxins that were in part driven by stream type suggest that some aspect of the tributaries that we did not capture in this study, such as benthic light intensities or sediment nutrients (Tee et al. 2020, Kirk et al. 2021), may promote proliferations of benthic *Microcoleus* and anatoxin production.

anaC genes as anatoxin indicators

We found strong relationships between *anaC* genes and anatoxin concentrations, suggesting that the concentrations of *anaC* genes suitably represented anatoxin concentrations. Some have found positive relationships between anatoxin synthesis genes and anatoxin detections (Wood

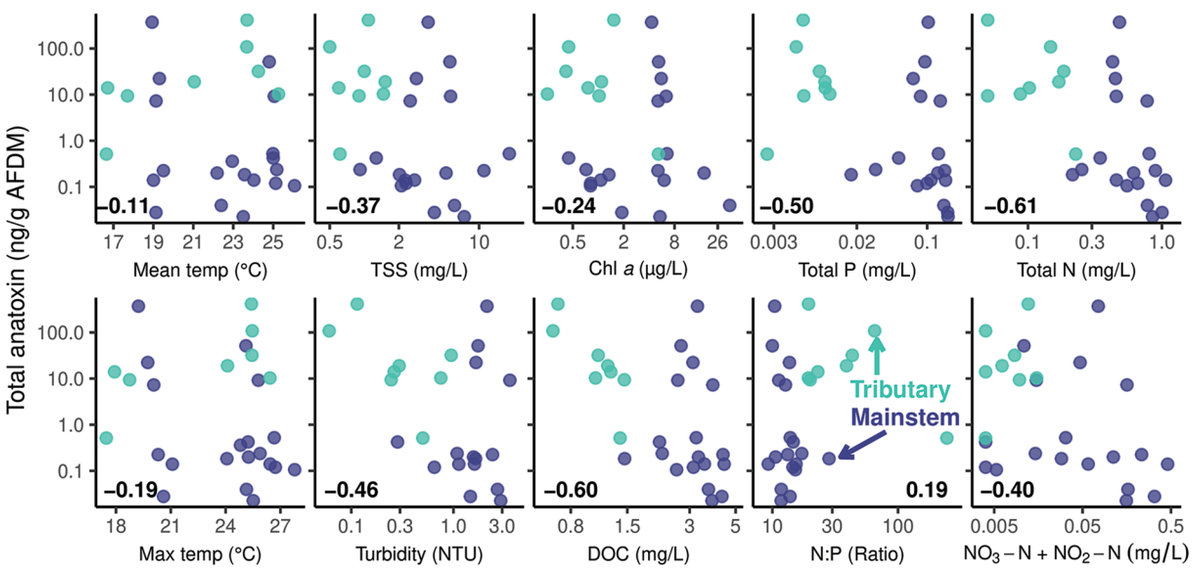

Figure 7. Correlations between anatoxin concentrations in transported coarse particulate organic matter (CPOM; ng anatoxins/g ash-free dry mass) and biological, physical, and chemical properties of water were weak in the lower Klamath River watershed in Northern California, USA. Where correlations were stronger, as expressed by Pearson's correlation coefficient (r-values displayed on each plot) of the log-transformed data (for all parameters except temperature), higher anatoxin concentrations in tributaries (blue points) and lower concentrations at mainstem sites (purple points) drove these relationships. Correlation analysis was performed on the full data set (tributary and mainstem sites combined). Chl a = chlorophyll a, DOC = dissolved organic C, max temp = maximum daily water temperature, mean temp = mean daily water temperature, TSS = total suspended solids.

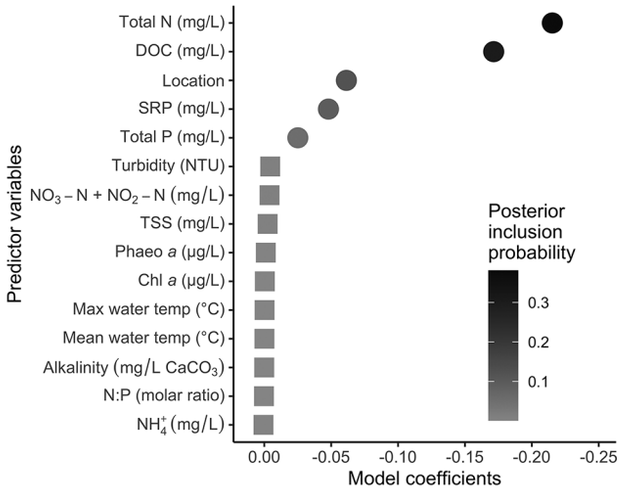

Figure 8. Results of sum of single effects linear regression model examining covariates of benthic anatoxin concentrations in the Klamath River watershed in Northern California, USA. Total N, dissolved organic C (DOC), location (tributary vs mainstem), soluble reactive P (SRP), and total P had posterior inclusion probabilities >0.05 and were included in the credible set (shown in circles), but 10 additional variables were not included in the final model (shown with squares). Coefficients of the centered, standardized predictor variables are on the *x*-axis. Chl *a* = chlorophyll *a*, max water temp = maximum daily water temperature, mean water temp = mean daily water temperature, Phaeo *a* = phaeophytin *a*, and TSS = total suspended solids.

et al. 2012, Shams et al. 2015, Kelly et al. 2018, Bouma-Gregson et al. 2019, Casero et al. 2019), but substantial variation has been found in the direction and strength of these relationships, including some studies with no congruence between genes and toxins (Sabart et al. 2015, Blahova et al. 2021). For example, in a study of planktonic cyanobacteria from North American rivers, 9 samples with *anaC* genes had undetectable anatoxin (Zuellig et al. 2021). In contrast, a study of >200 benthic mat samples resulted in >80% of samples with detection of anatoxin synthesis genes or anatoxins, but only 12% of samples had detections of both (Gaget et al. 2022). Very few studies have quantitatively related *anaC* gene copies to anatoxins (but see Kelly et al. 2018), which is needed to better understand how qPCR methods represent toxins and, thus, whether they can be used as a monitoring tool in other watersheds.

Despite the strong relationship between concentrations of *anaC* gene copies and anatoxins in our study, variation in the relationship was driven by the type of sample (i.e., CPOM, benthic mat). A higher intercept in the relationship of transported CPOM vs benthic mat samples indicates that CPOM samples had more anatoxins at a given concentration of *anaC* gene copies than did benthic mats. This difference may reflect the physiological status of the cells in the 2 sample types. Mats were more likely to include cells in their rapid growth phase, whereas cyanobacteria in CPOM, dislodged because of autogenic sloughing, were likely older (and no longer actively dividing) than those in the benthic mats (Vadeboncoeur et al. 2021). One lab experiment found total anatoxin concentrations tended to increase with time (Heath et al. 2016), which may be associated with the growth differentiation hypothesis, which proposes that cells not actively dividing are more likely to produce secondary metabolites (i.e., cyanotoxins, Herms and Mattson 1992). Understanding what drives variation in relationships between cyanotoxins and their synthesis genes will improve understanding of cyanotoxin ecology and will inform best practices for monitoring cyanotoxins.

Patterns in anatoxin occurrence

Benthic anatoxins were widespread in the Klamath River and tributaries in summer 2021. Conspicuous cyanobacterial mats occurred at 34 of 40 surveys, and 29 of those 34 mat samples had positive detections of *anaC* genes. There has not been widespread global documentation of anatoxins from benthic sources (Wood et al. 2020); however, some researchers have documented extensive benthic anatoxin production. In New Zealand rivers, anatoxins were detected in benthic *Phormidium* (closely related to *Microcoleus*) mats from 75% of 40 sampled rivers throughout the north and south islands (McAllister et al. 2016). In western North America, benthic anatoxins occurred in the Russian River, Eel River, and in rivers throughout Oregon (Bouma-Gregson et al. 2018, Fadness et al. 2022, Carpenter and Wise 2023) at frequencies similar to those in our study. Although benthic anatoxins are often identified in reaction to dog or wildlife illness or death (Krienitz et al. 2003, Bauer et al. 2020, McCarron et al. 2023), preventative monitoring and research is needed to understand the global distribution, extent, and public health risks associated with benthic anatoxins. On the Klamath River, we have only a single summer of extensive benthic anatoxin sampling, which prevents us from knowing if 2021, a drought year, was typical in its abundance of benthic cyanobacteria and associated anatoxins.

Documenting benthic anatoxins requires targeting the cyanobacteria that produce and contain anatoxins. We detected *anaC* genes in <50% of water grab samples and in <80% of targeted mat samples, and concentrations in mat samples averaged 10,000× higher than water samples. Water column grab samples are commonly used to sample cyanobacteria but were designed for plankton-dominated ecosystems and do a poor job of capturing benthic cyanobacteria and associated toxins (Wood et al. 2011, Kaminski et al. 2013, Quiblier et al. 2013, Tekwani et al. 2013). Composite mat samples are a recommended sampling technique for benthic cyanobacteria (Wood et al. 2020), but these methods can be challenging if surveyors are not trained in field identification or if rivers are too deep and swift to access the river bottom. Comparison of anatoxin concentrations in

composite mat samples and transported CPOM samples in this study and others (Wood et al. 2011, Genzoli and Kann 2020) showed that both of these sample types were better at detecting anatoxins than water column grab samples. Anatoxin concentrations in transported CPOM reflected concentrations in composite samples of benthic mats at a site, even when a relatively small area of river was surveyed, suggesting that both methods effectively captured anatoxins associated with benthic cyanobacteria (Fig. 4B). Our study indicates that the use of drift nets provides an additional tool to study cyanotoxins in nonwadeable rivers.

Anatoxins were more frequently detected and at higher concentrations toward the end of summer, aligning with other observations of benthic anatoxin production. Cyanobacterial mats in our study were most common in August, and anatoxin concentrations within those mats were also highest in August (Fig. 4A). Anatoxins in transported CPOM were highest in September at many of our mainstem sites and in August at tributary sites. These seasonal patterns are consistent with observations made elsewhere. In other Northern California rivers, anatoxins were highest in August and September and remained elevated through the autumn (Fadness et al. 2022). Higher cyanobacterial mat cover and higher anatoxin concentrations occurred in mid to late summer in rivers in Germany and New Zealand (Heath et al. 2011, McAllister et al. 2018, Bauer et al. 2022), where stable flows and warmer water temperatures may have supported proliferations of anatoxin-producing taxa.

Spatially, anatoxin concentrations varied substantially in our study, with tributary sites often having the highest anatoxin concentrations. Variation in anatoxins from benthic mats can occur on many scales, including differences among streams, among sites on the same stream, and within mats at a single location (Wood et al. 2010, McAllister et al. 2018). In some streams where cyanobacterial mats are the apparent dominant biomass, variation in anatoxins may be related to total attached algal biomass, but the proportion of toxin-producing cells within the algal mat likely varies, determining toxin concentration within the mat (McAllister et al. 2016). In the Klamath River and tributaries, cover of conspicuous cyanobacterial mats was low compared with other benthic primary producer assemblages, which were dominated by rooted macrophytes, filamentous green algae, and diatom-dominated biofilms. Transported CPOM samples showed that anatoxin-producing taxa were less common in areas with higher total biomass (where filamentous algae and rooted macrophytes dominated primary producer biomass), but more common where total biomass of all autotrophs was lower (i.e., in tributary streams where thin films of epilithon covered most of the stream bed; Fig. 5A–C). Understanding what drives the dominance of different taxa, including toxin-producing cyanobacteria, is essential for understanding what physical, chemical, and biological factors promote anatoxin production in a river.

Drivers of anatoxin variability

Differences between tributaries and Klamath River sites partially drove relationships between water quality constituents and anatoxin concentrations in this study. Most constituents, including nutrients, indicators of water clarity, and water temperature, were lower at tributary than mainstem sites despite higher anatoxin concentrations (Fig. 7). This consistent pattern among constituents, as well as inclusion of tributary status in the regularized regression model, suggests that unmeasured differences between tributary sites and Klamath River sites are associated with anatoxin production. Further, water column constituents interact with other environmental characteristics to influence benthic algae that can be more challenging to measure. Light reaching the riverbed (Kirk et al. 2021), nutrient delivery as influenced by water velocity (Townsend et al. 2012), sediment or groundwater nutrients (Tee et al. 2020), and biotic interactions (Bouma-Gregson et al. 2019, Vadeboncoeur et al. 2021) are unmeasured factors in our study that may promote benthic cyanobacterial proliferations.

Relationships between anatoxin concentrations and physical and chemical water quality constituents tended to be weak and partially driven by differences between tributary and mainstem sites, yet nutrient effects persisted after controlling for spatial differences between tributary and mainstem sites. In both the simple correlation analysis and the regularized regression analysis, both which tested for the effect of multiple variables, total N and DOC, and to a lesser extent P (SRP and total P), related with anatoxins, whereas relationships with water temperature, water clarity indicators, algal pigments, and nutrient ratios were weak. Counterintuitively, higher anatoxins occurred with lower nutrient concentrations. Although the extent of potentially toxic cyanobacterial mats may increase with increasing nutrients (Heath et al. 2016, Wood et al. 2017a), low nutrient concentrations may increase toxin quotas (toxin concentration/cell; Heath et al. 2014, 2016). A possible mechanism may be captured by the growth differentiation hypothesis, where cells not actively dividing can produce more secondary metabolites (Herms and Mattson 1992, Heath et al. 2016). That is, higher nutrients promote more cell growth, but these actively dividing cells produce fewer secondary metabolites. Additionally, identifying relationships between nutrients and the extent of cyanobacteria or cyanotoxins may be further complicated by nutrient acquisition and storage adaptations or biological interactions that allow cyanobacteria to rely on nutrients from sediments and associated microbes (Brasell et al. 2015, Wood et al. 2015, 2020, Bouma-Gregson et al. 2019). The correlations between nutrients and tributary status that we observed do not provide conclusive evidence about drivers of anatoxin production, but these correlations do suggest that nutrients, including N, P, and C, should be considered in future studies.

Taxa responsible for anatoxin production

Microcoleus was likely the primary anatoxin producer in the Klamath River and tributaries in summer 2021, adding more evidence that Oscillatoriales taxa are principal producers of anatoxins from benthic cyanobacterial mats. A previous study of water column grab samples in the Klamath River detected *anaC* genes from 32% of samples, and metagenomic analysis identified Oscillatoriales taxa as the source of anatoxins in that study (Otten 2017), corroborating our more recent findings. In other California rivers, anatoxins have been associated with other taxa (primarily *Anabaena*-dominated mats; Bouma-Gregson et al. 2018), but more recently, genetic source tracking studies have attributed anatoxin production to *Microcoleus* (Bouma-Gregson et al. 2019, Kelly et al. 2019, Conklin et al. 2020, Fastner et al. 2023). Similarly, in New Zealand, genetic source tracking and cultures of isolated strains have been used to identify *Microcoleus* (*Phormidium*) as the primary producer of anatoxins from benthic mats (Wood et al. 2012, 2017b). In other countries, including Canada and France, *Microcoleus* and other Oscillatoriales have been identified as anatoxin producers from benthic mats (Cadel-Six et al. 2007, Bauer et al. 2022, Valadez-Cano et al. 2023). Although planktonic taxa in other orders produce anatoxins (Ballot et al. 2018, Christensen and Khan 2020), Oscillatoriales taxa appear to be the most common producers of anatoxins from benthic mats, shedding light on which taxa should be prioritized for monitoring efforts in rivers where benthic algae are dominant.

Implications for monitoring and public health

Widespread detection of anatoxins by benthic cyanobacteria in our study and from previous work suggests that benthic habitats, even in large rivers, can drive ecological processes relevant to public health. Increased monitoring of benthic cyanobacterial proliferations and research that identifies factors promoting anatoxin production from benthic taxa are needed to assess current risks and to forecast trends of anatoxins in rivers. Although highly variable streambed habitats can make sampling feel futile, especially in rivers too deep and swift to wade across, multiple sampling strategies are available to overcome the limitations of traditional methods. Composite mat samples (Wood et al. 2010, 2020), nets capturing transported sloughed algal mats, and Solid Phase Adsorption Toxin Tracking samplers (SPATTs) are useful tools for documenting anatoxins in rivers (Kudela 2011, Wood et al. 2011, Bouma-Gregson et al. 2018).

Using multiple laboratory methods and increasing basic understanding of the ecology of benthic cyanobacteria can make monitoring more accessible. Tightly coupled relationships between anatoxins and *anaC* gene copies suggest that qPCR methods may be used as a quantitative indicator of anatoxins in lieu of more costly approaches such as ELISA (Kelly et al. 2018, Bouma-Gregson et al. 2019). Our data also indicate that visual surveys of benthic *Microcoleus* mats can also support anatoxin monitoring. Although anatoxins are not associated with all strains of *Microcoleus* (Bouma-Gregson et al. 2019, Tee et al. 2021, Valadez-Cano et al. 2023), visual observation of *Microcoleus* is an immediate indicator that anatoxins may be present. Research aimed at identifying specific strains or growth forms of anatoxin-producing cyanobacteria, linked with both microscopic and macroscopic characteristics (e.g., Conklin et al. 2020) will support improved identification of anatoxin-producing blooms via field observations. Increasing our understanding of when and where benthic cyanobacteria proliferate, and under what conditions, will help focus monitoring resources toward times and locations of greatest public health risk.

Informing river users on how to reduce contact with benthic cyanobacteria will help mitigate anatoxin exposure. In our study, highest concentrations of benthic cyanobacteria occurred in clear tributaries with high water quality that people swim in specifically to avoid poorer water quality conditions, including other cyanotoxins sourced from upstream reservoirs (Otten et al. 2015, Howard et al. 2023). Given these other concerns about water quality, some rivers with anatoxins may still be preferred for swimming. However, outreach to river users should warn them of the risk to dogs, which may accidentally ingest or be attracted to cyanobacterial scums or mats (Codd et al. 1992). To protect people, river users should watch small children closely, making sure that algae are not ingested. Taking extra precautions in the late summer and where mats are floating in the water or easily dislodged from the riverbed will reduce chances of ingesting toxins from benthic cyanobacteria.

Widespread detection of anatoxins from benthic algae in the Klamath River watershed in summer 2021 provides additional evidence that anatoxins may be more common than previously thought. Sampling that targets *Microcoleus* and similar taxa will help researchers and water managers focus their efforts on the taxa likely responsible for anatoxin production in benthic ecosystems. Clear, low-nutrient streams should be included in surveys, especially when possible anatoxin production is in areas with high recreational use. Although reports of human illness from anatoxins are rare (Colas et al. 2021), whether or not high benthic cyanobacteria cover and anatoxin concentrations are a longstanding occurrence or are a more recent phenomenon is not known (Quiblier et al. 2013, Vadeboncoeur et al. 2021). Continued monitoring of and research on benthic anatoxins will serve to protect public health and improve understanding of the extent to which currently observed anatoxin patterns are associated with stream ecosystem alterations. This new knowledge is needed to predict changes to benthic anatoxins associated with future impacts to and restoration of streams and rivers.

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