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Identification of *Planktothrix* (Cyanobacteria) Blooms and Effects on the Aquatic Macroinvertebrate Community in the Non-Tidal Potomac River, USA

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ABSTRACT

Using transverse cross-sectional transects, a survey of 31 km of the non-tidal Potomac River was conducted from White's Ferry, Virginia, to Brunswick, Maryland, USA, between June and September in 2013 through 2015 to assess a recurring benthic cyanobacteria bloom. Abundant benthic cyanobacteria blooms were detected during the 2014 and 2015 sampling seasons and the primary taxon was identified morphologically and molecularly as *Planktothrix* cf. *isothrix*. When present, *P*. cf. *isothrix* blooms were concentrated from river center to the Maryland shoreline. This pattern was correlated with significantly greater benthic chlorophyll-*a* and phycocyanin concentrations. In an apparent response to the *P.* cf. *isothrix* blooms in the study site, aquatic macroinvertebrate community assemblages were significantly different between areas with extensive benthic cyanobacterial growth compared to areas without cyanobacterial growth. Within the *P.* cf. *isothrix* mats, the percentage of pollution sensitive taxa was lower, and the percentage of pollution tolerant taxa was greater. These data suggest that *P*. cf. *isothrix* can act as an ecosystem disruptor through direct impacts to the aquatic

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macroinvertebrate abundance and community structure within this section of the freshwater, non-tidal Potomac River.

Keywords: aquatic insects, ecosystem disruption, harmful algal bloom, Hilsenhoff Biotic Index, water quality

INTRODUCTION

The role of aquatic macroinvertebrates in the ecosystem is well documented. In general, aquatic macroinvertebrates play crucial roles in riverine nutrient and carbon cycling as they are major contributors both as consumers and as prey (Wallace and Webster, 1996; Malmqvist, 2002) and are a valued source of nutrition in recreational fisheries (Losey and Vaughan, 2006). Aquatic macroinvertebrate community composition can be influenced by multiple factors such as physical habitat, flow, temperature, geomorphology, and land use (Malmqvist, 2002; Ashton et al., 2014). Because of these predictable influences, aquatic macroinvertebrates have proven to be reliable and comprehensive bioindicators of water quality due to individual tolerance and sensitivity thresholds (Mandaville, 2002).

Aquatic macroinvertebrate communities have been monitored annually within the non-tidal Potomac River watershed since 1976 by the Maryland Department of Natural Resources (MD DNR) (Friedman, 2009; Saville et al., 2014). Analyses of aquatic macroinvertebrate communities at sites within the non-tidal Potomac River basin sampled between 2000 through 2014 categorize the communities as "good" using both the Hilsenhoff Biotic Index (HBI) and the Index of Biotic Integrity (IBI) (Friedman, 2009; Saville et al., 2014). However, these broad surveys have not looked at the discrete relationships that may exist between the aquatic macroinvertebrate community and microhabitats.

While nutrient loading of freshwater systems is a worldwide problem with implications for both water quality and ecological integrity, reductions in both nitrogen (N) and phosphorous (P) have been well-documented for the freshwater Potomac River over the past three decades (Langland et al., 2012; Moyer et al., 2017). However, current nutrient levels can still promote the nuisance growth of aquatic plants (including cyanobacteria, algae, and submerged vegetation), especially in areas of the freshwater Potomac River where the shallow depth profile permits sunlight to contact the substrate (Moyer et al., 2017). Recently, in the lower non-tidal Potomac River algal blooms of a filamentous cyanobacteria species that cycles from the benthic to pelagic environment have resulted in unsightly and odorous mats that discourage recreational use.

On the United States west coast, benthic cyanobacteria blooms reported in several rivers have been responsible for domestic animal mortalities (Puschner et al., 2008; Bouma-Gregson et al., 2017) and have contributed to biotoxin loads in local and downstream waters (Fetscher et al., 2015). There is also increasing evidence that many cyanobacteria blooms negatively impact aquatic life by reducing community diversity and survival rates, resulting in increased impairment designations (Aboal et al., 2000; Suren et al., 2003a; Oberholster et al., 2008; Tourville-Poirier et al., 2010; Anderson et al., 2018). Globally, many cyanobacteria species cause concerns for human

health when they occur in high concentrations and/or produce toxic or taste and odor compounds (Paerl and Otten, 2013).

Proper identification of cyanobacteria bloom species is required to understand the risk these blooms pose to human and environmental health as different species can produce different toxins under different environmental regimes (Harke et al., 2016; Bouma-Gregson et al., 2019). The identification of cyanobacteria has traditionally been based on morphological and ecological characteristics; however, as morphological characteristics can be variable depending on environmental conditions (Komárek and Anagnostidis, 2005), recent investigations have recommended the incorporation of genetic sequence analyses to confirm identifications (Dvořák et al., 2015; Gaget et al., 2015).

This is the first report of *Planktothrix* cf. *isothrix* blooms in the non-tidal, freshwater portion of the Potomac River. An investigation along a 31 km stretch of the lower non-tidal Potomac River was conducted over the course of three summers (2013-2015) to: 1) confirm the identification of this benthic to pelagic cyanobacteria species using morphological, ecological, and phylogenetic characteristics; 2) determine the spatial and temporal extent of the benthic stage of this cyanobacteria species within the study area; and 3) examine changes to the local aquatic macroinvertebrate community in the presence of this benthic cyanobacterium.

MATERIALS AND METHODS

Study Area

The non-tidal Potomac River has a watershed which drains roughly 29,900 km², encompassing Pennsylvania, Maryland, West Virginia, and Virginia. The Chesapeake and Ohio (C&O) Canal parallels the non-tidal portion of the Potomac River in its entirety, offering a forested and protected buffer along the Maryland border. Adjacent borders to the river in West Virginia and Virginia are privately or commercially maintained and offer much less riparian protection. While sediment and nutrient loadings have been improving compared to previous decades, eutrophication and sedimentation continue to be problematic in this system (Murphy et al., 2011; Langland et al., 2012; Moyer et al., 2017). The non-tidal Potomac River is Maryland's most popular non-tidal fishing destination (MD DNR, 2017) and is widely used for other recreational activities.

Field Study Design

To investigate the impact of benthic cyanobacteria blooms on the aquatic macroinvertebrate community three transverse cross-sectional transects were established along a 31 km stretch at three geographical locations in the lower section of the non-tidal Potomac River: White's Ferry, Point of Rocks, and Brunswick (Fig. 1; Appendix Table 1). Each transect contained three plots: one 20 m from each shoreline (Maryland [MD] and Virginia [VA]), and river center (RC). Each plot was sampled biweekly by boat from May through September (2013 – 2015) and the following water quality variables were recorded: water temperature (^{o} C), conductivity (µS cm^{-3}), dissolved oxygen (DO, mg L^{-1}), pH, and the concentrations (as relative fluorescence units, RFU) of the accessory pigments chlorophyll-*a* (chl-*a*) and phycocyanin (PC) using a pre-calibrated YSI

EXO 1 sonde (YSI Incorporated, Yellow Springs, OH, USA) positioned horizontally on the river bottom. To estimate percent coverage of the cyanobacteria mats, a 1.0 m² quadrat was tossed out from the sampling points along the transect (Appendix Table 1) and the percent of the quadrat covered by cyanobacteria was visually estimated following the method described by Steneck and Dethier (1994).

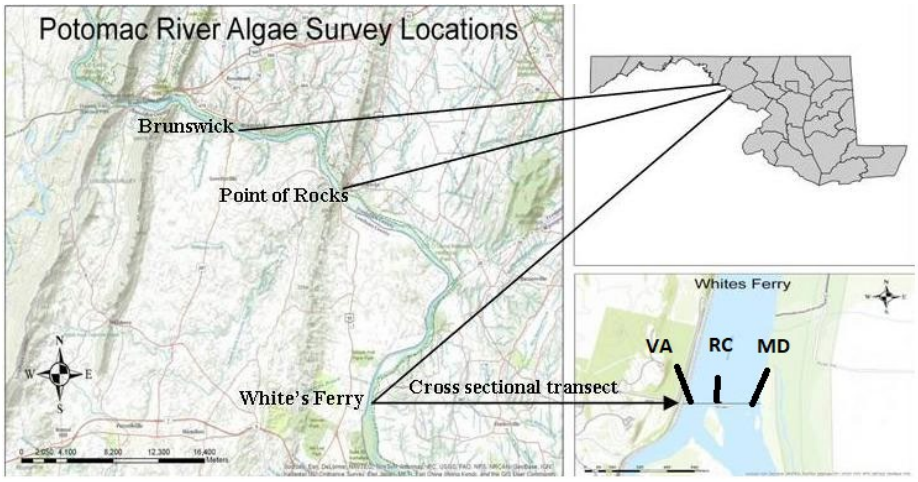

FIGURE 1. Survey locations in the lower non-tidal Potomac River, with an example of a cross-sectional transect used in the 2013-2015 survey.

Cyanobacteria Identification

Benthic algal mat samples collected in July 2014 from the Brunswick, Point of Rocks, and White's Ferry areas of the Potomac River were examined using a Zeiss Axiovert 200 inverted light microscope (Carl Zeiss, Thornwood, NY, USA) to determine the genus identification of *Planktothrix*, a filamentous, non-heterocystous cyanobacteria. Wet mounts of live and formalin-preserved material were examined at 400x and 640x magnification using the morphometric characteristics outlined by Suda et al. (2002) and Komárek and Anagnostidis (2005). Photomicrographs were acquired using an Olympus DP73 digital camera (Olympus America, Center Valley, PA, USA) for comparison with other known *Planktothrix* species. Throughout the course of the study benthic algal mats were collected at each sampling site to confirm the presence of *Planktothrix* using microscopy.

A 10 mL subsample from the benthic algal mat collected from Brunswick, MD in July 2014 was centrifuged at 15,000g to form a pellet of cyanobacterial cells. Total DNA was extracted from the pelleted cells following the manufacturer protocol for TRIzol (Invitrogen, Carlsbad, CA, USA). Universal cyanobacteria primers CYA106F and CYA781R (Appendix Table 2; Nübel et al., 1997) were used to amplify the 16S region of the rRNA gene cluster of *Planktothrix*. End point polymerase chain reaction (PCR) settings for *Planktothrix* amplification were 35 cycles at 94°C for 60 s, annealing for 60 s at 60°C, followed by extension at 72°C for 1 min. The presence of a ~616 base pair (bp) PCR product was confirmed using a 1% agarose gel electrophoresis and visualized using ethidium bromide staining. The PCR products were purified and cloned into

chemically competent *E. coli* using the Topo TA cloning vector (Invitrogen). Plasmid DNA was purified using the GenElute Plasmid Miniprep Kit (Sigma-Aldrich, St. Louis, MO, USA). Plasmid DNA was sent to the University of Maryland Center for Environmental Science at the Institute of Marine and Environmental Technology (Baltimore, MD, USA) for Sanger sequencing. The resulting 16S rRNA sequence was deposited in the National Center for Biotechnology Information (NCBI) GenBank with Accession number MH299785.

To identify the species of Potomac River *Planktothrix*, the resulting 16S rRNA sequence was compared to other known *Planktothrix* sequences (Appendix Table 3) using nucleotide BLAST (Altschul et al., 1990) to search the NCBI nonredundant database. Species were compared by creating a multi-alignment using the 616 bp region present in the sequenced clone and available sequences in GenBank using Clustalw. The alignment, formatted with the Boxshade 3.21 -ExPASy portal (Corpet, 1988; Artimo et al., 2012), indicated there was not a 100% match between the Potomac River *Planktothrix* and other known *Plankothrix* species. Using the 16S rRNA sequences, a phylogenetic consensus tree was constructed based on maximum likelihood (ML), maximum parsimony (MP), and neighbor joining (NJ) methods in Mega 7 (Kumar et al., 2016) to determine the relationship between the *Planktothrix* species. *Nostoc punctiforme* was used as an outgroup when constructing the consensus tree. The ML analysis was performed based on the Jukes-Cantor model, using all sites, with 1,000 bootstrap resampling per Kumar et al. (2016). The MP tree was based on the subtree-pruning regrafting algorithm with a search level of 1, with 10 random additions of sequences and 1,000 bootstrap replicates (Nei and Kumar, 2000). The NJ tree was computed with the Jukes-Cantor model and 1,000 bootstrap replicates.

Toxin Assays

Planktothrix benthic mat and overlaying water column samples from Brunswick, MD (29 July 2015) and White's Ferry, MD (17 August 2015) were collected and frozen at -20˚C to use for the analysis of the toxin microcystin. The frozen samples were subjected to three freeze/thaw cycles before using a commercially available enzyme-linked immunosorbent assay (ELISA) kit (#PN 520011, Eurofins Abraxis, Warminster, PA, USA) that tests for the ADDA moiety of microcystin. The ELISA toxin assays were performed according to the manufacturer's protocol.

Aquatic Macroinvertebrates

In 2013 and 2014 aquatic macroinvertebrate samples were collected once per month (June – September) at each survey point to identify possible relationships between macroinvertebrate populations and *Planktothrix* blooms. Aquatic macroinvertebrates were collected using three 30-second kicks at each sample plot (MD, VA, RC) according to the methodology described in MD DNR (2017). Briefly, sampling began at the downstream end of the plot and proceeded upstream. Using a 600-micron mesh D-net, three 30-second kicks were conducted by positioning the net and disturbing the bottom directly upstream of the net, using a kicking and stomping motion of the foot, dislodging the upper layer of cobble or gravel and scraping the underlying bed. The net contents were then placed into a 5-gallon bucket of water. Bucket contents were sieved through a three-sieve sorting system composed of a 13.2 mm, 1 mm, and 600 μm mesh sequence (U.S. Standard Sieves, W.S. Tyler, Inc., Mentor, OH, USA). Aquatic macroinvertebrates were handpicked in the field from each sieve and combined into vials containing 90% isopropyl alcohol

so that one sample was obtained for each sample plot (MD, VA, RC). The term macroinvertebrate for this study references those animals > 600 µm. The aquatic macroinvertebrates were identified at 100x using an Olympus 10x/22 dissecting microscope (Olympus America). Specimens were identified to the lowest taxon possible based on Merritt and Cummins (1984), Pennak (1978), Stewart and Stark (1988), and Wiggins (1977).

To investigate the impact of the benthic stage of this cyanobacterium on the aquatic macroinvertebrate community, we compared samples collected in areas with high cyanobacteria coverage to those devoid of cyanobacteria growth during the 2015 bloom season. Using the same aquatic macroinvertebrate collection procedures outlined above, during the cyanobacteria bloom peak in 2015 we collected samples within cyanobacteria mats and outside of cyanobacteria mats (n=6) along the Brunswick transect.

Field Data Analysis

The following calculations were used to summarize aquatic macroinvertebrate community data, per site along the transect each month:

1) Richness = number of species or taxa in the sample.

2) HBI = Hilsenhoff Biotic Index, formula as modified in Bode (1988).

Tolerance values were adapted from Maryland Biological Stream Survey (Southerland et al., 2005), assigning tolerance values to each species. The HBI formula calculates a rating value for the whole sample using the following:

$$HBI = \sum^{s} (n_t * T_t) / N$$

where s = taxa in the sample; N = total number of individuals in the sample, or sample size; n_t = number of specimens in each taxon; T_t = tolerance value for each specific taxon. The HBI values and interpretations are given in Appendix Table 4.

3) Diversity = the dispersion of the specimens among species in the sample. The analysis is derived using the Shannon-Wiener (US EPA, 1973) formula as follows for diversity of individual species:

$$H' = \sum pi\,(\ln\,(pi))$$

Where pi = relative abundance of each group of organisms; values above 3.00 indicate undisturbed waters while those less than 1.00 are severely degraded.

4) Equitability = results derived from Shannon-Wiener (H') are used as the numerator in the Shannon-Wiener based equitability formula to obtain a comparative analysis among the samples (species evenness). The equitability formula is as follows:

J = H' / H' max, where H' max = ln S (S = # of species)

These formulas compare the theoretical number of species associated with each diversity measure to the actual value. Equitability is measured on a scale of 0 to 1. The closer to 1, the closer the sample comes to the theoretical maximum. In general, the higher the equitability value, the healthier the population.

5) EPT Index Number = number of specimens in orders Ephemeroptera, Plecoptera, and Trichoptera in the sample. Reported as %EPT, these specific taxa are indicative of more pristine ecosystems, with larger numbers indicating healthier ecosystems (Lenat, 1988).

6) Functional Feeding Group Classification (FFG) = adapted from Maryland Biological Stream Survey (Southerland et al., 2005).

A principal component analysis (PCA) was performed on the water quality/accessory pigment data to help identify the years when cyanobacteria blooms occurred. The analysis generated axes of correlated habitat variables that represented the greatest spatiotemporal variation in water quality for this dataset. Following analysis of the Scree Plot, the first three principal component axes were retained for subsequent analysis because they represented the majority of variance in the dataset. Spatial and temporal differences in water quality were tested using a two-way analysis of variance (two-way ANOVA) of PCA scores (dependent variable) because cyanobacteria blooms differed in intensity across years and locations in the river. Location of sample (i.e., MD, RC, and VA) and year (2013, 2014, 2015) were independent variables.

Aquatic macroinvertebrate relative abundances were analyzed using ordination analyses to identify patterns of change in relation to *Planktothrix* blooms. Within-year changes in aquatic macroinvertebrates were examined with a non-metric multidimensional scaling analysis (NMS). This exploratory approach allowed for the analysis of the data and for testing the hypothesis that aquatic macroinvertebrate communities differed significantly in the presence of *Planktothrix* blooms. Prior to NMS analysis, aquatic macroinvertebrate abundances were transformed by the square-root to minimize variance. A general relativization by species, which is appropriate when using Sorensen (Bray-Curtis) distances to quantify site-specific dissimilarities in species abundances, was used. Ordination analyses were performed with PC-ORD for Windows (McCune and Mefford, 2018). Plots of NMS axes that fit the data well (i.e., had low stress) were examined to visualize changes in community structure throughout summer (June – September) and among sampling locations (MD, RC, and VA). To determine if community structure differed between years, which differed in bloom intensity, a perMANOVA was used to test for the effect of year. PerMANOVA is a non-parametric, multivariate analysis that computes sum of squares from distances among sample sites in a balanced sampling design (Anderson, 2001). The probability that an F-value from a randomized dataset was greater than or equal to that observed was computed from 10,000 permutations of randomizing the dataset.

Three sites were sampled for macroinvertebrates in 2015 where *Planktothrix* blooms occurred, and these data were compared with similar data from nearby areas where blooms did not occur. These data were used to qualitatively compare percentages of relative abundances of taxa between habitats. Additionally, multi-response permutation procedures (MRPP) were used to test whether communities differed between areas with blooms and those with no blooms. Similar to perMANOVA, MRPP is a nonparametric test that uses randomization to compare parameters from real datasets to those from null datasets that are expected from randomization. The MRPP was done with unweighted, non-relativized data and Bray-Curtis distances. Significant difference in community structure between habitats with and without *Planktothrix* blooms was determined with a test statistic (T) that represents the difference between the groups. The T-value, resulting from

observed and expected within group average Bray-Curtis distances, and the probability of obtaining that within-group distance from a randomized data set (p) were computed. Within-group agreement (A) is a measure of effect that is usually less than 0.1, with values of 0.3 considered high (McCune et al., 2002).

One-way analysis of variance (one-way ANOVA) was used to determine differences among individual water quality values and aquatic macroinvertebrate EPT and HBI results. Tukey-Kramer post-hoc tests were used to interpret results computed from one-way ANOVA.

A bivariate linear regression model was used to determine the relationship between water temperature and *Planktothrix* blooms, where PC values were the dependent variable. This model type was also used to illustrate the relationship between aquatic macroinvertebrate functional feeding groups (dependent variable) and PC values. Individual functional feeding group concentrations were log-transformed prior to analysis.

RESULTS

Cyanobacteria identification

The Potomac River bloom-forming cyanobacterium was identified as *Planktothrix* cf. *isothrix* based on morphology and life history. Diagnostic morphological characteristics of this cyanobacterium were straight, non-attenuating trichomes with rounded apical cells and slight constrictions at the crosswalls (Fig. 2a). Cells were shorter than wide or isodiametric with an average width of 7.2 (SD = 0.36) µm and length of 3.9 (SD = 0.49) µm (n = 40). Blooms initiated as dark green tufts attached to the benthos (Fig. 2b) before becoming free-floating in the water column, similar to the benthic to pelagic life history reported for *P. isothrix* in other locales (Komárek and Anagnostidis, 2005).

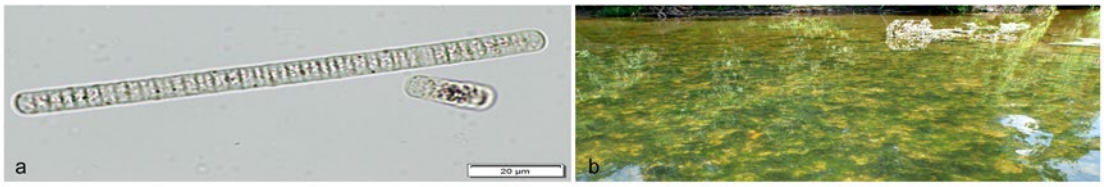

FIGURE 2. Light micrograph of *Planktothrix* cf. *isothrix* trichomes (a) and dark green tufts of *P.* cf. *isothrix* forming benthic mats in the Brunswick area of the Potomac River (b).

Additionally, a 616 bp region of 16S rRNA from the Potomac River *Planktothrix* was compared to known *Planktothrix* sequences. The BLAST sequence multi-alignment (not shown) indicated a 98% match between the Potomac River *Planktothrix* (MH299785) and *Planktothrix* sp. (strain UVFP1; AJ630646) from Spain (Camacho et al., 2005). A phylogenetic consensus tree generated using ML, MP and NJ analyses of 16S rRNA data (Fig. 3) shows the relationship between 18 known *Planktothrix* species and the Potomac River *Planktothrix*. Included in this

analysis was *Planktothrix agardhii* var. *isothrix* (AB045913; Suda et al., 2002). The Potomac River *Planktothrix* did not group with *P. agardhii* var. *isothrix* but formed a clade with *Planktothrix* sp. (strain UVFP1) from Spain (AJ630646; Camacho et al., 2005). Suda et al. (2002) synonymized their *P. agardhii* var. *isothrix* strains with *P. agardhii* based on morphological characteristics and a lack of genetic differentiation. Other *Planktothrix* species with benthic to pelagic life cycles, *P. clathrata*, *P. cryptovaginata*, and *P. tepida*, were not genetic matches with the Potomac River species. To our knowledge no genetically confirmed specimens of *P. isothrix* are available for comparison, hence the designation here as *P*. cf. *isothrix*.

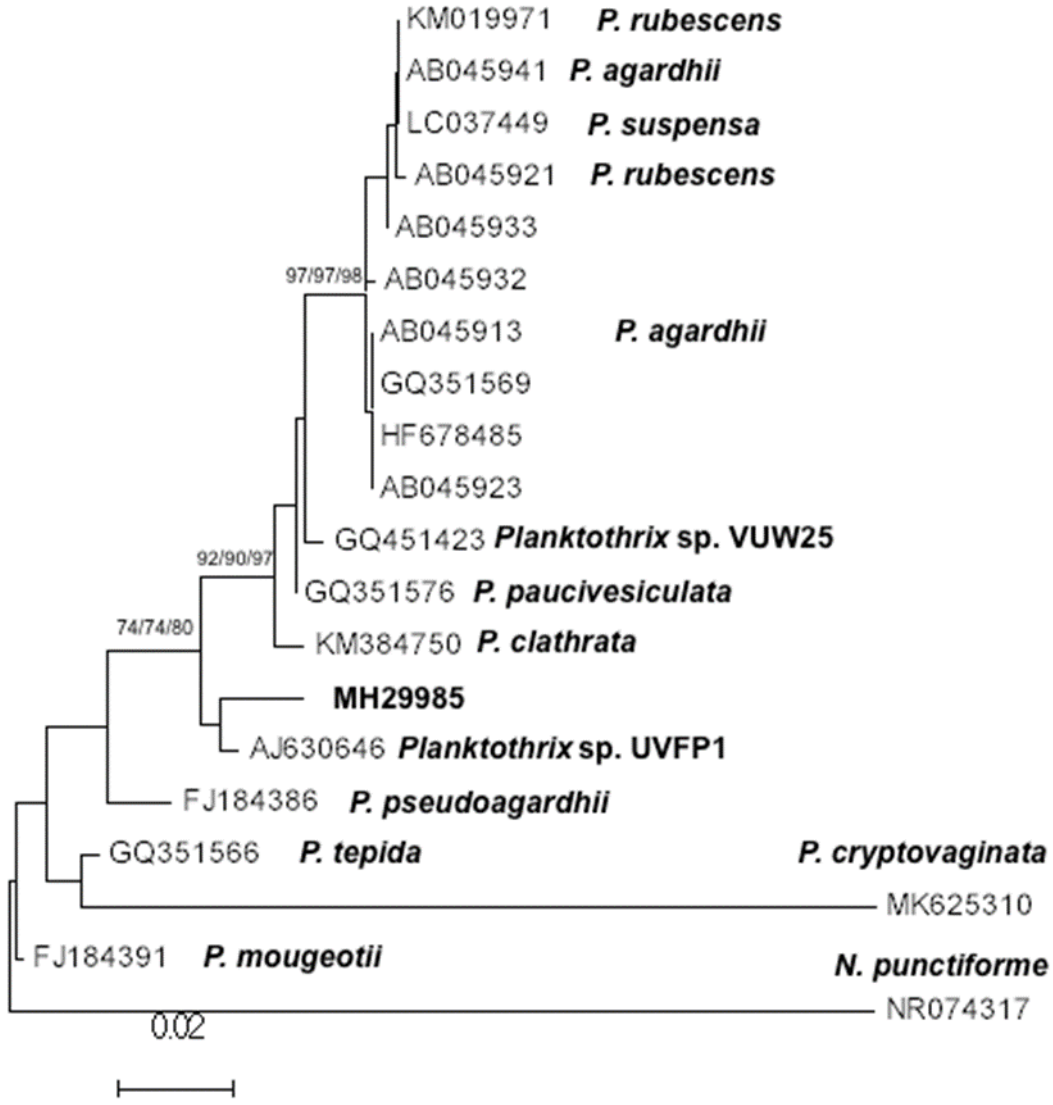

FIGURE 3. Maximum likelihood phylogeny of the 16S ribosomal RNA region for 19 *Planktothrix* sequences, representing 8 species groups and one out-group (*Nostoc punctiforme*). The numbers above the branches represent ML/NJ/MP bootstraps values (each branch represents 1000 replicates). Each sequence is identified by the GenBank Accession number. Sequence from this study is in bold.

Toxin analysis

The two *P.* cf. *isothrix* mat samples collected in 2015 tested positive for the presence of the hepatotoxin microcystin. Using ELISA, trace amounts of microcystin were detected (0.49 and 0.81 μg L^{-1}, expressed per 5 g wet weight). Whole water samples collected from the water column overlaying the *Planktothrix* mats were negative for microcystin.

Planktothrix cf. *isothrix* and Water Quality Variables

The 2013 dataset represented a year when nuisance levels of *P*. cf. *isothrix* did not occur versus 2014 and 2015 when *P.* cf. *isothrix* proliferated throughout the 31 km study area in the lower, non-tidal Potomac River. Benthic blooms of *P*. cf. *isothrix* were observed (within quadrats) 12 times at both Brunswick and Point of Rocks, and five times at White's Ferry in 2014 and 2015. Estimates of quadrat coverage data are presented in Table 1. It should be noted that low visibility on the Maryland shoreline prevented accurate observations of the benthos during most of July 2014 which likely underestimated coverages. These data revealed spatial and seasonal trends in pigment concentrations (chl-*a* and PC) and *P.* cf. *isothrix* distribution/coverages (Table 1). There was a relationship between PC pigment RFU and quadrat coverage estimates (Spearman's rank correlation, $r = 0.13$; $p = 0.04$), indicating that PC pigment concentration is a viable surrogate for estimating benthic cyanobacteria coverages.

TABLE 1. Mean spatial and temporal accessory pigment concentrations (chlorophyll-*a* and phycocyanin, measured as relative fluorescence units [RFU]) and quadrat coverage estimates for Maryland (MD), River Center (RC) and Virginia (VA) sites in the non-tidal Potomac River. Data was collected between June and September of 2013 through 2015 with observation and RFU values obtained from the river bottom.

Year	Shore-line	Sample size (N)	Non-detects (# occurrences)	Algae present (# occurrences)	Max quadrat (%)	Mean quadrat (%)	Mean phycocyanin	Mean chlorophyll-*a*
2015	MD	31	17	14	90	9.8	0.52	0.40
	RC	31	23	8	65	6	0.55	0.51
	VA	31	17	14	80	7.4	0.41	0.35
2014	MD	18	11	7	35	3.9	0.53	0.3
	RC	18	11	7	50	5.8	0.58	0.42
	VA	18	15	3	40	2.8	0.36	0.01
2013	MD	36	35	1	5	0.01	0.41	-0.13
	RC	36	34	2	5	0.03	0.42	-0.09
	VA	36	29	7	50	4	0.41	-0.12

Three principal component axes accounted for 75.2% variance in the water chemistry dataset. Of those, the first principal component (PC1) accounted for 32.5% of the variance in the original dataset and was highly correlated with chl-*a* and PC (Table 2). These data illustrated significant temporal and spatial differences in a gradient highly correlated with accessory pigments associated with algal blooms (Table 3). Differences existed temporally between years, where PC values were significantly higher in 2014 and 2015 than in 2013 (two-way ANOVA: $F = 33.6$, $p = <0.0001$) (Fig. 4). Significant spatial differences, across cross sectional transects (MD, VA, and RC), were also detected in those years when blooms occurred (one-way ANOVA: 2015: $P = 0.005$; 2014: $p = <0.0001$; 2013: $p = 0.3$) (Fig. 4). The chl-*a* and PC concentrations in 2014 and 2015 differed significantly (one-way ANOVA: $F = 30.1$, $p = <0.0001$; Tukey-Kramer Post-Hoc = 0.001) between the Maryland and Virginia shorelines (Fig. 5), with higher levels measured along the Maryland shoreline.

TABLE 2. Principal component analysis (PCA) axis loadings for water quality monitoring data collected in the summers of 2013 through 2015. Bold numbers represent the two main parameters for each axis.

Water Quality Parameter	PC1	PC2	PC3
Temperature °C	-0.1340	-0.1715	**0.7264**
Conductivity µS/cm	0.1670	0.0469	**0.6529**
Dissolved Oxygen mg/L	-0.2278	**0.7142**	0.0334
pH	-0.4330	**0.5242**	0.1105
Chlorophyll-*a*	**0.6080**	0.2657	0.1576
Phycocyanin	**0.5874**	0.3359	-0.0885

TABLE 3. The two-way ANOVA results for PC1 scores (Accessory Pigment Axis – Chlorophyll-a, Phycocyanin) for water quality monitoring data collected in the summers of 2013 through 2015.

	Sum of sqrs	df	Mean square	F	P
Temporal	104.06	2	52.03	36.6	< 0.0001
Spatial	27.34	2	13.67	9.6	< 0.0001
Interaction	12.10	4	3.03	2.1	0.08
Within	245.58	243	1.42		
Total	489.08	251			

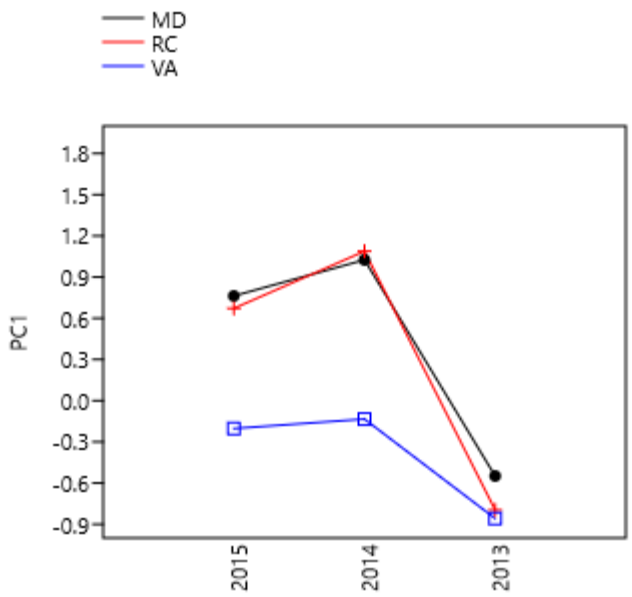

FIGURE 4. Plot of mean PC1 scores (chlorophyll-*a* and phycocyanin values) by year (2013, 2014, and 2015) and location (MD, RC, and VA). MD, RC, and VA signify Maryland, River Center, and Virginia sample locations, respectively.

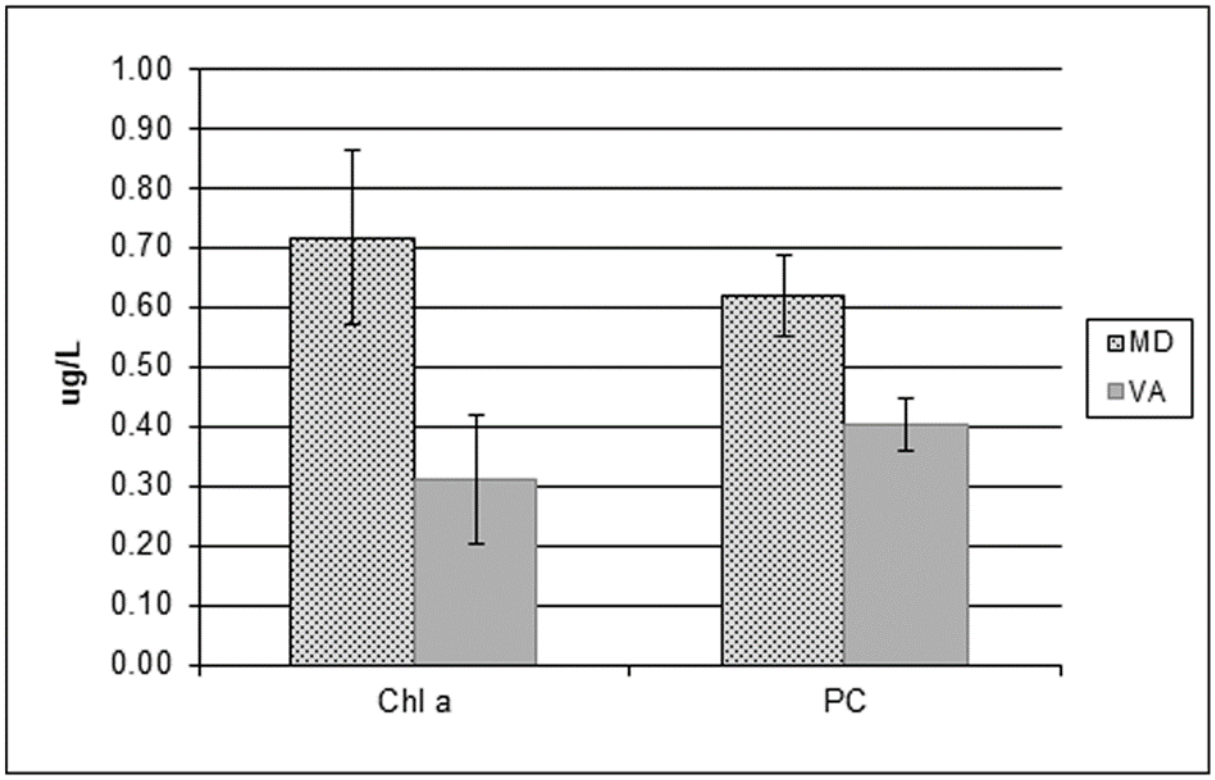

FIGURE 5. Annual mean accessory pigment values for *Planktothrix* monitoring at MD (n = 26) and VA (n = 26) Potomac River sampling stations. Error bars represent 95% confidence interval.

The second principal component (PC2) accounted for 23.2% of the variance in the original dataset. The PC2 was correlated with DO and pH (Table 2). The PC2 scores differed among years (two-way ANOVA: $F = 7.3$, $p = 0.001$) and between shorelines (two-way ANOVA: $F = 5.8$, $p = 0.004$), following similar trends to that observed in PC1.

The third principal component (PC3) accounted for 19.5% of the variance in the original dataset and was highly correlated with conductivity and temperature (Table 2). Similar to PC1, PC3 scores illustrated significant spatial (two-way ANOVA: $F = 4$, $p = 0.02$) differences. A difference in temperature and conductivity values was evident across transects. These values were significantly higher in 2014 and 2015 than 2013 (one-way ANOVA: $F = 6.1$, $p = <0.05$; Tukey-Kramer Post-Hoc: $P = 0.002$), coinciding with the trends documented for *P.* cf. *isothrix*.

Aquatic Macroinvertebrates

During the course of this study, 17,433 aquatic macroinvertebrates from 67 taxa were collected and identified (Appendix Table 5). This data showed that aquatic macroinvertebrate communities differed between early summer (June – July) and late summer (August – September) (Fig. 6) (NMS final stress = 20.41; $p = 0.004$ for first three axes). In contrast, macroinvertebrate

communities appeared to be relatively similar among locations (MD, RC, VA). Within-year differences in community structure were related to the distribution of EPT taxa and changes in species richness. The HBI values were significantly higher in June and July when compared to August and September (mean values = 5.23 [SD = 0.27] and 4.24 [SD = 0.32], respectively). Percent abundance of EPT taxa was greater in August and September (average = 72%; SD = 0.09) than in June and July (average = 49%; SD = 0.09; one-way ANOVA, p = 0.0004). Taxa richness and diversity was also significantly greater in August and September (p = 0.006 and p = 0.02, respectively).

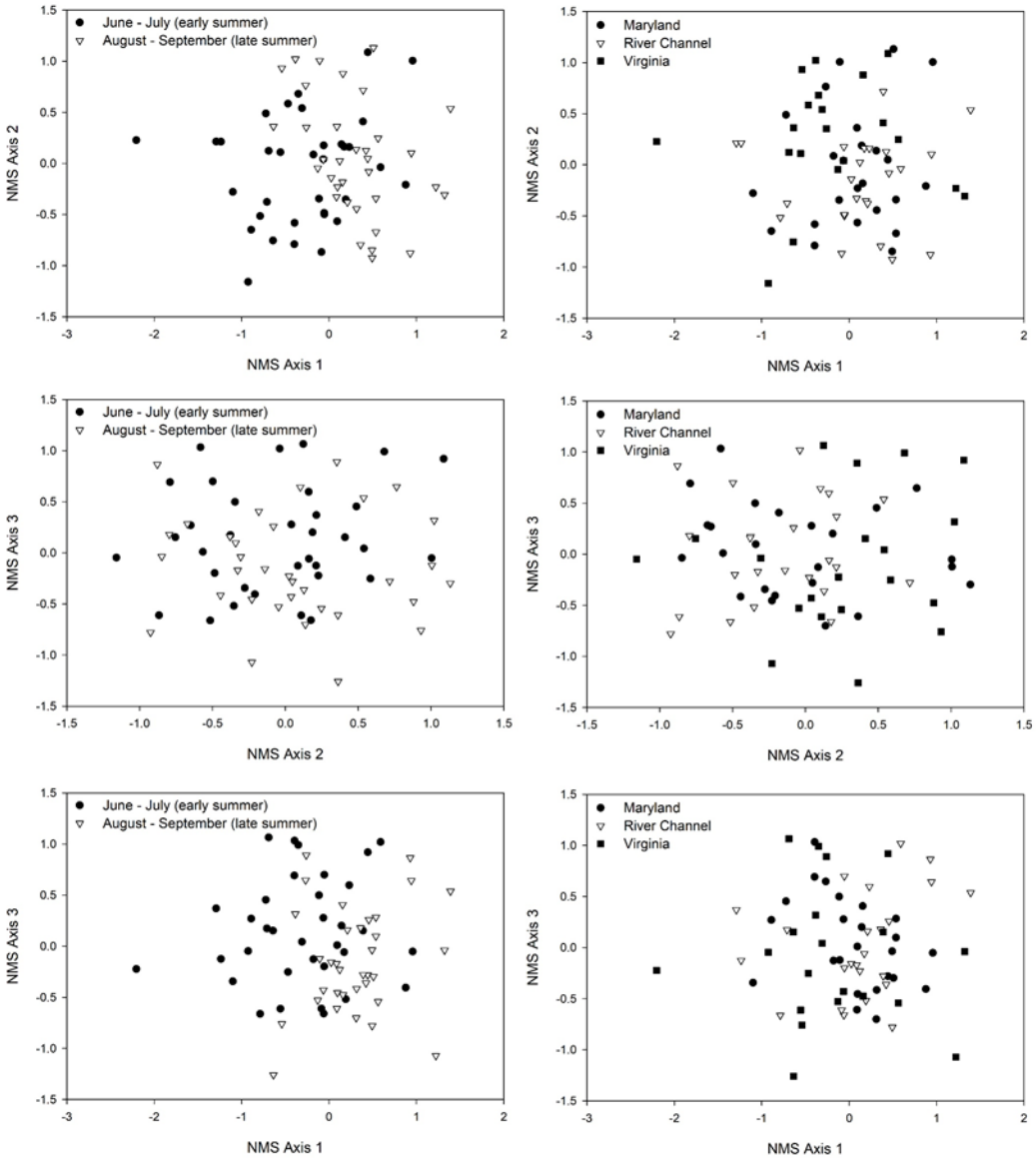

FIGURE 6. Plots of mean NMS scores (2013 and 2014). Plots on the left are temporal comparisons between early summer (June and July) versus late summer (August and September) and plots on the right are spatial (MD, RC, and VA). MD, RC, and VA signify Maryland, River Center, and Virginia sample locations, respectively.

Aquatic macroinvertebrate communities sampled during the *P*. cf. *isothrix* bloom in 2014 significantly differed from those in 2013, when no bloom occurred (F = 2.64, p = 0.001). This pattern was also evident in 2015. In 2015, macroinvertebrate communities collected from sites that experienced a *P.* cf. *isothrix* bloom differed significantly from those sites that did not experience blooms (T = -2.88, A = 0.24, p = 0.02). The %EPT and tolerant taxa differed between areas with and without benthic *P.* cf. *isothrix* blooms in 2015 (Table 4). In sites without *P*. cf. *isothrix*, the EPT taxa represented 86% of the community, whereas sites with *P.* cf. *isothrix* EPT taxa were 45% of the community. Similarly, locations without *P*. cf. *isothrix* were classified as "very good" by the HBI but only as "good" in locations with benthic *P*. cf. *isothrix* mats. When PC values indicated the presence of *P.* cf. *isothrix*, we observed proportionately more shredders than in areas without *P*. cf. *isothrix* (Fig. 7; p = 0.01).

TABLE 4. Summary of the benthic macroinvertebrate analyses for samples collected at the Brunswick, MD site in 2015. Data compares areas within the site that had a *Planktothrix* cf. *isothrix* bloom (Effect) to areas within the site that did not have an obvious *P.* cf. *isothrix* bloom (Control). Bold numbers indicate statistically significant ($p<0.05$) values. C = Control; E = Effect; BRRC – River Center station; BRMD – Maryland shoreline station.

Sample	Diversity	Equitability	Richness	Number	EPT%	Mean EPT%	HBI	Mean HBI
BRRC-C1	1.18	0.46	13	178	**88**	86%	**3.54 (very good)**	3.68 (very good)
BRMD-C1	1.79	0.72	12	152	**84**		**3.86 (very good)**	
BRMD-C2	1.99	0.72	16	98	**85**		**3.65 (very good)**	
BRMD-E1	1.68	0.64	14	180	43	45%	5.26 (good)	5.16 (good)
BRMD-E2	2.02	0.7	18	425	55		4.82 (good)	
BRRC-E1	1.94	0.7	16	163	36		5.4 (good)	

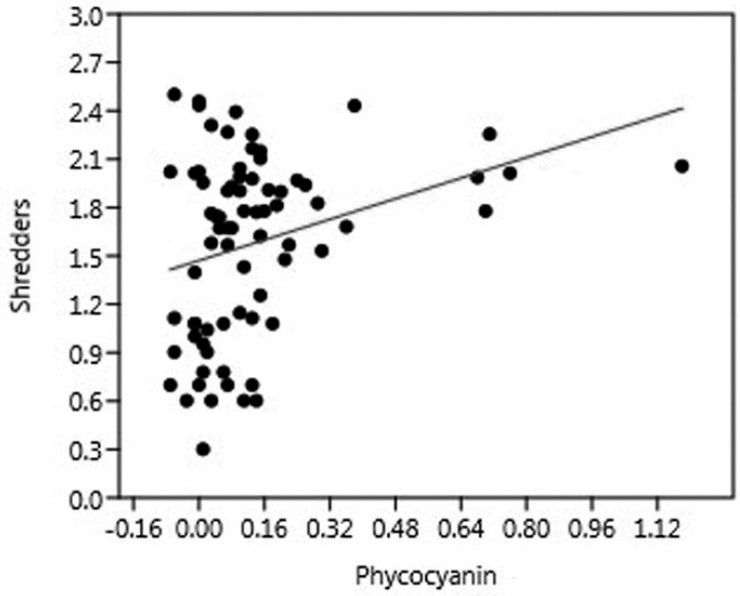

FIGURE 7. Bivariate linear regression model of the proportion of shredders (n = 5362; independent variable) and phycocyanin (n = 74; dependent variable), illustrating an increase in the shredders with an increase in phycocyanin. Data used for this analysis were collected throughout the entire sampling period (2013 through 2015).

DISCUSSION

The identification of a novel nuisance, and potentially toxic, cyanobacteria species in the lower non-tidal Potomac River was made using a combined morphological and phylogenetic analysis. This analysis identified the species as *Planktothrix* cf. *isothrix* based on the apical cell shape, cell dimensions, and a benthic to pelagic life cycle, as described by Komárek and Anagnostidis (2005). The Potomac River species is most closely related to *Planktothrix* sp. (strain UVFP1) from Spain. *Planktothrix* sp. (strain UVFP1) was reported by Camacho et al. (2005) to occur in shallow, well-oxygenated, low flow areas between Fuente Podrida spring and the Cabriel River. Similar to the *P*. cf. *isothrix* from the Potomac River, *Planktothrix* sp. (strain UVFP1) mats start to form after the spring floods subside when water levels are low, and temperatures are above 17ºC. Unfortunately, no morphological description for *Planktothrix* sp. (strain UVFP1) accompanied the report by Camacho et al. (2005). The phylogenetic relationship of the Cabriel River and Potomac River *Planktothrix*, as well as other benthic to pelagic *Planktothrix* species, should be further explored as cryptic speciation within the genus *Planktothrix* has been demonstrated by Lin et al. (2010) and Gaget et al. (2015) and documenting within-strain variability is crucial to understanding climate change driven responses (Burford et al., 2019). This appears to be the first record of *P*. cf *isothrix* in this region as only *P. agardhii* appears in species lists generated from freshwater surveys conducted by Marshall (2013; 2014). During the course of this study the benthic cyanobacteria mats appeared to be monospecific using light microscopy techniques; however, as stated by Bouma-Gregson et al. (2017), speciation within benthic habitats

has only recently become an area of focused efforts. Continued monitoring of the benthic cyanobacteria community in this region should be conducted to elucidate cryptic species or species shifts that may be the result of changes to flow regimes, temperature, light attenuation, and available nutrient pools.

Cyanotoxins are a focus of environmental monitoring programs world-wide (de Figueiredo et al., 2004; Paerl and Otten, 2013; Harke et al., 2016). These toxins are known to negatively impact aquatic macroinvertebrate abundance and community structure in lakes (Oberholster et al., 2008) and streams (Aboal et al., 2000; Wood et al., 2014b), and cyanotoxins, including microcystin, inhibit growth and cause high levels of mortality in aquatic macroinvertebrates (Ferrão-Filho and Kozlowsky-Suzuki, 2011). Within the Chesapeake Bay region, microcystins, produced by pelagic *Microcystis* blooms in the James River, have been found in emerging aquatic macroinvertebrates (i.e., EPT and chironomid genera) and in their avian (Moy et al., 2016), pelagic fish, shellfish, and blue crab (Wood et al., 2014a) consumers. Exposure to microcystins from pelagic cyanobacteria blooms have been shown to suppress fish health, behavior, development, and growth, and can cause endocrine system disruption (Ernst et al., 2006; Rogers et al., 2011; Marie et al., 2012). However, benthic cyanobacteria species are noted as different from their pelagic counterparts because of the dense and persistent nature of benthic blooms (Bouma-Gregson et al., 2017). In 2015, microcystin production by the Potomac River *P.* cf. *isothrix* populations found in mats collected at the Brunswick and White's Ferry sites was well below the US EPA Human Health Recreational Ambient Water Quality Criteria of 10 ppb (US EPA, 2016). Since 2017, limited sampling in the region suggests that *P.* cf. *isothrix* blooms have occurred annually (J. Wolny, unpublished data) and with increased toxin concentrations (C. Luckett and P. Brady, unpublished data). The threat posed to recreational users and drinking water sources from benthic cyanobacteria blooms is poorly understood and consequently guidelines are lacking (Wood et al., 2020). The intense use of the Potomac River for recreational activities (MD DNR, 2017), particularly during the summer period when *P.* cf. *isothrix* blooms, and as a source of drinking water, with > 65 water intakes servicing numerous water treatment facilities (Weidner, 2009), highlights the need for routine investigations of toxin-production by this region's benthic cyanobacteria community. Bioaccumulation and the environmental variables, such as light availability and nitrogen cycling, which influence microcystin production in *Planktothrix* species (Tonk et al., 2005; Chaffin et al., 2018), were not examined during this study but should be considered as part of future monitoring efforts to determine if toxin presence and concentration contributes to changes in the macroinvertebrate community structure that could lead to further disruption in the food web, as well as to assess human health risks.

In the absence of benthic cyanobacteria blooms in 2013, algal pigments were found uniformly distributed with increasing pigment concentrations from the study area's most upstream site to the most downstream site. However, when nuisance levels of *P.* cf. *isothrix* were present in 2014 and 2015 "hot spots" of elevated algal pigments were documented along the Maryland shoreline and river center. Observations made upstream of the study area indicate that conditions for algal blooms are present in a larger geographic area than the 31 km stretch of this study (data not shown). The "hot spot" or patchy spatial distribution of *P.* cf. *isothrix* might be explained by the nutrient contributions of tributaries and/or transition(s) of surrounding land use, in addition to

microhabitats within the river that could impact flow regime or light attenuation making conditions more or less suitable for *Planktothrix* proliferation. Our study area was too small to draw parallels with major riverine ecological models, but the significant differences between the occurrence of *P*. cf *isothrix* on the Maryland versus Virginia shorelines indicates more than just longitudinal river flow is a factor. Selckmann et al. (2018) reported similar findings when examining filamentous green algae (FGA) in an area just north of our Brunswick, Maryland site. In their study, Selckmann et al. (2018) found that FGA and an unidentified cyanobacteria bloomed on one shoreline but not the other despite there being no differences in water chemistry or habitat. The incorporation of riparian land use characteristics, climatic (specifically temperature) changes, and pulsed flood/flow events (man-made or natural) to the River Continuum Concept (Vannote et al., 1980) have been encouraged by Maiolini and Bruno (2007). Locally, Selckmann et al. (2018) and Shull et al. (2019) have demonstrated that water quality and biological communities in large rivers, such as the Potomac River, are subject not only to influence from shoreline land use but from tributaries as well and that increasing the spatial and temporal extent of samples, as well as the number of biological samples collected, in response to upstream tributaries increases the likelihood of distinguishing between impaired and non-impaired environments. An examination of a larger portion of the non-tidal Potomac River ecosystem along a broader temporal and spatial scale with these modeling concepts in mind should be conducted to better understand the environmental conditions that can promote benthic cyanobacteria blooms and changes in the aquatic macroinvertebrate community. The data collected during this geographically-limited survey showed that the local aquatic macroinvertebrate community was impacted by the presence of *P*. cf. *isothrix*. However, the extent to which both the aquatic macroinvertebrates and presence or toxicity of *P*. cf. *isothrix* was compounded by in-flow from the closely co-located Shenandoah River, which has a history of recurring algal blooms (Griggs et al., 2015), or from nutrient pools in the forested headwaters of the Potomac River was not examined.

Blooms of *P*. cf. *isothrix* proliferated in June and reached peak biomass from mid-to-late July when water temperatures were 21-28 °C, the optimal growth range reported for this species (Halstvedt et al., 2007). In addition to water temperature, blooms were also affected by stream flow. Stanfield (2018) determined that *P*. cf. *isothrix* blooms only occurred when flows were < 5,000 ft^3/sec. The genetically related *Planktothrix* sp. documented by Camacho et al. (2005) also had a preference for lower flow environments. The relationship between river flow and benthic cyanobacteria mat proliferation appears to be species-specific (see Suren et al., 2003b; Wood et al., 2017; Bouma-Gregson et al., 2019). In this region, climate change is predicted to create more intense precipitation and drought events, which will inevitably affect flow regime and the retention of N and P pools that may favor the growth of different benthic algal species (Bukaveckas et al., 2018; Reidmiller et al., 2018). This highlights the need for multi-faceted monitoring plans for ecosystems impacted by potentially harmful benthic algae and cyanobacteria.

Seasonal differences in the aquatic macroinvertebrate communities were evident throughout the study period and were largely attributed to cyclic patterns that were not directly influenced by the blooms of *P.* cf. *isothrix*. However, we found that benthic *P*. cf. *isothrix* blooms had a direct effect on the local aquatic macroinvertebrate communities as indicated with a decrease in %EPT and increased HBI designation. Freidman (2009) reported stable or improving trends in

aquatic macroinvertebrate communities from the non-tidal Potomac River. Our study suggests that impacts to the aquatic macroinvertebrate community due to the presence of *Planktothrix* may occur on a fine spatial scale, only noted when sample collections are designed to cover both within and outside of bloom patches. However, persistent alterations of the freshwater non-tidal Potomac River aquatic macroinvertebrate community, such as the decrease in %EPT and increase in HBI due to ecosystem disruptive cyanobacteria blooms and/or toxin production could have long-term, negative impacts for the recreational fisheries found here. While toxin production was low, natural mortality or emigration by aquatic macroinvertebrates in response to all aspects of the bloom could account for changes in the aquatic macroinvertebrate communities we documented. For the aquatic macroinvertebrates that remained associated with the *Planktothrix* mats, bioaccumulation of toxins could ultimately influence their own growth or have sub-lethal effects on their major predators, fishes, as has been documented with other cyanobacteria blooms by Wood et al. (2014a, b). A need for additional research regarding the total toxin production of this taxon, and any associated bioaccumulation and trophic transfer to river fauna exists and should be the focus of future efforts. Resource managers should account for the spatial and temporal distribution of benthic cyanobacteria mats when planning aquatic macroinvertebrate surveys to avoid bias and to accurately document changes that may be occurring within benthic flora and fauna communities.

Climate change and eutrophication are predicted to cause increases in the occurrence and toxicity of cyanobacteria blooms (Paerl and Huisman, 2009); future drought periods are likely to exacerbate the proliferation of cyanobacteria blooms and toxin production, which could degrade water quality and the aquatic macroinvertebrate community. If freshwater riverine systems change as predicted, then we encourage continued or even increased routine water quality monitoring coupled with detailed examinations of benthic flora and fauna in the interest of safeguarding human health and providing sound fisheries management.

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STATEMENT OF RESPONSIBILITIES

Joshua Henesy and John Mullican designed the study. All authors contributed to data collection and analysis. The first draft of the manuscript was written by Joshua Henesy and Jennifer Wolny and all authors provided edits to subsequent versions. All authors read and approved the final manuscript. Data used in this study is available at http://eyesonthebay.dnr.maryland.gov/eyesonthebay/DataInfo.cfm (accessed 10/25/2020).

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APPENDICES

APPENDIX TABLE 1. GSP coordinates for sampling locations on the Maryland shoreline (MD), river center (RC), and Virginia shoreline (VA) at Brunswick (BR), Maryland, Point of Rocks (PR), Maryland, and White's Ferry (WF), Virginia.

BRMD	39.31602N	-77.64895W
BRRC	39.31263N	-77.65401W
BRVA	39.31383N	-77.65172W
PRMD	39.27270N	-77.54175W
PRRC	39.27187N	-77.54287W
PRVA	39.27105N	-77.54327W
WFMD	39.15057N	-77.52048W
WFRC	39.15042N	-77.52261W
WFVA	39.15083N	-77.52390W

APPENDIX TABLE 2. Primer sequences used to examine *Planktothrix* from the non-tidal Potomac River.

Primer	Sequence (5' to 3')	Reference
CYA 106F	CGG ACG GGT GAG TAA CGC GTG A	Nübel et al. (1997)
CYA 781R (a)	GAC TAC TGG GGT ATC TAA TCC CAT T	Nübel et al. (1997)

APPENDIX TABLE 3. List of species, isolate designation, sampling locations and Genbank Accession numbers used in the sequence alignment analysis of *Planktothrix. Nostoc punctiforme* was used as outgroup. Sequence from this study is in bold.

Species	Isolate	Sampling location	Accession no.
P. agardhii	NIVA-CYA 11	Norway	AB045913
P. agardhii	NIVA-CYA 15	Norway	AB045923
P. agardhii	NIVA-CYA 313	Germany	AB045933
P. agardhii	NIVA-CYA 64/6	Norway	AB045941
P. agardhii	CCAP 1459/21	United Kingdom	AB045900
P. agardhii	NIVA-CYA	Norway	AB045932
P. cryptovaginata	NK1-11	China	MK625310
P. rubescens	NIVA-CYA	Denmark	AB045921
Planktothrix sp.	UVFP1	Spain	AJ630646
P. agardhii	PCC 9637	France	GQ351569
P. agardhii	CCAP 1460/133	Switzerland	HF678485
P. clathrata	PUPCCC	Northwestern Himalayas	KM384750
P. cf. *isothrix*	BRVA072915	Maryland, USA	**MH299785**
P. mougeotii	HAB626	China	FJ184391
P. paucivesiculata	PCC 8954	France	GQ351576
P. pseudagardhii	HAB2310	China	FJ184386
P. rubescens	SAG: 5.89	United Kingdom	KM019971
P. suspensa	NIES-3736	Japan	LC037449
P. tepida	PCC 9214	Central African Republic	GQ351566
Planktothrix sp.	VUW25	New Zealand	GQ451423
N. punctiforme	PCC 73102	Spain	NR074317

APPENDIX TABLE 4. Hilsenoff Biotic Index (HBI) Values and comments used for macroinvertebrate communities collected from the non-tidal Potomac from 2013-2015.

HBI Value Ranking	Comments
0.00 to 3.50 (excellent)	No apparent organic pollution
3.51 to 4.50 (very good)	Possible slight organic pollution
4.51 to 5.50 (good)	Some organic pollution
5.51 to 6.50 (fair)	Fairly significant organic pollution
6.51 to 7.50 (fairly poor)	Significant organic pollution
7.51 to 8.50 (poor)	Very significant organic pollution
8.51 to 10.00 (very poor)	Severe organic pollution

APPENDIX TABLE 5. Aquatic macroinvertebrate taxonomic list from samples collected from the lower, non-tidal Potomac River between June and September of 2013 through 2015. * indicates individuals where subsampling took place. ** signifies a lower taxonomic resolution (i.e., class instead of genera).

Order	Family (genera)	Total Abundance	Functional Feeding Group
Ephemeroptera	Potamanthidae (1)	4443	Filterer
	Baetidae (3)	1451	Collector
	Heptagenidae (4)	1063	Scraper
	Ephemerellidae (2)	12	Collector
	Ephemeridae (1)	21	Collector
	Caenidae (1)	244	Collector
	Tricorythidae (1)	1700	Collector
	Isonychiidae (1)	35	Filterer
	Polymitarcyidae (1)	1	Collector
Plecoptera	Perlidae (3)	35	Predator
Trichoptera	Hydropsychidae (4)	423	Filterer
	Leptoceridae (1)	12	Collector
	Brachycentridae (1)	28	Filterer
	Glossosomatidae (1)	44	Scraper
	Hydroptilidae (2)	11	Scraper
	Polycentropodidae (1)	6	Filterer
	Psychomyiidae (1)	11	Scraper
	Philopotamidae (1)	43	Filterer
*Amphipoda	Gammaridae (1)	5329	Shredder
Coleoptera	Elmidae (4)	1132	Scraper
	Haliplidae (1)	1	Shredder
	Dryopidae (1)	2	Scraper
	Psephenidae (2)	15	Scraper
	Gyrinidae (2)	2	Predator
Odonata	Coenagrionidae (3)	112	Predator
	Calopterygidae (1)	2	Predator
	Corduliidae (2)	5	Predator
	Gomphidae (4)	28	Predator
Megaloptera	Corydalidae (2)	9	Predator
	Sialidae (1)	23	Predator
Lepidoptera	Pyralidae (1)	6	Shredder
Diptera	Simuliidae (1)	123	Filterer
	Chironomidae (4)	800	Collector
	Tipulidae (2)	12	Shredder
	Empididae (1)	2	Predator
Platyhelminthes	Turbellaria (1)	172	Predator
**Class	**Oligochaeta (1)	45	Collector
**Class	**Hirudinea (1)	30	Predator