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UNEXPECTED PRESENCE OF THE NITROGEN-FIXING SYMBIOTIC CYANOBACTERIUM UCYN-A IN MONTEREY BAY, CALIFORNIA[1]

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In the last decade, the known biogeography of nitrogen fixation in the ocean has been expanded to colder and nitrogen-rich coastal environments. The symbiotic nitrogen-fixing cyanobacteria group A (UCYN-A) has been revealed as one of the most abundant and widespread nitrogen-fixers, and includes several sublineages that live associated with genetically distinct but closely related prymnesiophyte hosts. The UCYN-A1 sublineage is associated with an open ocean picoplanktonic prymnesiophyte, whereas UCYN-A2 is associated with the coastal nanoplanktonic coccolithophore *Braarudosphaera bigelowii*, suggesting that different sublineages may be adapted to different environments. Here, we study the diversity of *nifH* genes present at the Santa Cruz Municipal Wharf in the Monterey Bay (MB), California, and report for the first time the presence of multiple UCYN-A sublineages, unexpectedly dominated by the UCYN-A2 sublineage. Sequence and quantitative PCR data over an 8-year time-series (2011–2018) showed a shift toward increasing UCYN-A2 abundances after 2013, and a marked seasonality for this sublineage which was present during summer-fall months, coinciding with the upwelling-relaxation period in the MB. Increased abundances corresponded to positive temperature anomalies in MB, and we discuss the possibility of a benthic life stage of the associated coccolithophore host to explain the seasonal pattern. The dominance of UCYN-A2 in coastal waters of the MB underscores the need to further explore the habitat preference of the different sublineages in order to provide additional support for the hypothesis that UCYN-A1 and UCYN-A2 sublineages are different ecotypes.

Key index words: **cyanobacteria; *nifH*; symbiosis; UCYN-A; *Braarudosphaera bigelowii*; nitrogen fixation**

***Abbreviations*: UCYN-A, unicellular cyanobacterial group A; *nifH*, nitrogenase; MB, Monterey Bay; SCMW, Santa Cruz Municipal Wharf; N, nitrogen**

Marine microbes catalyze chemical transformations that are essential for maintaining ocean ecosystems. Among them, photosynthetic microorganisms, comprised of diverse eukaryotic and cyanobacterial taxa, are responsible for about half of primary production on Earth (Field et al. 1998, Falkowski and Raven 2007). Primary productivity in marine ecosystems is frequently limited by the availability of nutrients, of which nitrogen (N) is one of the most important due to widespread N-limitation (Gruber 2004, Moore et al. 2013). In the ocean, some microorganisms, called diazotrophs, are able to fix gaseous dinitrogen (N_2) into biologically available ammonia through the nitrogenase enzyme, providing an important source of N for microbial communities and food webs (LaRoche and Breitbarth 2005, Gruber and Galloway 2008). Conventionally, cyanobacterial taxa have been considered the most important marine N_2-fixers, following the demonstration of N_2 fixation associated with *Trichodesmium* (Dugdale et al. 1961) and diatom symbionts (Villareal 1990). Based on phylogenetic studies of the *nifH* gene (which encodes the iron protein of the nitrogenase enzyme), we now know that diazotrophs are comprised of cyanobacterial taxa and a large diversity of heterotrophic (or photoheterotrophic) archaea and bacteria mostly represented by proteobacterial groups (Zehr et al. 2003, Farnelid et al. 2011, Bombar et al. 2016). Additional cyanobacterial and non-cyanobacterial N_2-fixing taxa have recently been discovered and some are proposed to be equally important (Bombar et al. 2016, Martínez-Pérez et al. 2016). One of these is the unicellular Cyanobacterium UCYN-A (*Candidatus Atelocyanobacterium thalassa*; Zehr et al. 2001), which has extreme metabolic reduction (Zehr et al. 2008, Tripp et al. 2010) and is symbiotic with eukaryotic hosts closely

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related to the prymnesiophyte alga *Braarudosphaera bigelowii* (Thompson et al. 2012, 2014).

UCYN-A is more broadly distributed than other marine N_2-fixing cyanobacteria, being found in cooler waters (Needoba et al. 2007, Rees et al. 2009) and N-enriched waters, including polar seas (Harding et al. 2018, Shiozaki et al. 2018), coastal waters (Mulholland et al. 2012, 2019, Bentzon-Tilia et al. 2015), and upwelling regions (Agawin et al. 2014, Moreira-Coello et al. 2017, 2019). Based on the genetic diversity of *nifH*, four main sublineages have been defined and are globally distributed (Thompson et al. 2014, Farnelid et al. 2016). UCYN-A1 and UCYN-A3 sublineages are typically found in oligotrophic waters, whereas UCYN-A2 and UCYN-A4 are associated with coastally influenced waters, suggesting sublineages may be different ecotypes (Turk-Kubo et al. 2017). The different sublineages are also associated with genetically distinct prymnesiophyte hosts: UCYN-A2 is associated with *Braarudosphaera bigelowii* sensu stricto (Gran and Braarud 1935, Deflandre 1947) which is typically 5–10 μm in cell diameter; UCYN-A1 is associated with a smaller (1–3 μm diameter) closely related prymnesiophyte (Thompson et al. 2012, 2014, Hagino et al. 2013). The other hosts have not yet been taxonomically identified.

The UCYN-A2 host, *Braarudosphaera bigelowii,* similar to other coccolithophore species, alternates between a calcified non-motile form and a non-calcified motile form (Billard 1994, Houdan et al. 2004). This has been verified through molecular approaches (Hagino et al. 2013) and more recently by direct microscopic observation (K. Hagino, pers. comm.). Interestingly, the fossil record of the calcified form extends from the early Cretaceous to the present and it has been largely confined to neritic sediments (Takayama 1972, Peleo-Alampay et al. 1999, Bown et al. 2004), providing supporting evidence that the UCYN-A2/*B. bigelowii* symbiosis may be coastally adapted.

Coastal areas have been generally disregarded as important regions for N_2 fixation because they are often less N-limited than the oligotrophic tropical and subtropical oceanic areas and the presence of dissolved inorganic N (DIN) was thought to inhibit the growth and activity of diazotrophs (Flores and Herrero 1994, Holl and Montoya 2005). In the last decade, several studies have broadened the known biogeography of N_2 fixation to N-rich coastal environments from temperate, tropical, and subtropical regions (Grosse et al. 2010, Mulholland et al. 2012, 2019, Bentzon-Tilia et al. 2015), including upwelling systems (Benavides et al. 2014, Fernandez et al. 2015) and polar waters (Harding et al. 2018, Shiozaki et al. 2018) and there is now a focus on characterizing the diversity and activity of diazotroph assemblages in these areas.

Monterey Bay (MB) is a semi-enclosed embayment along the central California coast in the Northeast Pacific Ocean, located within the broader California Current System. The hydrography of the MB is characterized by seasonal cycles of upwelling (ca. February to August, peaking between April and June), periods when upwelling-favorable winds relax (ca. August to November), and periods when the poleward Davidson current surfaces (ca. November to February; Rosenfeld et al. 1994, Graham and Largier 1997, Pennington and Chavez 2000). In upwelling-relaxation periods, also known as the oceanic period, the California Current moves onshore, bringing warmer sea surface temperatures and lower salinity, creating water column stratification in northern MB. During periods of upwelling, these warm waters are sheltered from upwelling by an orographic shadow and often form a warm cyclonic feature separated from the rest of the MB by strong frontal gradients. This is referred to as an upwelling shadow (Graham et al. 1992). The hydrological conditions within the upwelling shadow allow high phytoplankton productivity (Ryan et al. 2005, 2014, Kudela et al. 2008), and numerous diatom and dinoflagellate blooms occur (Ryan et al. 2009, Schulien et al. 2017).

In the present study, we examine diazotroph diversity based on the *nifH* gene over an 8-year time-period (2011–2018) at a near-shore station located at the Santa Cruz Municipal Wharf in MB, and report for the first time the presence of the cyanobacterium UCYN-A. Prior to this study, UCYN-A had been reported in several regions of the Southern California Bight, including the San Pedro Ocean time-series (SPOT; Hamersley et al., 2011), Catalina Island (Needham et al. 2018), and the Scripps Institute of Oceanography pier (SIO, La Jolla/San Diego; Thompson et al. 2014, Cornejo-Castillo et al. 2019), but it was not previously detected in the *nifH* surveys of MB (Turk-Kubo et al. 2017). Our findings show that UCYN-A assemblages in coastal MB waters are mainly dominated by the UCYN-A2 sublineage, have seasonal patterns in abundance, and were detected much more frequently and at higher abundances when climatological conditions in the MB shifted from cold to warm. Overall, its occurrence is associated with warmer temperatures and lower nutrient concentrations, but no statistically significant correlations were found between environmental parameters and UCYN-A abundances.

MATERIALS AND METHODS

Sample collection. Weekly samples were collected from the Santa Cruz Municipal Wharf (SCMW; 36°57'28.7136" N, 122°01'03.2124" W; Fig. 1, inset). Equal volumes of water from three depths (0 m, 1.5 m, and 3 m) were combined to yield an integrated water sample. Water temperature was recorded on site and the sample was maintained at ambient temperature until processed (within 2 h of collection). Chlorophyll a samples were vacuum filtered onto Whatman GF/F filters (nominal pore size 0.7 μm) and analyzed using

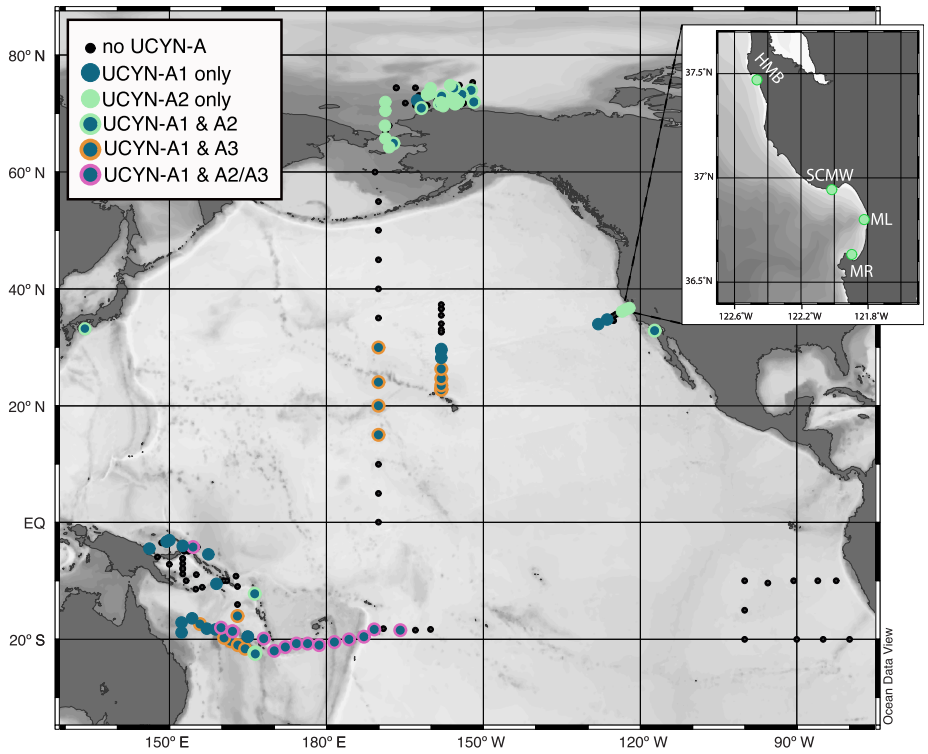

FIG. 1. Map of distribution of UCYN-A in the Pacific and Arctic Oceans. The inset shows location of the Santa Cruz Wharf Time-Series (SCMW) in the Monterey Bay (California). Only data sets where both UCYN-A1 and UCYN-A2/A3 were characterized using qPCR were included. Other locations around the MB sampled in September 2018 included Half Moon Bay (HMB), Moss Landing (ML), and the Monterey (MR) Harbor. For most sample sets, complementary sequence data were available to determine whether UCYN-A2 or UCYN-A3 was present. This figure was modified from Farnelid et al. 2016, and updated to include data from Hagino et al. 2013, Shiozaki et al. 2017, 2018, Stenegren et al. 2018, Berthelot et al. 2017, Henke et al. 2018, Harding et al. 2018, Gradoville et al. 2020, and this work.

the non-acidification fluorometric technique on a Turner Designs 10-AU fluorometer (Welschmeyer 1994). Filtrate from water passed through a Whatman GF/F (Cytiva, Marlborough, MA, USA) filter was collected for nutrient analyses. Nitrite + nitrate (hereafter referred to as NO_3^-), phosphate (PO_4^{3-}), and silicate (SiO_4^{2-}) were analyzed on a Lachat QuikChem 8000 series (Hach; Smith and Bogren 2001, Knepel and Bogren 2002). Ammonium (NH_4^+) was analyzed on a fluorometer (TD700 Turner Designs, Sunnyvale, CA, USA) following the method described by Holmes et al. (1999). Additional surface samples for qPCR surveys were collected in May 2018 at Haruno Harbor off Kochi, Japan (33°28'6.7008'' N, 133°30'23.1984'' E) using a manual pump and in September 2018 along the MB coast (Moss Landing Harbor, 36°48'5.9544'' N, 121°47'6.9684'' W; Monterey Municipal Wharf II, 36°36'17.8704'' N, 121°53'21.7104'' W; and Half Moon Bay Harbor, 37°30'7.1784'' N,122°29'0.996'' E) using a bucket (Fig. 1, inset).

DNA sample collection and processing. For SCMW DNA samples, 150 mL of seawater was filtered through 0.2 µm pore size Supor-200 filters (25 mm diameter; Pall Corporation, Port Washington, NY, USA) using vacuum filtration. For Japan and MB September 2018 samples, 4 and 1 L volumes, respectively, were sampled onto 0.2 µm Supor filters in swinnex using gentle peristaltic pumping. Filters were stored in sterile bead-beating tubes with glass beads (BioSpec Products, Bartsville, OK, USA) at − 80°C until DNA was extracted. DNA was extracted using a Qiagen DNeasy Plant kit (Valencia, CA, USA), with protocol modifications (Moisander et al. 2008) including additional freeze–thaw cycles, bead-beating agitation, and proteinase K treatment to maximize DNA recovery. The final on-column wash steps were automated using a QIAcube (classic; Qiagen). For quality assessment, DNA extracts were analyzed using a NanoDrop (Thermo Scientific, Waltham, MA, USA) and were stored at − 20°C.

Analysis of diazotroph community composition by sequencing of nifH *gene amplicons.* The diversity of diazotrophs was assessed in SCMW monthly samples (n = 88) using a universal nested *nifH* PCR assay (Zehr and McReynolds 1989, Zani et al. 2000). Briefly, first round reactions (20 µL) contained Platinum™ Taq DNA polymerase (6 units; Invitrogen, Carlsbad, CA, USA), 1X PCR Buffer (-$MgCl_2$), 4 mM $MgCl_2$, 400 µM dNTP mix, 0.5 µM of each outer primer (nifH3/nifH4), and 2 µL of template DNA. Second round reactions (15 µL) contained 10 units of Platinum™ Taq DNA polymerase (Invitrogen), 1X PCR Buffer (-$MgCl_2$), 4 mM $MgCl_2$, 200 µM dNTP mix, 1 µM of each outer primer (nifH1/nifH2), and 2 µL of the first round reactions. Thermocycling parameters were as follows: initial denaturation at 95°C for 3 min, followed by 25 cycles (round 1) or 30 cycles (round 2) of denaturation at 95°C for 30 s, annealing at 55°C (round 1) or 57°C (round 2) and elongation at 72°C for 45 s. All samples were amplified in triplicate, visualized by gel electrophoresis, and pooled prior to sequencing. Second round primers (nifH1/nifH2) were modified to contain common sequence linkers (Moonsamy et al. 2013) used to create barcoded libraries by the DNA Service Facility at the University of Illinois at Chicago using the targeted amplicons sequencing approach described in Green et al. (2015). Multiplexed amplicons were bidirectionally sequenced (2 × 300 bp) using the Illumina MiSeq platform at the W.M. Keck Center for Comparative and Functional Genomics at the University of Illinois at Urbana-

Champaign. Samples were multiplexed to achieve ca. 40,000 high-quality merged reads per sample (ca. 364 bp).

De-multiplexed raw paired end reads were processed as described in Turk-Kubo et al. (2015). Briefly, raw reads were merged and quality filtered (Phred score 20, using the argument-q 19) using PEAR (Paired-End reAd mergeR; Zhang et al. 2014); merged reads < 300 and > 400 were filtered out at this stage (-n 300, -m 400). Primers were trimmed in CLC Genomics workbench (Qiagen). Chimeric sequences were removed using UCHIME (Edgar et al. 2011) and operational taxonomic units (OTUs) were determined using Usearch6.1 at 97% nucleotide identity (Edgar 2010) in QIIME (Caporaso et al. 2010). Representative nucleotide sequences from OTUs with greater than 100 sequences were imported into ARB (Ludwig et al. 2004), translated into amino acid sequences, and representative sequences with frameshifts or non-*nifH* related were discarded. After quality control steps, a total of 4035 OTUs remained (representing 2,469,962 sequences; 68% of total sequences that passed merging and quality filtering steps in PEAR). There were seven samples from sampling year 2013 that had less than 10,000 reads. In order to include as many of these samples in the analysis, the resulting OTU table was randomly subsampled to 2464 reads per sample, using QIIME script multiple_rarefactions_even_depth.py which resulted in the loss of three samples. Correlation analysis between Bray–Curtis distances obtained with raw and subsampled datasets (Mantel test, $R = 0.94$, $P < 0.001$) showed that subsampling did not have a negative impact on the overall beta-diversity of the dataset (Fig. S1 in the Supporting Information). After subsampling, 4033 OTUs from 85 samples remained and were used for downstream analyses. QIIME script exclude_seqs_by_blast.py was used post-rarefaction to check for OTUs with > 92% nucleic acid identity to known reagent contaminants, and there were no positive hits. Taxonomy was assigned using a curated *nifH* database containing genome-derived *nifH* sequences where *nifH* cluster designations have been assigned based on the convention outlined in Zehr et al. (2003). For each OTU, the closest full-length *nifH* sequence was determined using blastx, and the OTU was assigned the *nifH* cluster based on the top hit. Percent similarity to the top hit in the reference database is reported in Table S1 in the Supporting Information (can be downloaded from https://figshare.com/articles/Table_S1_xlsx/11950662). To finely resolve UCYN-A sublineages and *nifH* phylotypes within them, oligotyping analysis (which uses alternative taxonomic unit determination that results in fine phylogenetic resolution; Eren et al. 2013) was performed using the pipeline described in Turk-Kubo et al. (2017). Sequences are available from NCBI (accession PRJNA605009).

Quantitative PCR and microscopy visualization of the symbiosis. Current quantitative PCR (qPCR) assays targeting the UCYN-A *nifH* gene do not differentiate between known UCYN-A/prymnesiophyte sublineages due to cross reactivity between assays (Farnelid et al. 2016). At this time, one assay targets UCYN-A1 *nifH* (Church et al. 2005) and a second assay amplifies UCYN-A2/A3/A4 *nifH* (Thompson et al. 2014). UCYN-A1 and UCYN-A2/A3/A4 abundances were quantified using these previously described Taqman® qPCR assays. Primer and probe sequences for the UCYN-A1 *nifH* gene assay are as follows: Forward, 5′-AGCTATAACAACGTTTTATGCGTTGA-3′; Reverse, 5′-ACCACGACCAGCACATCCA-3′; Probe, 5′-FAM-TCTGGTGGTCCTGAGCCTGGA-TAMRA-3′. For the UCYN-A2/A3/A4 *nifH* assay, the same reverse primer is used and the other sequences are as follows: Forward, 5′-GGTTACAACAACGTTTTATGTGTTGA-3′; Probe, 5′-FAM-TCTGGTGGTCCTGAGCCCGGA-TAMRA-3′. All samples, standards, and inhibition tests were duplicate 20 µL qPCR reactions, and no template controls were included with each assay. qPCR efficiency was always > 96% in the tested samples for both targets. Generation of *nifH* standards, reaction conditions, and thermocycling parameters are described elsewhere (Goebel et al. 2010, Thompson et al. 2014), but note that primer probe specificity was ensured by using annealing temperatures of 60°C and 64°C for the UCYN-A1 and UCYN-A2/A3/A4 assays, respectively. Based on the volumes of seawater filtered, DNA extract and DNA template volumes used in the qPCR reactions, the limit of detection (LOD) and limit of quantitation (LOQ) for the SCMW samples were 333 and 2667 *nifH* gene copies L^{-1}, respectively. LOD and LOQ for the auxiliary samples were lower (13 and 100 *nifH* gene copies $\cdot L^{-1}$, respectively, for the Japan samples; 50 and 400 for the MB September 2018 samples). Samples with abundances in between these limits are indicated as "detected not quantified" (DNQ).

In order to visualize the UCYN-A/prymnesiophyte symbiosis detected at the SCMW, a CARD-FISH sample (100 mL of seawater fixed with formaldehyde at 1.8% final concentration) was collected in September 2018 and filtered onto a 0.6 µm pore-size polycarbonate filter. The UCYN-A2 association was targeted using a double CARD-FISH hybridization with specific probes UCYN-A2-732 and UBRADO69 and competitors as described elsewhere (Cabello et al. 2016, Cornejo-Castillo et al. 2019). Samples were inspected under epifluorescence on a Leica DM500B epifluorescence microscope (University of California, Santa Cruz Life Sciences Microscopy Center) and micrographs were taken under ultraviolet (DAPI signal of the nucleus), blue (green labeled host with Alexa 488 stain), and green (red labeled UCYN-A with Cy3) excitation wavelengths using LAS X 3.6 software.

RESULTS AND DISCUSSION

Previous nitrogenase (*nifH*) gene surveys in MB did not detect any cyanobacteria *nifH* genes, except in offshore waters of the California current (Needoba et al. 2007, Turk-Kubo et al. 2017). Size-fractionated DNA samples collected monthly between June 2009 and August 2010 from four stations located in the MB (in an inshore to offshore transect) were screened using UCYN-A *nifH*-specific PCR assays (Turk-Kubo et al. 2017) with negative results (Fig. S2 in the Supporting Information). However, *nifH* sequences related to UCYN-A were detected in DNA samples collected from the SCMW on MB in 2018, which raised the question of how long UCYN-A had been present in the MB. Using a subset of the weekly time-series samples (spanning 2011–2018), we analyzed the composition of the N_2-fixing assemblage and quantified UCYN-A abundances to determine when it was first detected in the MB. Analysis of the 8 y of monthly samples showed that UCYN-A was present in samples dating back to 2011, but has increased in abundance in recent years. Additional qPCR surveys along the MB and central California coast (Moss Landing Harbor; Monterey Municipal Wharf II and northern Half Moon Bay Harbor) in September 2018 showed that UCYN-A was widely distributed in coastal waters during the survey period (Fig. 1, inset).

nifH *diversity and diazotroph assemblages.* In order to determine the diversity of N_2-fixing microorganisms present at the SCMW time-series site, *nifH* gene

diversity was assessed via high-throughput sequencing of amplified partial *nifH* gene sequences. Diazotroph assemblages were comprised mainly of putative proteobacterial taxa from *nifH* clusters 3 and 1A (putative delta-proteobacteria, 77% of total sequences), 1G (putative gamma-proteobacteria, 10.3%), and 1J/1K (putative alpha- and beta-proteobacteria, 2.8%) and the unicellular cyanobacterium UCYN-A (4.3%; Fig. 2, Table S2 in the Supporting Information). Notably, the diazotroph assemblage at the SCMW shifted over the course of the study (Fig. 2): alphaproteobacteria were detected more frequently and comprised a greater percentage of total sequences in samples from the period prior to 2014, while UCYN-A was detected much less frequently and had lower relative abundances. Delta- and gamma-proteobacteria appeared throughout the time-series. Minor components of the SCMW communities were affiliated with other proteobacterial groups (*nifH* clusters 1O/1P, 1F, 1C, 4, 2; 5% of total sequences) and other cyanobacteria (0.5% of total sequences). These cyanobacterial sequences were mostly related to *Trichodesmium* sp. and heterocyst-forming species that were only detected in single samples (Table S1; see Fig. S3 in the Supporting Information for a phylogenetic tree).

Although sequences affiliating with UCYN-A only accounted for 4.3% of the total *nifH* library, the most abundant single OTU in the rarefied dataset was UCYN-A (7094 sequences). UCYN-A is comprised of several lineages (A1, A2, A3, and A4), each of which are comprised of distinct sequence types (oligotypes). A detailed analysis of all UCYN-A *nifH* sequences showed that UCYN-A2 was the dominant lineage in SCMW samples (94.8% of all UCYN-A sequences) and was represented by several oligotypes, with oligo_3 having the highest relative abundance (90.7%), as previously observed in other datasets (Turk-Kubo et al. 2017). Oligotypes affiliated with UCYN-A4 (oligo_4) and UCYN-A1 (oligo_1) sublineages were sporadically detected in the dataset at 2.0% and 1.1% of total UCYN-A sequences, respectively. It has been hypothesized that UCYN-A2 is a coastal strain or ecotype that sometimes co-occurs with UCYN-A4, while UCYN-A1 and UCYN-A3 are open ocean strains (Turk-Kubo et al. 2017). However, UCYN-A1 and UCYN-A2 have been reported to co-occur in coastal waters in the Southern California Bight (Needham et al. 2018), the Scripps Pier (Thompson et al. 2014, Cornejo-Castillo et al. 2019), in the Western Tropical South Pacific (Turk-Kubo et al. 2015, Messer et al. 2016, Henke et al. 2018, Stenegren et al. 2018), and in the Bering Sea off Nome, Alaska (Harding et al. 2018; Fig. 1). It is still not clear if the occurrence of UCYN-A1 in these coastally influenced waters is the result of intrusion of oligotrophic waters or habitat adaptation. The UCYN-A2 lineage has only been reported at high relative abundances in some mesohaline estuarine waters of the Danish strait (Bentzon-Tilia et al. 2015, Turk-Kubo et al. 2017), and in hypersaline estuarine waters in the South Australian Bight (Messer et al. 2015). Thus, the dominance observed for the UCYN-A2 sublineage at SCMW was unexpected and might support the hypothesis that UCYN-A1 and UCYN-A2 sublineages represent different ecotypes. The environmental factors selecting for different ecotypes are not well understood, but these results provide more data that might ultimately elucidate physiological differences related to habitat differences.

Other major *nifH* sequence types detected were affiliated to non-cyanobacterial diazotophs (NCDs) from the delta-proteobacterial *nifH* clusters 3 and 1A (4247 and 3850 total sequences, respectively), the gamma-proteobacterial *nifH* cluster 1G (4968 sequences), and clusters 1K/J related to alpha- and beta-proteobacteria (427/108 sequences). It is noticeable that the cosmopolitan Gamma A *nifH* phylotype was not detected in the SCMW assemblages. Although more commonly found in tropical and subtropical oligotrophic regions, such as Station ALOHA (Langlois et al. 2015), this group has been

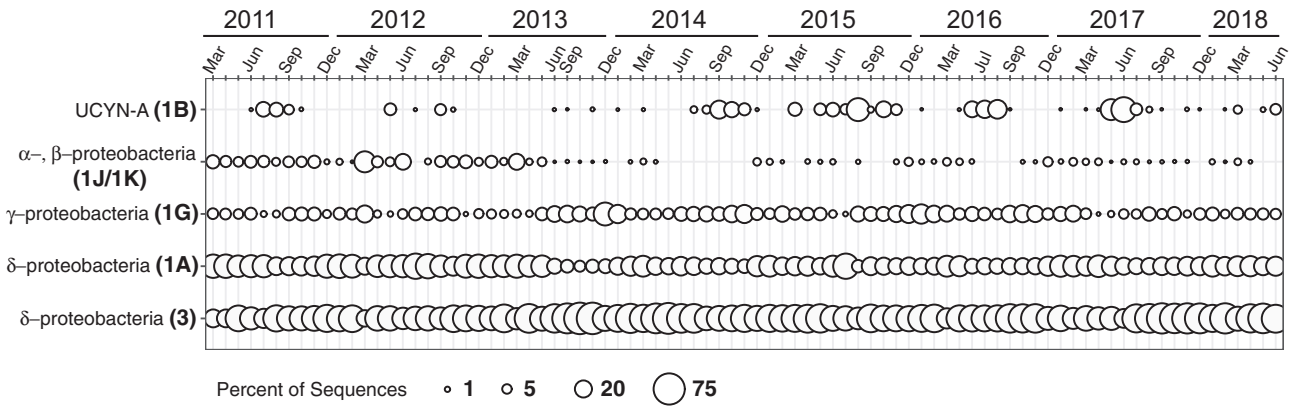

FIG. 2. Diazotroph assemblage composition in the SCMW time-series based on *nifH* gene sequencing. Groups represented account for 95% of total sequences in the subsampled dataset. *nifH* clusters are indicated in parenthesis and follow the convention established by Zehr et al. 2003.

reported from coastal environments such as the South China Sea (Moisander et al. 2014, Bombar et al. 2016), the Southern Californian Bight (Hamersley et al. 2011), and the upwelling region off the Northwest Iberian Peninsula (Moreira-Coello et al. 2017). Nevertheless, our results are in agreement with the minor contribution of Gamma A *nifH* sequences along a northwest Pacific coastal transect (Gradoville et al. 2017). Our dataset was dominated by delta-proteobacteria (clusters 1A and 3), which are groups that have been widely reported in both pelagic and sediment samples from other temperate coastal regions including the Southern California Bight (Hamersley et al. 2011), Western North Atlantic (Moisander et al. 2007, Mulholland et al. 2012, Newell et al. 2016), Pacific Northwest (Gradoville et al. 2017), English Channel (Rees et al. 2009), and North Sea and Danish Straits (Bentzon-Tilia et al. 2015, Fan et al. 2015, Pedersen et al. 2018). Their presence at the SCMW may be a signature of sediment resuspension due to the shallow sampling location (12 m depth). It is notable that non-cyanobacterial diazotrophs (NCDs) accounted for such a large percentage of the sequence libraries from the SCMW time-series; however, it is difficult to ascertain their potential biogeochemical importance without direct evidence of their N_2-fixing activity (Zehr and Capone 2020). Further research is needed to determine whether the NCDs described in this study are active and fixing N_2.

Microscopic visualization of the UCYN-A2 symbiosis at SCMW. The presence of UCYN-A was confirmed by species-specific FISH hybridization using UCYN-A2-specific ribosomal RNA (rRNA) probes for UCYN-A2 and the haptophyte host of UCYN-A2 (Fig. 3). The images show that the size of *Braarudosphaera bigelowii* is larger (ca. 20 μm) than previous reports (5–10 μm) as well as the size of the UCYN-A2 cyanobacteria cell, approximately 7 μm in diameter (Thompson et al. 2014, Cornejo-Castillo et al. 2019). As CARD-FISH hybridizations require active cells with many rRNA molecules, these images also verify that these cells were alive and active. The morphology and ultrastructure of the calcified form of *B. bigelowii* have been studied in coastal waters of Japan, where the uncalcified flagellated form is also known to exist (Hagino et al. 2013, 2016). In those studies, it is clear that the UCYN-A2 is intracellular, and although such conclusion cannot be reached from FISH images, it can be assumed that the morphology of the flagellated form in Californian coastal waters is similar to those observed by light microscopic techniques in Japan waters (K. Hagino, pers. comm.). UCYN-A1, which has primarily been observed by FISH (Cabello et al. 2016, Cornejo-Castillo et al. 2016, 2019), is likely to also be intracellular but this has yet to be demonstrated conclusively. Electron microscopy images in another study, although misinterpreted as *Prochlorococcus* cells being grazed, are actually some of the best images of UCYN-A1 symbiosis and they suggest but do not prove that the prymnesiophyte host membrane surrounds the symbiont (Kamennaya et al. 2018).

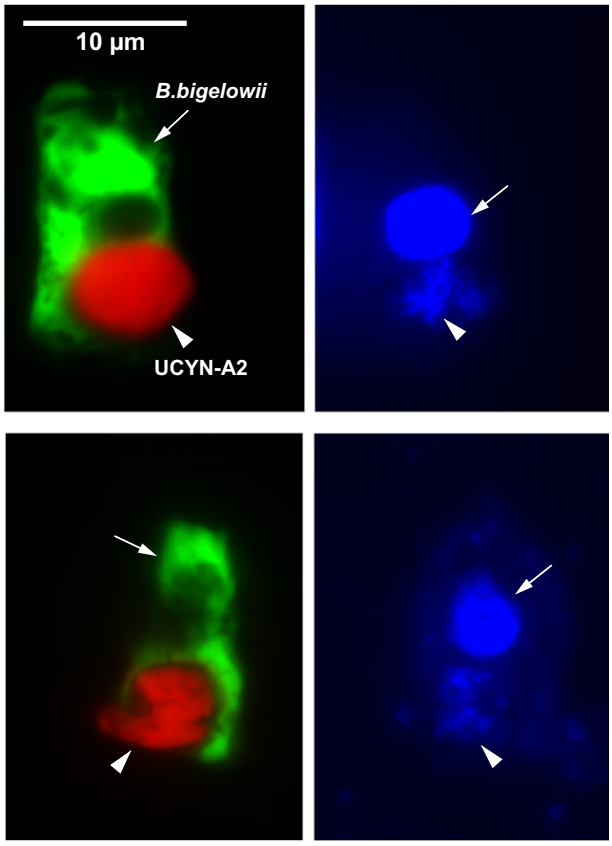

FIG. 3. Epifluorescence microscopy images of the UCYN-A2 symbiosis present at the Santa Cruz Wharf on September 24, 2018 using CARD-FISH UCYN-A2-specific probes. Left panels show the combined signal of *Braarudosphaera bigelowii* cells (arrows) labeled in green and UCYN-A2 cells (arrow heads) labeled in red. Right panels show the corresponding DAPI signal (blue-labeled nucleous) of symbiont (arrow head) and host cells (arrow).

UCYN-A2 abundances and seasonality in the time-series. The annual detection of UCYN-A based on *nifH* sequencing was consistent with its detection by qPCR of the same gene (Fig. 4A). The presence and abundance of UCYN-A2 at SCMW varied annually and seasonally. Abundance data showed a seasonal cycle with highest abundances in late spring and summer in all years sampled. It can be detected in the water column until late autumn but is not detected during winter (December to February) when circulation in MB is dominated by the poleward-flowing Davidson current. The presence of the UCYN-A1 sublineage also varied annually similar to the pattern observed for UCYN-A2 but it was always detected below the limit of quantification ($<2.7\text{x}10^3$ *nifH* copies $\cdot$ L^{-1}; Fig. 4A; Table S3 in the Supporting Information).

Maximum abundances of UCYN-A2 in this data set ($\sim 3 \times 10^6$ *nifH* copies $\cdot$ L^{-1}) were measured in

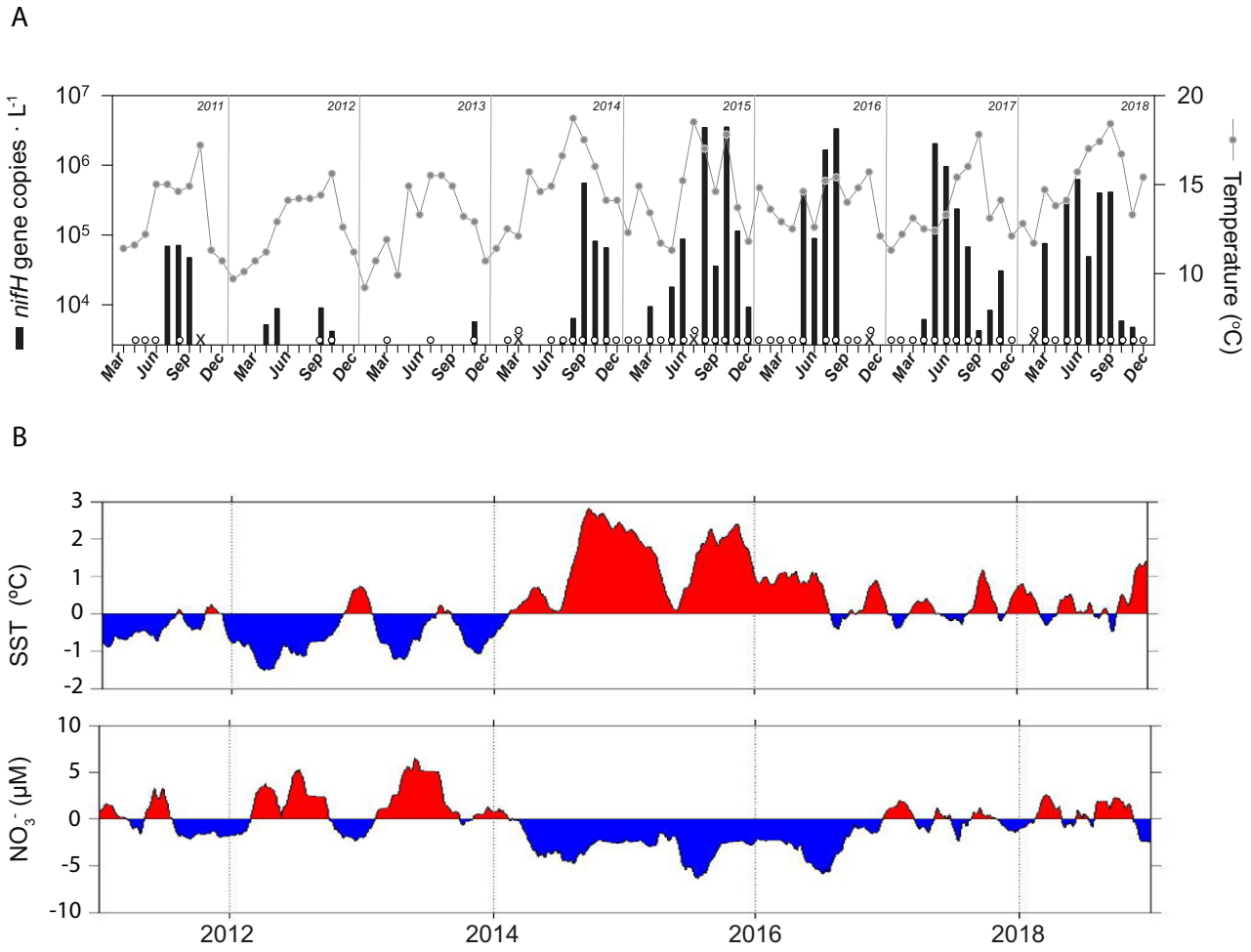

FIG. 4. (A) Monthly time-series of temperature and UCYN-A2 abundances estimated with qPCR during 7-y period (2011–2018) at the SCMW. Cross marks (**x**) and circles (○) indicate detection below the quantitation limit (DNQ), respectively, for UCYN-A2 and UCYN-A1. (B) Sea surface temperature (SST) anomalies and 0 m NO_3- concentration anomalies at the M1 mooring station in MB (Fig. S2) for the period of study (please notice that values are relative to long-term data from Monterey Bay Aquarium Research Institute).

August and October 2015, August 2016 and May 2017. To our knowledge, these are the highest abundances ever reported for this lineage (orders of magnitude higher than in previous studies; Thompson et al. 2014, Moreira-Coello et al. 2017, Shiozaki et al. 2017, 2018, Henke et al. 2018). It is not clear why abundances were higher in these years. Annual patterns of temperature, chlorophyll concentration, and nutrient concentrations for the period of study are in agreement with previous observations (Schulien et al. 2017, from 2006 to 2013; Fig. S4 in the Supporting Information). Highest annual temperatures usually occur in the period from July to October (mean = 15.82°C, range [12.1–18.7]). Nutrient concentrations vary inversely relative to temperature with lower concentrations of NO_3^-, SiO_4^{2-}, and PO_4^{3-} measured from June to September/November (mean = 1.66; 9.90; 0.58 µM respectively, range [≤ 0.33–9.82; 41.45; 1.67, respectively]) and higher concentrations from January to May (mean = 5.58; 16.23; 0.93 µM, respectively, range [≤ 0.48–22.86; 91.95; 4.7, respectively]). Chlorophyll *a* concentrations had two annual peaks, the first in spring (mean = 7.58 $\mu g \cdot L^{-1}$; range [1.61 – 42.56]) during the early upwelling season and the second in October/November (mean = 14.67 $\mu g \cdot L^{-1}$; range [1.34 – 137.55]) during the oceanic period. No statistically significant correlations were found between UCYN-A2 presence/abundance and environmental factors in this study (p values> 0.05, Spearman's rank analysis; Fig. 5). Generally, UCYN-A2 was detected (and also undetected) along the entire range of nutrients and chlorophyll concentrations, and temperature values (from 11.2 to 18.7°C) measured at the SCMW.

The temperature range where UCYN-A2 is detected at the SCMW is in agreement with those reported in other oceanographic areas (Thompson et al. 2014, Moreira-Coello et al. 2017, Mulholland et al. 2019). Recently, the niche of temperature for

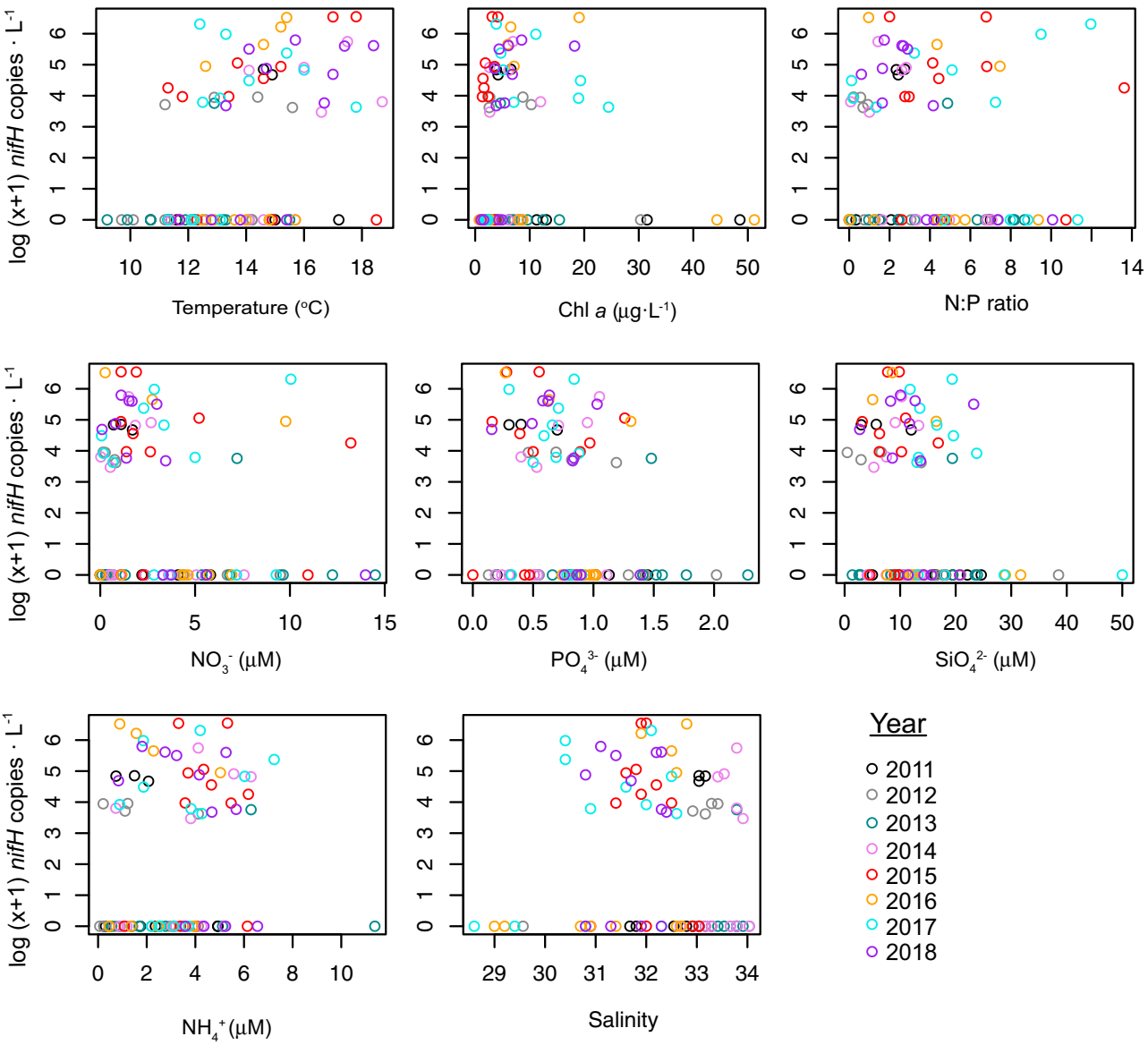

FIG. 5. UCYN-A2 *nifH* gene abundances versus temperature, chlorophyll a (Chl *a*), N:P ratio, nitrate + nitrite (NO_3^-), phosphate (PO_4^{3-}), silicate (SiO_4^{2-}), ammonium (NH_4^+), and salinity in the SCMW time-series.

this sublineage has been broadened below 0°C (in the Arctic, with N_2-fixation activity at 10°C; Harding et al. 2018, Shiozaki et al. 2018) and some studies have shown that they are mostly absent at temperatures above 25°C (Berthelot et al. 2017, Henke et al. 2018) confirming initial observations for UCYN-A (Moisander et al. 2010).

While environmental parameters did not explain the annual and seasonal variability observed for UCYN-A in the time-series, we observed that higher *nifH* gene abundances overlapped in large part with higher temperatures and lower concentrations of NO_3^-, SiO_4^{2-}, and PO_4^{3-} (Fig. S5 in the Supporting Information). UCYN-A2 was only detected sporadically (in 8 out of 34 samples) and at lower abundances (4.2×10^3 to 7.1×10^4 *nifH* copies · L^{-1}; median = 8.9×10^3) in years prior to 2014. After 2013, UCYN-A2 occurrence at the SCMW was detected in 55% of the samples (n = 60) and at higher abundances (median = 8.1×10^4 *nifH* copies · L^{-1}). This period corresponded to higher sea surface temperatures (mean = 14.39°C; Wilcoxon Rank Sum test p value < 0.001) compared to those measured for 2011–2013 (mean = 12.88°C). The SCMW time-series covers the transition of a climate shift from cold to warm in the Northeastern Pacific Ocean related to high positive sea surface temperature anomalies during the 2013–2014 winter (named "The warm Blob") that persisted through the 2015–2016 ENSO (Peterson et al. 2017,

Gómez-Ocampo et al. 2018). As a consequence, warm anomalies and negative NO_3^- concentration anomalies have been registered after 2013 in the MB (and in the California Current System) until present (Fig. 4B; Chavez et al. 2017). The shift observed after 2014 in the diazotroph community composition and in the presence/abundances of UCYN-A is consistent with the warm anomalies and the overall changes reported in phytoplankton community composition related to the same conditions (Peterson et al. 2017, Gómez-Ocampo et al. 2018). These changes included an increase in diatom and dinoflagellate species richness, as well as in other planktonic groups such as copepods. Based on these observations, we suggest that biotic or abiotic factors (such as lower NO_3^- concentrations) related with warmer conditions in the MB might have favored the increased UCYN-A abundances in this area.

The seasonality observed for UCYN-A2 at SCMW may be explained by the life cycle patterns of the UCYN-A2 host, *Braarudosphaera bigelowii,* that alternates between calcified and flagellated forms (Hagino et al. 2013). Several observations strongly suggest that the calcified form has a benthic stage in its life cycle. Light and scanning electron microscopy observations show that the coccolithophore is non-motile (do not present flagella) and presents an haptonema that can be used for adhesion to external substrates (Hagino et al. 2016), which makes plausible the idea of sinking and/or adhering to sinking substrates or sediments. Moreover, the calcified form has been recently recovered from coastal sediment samples (Kim et al. 2016, no specimen identification) and external oyster-shell scrapes (Jordan and Riaux-Gobin 2019) supporting the observations of Hagino et al. (2016). We have not yet verified that *B. bigelowii* has a benthic life stage at coastal MB, but we hypothesize that after entering in the calcified stage (suspected to take place in late autumn in these waters) it sinks to the seafloor and is no longer detectable in the water column until the motile phase reappears in late spring/summer. Microscopic studies in coastal waters of Japan over a 9-y period showed that the calcified form can only be observed in the water column for a short period (5–15 d) in mid-June to early July as sea surface temperature increases from 19 to 26°C, and then it is no longer detectable (Hagino et al. 2015, 2017). Hagino et al. (2015, 2017) do not report seasonal abundance of the motile form but it is observed in the water column prior to observing the calcified form (May–June; K. Hagino pers. comm.). The environmental factors leading to coccolithophore calcification are not well understood (Raven and Crawfurd 2012, Sett et al. 2014) and it is unknown if temperature could drive life cycle phase change and calcification of *B. bigelowii.* Further analysis of seasonal UCYN-A abundances including sediment samples combined with light microscopy for identification of calcified *B. bigelowii* would shed light on the calcification dynamics and life cycle of this symbiosis.

UCYN-A2 seasonality differs from that observed for UCYN-A1 in oligotrophic oceanic regions such as Station ALOHA (Church et al. 2009). UCYN-A1 generally shows increasing abundance in the late winter-early spring and are present throughout the other seasons (Church et al. 2009), being the dominant diazotroph from March to June (Böttjer et al. 2014, Gradoville et al. 2017). Based on the unique dodecahedral form of calcified *Braarudosphaera bigelowii,* high sinking rates have been estimated to be positively correlated with temperature (between 3.4 and 8.5 $m \cdot d^{-1}$ at 15°C; Larsen 2018). In warmer open ocean waters as at Station ALOHA, sinking rates increase, and it might not be advantageous to an form coccoliths with the risk of sinking below the pycnocline (Larsen 2018). Thus, it could be suggested that the host of UCYN-A1 is primarily in the motile form at Station ALOHA and this may explain the detection of UCYN-A1 in the water column at Station ALOHA year-round.

Although variable between years, overall the seasonal timing of UCYN-A's appearance at the SCMW coincides with the upwelling-relaxation period, and highest abundances (>10^5 *nifH* gene copies $\cdot L^{-1}$) usually occurred between May and September (2014–2018). This is consistent with findings in another upwelling system off the NW Iberian Peninsula (Moreira-Coello et al. 2017, 2019) where maximum abundances (ca. 10^3 *nifH* gene copies $\cdot L^{-1}$) were also measured in the relaxation period (July/September), although the lower detection limit in this study allowed quantification of low abundances of UCYN-A2 (ca. 10^2 *nifH* gene copies $\cdot L^{-1}$) during upwelling (April/May/June) and downwelling periods (November/December/February). This seasonal pattern was also observed for other phytoplankton groups at the SCMW. Previous time-series studies of phytoplankton dynamics at this time-series (Schulien et al. 2017) showed that half of the bloom events recorded for the period between 2006 and 2013 occurred within the oceanic season (ca. August to November), when warmer waters of the California current flush the coast of the MB, leading to optimal hydrological conditions for phytoplankton growth and biomass accumulation. These blooms corresponded mainly to dinoflagellates, but the cyanobacteria *Synechococcus* and bacterial heterotrophs also peak in abundances within this period. Whether the intrusion of the California current waters could transport UCYN-A into the MB seems unlikely regarding its lack of detection in *nifH* UCYN-A-specific high-throughput sequencing datasets from more than 60 samples screened within this system (Turk-Kubo et al. 2017). Interestingly, photosynthetic picoeukaryotes do not show a clear seasonality and blooms are recorded throughout the year except in winter months. Diatoms, in turn, generally dominate phytoplankton assemblages from January

to August, and are responsible for spring blooms during the early upwelling season. Also, it is noticeable that no *Synechococcus* blooms were recorded from 2011 to 2013 (2006–2013 dataset; Schulien et al. 2017) corresponding with the period in which UCYN-A was barely detected throughout the year and in abundances below 10^5 cells $\cdot$ L^{-1}.

We do not know why UCYN-A was undetected in *nifH* gene surveys conducted prior to 2011 in the MB (Turk-Kubo et al. 2017). These negative results leave open the question of whether the symbiosis was always present at low abundances and the environment shifted to support increased abundance or we missed detecting it because of the location of the sampling stations (Fig. S2) and the preference/affinity of *Braarudosphaera bigelowii* for neritic environments (Takayama 1972, Tanaka 1991, Konno et al. 1997). These stations were located in sites where bottom depth is more than 100 m, rather than in shallow neritic areas (i.e., wharfs, harbors). Simultaneous screening of SCMW samples and samples from the center of the MB would help to address this question.

The results of this study demonstrate that the geographic and environmental constraints of marine N_2-fixing cyanobacteria are poorly understood, and highlight the value of long-term data. Recent studies in the Western Atlantic coast have revealed the importance of N_2 fixation in coastal areas and suggested cyanobacterial diazotrophs, particularly UCYN-A, as major contributors (Mulholland et al. 2019, Tang et al. 2019). Future studies in these areas including measurements of cell-specific N_2-fixation rates would link bulk N-fixation rates to specific microorganisms. Although the presence of N_2-fixing cyanobacteria in habitats such as the Arctic and Monterey Bay is unlikely to be significant in current ecosystem budgets, they are of ecological significance and expand the domain of these diazotrophs to N-rich coastal waters.

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web site:

Figure S1. Correlation analysis between Bray-Curtis distances obtained with raw (not subsampled) and subsampled (2464 reads per sample) datasets. Mantel test statistic based on Pearson's product moment correlation was calculated ($R = 0.94$, $P < 0.001$) using Vegan package 2.5-6 in R version 3.6.2.

Figure S2. Location of the sampling stations (in red) in the Monterey Bay that were screened for UCYN-A between 2009 and 2010 using *nifH* specific PCR assays. Specific sampling dates are indicated. Map adapted from http://www3.mbari.org/bog/roadmap/major_stations.htm.

Figure S3. Maximum likelihood phylogenetic tree derived from partial *nifH* amino acid sequences from the SCMW time-series. Only representative sequences from each operational taxonomic unit (OTU) containing > 100 reads in the subsampled dataset are displayed (342 OTUs representing 57.6% of total reads). *nifH* clusters and their closest cultured representatives are indicated in different colors. Phylogenetic analysis was performed using MEGA6 (Tamura et al. 2013) and the tree was produced using the Interactive Tree of Life (http://itol.embl.de/; Letunic and Bork 2016).

Figure S4. Weekly time-series (left panels) and monthly means (right panels) of temperature, chlorophyll *a*, salinity, nitrate + nitrite, phosphate, ammonium and silicate for the period of study (2011-2018). Error bars indicate standard deviations.

Figure S5. SCMW time-series samples as represented by their UCYN-A2 abundances (nifH gene copies $\cdot$ L^{-1}) and environmental variables. Variables (all rows) were $\ln(x + 1)$-transformed, converted to z-scores, and then hierarchically clustered (average linkage), with distances based on Pearson correlations between variables ($1 - \rho$ [v1,v2]). Samples (columns) were not transformed or scaled, but were clustered as just described. Bootstrap percentages > 70 are shown (1000 bootstraps).

Table S1. Final subsampled OTU table showing the total of sequences within each OTU along the time-series, the percentage of identity (% ID) of each OTU reference sequence to the closest GenBank reference and its taxonomic assignment.

Table S2. Relative contributions of the different *nifH* clusters to total sequences in the final subsampled dataset at the SCMW time-series. The number of OTUs containing > 100 reads whose representative sequences were used for phylogeny (Fig. S3) and their relative contribution within each cluster are also indicated.

Table S3. UCYN-A sublineages qPCR abundances at SCMW, other locations along the Monterey Bay and central California coast (Moss Landing Harbor; Monterey Municipal Wharf II and northern Half Moon Bay Harbor) and Japan (Haruno Harbor, off Kochi). UD: Undetected, DNQ: Detected below the limit of quantitation.