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# Rise and fall of toxic benthic freshwater cyanobacteria (*Anabaena* spp.) in the Eel river: Buoyancy and dispersal

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ABSTRACT

Benthic cyanobacteria in rivers produce cyanotoxins and affect aquatic food webs, but knowledge of their ecology lags behind planktonic cyanobacteria. The buoyancy of benthic *Anabaena* spp. mats was studied to understand implications for *Anabaena* dispersal in the Eel River, California. Field experiments were used to investigate the effects of oxygen bubble production and dissolution on the buoyancy of *Anabaena* dominated benthic mats in response to light exposure. Samples of *Anabaena* dominated mats were harvested from the South Fork Eel River and placed in settling columns to measure floating and sinking velocities, or deployed into *in situ* ambient and low light treatments to measure the effect of light on flotation. Floating and sinking occurred within minutes and were driven by oxygen bubbles produced during photosynthesis, rather than intracellular changes in carbohydrates or gas vesicles. Light experiment results showed that in a natural ambient light regime, mats remained floating for at least 4 days, while in low light mats begin to sink in <24 h. Floating *Anabaena* samples were collected from five sites in the watershed and found to contain the cyanotoxins anatoxin-a and microcystin, with higher concentrations of anatoxin-a (median 560, max 30,693 ng/g DW) than microcystin (median 30, max 37 ng/g DW). The ability of *Anabaena* mats to maintain their buoyancy will markedly increase their downstream dispersal distances. Increased buoyancy also allows toxin-containing mats to collect along shorelines, increasing threats to human and animal public health.



## 1. Introduction

Cyanobacterial harmful algal blooms (cyanoHABs) have become increasingly common phenomena in many freshwater systems (Paerl and Huisman, 2009; Carey et al., 2012; Taranu et al., 2015). Most of these are nuisance blooms of planktonic species and occur in lakes, estuaries, or regulated lowland rivers, but benthic cyanobacteria can also produce cyanotoxins (Quiblier et al., 2013). Benthic cyanobacterial species are generally different from planktonic species, forming dense mucilaginous mats on bottom substrates. Benthic cyanoHABs are caused by species of *Anabaena* (Mohamed et al., 2006), *Phormidium* (McAllister et al., 2016), *Oscillatoria* (Edwards et al., 1992), *Lyngbya* (Cowell and Botts, 1994), and *Nodularia* (Lyra et al., 2005). Toxic benthic cyanobacteria in rivers have been documented in many countries including Egypt, Spain, France, California, and New Zealand (Mohamed et al., 2006; Sabater et al., 2003; Cadel-Six et al., 2007; Fetscher et al., 2015; McAllister et al., 2016; for review Quiblier et al., 2013).

Different hydraulic and physicochemical environments between benthic and planktonic cyanobacteria have led to different ecological interactions and strategies for cyanobacteria growing in each habitat (Scott and Marcarelli, 2012). In benthic cyanobacterial mats, light attenuates rapidly through the mat (Jorgensen et al., 1987), strong geochemical gradients develop (Wood et al., 2015), and nutrients are acquired from within the mat, from overlying water, and sometimes from substrates (Aristi et al., 2017; Stevenson et al., 1996). Planktonic cyanobacteria, on the other hand, often experience a spatial separation between light and nutrients availability in stratified waters, with high light and low nutrients near the water surface, and high nutrients and low light in deeper waters. To access these essential resources, many planktonic cyanobacteria migrate between surface and bottom waters by regulating their buoyancy with the formation of gas vesicles to increase buoyancy (Reynolds, 1972; Walsby, 1975) and the formation of dense carbohydrate granules to decrease

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buoyancy (Kromkamp and Mur, 1984; Reynolds et al., 1987). Colonial species, like *Microcystis,* and filamentous species, like *Dolichospermum,* (formerly *Anabaena*, (Komárek, 2010; Wacklin et al., 2009)), use these mechanisms to move up and down through the water column (Bormans et al., 1999; Visser et al., 1997). While dispersal mechanisms have been well-studied in river phytoplankton (Bormans and Condie, 1997; Maier et al., 2001), much less is known about dispersal by benthic cyanobacteria (or other benthic algae) in rivers. Studies on benthic species dispersal have been on detached and floating green algae (Mendoza-Lera et al., 2016; Power, 1990) or diatoms (Stevenson and Peterson, 1991, 1989), and more recently on the cyanobacterium *Phormidium*, which also poses a public health risk (McAllister et al., 2016). To better understand benthic algal dispersal, this paper reports observations on the buoyancy of *Anabaena* spp. in the Eel River of northwestern California, where it grows epiphytically on the green macroalga, *Cladophora glomerata* (Power et al., 2015).

Benthic *Anabaena* produce no gas vesicles (Komarek, 2013; Li et al., 2016), and are not able to regulate their density via intracellular mechanisms used by planktonic cyanobacteria. There are, however, other potential mechanisms for dispersal of benthic *Anabaena*: 1) trichome motility via gliding (Hoiczyk, 2000); 2) vegetative overgrowth of host algae; 3) detachment of individual trichomes from the mat (Otten et al., 2015); 4) akinete formation and dispersal, followed by germination (Cirés et al., 2013); and 5) detachment of macroscopic floating clumps advected downstream by the river flow (Cadel-Six et al., 2007; McAllister et al., 2016).

This paper focuses on downstream dispersal of floating detached clumps (mechanism 5), which likely accounts for long-range dispersal from the original mat location. Benthic cyanobacteria are known to produce oxygen bubbles from photosynthesis (Bosak et al., 2010; Wilson, 1965). These bubbles become trapped in the intercellular mucus of the mats, lifting the top of the mat to form a vertical, spire-like shape, which is a common morphology for benthic cyanobacterial mats (Figs. 2 A,B, 3 C; Bosak et al., 2010; McGregor and Rasmussen, 2008). In the Eel River, California, *Anabaena* mats are delicate, and easily detached by flows of >5 cm $s^{-1}$ or by gentle water agitation (Bouma-Gregson, personal observation). When these mats disintegrate, the bubbles in the mucus cause the majority of an *Anabaena* mat to float to the water surface as many small clumps (1–5 cm diameter). During summer, floating clumps of *Anabaena* are frequently observed in the river, moving hundreds of meters, primarily in a downstream direction unless wind-driven over slow pools. Floating clumps often concentrate at channel margins and recreational swimming areas, and dogs have died in the Eel River due to anatoxin-a poisoning after ingesting cyanobacterial mats (Puschner et al., 2008), making toxic benthic cyanobacteria a public health concern each summer.

Given the consequence for water quality and animal and public health from cyanotoxin exposure, more knowledge is needed about how filaments, clumps, or mats of benthic cyanobacteria disperse in rivers during summer low flow periods. Combined with the river flow velocity, the time scales of floating and sinking will control the distance scales of dispersal of floating clumps and mats in the river. To understand these time-scales and the processes responsible for buoyancy, field experiments were used to 1) study the effects of light on macroscopic oxygen bubble production, and 2) the effect of bubbles on the buoyancy of benthic *Anabaena* dominated mats.

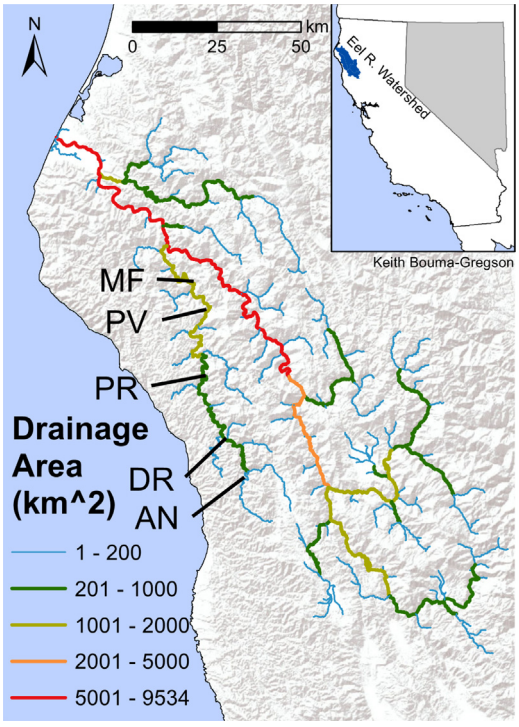


**Fig. 1.** Map of the Eel River watershed showing sites along the South Fork Eel River where floating *Anabaena* spp. clumps were collected in 2014. Field experiments were conducted at the AN site in 2016. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

## 2. Material and methods

### 2.1. Study system and site description

The Eel River, draining 9546 $km^2$, is located in the coastal mountains of Northern California (Fig. 1). Forestry has been the principal land-use since European settlement, with dairy and small-scale agriculture near the estuary. A history of logging and dense networks of unpaved roads along with two large floods (1955 and 1964) have loaded massive amounts of fine sediments into channels, rendering them wider, shallower, and easier to warm or de-water at low flow (Lisle, 1990). The Eel is in a Mediterranean climate with rainy winters and seasonal summer droughts. Daily average river temperatures in the summer range from 20 to 25 °C, and daily maximum temperatures can exceed 30 °C along shallow channel margins. Experiments took place at the Angelo Coast Range Reserve (angelo.berkeley.edu), a 3100 ha reserve on the South Fork (SF) Eel River where the channel drains 150 $km^2$ (Fig. 1).

*Anabaena* spp. appear in June-August in sunny, slow-flowing (<10 cm $s^{-1}$) river reaches, first as small, blue-green epiphytic tufts near the top of fresh or senescent, diatom-covered *Cladophora* proliferations (Power et al., 2015). Over the following weeks or months, if flows remain slow (<10 cm $s^{-1}$) and relatively warm (20–25 °C at midday), *Anabaena* mats can spread, turning several square meters of *Cladophora*/diatom assemblages a blue-green color (Fig. 2). There are at least three common species of mat forming *Anabaena* in the Eel River: *Anabaena oscillarioides*, *A. cylindrica*, and *A. sphaerica* (Komarek, 2013). These species are difficult to differentiate based on macroscopic mat morphology and all grow in similar habitats.

### 2.2. Light experiment

To investigate the effect of light on flotation and bubble production, an *in situ* field experiment was conducted during June 20–24, 2016. Vertically-placed PVC pipes (25 cm diameter and 1 m length, 10 pipes total) were set into the SF Eel River, with the top 2–6 cm of the pipe above the river surface (Fig. 3). Mesh windows (0.3 × 0.3 mm, dia. 9 cm) on the sides of each pipe allowed enough

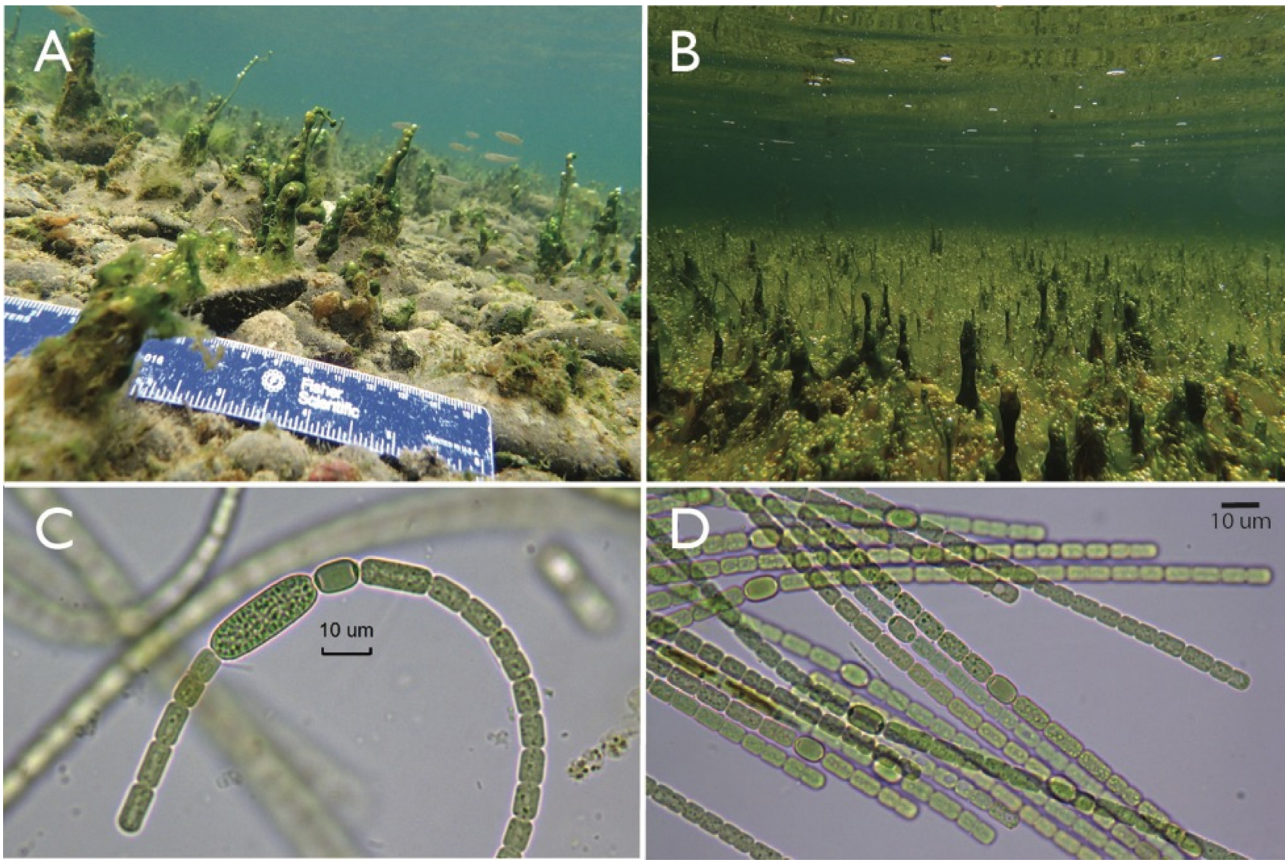


**Fig. 2.** A-B: Underwater photographs of *Anabaena* spp. spires. The dark green patches are *Anabaena* spp. growing on top of Cladophora glomerata filaments. Note the trapped bubbles in the *Anabaena* mucus. C-D: *Anabaena* cylindrica trichomes at 400×, panel C shows a developing akinete.

water exchange to keep inside temperatures at ambient levels. Five replicate pipes each were used for Ambient-Light and Low-light treatments. Low-light treatment pipes were wrapped in black polyurethane plastic to decrease light intensity inside the pipe. A spoon was used to harvest benthic *Anabaena* from nearby naturally occurring mats without disturbing bubbles, and three *Anabaena* clumps (3–48 × 7–80 mm) were placed in each pipe (Fig. 3). All clumps were floating when placed in the pipes. Then an aluminum foil covering was placed over the Low-light treatment pipes. Clumps were placed on the afternoon of day 0. Beginning on day 1, every morning (∼06:00, before direct sunlight hit any pipes) and afternoon (14–16:00, at the hottest time of the day) clumps were observed to see if they were still afloat or had sunk. The number of bubbles in each clump was also counted and measured to the nearest millimeter. The experiment concluded after the morning measurement on day 4. In some pipes, two clumps merged together to form a single clump, therefore the proportion of clumps floating at each measurement was used as the response variable.

To determine the light reduction in the Low-light treatment pipes, Hobo Pendant temperature and light sensors (UA-002-08; Onset, Bourne, Massachusetts, USA) were deployed on day 4 after the *Anabaena* clumps were removed from the pipes. In each of the five Low-light and Ambient-light pipes, two sensors were deployed for 24 h at the water's surface, one with the light sensor pointing upwards to the sky, and the other with the sensor pointing downwards into the water. After positioning the sensors, Low-light pipes were again covered with aluminum foil.

At deployment and on day 4, samples from each clump were collected and stained with SYTOX green (Sigma-Aldrich, S7020) to test for cell wall integrity. If flotation was stressful for the cyanobacteria, an increase in cell wall degradation might be expected at the end of the experiment. An *Anabaena* clump subsample was placed in a 2 mL microfuge tube filled with ∼1 mL of river water. Then, 20 μL of 5 μM SYTOX green was added and the total volume brought to 2 mL for a final SYTOX concentration of 0.5 μM. The SYTOX solution was briefly vortexed and incubated for 10 min in the dark at room temperature. Stained cells, indicating degraded cell walls, were counted at 200× with epi-fluorescent microscopy (Nikon Optiphot 2, 490/520 nm ex/em λ). The species identification and cell condition for all samples were determined by microscopy on fresh material using the same Nikon Optiphot 2.

### 2.3. Buoyancy experiments

To determine floating and sinking velocities of *Anabaena* clumps from different algal assemblages, subsamples of algal mats from different origins were collected: floating *Cladophora glomerata* mats detached from bottom substrates, floating *Cladophora* mats still attached to bottom substrates, and *Anabaena* spires attached to bottom substrates. Sub-samples from the *Cladophora* mats were divided into two categories: clumps from *Cladophora* mats dominated by *Cladophora*, and clumps from *Cladophora* mats dominated (>50%) by epiphytic *Anabaena* growing on the *Cladophora*. Experiments were performed on June 22 and July 20–21, 2016. Glass Pyrex graduated cylinders (1000 mL) filled with river water and exposed to natural sunlight were used as settling columns to measure velocities. Floating and sinking of clumps was recorded with a video camera, using time elapsed and the graduated scale on the cylinder to quantify upwards and downwards velocities. The temperature of the river water was also measured.

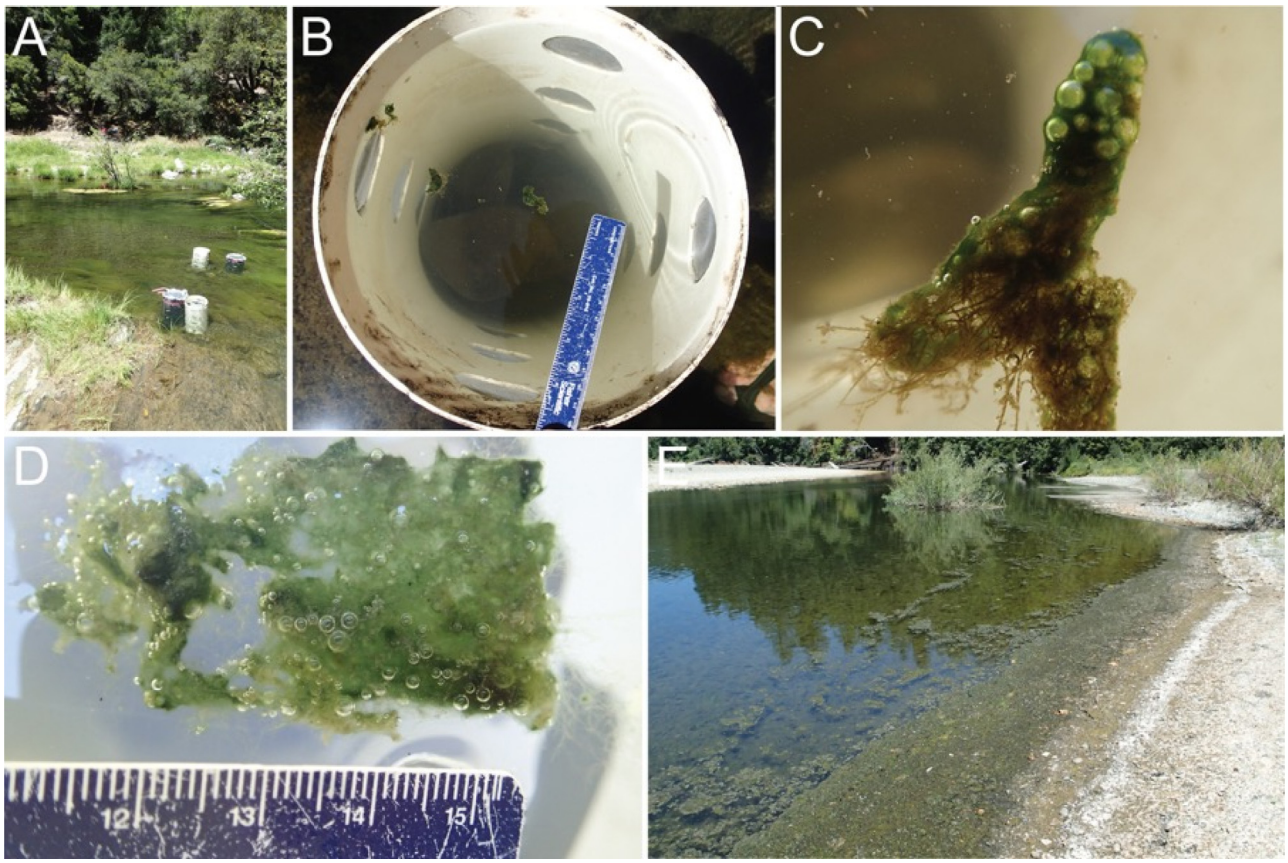


**Fig. 3.** A) Two of the replicate Low-light and Ambient-light treatment pipes. B) Floating *Anabaena* spp. clumps in a flow through pipe. C-D) Floating *Anabaena* spp. clumps showing the accumulation of bubbles within extracellular mucus. E) Accumulation of floating *Anabaena* spp. clumps along the river margin in July 2016.

Bubbles were removed from floating clumps by either manual pinching or by shaking the cylinders. Subsamples of mats collected *in situ* were either directly exposed to natural sunlight or first placed in the dark for 12 h to remove bubbles and ensure that clumps sank. Once clumps had sunk, the graduated cylinder was placed in the light until bubbles formed and the clumps floated to the surface.

### 2.4. Cyanotoxin analyses

To determine if floating *Anabaena* clumps contained cyanotoxins, twenty samples of floating *Anabaena* spp. were collected in late June through mid-September 2014 from five different locations on the SF Eel River (Fig. 1) and analyzed for anatoxin-a (ANTX) and microcystins (MC) using liquid chromatography and mass spectrometry (LC–MS). Fifteen of these samples came from weekly sampling events at the monitoring site PV. The remaining five samples, were collected on different days in July 2014 at four additional sites MF, PR, DR, and AN. Floating samples were collected with a bulb syringe or skimmed off the surface with an 80 μm plankton net, transferred into glass sample jars, placed in a cooler, and within 6 h returned to the laboratory for overnight storage in the dark at 4 °C. The next day samples were homogenized and sub-samples frozen at −20 °C for dry weight and cyanotoxin analysis. To measure the dry weight, samples were dried at 55 °C for 24 h and then weighed to the nearest 0.1 mg. A subsample was also collected for microscopic analysis to identify the dominant species of cyanobacteria in the sample.

For cyanotoxin analysis, samples were thawed, then sonicated for 30 s (Fisher Sonic Dismembrator 100) in 6 mL of 50% methanol (Fisher A452), then centrifuged (Model IEC Centra CL2; Thermo Fisher Scientific, Massachusetts, USA) for 5 min at 1083 rcf, and the supernatant sampled. For ANTX analysis, a 1 mL subsample was filtered (0.2 μm) into a LC–MS vial. Samples for MC analysis were cleaned using a Baker C18 solid phase extraction column, and 1 mL of cleaned sample was transferred to an LC–MS vial.

MC and ANTX were analyzed separately by liquid chromatography coupled with a mass spectrometer on a Single Quadrupole Agilent 6130 LC–MS (Agilent Technologies). ANTX analysis followed Cogent method 141 (MicroSolv Technology Corporation, Leland, NC, USA; http://kb.mtc-usa.com/getAttach/1114/AA-00807/No+141+Anatoxin-a+ANTX-A.pdf). Briefly, a Cogent diamond hydride column (100A, 4um, 100 × 2.1 mm) was used with a gradient elution of 50% MeOH with 0.1% formic acid, and 100% acetonitrile with 0.1% formic acid with the MS in Select Ion Mode (SIM) for MW 166.1 and 149.1. Quantification was based on standard curves (run daily) with a CRM-ATX standard from National Resource Council Canada (http://www.nrc-cnrc.gc.ca/eng/solutions/advisory/crm/list_product.html#B-CT). MC analyses followed the method in Gibble and Kudela (2014), which was adapted from Mekebri et al. (2009). Briefly, a gradient-elution method was used as the mobile phase, with HPLC water (solvent A) and LC–MS acetonitrile (solvent B), both acidified with 0.1% formic acid. The gradient starts with 95:5 of solvent A:B and ends with 25:75 at 19 min, is held for 1 min, then followed by a 5 min equilibration at initial conditions prior to injection of the next sample. Standard curves (for each batch of samples) using pure standards (Fluka 33578 and Sigma–Aldrich M4194) were used to calibrate samples. For sample runs lasting more than 8 h, standards were run again at the end of the run. The LC–MS measured four microcystin congeners (−LR, −YR, −RR, and −LA), and their values were summed to give total microcystin (MC). The limit of detection for ANTX and MC are 0.25 and 0.01 ppb, respectively.

### 2.5. Statistics

For the light experiment, generalized linear mixed models (glmms), with replicate pipe as a random effect, were used to model the effect of light treatment, day, and time of measurement (AM/PM) on the flotation of clumps (binomial distribution) and the number of bubbles (Poisson distribution). For the buoyancy experiment, one-way ANOVA was used to test the effect of algal assemblage on sinking and floating velocities. Likelihood ratio or F ratio tests between full and reduced models were used to estimate the statistical significance of model parameters (alpha = 0.05). All statistics were performed in the R environment, version 3.3.2 (R Core Team, 2016), with the lme4 package (Bates et al., 2015) used for glmms.

## 3. Results

### 3.1. Anabaena cell condition

Samples for light and buoyancy experiments were dominated by *Anabaena cylindrica*, with <1% of trichomes being *A. oscillarioides* (Fig. 2). At the time of collection, all *Anabaena* trichomes were fully pigmented, had heterocytes, and <1 akinete per trichome, as well as containing many dividing cells, indicating a relatively healthy physiological status. SYTOX green staining from light experiment samples indicated <<1 cell per trichome had a cell wall permeable to SYTOX green on samples from Day 0 and Day 4, a further indication of healthy cells.

### 3.2. Light experiment

Light intensity data showed that Low-light treatment had values <0.01% of the Ambient-light treatment with the mean maximum intensity from the upward facing sensor for the Ambient-light and Low-light treatments being 235,877 ± 12,567 and 58 ± 31 lumens/$m^2$, respectively. The diel range in river surface temperatures was 16.5-23.5 °C, with Low-light treatments being 0.5–1 °C warmer than Ambient-light treatments in the afternoon and having the same temperatures in the morning.

All clumps were floating at the onset of the light experiment. Clumps began sinking within 24 h in the Low-light treatments (Fig. 4). By day four, all the clumps in four of the five Low-light treatment replicates had sunk. In the Ambient-light treatment, by day 4 only one individual clump had sunk. Any clump that sunk in the Low-light treatment never re-floated, whereas the clump in the Ambient-light treatment that sunk after the Day 1: PM measurement, refloated on day two due to bubble production (Fig. 4). This clump then remained floating for two more days until sinking on day four. The binomial glmm found a significant positive effect of light treatment ($p < 0.005$), a significant negative effect of day ($p < 0.001$), and a positive, but non-significant, treatment*day interaction effect ($p = 0.092$) on flotation.

The number of bubbles in the clumps were similar at deployment on Day 0 (Fig. 5). Bubble numbers subsequently decreased in the Low-light treatments, but remained relatively constant in Ambient-light treatments. The glmm model showed a significant treatment effect ($p < 0.005$) and day*treatment interaction effect ($p < 0.005$) on the sum of bubbles in each replicate pipe during the experiment, with the Ambient-light treatment having more bubbles than the Low-light treatment. There was also a significant positive interaction effect between the time of day (AM/PM) and light treatment ($p < 0.005$) indicating more bubbles at each of the afternoon (PM) measurements in the light treatment compared to the previous morning's (AM) measurement (Fig. 5), while in the Low-light treatment there was no difference in bubbles between morning and afternoon.

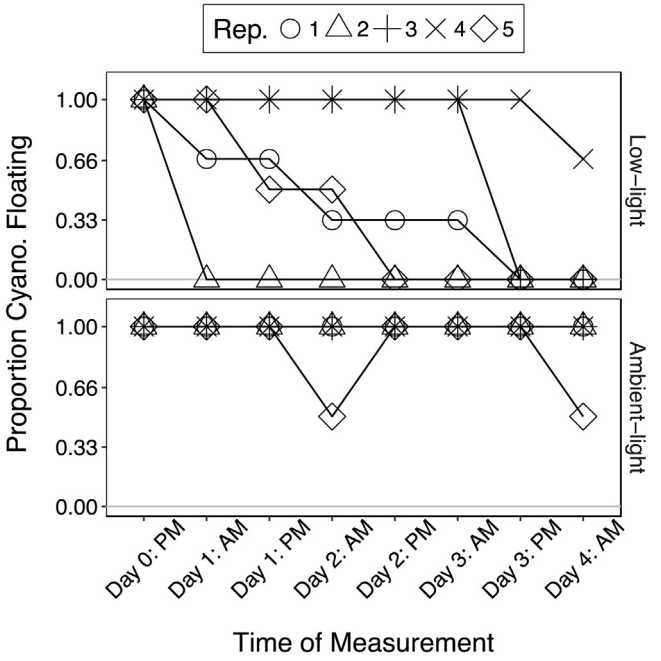


**Fig. 4.** Proportion of floating *Anabaena* spp. clumps in each of the five replicates of the Low-light and Ambient-light treatments at each morning (AM) and afternoon (PM) measurement over the 4 days of the experiment.

### 3.3. Buoyancy experiment

All small *Anabaena* clumps (1–3 $cm^3$) collected from bottom *Anabaena* spires *in situ* in mid-afternoon on June 22, 2016 floated at the surface of the glass cylinder placed in sunlight (water temp. 18.5 °C). Oxygen bubbles were observed in all clumps, some on the outside of the clumps, but mostly within the mucus of the mat. The bubbles were manually removed by pinching to assess the role of oxygen on the buoyancy of the clumps. Following the removal of oxygen bubbles, all clumps began to sink within 60 s, with mean velocities of 0.7 cm $s^{-1}$ (Fig. 6). After around 30 min in full sun, they began to rise again with mean velocities of 0.9 cm $s^{-1}$(Fig. 6). Slightly different floating velocities were observed depending on the morphology, smaller clumps (~1 $cm^3$) floating faster than larger ones (~3 $cm^3$).

Subsamples collected *in situ* in late afternoon on July 20, 2016 from detached floating *Cladophora* mats epiphytized by *Anabaena* stayed at the surface of the glass cylinders placed in sunlight (water temp 19.5 °C). All of them displayed visible oxygen bubbles. The water was gently mixed in the cylinder by manual shaking for a few seconds and this disrupted the mat, which disintegrated into small sinking clumps. Within minutes after sinking to the bottom of the cylinder, these *Cladophora-Anabaena* clumps started to float and to re-aggregate. *Cladophora* clumps heavily overgrown by *Anabaena* floated more rapidly than *Cladophora* clumps with sparser (<50%) epiphytic *Anabaena* cover (ANOVA $F_{(2,33)} = 49.67$, $p < 0.01$; Tukey-Kramer *post hoc* test $p < 0.05$) (Fig. 6). Oxygen bubbles were observed during the rise of the mats, with larger bubbles associated with the *Anabaena* and smaller bubbles with *Cladophora*.

After being in the dark for 12 h at 19 °C, all samples from the three different source mats sank to the bottom of cylinders, and no obvious oxygen bubbles were observed initially. Once placed in the sunlight, bubbles formed after 30–40 min, and the mats began to float. Again, clumps dominated by *Anabaena* rose faster than clumps dominated by *Cladophora* (ANOVA $F_{(2,7)} = 13.23$, $p < 0.01$; Tukey-Kramer *post hoc* test $p < 0.05$) (Fig. 6). After gentle mixing of the cylinders by shaking them manually for a few seconds, the

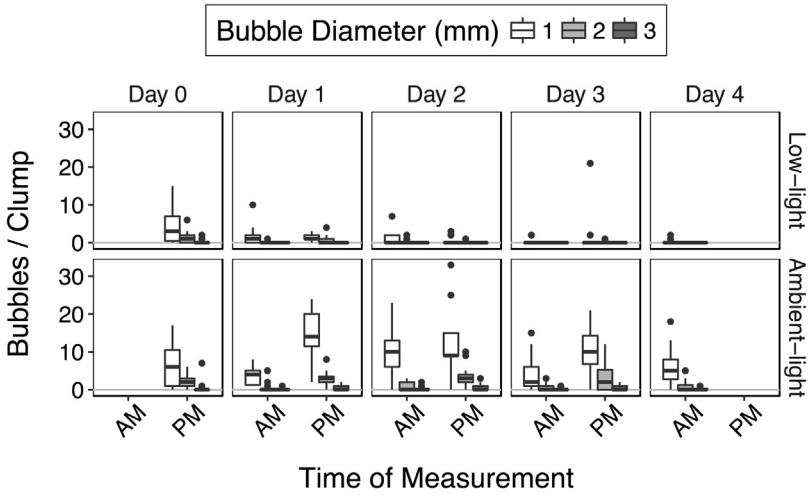


**Fig. 5.** Boxplots of number of bubbles in *Anabaena* spp. clumps in the Low-light and Ambient-light treatments at each measurement time. Bubble diameter was measured to the nearest millimeter.

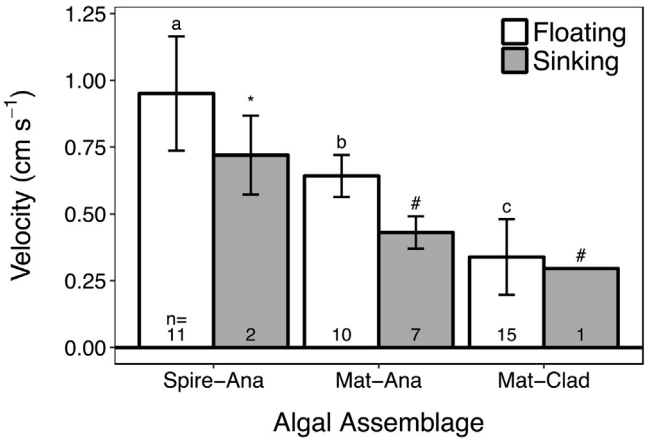


**Fig. 6.** Combined results from the buoyancy experiments showing the mean ± SD floating and sinking velocities of clumps from different algal assemblages. Spire-Ana: clumps sampled directly from *Anabaena* spp.spires that were almost exclusively *Anabaena* spp.; Mat-Ana: clumps from *Cladophora glomerata* mats overgrown by epiphytic Anabaena spp. overgrowth; and Mat-Clad: clumps from Cladophora mats dominated by Cladophora, with less than 50% Anabaena. Number of replicates (n) indicated at the bottom of each bar. Different letter superscripts indicate statistically significant differences in mean floating velocities and different symbol superscripts indicate statistically significant differences in mean sinking velocities between algal assemblages based on one-way ANOVA and Tukey-Kramer post hoc tests (alpha = 0.05).

mats disintegrated into small clumps that settled to the bottom of the cylinder. The *Anabaena* broke apart more easily than the *Cladophora*, formed much smaller clumps and sank faster. The *Cladophora* did not disintegrate and sank more slowly than *Anabaena* (Fig. 6). Regardless of the habitat and algal taxa within the mat assemblages, floating velocities were consistently higher than sinking velocities. However, the observed range between 0.25 and 1 cm $s^{-1}$ suggested that clumps would rise and fall in a 50 cm deep still river pool, representative of much of the South Fork Eel habitat during the low flow summer season, within less than five minutes.

### 3.4. Cyanotoxins

Both ANTX and MC were detected in the floating *Anabaena* samples collected in the SF Eel watershed (Table 1). Fourteen of the 20 samples contained ANTX (median and mean concentrations of 560 and 3184 ng ANTX/g DW, respectively). In contrast, only 3 samples tested positive for MC and all had much lower MC concentrations (<50 ng MC/g DW). All three positive MC samples also contained ANTX. Toxin concentrations varied weekly at the Phillipsville (PV) site, though due to the movement of floating clumps the same mat was not sampled each week. ANTX concentrations also varied spatially, the difference in ANTX concentrations between duplicate samples collected on the same date from the PV site ranged from 0 to 10,424 ng ANTX/g DW.

**Table 1**
Concentration of cyanotoxins anatoxin-a (ANTX) and microcystin (MC) in floating *Anabaena* spp. clumps collected from the South Fork Eel River. Microcystin congeners −LR, −YR, −RR, and −LA were summed together to calculate the MC value.

| Date Collected | Site | Collection Method | ANTX (ng/g DW) | MC (ng/g DW) |
|---|---|---|---|---|
| 07-Jul-14 | AN | bulb syringe | NA[a] | NA[a] |
| 18-Jul-14 | DR | bulb syringe | 2744 | 0 |
| 12-Jul-14 | MF | bulb syringe | 172 | 0 |
| 18-Jul-14 | PR | bulb syringe | 30,693 | 0 |
| 24-Jul-14 | PR | bulb syringe | 0 | 0 |
| 29-Jun-14 | PV | plankton net | 0 | 0 |
| 12-Jul-14 | PV | plankton net | 647 | 0 |
| 18-Jul-14 | PV | plankton net | 3184 | 0 |
| 18-Jul-14 | PV | bulb syringe | 3703 | 0 |
| 24-Jul-14 | PV | plankton net | 0 | 0 |
| 02-Aug-14 | PV | bulb syringe | 15 | 0 |
| 02-Aug-14 | PV | plankton net | 4985 | 0 |
| 07-Aug-14 | PV | bulb syringe | 0 | 0 |
| 07-Aug-14 | PV | plankton net | 0 | 0 |
| 16-Aug-14 | PV | bulb syringe | 560 | 0 |
| 16-Aug-14 | PV | plankton net | 416 | 30 |
| 24-Aug-14 | PV | bulb syringe | 11,203 | 9 |
| 24-Aug-14 | PV | plankton net | 780 | 37 |
| 31-Aug-14 | PV | bulb syringe | 6465 | 0 |
| 19-Sep-14 | PV | bulb syringe | 3108 | 0 |

[a] Dry weight was not measured for this sample. However, the LC–MS detected MC, but no ANTX, in the sample.

## 4. Discussion

### 4.1. Processes responsible for regulating buoyancy

Unlike planktonic cyanobacteria, in which buoyancy is regulated via intracellular processes including formation of gas vesicles (Walsby et al., 1991) and carbohydrates (Visser et al., 1997), in

benthic *Anabaena* mats, the results of this study indicate that buoyancy is regulated by photosynthetic oxygen production and respiration. Oxygen bubble production by photosynthesis has been suggested as responsible for algal lift in rivers (Mendoza-Lera et al., 2016), for laboratory-generated bloom formation in planktonic cyanobacteria *Microcystis* after gas vesicle collapse (Dervaux et al., 2015), and for persistent buoyancy of *Microcystis* colonies leading to cyanobacterial scums on lakes (Medrano et al., 2016). For benthic *Anabaena* bubbles cause floating and sinking to occur within minutes and flotation to be maintained for days.

Light experiment results show that in low light, the number of macroscopic bubbles decreased, but under ambient diel summer light regimes, bubble numbers initially increased on day 1, and then numbers remained relatively constant for the duration of the experiment. Indeed, Bosak et al. (2010) showed bubbles could be stable for weeks in cyanobacterial mats. The buoyancy experiments demonstrated the direct flotation of clumps collected in mid-afternoon on a sunny summer day, as well as the flotation within minutes upon exposure to natural sunlight of negatively buoyant clumps held for 12 h in darkness. Intracellular buoyancy mechanisms cannot explain the *Anabaena* flotation observed in these experiments, which were dominated by *Anabaena cylindrica*. Indeed, benthic *Anabaena cylindrica* and *Anabaena oscillarioides* do not form gas vesicles (Komarek, 2013; Li et al., 2016), and no intracellular gas vesicles in *Anabaena* trichomes were observed under microscopic examination. Over the short time scale of minutes when flotation was observed to occur, there would not be time for either lipids nor carbohydrates to be produced, as both types of molecules fluctuate at longer, daily, time scales (Chu et al., 2007; Ibelings et al., 1991).

In the graduated cylinders, the artificial manual removal of bubbles led to a sinking rate of clumps between 0.3–0.9 cm $s^{-1}$ (Fig. 6). This suggests that without the macroscopic oxygen bubbles, *Anabaena* clumps will sink at speeds of the order of 1 cm $s^{-1}$. As with flotation, intracellular processes cannot account for the rapid shift from floating to sinking. As *Anabaena* clumps float downstream, bubbles are most likely removed through physical disturbance, rather than cellular respiration or gas diffusion through the extracellular mucus. It is therefore likely that bubble removal primarily occurs through hydraulic turbulence, such as when clumps flow through a riffle. Though respiration consumes oxygen in the bubbles and decreases buoyancy, the light experiment results show that under a natural summer light regime, oxygen consumption from respiration is not sufficient to induce sinking. Only under several days of low light, when photosynthesis is suppressed, do respiration and diffusion affect buoyancy enough to cause sinking (Fig. 4). However, the positive day by treatment interaction effect from the glmm model was not statistically significant ($p = 0.092$), suggesting no difference in flotation between light treatments. This statistical result was likely due to low replication and less power inherent to binomial models. Considering that the direction of all parameter estimates matched our hypotheses and that there was minimal variation in the floating or sinking response of clumps in the treatments, with more replicates or a longer experiment it is expected that the interaction would be statistically significant.

Although these data were not sufficiently precise to test the size dependence of the clumps on the floating and sinking rates, qualitative observations indicated that smaller clumps of equivalent algal composition sank faster than larger ones. If they are spherical, larger clumps of similar density sink faster than smaller ones, therefore this result suggests that the elongated shape of the clumps, which acts as resistance to floating and sinking, is more important than the actual size of the clumps in accordance with the modified Stoke's law (Fraisse et al., 2015; Jaworski et al., 1988; Padisak et al., 2003; Walsby and Holland, 2006).

Attached *Anabaena* mats *in situ* consistently have a spire-like morphology with visible bubbles in the spires (Fig. 2). The processes that initiate flotation remain unknown. One likely explanation is that as growth rates increase, photosynthetic oxygen production creates enough bubbles that the buoyancy force exceeds the tensile strength of part of the *Anabaena* clump or entangled attachments of host *Cladophora*, detaching it from the substrate. *Anabaena* spires are fragile and easily detached by hydraulic turbulence or other physical disturbance. For example, walking slowly through a large proliferation will generate pressure waves that detach many spires, even meters away.

### 4.2. Cyanotoxin productions

Results from this study provide further evidence for the presence of microcystin and anatoxin-a in the Eel River watershed. Freshwater benthic *Anabaena* have been documented to produced microcystins (Mohamed et al., 2006), but the authors are not aware of published studies of benthic freshwater *Anabaena* producing anatoxin-a. The floating samples analyzed for cyanotoxins were not pure cultures of *Anabaena*, and so it is possible that other taxa could be producing anatoxin-a. Though the occasional *Oscillatoria*, *Cylindrospermum*, or *Nodularia* may occur, based on microscopic observations *Anabaena* was >100x more abundant than other cyanobacterial taxa in the samples. Given the high concentrations of anatoxin-a in the samples, it is unlikely that they would originate from other cyanobacterial taxa than *Anabaena*. Creating pure cultures of *Anabaena* spp. from the Eel River and testing them for cyanotoxins will be necessary to definitively conclude that *Anabaena* are producing anatoxin-a. Additionally, with the presence of anatoxin-a in the watershed confirmed, future sampling could investigate the presence of the homologue homoanatoxin-a and the anatoxin-a degradation product dihydroanatoxin-a in the watershed (Osswald et al., 2007). The results from these samples supports previous data, which identified anatoxin-a poisoning as the cause of dog deaths in the Eel River watershed (Puschner et al., 2008), though the cyanobacterial species producing anatoxin-a were not identified in that study.

### 4.3. Consequence for dispersal

Since entrapped oxygen bubbles prevent *Anabaena* from sinking, *Anabaena* will likely travel further downstream than less buoyant algae. The light experiment showed that after four days of floating, *Anabaena* trichomes appeared healthy. If growth rates in floating *Anabaena* mats remain high, then instead of *Anabaena* being isolated in discrete benthic mats throughout the watershed, the release of floating clumps from mats results in a semi-continuous presence at the water's surface at the kilometer scale. Therefore, when floating clumps do eventually sink after travelling through riffles which have been observed to dislodge bubbles, they could grow and form a new benthic *Anabaena* mat at that location. This phenomenon of new colonization could contribute to the widespread distribution of *Anabaena* mats in the SF Eel watershed in summer. Additionally, the downstream fate of floating *Anabaena* clumps will likely be controlled by hydraulics and winds. Clumps advected by currents and blown by winds will tend to accumulate along channel margins and in backwater eddies. Once trapped, cells could become stressed although accumulations of floating Anabaena have been observed to last several weeks. Considering that floating *Anabaena* clumps contain anatoxin-a (Table 1), flotation also poses public health concerns, since clumps accumulate in slow flowing pools, including popular recreational swimming locations, and channel margins. Freshwater cyanotoxins can also affect nearshore marine ecosystems (Gibble and Kudela, 2014; Miller et al., 2010) and flotation increases the

probability of cyanobacteria being transported from rivers to oceans. Future research on downstream dispersal could use 2-dimensional hydraulic models of river profiles to estimate the number of riffles/km or backwater pools, and produce flow-specific estimates of the effect of riffles on removing bubbles and effects of backwater pools on entraining floating mats over specific mapped reaches. Understanding how buoyancy and dispersal mechanisms differ between benthic and planktonic cyanobacteria is needed to manage for public health and water quality in freshwater environments where toxic benthic cyanobacteria occur.

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