Scientific Research Publishing

Journal of Water Resource and Protection, 2023, 15, 413-423
https://www.scirp.org/journal/jwarp
ISSN Online: 1945-3108
ISSN Print: 1945-3094

Isolation of Picocyanobacteria (Order *Synechococcales*) and Occurrence of Cyanotoxins (Anatoxin-a) in Saline Microhabitats at Martha's Vineyard, MA

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How to cite this paper: Leland, N.J., Caseau, S., Caseau, E., Miller, J.T., Watts, A. and Haney, J.F. (2023) Isolation of Picocyanobacteria (Order *Synechococcales*) and Occurrence of Cyanotoxins (Anatoxin-a) in Saline Microhabitats at Martha's Vineyard, MA. *Journal of Water Resource and Protection*, **15**, 413-423.
https://doi.org/10.4236/jwarp.2023.159024

Received: July 16, 2023
Accepted: September 10, 2023
Published: September 13, 2023

Abstract

We have used serial filtration to isolate picocyanobacteria from brackish and marine microhabitats for analysis. We used 16s metabarcoding to confirm the picocyanobacteria as members of the Order *Synechococcales*, Genus *Cyanobium* 6307 (Upper Chilmark Pond) and differing abundances of *Cyanobium* 6307 and *Synechococcus* 9902 (Chilmark Pond, Edgartown Great Pond, Tisbury Great Pond and Tashmoo Pond). The proportion and composition of (pico)cyanobacteria in water samples were influenced by the salinity concentrations at various sites, as evidenced by fluorometry and 16s metabarcoding analysis. The cyanobacterial neurotoxin anatoxin-a was present in the picocyanobacterial samples from all studied sites. Additional analyses using fluorometry and 16s metabarcoding described members of the Order *Nostocales*, including a halotolerant population of *Dolichospermum* sp., *Sphaerospermopsis* spp. and *Nodularia* spp. in Upper Chilmark Pond. We were able to establish a positive linear correlation between cyanobacterial biomass (phycocyanin) and anatoxin-a concentrations using samples taken from Upper Chilmark Pond.

Keywords

Picocyanobacteria, Anatoxin-a, 16s Metabarcoding, Phycocyanin, Phycoerythrin

1. Introduction

Picocyanobacteria are known to be a diverse and widely distributed group of cyanobacteria that occupy a wide range of ecological niches including freshwater, brackish and marine microhabitats [1] [2]. In general, cyanobacterial populations can be described using photosynthetic accessory pigments and size-structure analysis [1] [3] to provide detailed descriptions of these populations. The picocyanobacteria can produce secondary metabolites including microcystin (MC) and its variants and B-Methylamino-L-alanine (BMAA) [4] [5]. Recent genomic [6] and 16s metabarcoding [7] analysis has confirmed that commonly found picocyanobacteria (Order *Synechococcales*) can produce anatoxin-a. As described elsewhere [7], the presence of (pico)cyanobacteria can be heavily influenced by salinity.

The purpose of this project was to verify methods necessary to collect (floatation and serial filtration) and analyze (light microscopy, fluorometry and 16s metabarcoding) cyanobacterial populations in salinity microhabitats with a focus on picocyanobacteria. The 16s metabarcoding portion of this project was utilized to determine where this method could provide "value-added" information to our interpretations of cyanobacterial population dynamics. By confirming the presence/absence of cyanotoxins, specifically anatoxin-a, at varying concentrations from these populations we hoped to develop an initial profile of the potential for exposure.

2. Materials and Methods

Field Sampling

Samples of whole lake water (WLW) and net (NET) water samples were collected from five different sites representing a range of saline environments on a series of dates between May and October from the shoreline (maximum depth 1m) (**Figure 1**), including Upper Chilmark (CHP-Up) (lat./long. 41.3419907, 70.7188816), Chilmark-2 (CHP2) (lat./long. 41.3501158, 70.6936283), Edgartown Great Pond-9 (EGP-9) (lat./long. 41.3741377, 70.5608743), Tisbury Great Pond-7 (TGP-7) (lat./long. 41.3502175, 70.6516254) and Tashmoo Pond (TSH-Sen) (lat./long. 41.4507261, 70.6240343). Samples were placed in 500 mL brown amber bottles, placed on ice and transported to the laboratory for further analysis. Size fractionation using floatation, serial filtration with gravity and serial filtration with pressure were used for sample processing and collection [7]. Samples were stored at −4˚C prior to cyanotoxin and fluorometric analysis. Samples for 16s meta barcoding analysis were collected by passing a known volume of either <5 μm, WLW or NET water samples through a Sterivex filter (0.2μm pore size) using a 50-mL syringe and stored at −80˚C prior to analysis.

Quantification of (pico)cyanobacterial biomass

All water samples were prepared, preserved, stored and analyzed as previously described [7]. Prior to fluorometric analysis, the hand-held fluorometry units were calibrated [8] for both phycocyanin (PC) and phycoerythrin (PE), accessory

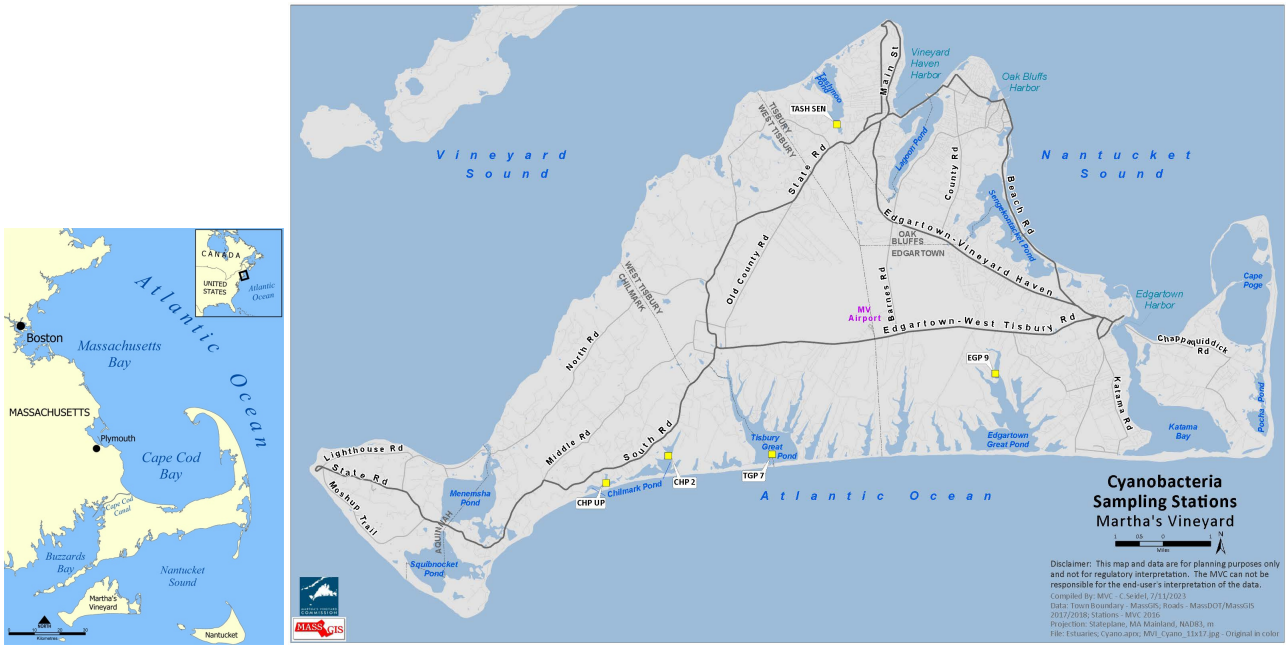

Figure 1. Location of sample collection sites, Martha's Vineyard, MA.

pigments primarily associated with cyanobacterial (phycocyanin) and picocyanobacterial (phycoerythrin) biomass. The whole lake water sample (WLW) was fractionated (<50 μm, <10 μm, <5 μm, <0.2 μm) according to the project QAPP and samples frozen for a single freeze-thaw extraction (SFT) prior to fluorometric analysis using the handheld Fluoroquik™ fluorometer (excitation wavelengths 595 nm for phycocyanin and 545 nm for phycoerythrin) using a quantitation limit for phycocyanin (PC) at 3 $\mu g \cdot L^{-1}$ and phycoerythrin (PE) at 0.1 $\mu g \cdot L^{-1}$. The 16s meta-barcoding results were used as a quality assurance/quality control (QA/QC) measure as previously described [7]. The number of reads was used as a measure of (pico)cyanobacterial abundance.

Quantification of (pico)cyanobacterial cyanotoxin

The samples for cyanotoxin analysis were collected, prepared and stored following the protocol that was used for fluorometric analysis with two additional freeze-thaw cycles Samples were vortexed on high speed for 3 minutes followed by sonication for 3 minutes to complete the triple freeze-thaw extraction process as previously described [7] Leland N.J. *et al.* 2023). Water samples were prepared for enzyme-linked immunosorbent assay (ELISA) anatoxin-a (ATX) analysis using vacuum centrifugation (*i.e.* 10X) as previously described [7]. Replicate (n = 2) anatoxin-a concentrations are reported, where the detection limit for the ELISA analysis [9] was 0.15 $\mu g \cdot L^{-1}$.

Salinity Interference with anatoxin-a ELISA analysis

Potential interference when sample concentration would be required was evaluated using a concentration series (**Figure 2**), where a stock sample of seawater at 30 ppt was prepared. The samples were placed in microcentrifuge tubes and vacuum centrifuged to the desired concentration factors of 5X, 10X and 20X

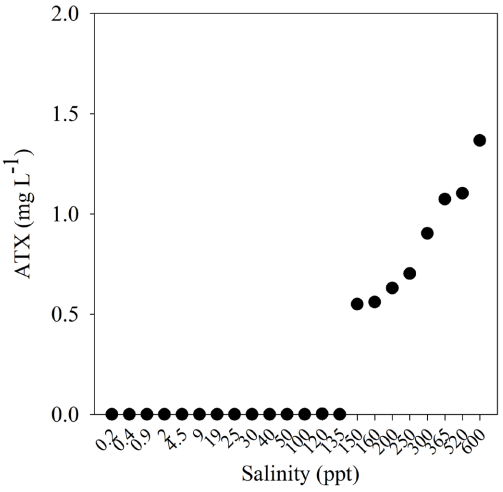

Figure 2. Validation of anatoxin-a concentrations using ELISA analysis along a salinity gradient.

for final blank salinity values that approximated 150 ppt, 300 ppt, and 600 ppt. The data confirm that salinity interference will occur for samples (*i.e.* dissolved cyanotoxins) at 30 ppt that are subsequently concentrated (5X, 10X and 20X) prior to ATX analysis. To refine of this analysis, a stock sample of seawater at 23.7 ppt was prepared, samples placed in microcentrifuge tubes and vacuum centrifuged to the desired concentrations factors of 1.2X, 2X, 4X, 5X, 6X, 7X, 8X, 10X, 12X, and 15X for final blank salinity values of 31 ppt, 50 ppt, 101 ppt, 117 ppt, 134 ppt, 162 ppt, 207 ppt, 250 ppt, 298 ppt and 365 ppt. The data confirm that salinity interference will occur for ELISA analysis of anatoxin-a at varying salinities. Specifically, we found that interference may occur for anatoxin-a at salinities greater than 150 ppt (**Figure 2**).

16s metabarcoding

The DNA was extracted from a 0.22-micron filter for each size fraction of water samples collected for this study. The total DNA for each sample fraction was amplified with 16s primer 515 – 926 R [10]. Amplicons were then sequenced on the Novaseq platform (250 bp paired-end reads). Sequences were trimmed, denoised, and assigned taxonomy with Qiime2 [11]. Each ASV (unique sequences found among all samples) was assigned a taxonomy with the sklearn algorithm using a feature classifier generated from the Silva reference database (v132, 99 OTU). To compare the total number of sequences that were assigned to either *Nostocales* or *Synechococcales*, we collapsed the taxonomic assignments down to the level of species.

Statistical data analysis

Statistical analysis of cyanotoxin and fluorometric data was performed using Sigma Plot Version 14 software. Parametric analysis (Pearson's correlation coefficients and linear regression analysis) were used to describe relationships between variables. For linear regression analysis autocorrelation (Durbin-Watson = 2.0), leverage (Studentized deleted residuals: SDR > 2) collinearity (VIF > 3), and influence (Cooks distance: Cd > 4/n and Difference in Fits: DFFits = 2 ×

sq.rt. [(p + 1)/(n − p − 1)] where n = number of observations, p = number of variables (including the constant) were examined.

(Pico)cyanobacterial detection and biomass quantification

This project used photosynthetic accessory pigments phycocyanin (PC) (**Table 1**) and phycoerythrin (PE) (**Table 2**) to quantify cyanobacterial biomass where PC is cyanobacterial specific and PE is considered a "signature" pigment for picocyanobacteria and potentially other cyanobacterial genus [12] [13]. With the exception of PC in Tashmoo Pond, all samples were above the limit of detection for both PC and PE. Analysis of cyanobacterial accessory pigments PC and PE have been used in marine environments to aid in the classification to species level [2] [14]. The range of PC concentrations (**Table 1**) for our samples was similar to those previously observed for *Dolichospermum* spp., from freshwater mesotrophic ponds [3] [7] that were confirmed using light microscopy. *Dolichospermum* sp. has been shown to tolerate salinity concentrations similar to those observed in Chilmark Pond [15] [16]. The picocyanobacteria (<5 μm) being reported here could be described as PC-rich or PE-rich based on habitat, where PC-rich are found in near-shore waters and PE-rich are more representative of open marine systems [1] [2].

Table 1. Phycocyanin concentrations (μg·L^{-1}) and salinity in water samples from Martha's Vineyard sites.

			Size fraction							
Site	Date	Salinity (ppt)	<0.2 μm	<0.2 μm SEM	<5 μm	<5 μm SEM	WLW	WLW SEM	BFC	BFC SEM
CHP- Up	8/1/2022	2.0	0.10	0.00	78.40	1.17	192.98	18.36	124.17	23.68
	8/10/2022	1.5	8.22	0.12	17.70	1.26	22.75	0.48	34.41	3.62
	8/24/2022	1.2	12.15	0.61	96.71	1.68	255.84	4.89	2455.64	281.10
	9/9/2022	1.0	5.59	0.20	29.11	1.00	76.64	3.64	761.41	55.34
	9/14/2022	0.5	9.18	0.17	30.50	0.66	203.24	6.47	5611.61	1483.91
CHP- 2	8/1/2022	8.5	0.10	0.00	17.54	1.89	16.37	1.07	3.93	0.65
	8/10/2022	8.0	8.03	0.16	23.49	0.44	26.31	0.40	17.06	0.27
	8/24/2022	8.0	1.62	0.13	10.31	0.16	14.04	0.39	16.04	0.53
	9/9/2022	6.0	1.68	0.13	31.36	1.95	76.64	3.64	61.01	1.39
	9/14/2022	6.7	23.10	3.13	29.93	0.77	33.79	2.13	42.53	3.21
EGP-9	8/30/2022	13.6	4.54	0.49	16.51	1.17	20.31	1.00	n/a	n/a
	9/15/2022	13.0	1.03	0.26	34.44	2.21	32.53	0.06	n/a	n/a
	9/30/2022	13.6	5.61	0.46	31.78	0.82	28.47	2.34	n/a	n/a
TGP-7	8/29/2022	17.3	3.12	0.06	30.12	0.11	34.36	0.23	n/a	n/a
	9/13/2022	15.7	4.00	0.00	20.50	0.62	21.33	1.46	n/a	n/a
	9/29/2022	16.0	4.07	1.04	14.97	0.95	16.74	0.94	n/a	n/a
Tashmoo Pond	7/11/2022	31.5	1.06	0.06	1.87	0.17	1.87	0.06	n/a	n/a
	8/7/2022	31.7	3.87	0.32	5.40	0.93	3.75	0.10	n/a	n/a
	8/24/2022	31.1	1.89	0.28	2.06	0.16	1.67	0.08	n/a	n/a

Table 2. Phycoerythrin concentrations ($\mu g \cdot L^{-1}$) and salinity in water samples from Martha's Vineyard.

			Size fraction							
Site	Date	Salinity (ppt)	<0.2 µm	<0.2 µm SEM	<5 µm	<5 µm SEM	WLW	WLW SEM	BFC	BFC SEM
CHP-Up	8/1/2022	2.0	0.05	0.00	0.66	0.03	1.30	0.13	0.68	0.07
	8/10/2022	1.5	2.07	0.08	4.11	0.33	5.50	0.08	3.46	0.21
	8/24/2022	1.2	2.42	0.03	3.32	0.02	3.04	0.05	7.18	0.32
	9/9/2022	1.0	1.37	0.06	2.59	0.42	2.62	0.08	2.64	0.02
	9/14/2022	0.5	2.21	0.07	3.14	0.02	3.26	0.04	9.82	0.36
CHP-2	8/1/2022	8.5	0.05	0.00	0.12	0.03	0.14	0.05	0.12	0.05
	8/10/2022	8.0	2.00	0.02	2.15	0.03	2.44	0.05	2.77	0.02
	8/24/2022	8.0	0.83	0.16	1.34	0.07	1.94	0.05	2.48	0.09
	9/9/2022	6.0	1.12	0.02	1.86	0.05	2.62	0.08	2.49	0.02
	9/14/2022	6.7	1.21	0.03	1.30	0.08	1.59	0.13	1.50	0.13
EGP-9	8/30/2022	13.6	0.90	0.10	0.92	0.14	0.83	0.03	n/a	n/a
	9/15/2022	13.0	0.25	0.09	0.77	0.06	1.12	0.05	n/a	n/a
	9/30/2022	13.6	0.84	0.14	1.58	0.05	1.17	0.09	n/a	n/a
TGP-7	8/29/2022	17.3	1.17	0.02	1.73	0.12	2.12	0.06	n/a	n/a
	9/13/2022	15.7	0.71	0.03	1.32	0.09	1.81	0.22	n/a	n/a
	9/29/2022	16.0	0.42	0.02	0.70	0.06	0.84	0.09	n/a	n/a
Tashmoo Pond	7/11/2022	31.5	0.12	0.05	0.58	0.02	0.70	0.10	n/a	n/a
	8/7/2022	31.7	0.58	0.05	1.12	0.29	1.14	0.20	n/a	n/a
	8/24/2022	31.1	0.43	0.00	0.94	0.06	0.83	0.00	n/a	n/a

We verified the shifts in population structure and composition using two different analytical techniques including fluorometry and 16s metabarcoding. The fluorometric analysis using phycocyanin (PC) confirmed a positive correlation ($r = 0.738$, $p < 0.001$) between salinity and cyanobacterial community structure as shown in **Figure 3**, where picocyanobacteria were measured as a proportion of the whole lake water (<5 µm/WLW%). A similar analysis using phycoerythrin (PE) had a weak negative correlation ($r = -0.09$, $p = 0.683$). The principal component analysis (**Figure 4**) identified the degree to which variables influenced the structure and composition of the cyanobacterial populations with salinity and two principle components (PC1 = 39% and PC2 = 23%). These results are similar to those described for Lake Ellesmere/Te Waihora [17] where salinities ranged from 5 - 13.5 ppt and halotolerant picocyanobacteria dominated. Both approaches (fluorometry and metabarcoding) verified unique populations at the sites that were tested.

The 16s metabarcoding confirmed the particulate material collected from the picocyanobacteria sample (<5 µm) from Upper Chilmark Pond (CHPUp) as belonging to the Order *Synechococcales*, specifically *Cyanobium* PCC-6307. Analysis of samples collected from Chilmark-2 (CHP-2), Edgartown Great Pond (EGP-9), Tisbury Great Pond (TGP-7), and Tashmoo Pond (TSH-Sen) confirmed varying abundance of *Cyanobium* PCC-6307 and *Synechococcus* 9902 (**Figure 5**). These findings are similar to the previous reports on the diversity of

picocyanobacteria in this coastal area [2] confirming the influence of salinity microhabitats on the structure and composition of cyanobacterial populations. Absolute abundance of the top seven genus (Figure 5) confirmed the presence of *Dolichospermum* spp. and *Sphaerospermopsis* spp. in Upper Chilmark Pond. While halotolerant *Dolichospermum* spp. is known to produce anatoxin-a, *Sphaerospermopsis* spp. has been shown to produce guanitoxin, formerly known as anatoxin-a(s). [18]

(Pico)cyanobacterial populations and production of anatoxin-a

Anatoxin-a was detected in all of the water samples collected throughout the entire sampling season (Table 3). The concentrations are similar to those previously reported for anatoxin-a concentrations in picocyanobacteria [6] [7] using ELISA analysis. The similarity in anatoxin-a concentrations may be influenced by the high proportion of picocyanobacteria within the samples (Figure 3). In Chilmark Pond (CHP-Up) which contains the halotolerant BFC *Dolichospermum* sp., and picocyanobacteria, we confirmed a positive correlation between cyanobacterial biomass, as PC, and anatoxin-a concentrations (Figure 6) where $r = 0.438$, $p = 0.0536$ and a positive correlation using PE as a measure of cyanobacterial biomass ($r = 0.348$, $p = 0.133$).

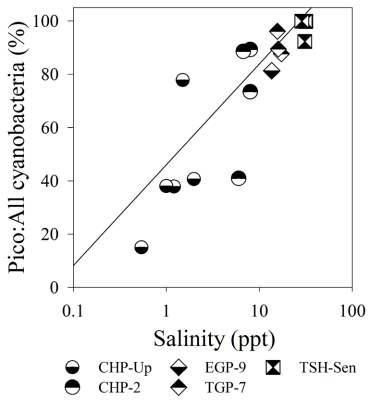

Figure 3. Correlation between salinity (ppt) and cyanobacterial community structure as described using phycocyanin from Martha's Vineyard sites. ($r = 0.738$, $p < 0.001$).

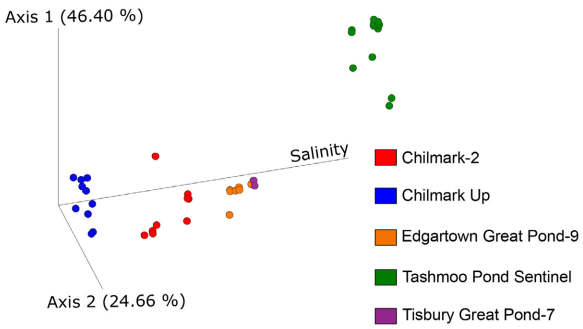

Figure 4. Principle component analysis of net (BFC), whole lake water (WLW) and <5 μm (<5) using 16s metabarcoding samples from Martha's Vineyard sites. Variable weights of influence after accounting for salinity, PC1 = 39%, PC2 = 23%.

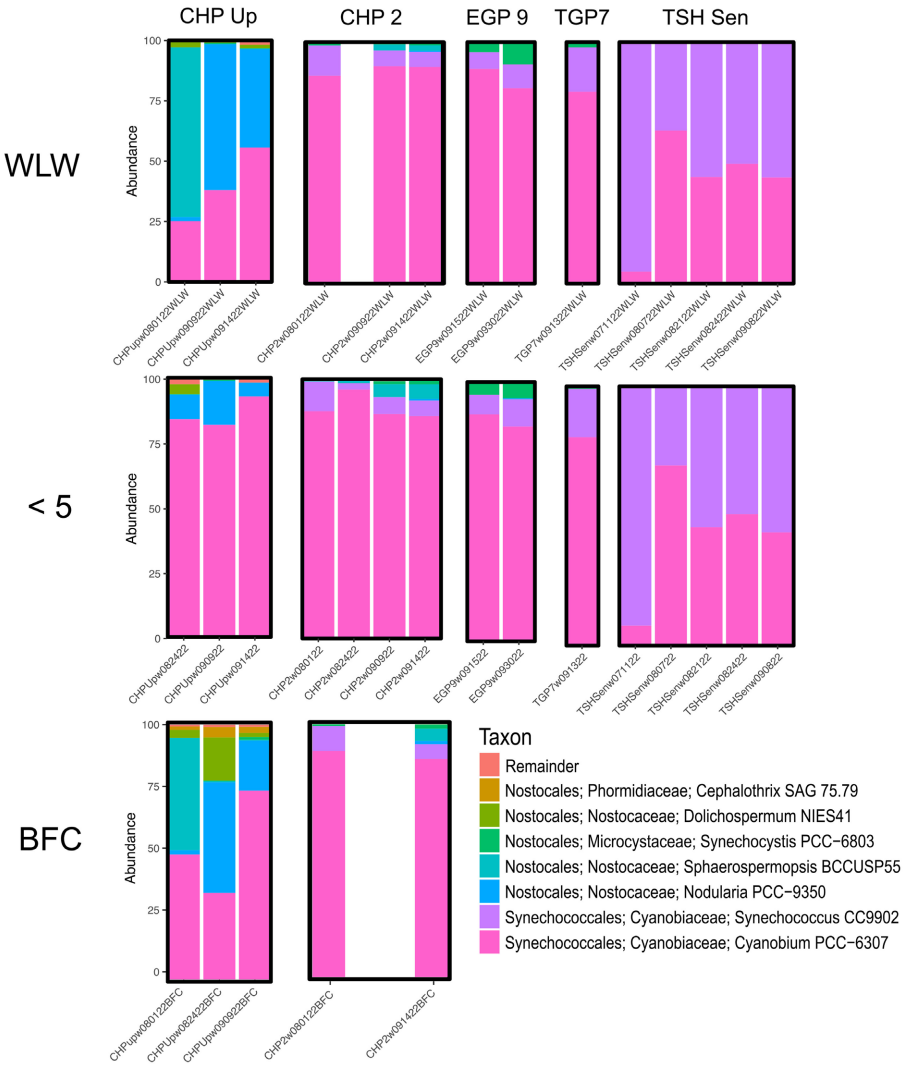

Figure 5. Relative abundance of cyanobacterial species in samples from Martha's Vineyard sites as determined by 16s metabarcoding.

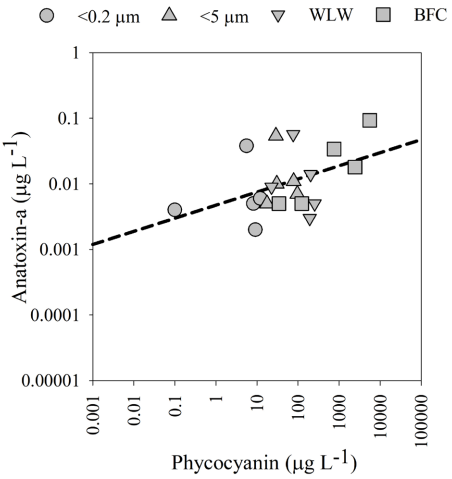

Figure 6. Correlation analysis of (pico)cyanobacterial biomass and anatoxin-a in Upper Chilmark Pond ($r = 0.438$, $p = 0.0536$).

Table 3. Anatoxin-a concentrations ($\mu g \cdot L^{-1}$) in water samples from Martha's Vineyard sites.

				Size fraction			
Site	Date	Temperature (˚C)	Salinity (ppt)	<0.2 µm	<5 µm	WLW	BFC
CHP-Up	8/1/2022	Unk	2.0	0.004	0.011	0.003	0.005
	8/10/2022	Unk	1.5	0.005	0.005	0.009	0.005
	8/24/2022	24.7	1.2	0.006	0.007	0.005	0.018
	9/9/2022	Unk	1.0	0.038	0.054	0.057	0.034
	9/14/2022	21.0	0.5	0.002	0.010	0.014	0.093
CHP-2	7/24/2022	28.8	8.5	0.017	0.040	0.022	0.028
	8/1/2022	Unk	8.0	0.011	0.008	0.028	0.023
	8/10/2022	Unk	8.0	0.009	0.008	0.017	0.022
	8/24/2022	25.5	6.0	0.044	0.052	0.022	0.046
	9/9/2022	Unk	6.7	0.023	0.013	0.012	0.031
	9/14/2022	21.7	13.6	0.022	0.025	0.028	0.029
EGP-9	8/30/2022	26.6	13.0	0.012	0.016	0.006	n/a
	9/15/2022	22.6	13.6	0.015	0.026	0.029	n/a
	9/30/2022	18.3	17.3	0.011	0.017	0.036	n/a
TGP-7	8/29/2022	26.1	15.7	0.017	0.019	0.022	n/a
	9/13/2022	22.8	16.0	0.021	0.035	0.042	n/a
	9/29/2022	Unk	31.5	0.028	0.022	0.051	n/a
Tashmoo Pond	7/11/2022	23.7	31.7	0.081	0.069	0.092	n/a
	8/7/2022	25.3	31.1	0.056	0.076	0.050	n/a
	8/24/2022	25.0	31.1	0.079	0.008	0.021	n/a

3. Conclusions

Our investigation has demonstrated that serial filtration using gravity and pressure is an effective field and laboratory method to isolate picocyanobacteria. It was demonstrated that salinity had an influence on the cyanobacterial community structure and composition, where the halotolerant bloom forming cyanobacterium *Dolichospermum* spp. (Order *Nostocales*) was found in salinity microhabitats up to 6 ppt (Upper Chilmark Pond and Chilmark Pond 2) in addition to picocyanobacteria (Order *Synechococcales*). Salinity microhabitats between 6 - 30 ppt, including Edgartown Great Pond-9 and Tisbury Great Pond-7 supported colonial and single-celled picocyanobacteria, while Tashmoo Pond exclusively supported picocyanobacteria (Order *Synechococcales*). The picocyanobacteria *Cyanobium* 6307 was exclusive to Upper Chilmark Pond, while mixtures of *Cyanobium* 6307 and *Synechococcus* 9902 were found at higher salinities in Chilmark-2, Edgartown Great Pond-9, Tisbury Great Pond-7, and Tashmoo Pond. These findings were verified using both fluorometric and 16s metabarcoding techniques.

A range of anatoxin-a concentrations were documented from sites of varying salinity, ranging from 1 ppt - 31 ppt. We were able to identify the picocyanobacteria to the species level using 16s metabarcoding techniques, where *Cyanobium* 6307 was present (exclusively) in Upper Chilmark Pond. Varying proportions of *Cyanobium* 6307 and *Synechococcus* 9902 were found in Chilmark Pond, Edgartown Great Pond-9, Tisbury Great Pond-7 and Tashmoo Pond. We were able to affirm a positive linear correlation between cyanobacterial biomass (PC) and anatoxin-a in Upper Chilmark Pond.

Acknowledgements

This research was supported by contributions of field collection and processing by the water quality staff of The Martha's Vineyard Commission (Sheri Caseau and Eleah Caseau). Financial support was provided by the Martha's Vineyard Commission. Use of laboratory space and equipment was provided by UNH-Center for Freshwater Biology and Ecotoxicology. The project 16s metabarcoding was supported by a grant from the United States Geological Survey #G20AP00034.

Conflicts of Interest

The authors declare no conflicts of interest regarding the publication of this paper.

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