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Microbial diversity and diazotrophy associated with the freshwater non-heterocyst forming cyanobacterium *Lyngbya robusta*

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Abstract Lake Atitlan, Guatemala, a freshwater lake in South America, experiences annually recurring blooms comprised of the planktic filamentous cyanobacterium *Lyngbya robusta*. Previous physiochemical characterisation of the bloom identified diurnal nitrogenase activity typical of non-heterocystous cyanobacteria, in addition to the low-level detection of the cyanotoxins cylindrospermopsin and saxitoxin. A molecular approach, combining deep sequencing of the 16S rRNA and *nifH* genes, was applied to a cyanobacteria-dominated sample collected during the extensive 2009 bloom. *Lyngbya* accounted for over 60 % of the total 16S rRNA sequences with the only other cyanobacterial species detected being the picophytoplankton *Synechococcus*. The remaining bacterial population was comprised of organisms typical of other eutrophic freshwater bodies, although the proportionate abundances were atypical. An obligate anaerobe *Opitutus*, not typically found in freshwater systems, was identified within the community which suggests it may have a role in enhancing nitrogen fixation. Primary nitrogen fixation was attributed to *Lyngbya*, with other putative nitrogen fixers, *Desulfovibrio*, *Clostridium* and *Methylomonas*, present at very low abundance.

Keywords Cyanobacteria · Freshwater · Nitrogenase · Bloom

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Introduction

Cyanobacteria are well established as organisms that demonstrate a wide array of morphological, physiological and chemical diversity (Tan 2007). Globally, cyanobacteria pose a number of threats to both humans and the environment through the formation of toxic blooms (Eiler and Bertilsson 2004; Rinta-Kanto et al. 2005; Pearson et al. 2008). The increasing occurrence and, in some instances, intensity of these toxic blooms have been noted over the last 10 years. Blooms composed of toxic cyanobacteria, such as *Microcystis*, *Anabaena* and *Cylindrospermopsin*, receive much attention, often due to their proximity to urban areas and drinking water supplies, with a number of molecular assays available to monitor their proliferation (Pearson and Neilan 2008; Al-Tebrineh et al. 2012). Blooms comprised of organisms, such as *Oscillatoria*, *Planktothrix* and *Lyngbya*, filamentous forms lacking heterocysts, are being increasingly reported as a consequence of increasing anthropogenic contamination (Sivonen et al. 1990; Prakash et al. 2009).

Cyanobacterial blooms are complex microbial assemblages, comprising many representatives from characterised phyla (Pope and Patel 2008; Li et al. 2011; Wilhelm et al. 2011). The morphological features of organisms, such as *Lyngbya* and *Oscillatoria*, specifically the arrangement of the thallus and production of exopolysaccharide mucilage, allow for the formation of associative microbial assemblages analogous to biofilms (Zehr et al. 1995; Reid et al. 2000; Omoregie et al. 2004; Burke et al. 2011). Cyanobacteria-dominated microbial mats, which are common in aquatic systems, have also been characterised (Zehr et al. 1995; Ferris et al. 1996; Steppe et al. 1996; Ward et al. 1998; Neilan et al. 2002; Dupraz and Visscher 2005; Steppe and

Paerl 2005). In particular, several studies have demonstrated interactions between cyanobacteria and their associated microbial community with respect to carbon, nitrogen and sulphur cycling, often exhibiting diurnal patterns (Fründ and Cohen 1992; Steppe et al. 1996; Teske et al. 1998; Visscher et al. 1998; Steppe and Paerl 2005).

In aquatic systems where blooms comprising planktic organisms generally dominate, the precise nature of these microbial associations is not understood. Bloom initiation, maintenance and subsequent decline have been shown to depend to a large extent on the availability of nitrogen (N) and phosphorus (P) within the system (Levich 1996; Sañudo-Wilhelmy et al. 2001). Furthermore, it has been demonstrated that certain ratios of N and P may select for organisms capable of fixing atmospheric nitrogen over those which lack this physiology (Klausmeier et al. 2004). As the incidence of *Lyngbya/Oscillatoria/Planktothrix* blooms increases worldwide, there is a need to further our understanding of the role of nutrient availability during proliferation of these organisms.

Lake Atitlan in Guatemala has experienced three major *Lyngbya robusta* bloom incidences, with the largest reaching its peak in November of 2009. The bloom of December 2008 was the first reported global observation of the planktic *L. robusta* (Rejmánková et al. 2011). Preliminary investigations indicated that bloom initiation occurred as a consequence of high levels of P and low N/P ratios that are indicative of nitrogen limitation. Diurnal patterns of nitrogen fixation indicated light-dependent nitrogen fixation in the presence of non-heterocystous cyanobacteria (Rejmánková et al. 2011). High rates of diurnal nitrogen fixation, oxygenic photosynthesis and the unique morphological characters of *Lyngbya* provide a specific niche for associated heterotrophic bacteria not found amongst freshwater cyanobacteria.

In this study, molecular techniques were used to determine the microbial composition of the December 2009 *L. robusta*-dominated bloom in an attempt to identify and understand the role of the heterotrophic population associated with this planktic freshwater filamentous cyanobacterium. In particular, tagged amplicon sequencing was used to determine the identity and composition of microbial taxa associated with the *L. robusta* bloom. Furthermore, taxa identified by this method were correlated with the identification of genes encoding bacterial nitrogenases to identify sources of primary and secondary nitrogen fixation and their relative compositions.

Methods

Sample collection, preparation and microscopy

Cyanobacterial bloom material was collected from four distinct locations around Lake Atitlan on November 8, 2009. Within a few hours of sampling, the samples were air dried in a nearby facility under sterile conditions before being transported to the laboratory. Samples were hydrated with sterile phosphate-buffered saline (pH 7.0) prior to microscopic analysis. Microscopic inspection was performed with the use of an Olympus BX51 fixed stage fluorescence microscope. Bloom material was stained with 300 nM 4′,6-diamidino-2-phenylindole (DAPI) and observed under bright field and using two fluorescence filter sets. Observation of DAPI fluorescence was made with a U-MNUA2 (excitation 360–370 nm, emission 420–460 nm) mirror unit. Genomic DNA was extracted from 100 mg of dried bloom material using the Soil DNA Extraction kit (MP Biosciences) according to manufacturer's specifications.

Ribosomal tag pyrosequencing

Amplicon sequencing of partial 16S rRNA gene sequences was performed on extracted bloom DNA at the Research and Testing Laboratory (Lubbock, Texas). Primers 27f and 519r (Weisburg et al. 1991) were used to PCR amplify a 500-bp product spanning the 16S rRNA variable regions V1–V3. Sequencing was performed using a Roche GS FLX Titanium machine. Initial quality control measures used to ensure sequence fidelity included the removal of short sequences (<100 bp), any sequence with a discrepancy to the 5′ primer or any sequence containing an unresolved nucleotide (N). This provided ~10,000 reads upon which further quality control was performed using the Mothur package (Schloss et al. 2009). A 2 % pre-clustering step was performed to remove other potential errors in sequence data. Chimeric sequences were identified and removed using the program chimera.slayer with minsnp = 100. Sequences containing long (>7) homopolymers and those that did not align within the appropriate rRNA gene variable region were also removed. The remaining 7,949 reads, representing 1,333 unique sequences, were taxonomically characterised using a highly curated database from the Ribosomal Database Project (http://rdp.cme.msu.edu/). This dataset is publically available online at the MG-RAST server (http://metagenomics.anl.gov) under the project title "Lake_Aitlan_Bloom_16S". The depth of sampling of community members was examined using rarefaction analysis of operation taxonomic units (OTUs) defined by a similarity of >97 %.

Characterisation of the nifH-containing community

The dinitrogenase reductase gene, *nifH*, was amplified using *Pfu* polymerase (Promega) as previously described (Jungblut and Neilan 2009). The amplicons were A-tailed and cloned into pGEM-T-Easy vector (Promega) as per manufacturer's instructions. This ligation was transformed into NEB 5-alpha high efficiency chemically competent

Escherichia coli (New England Biolabs). White colonies were picked and grown overnight with ampicillin selection, and the plasmids were extracted using a PureLink Quick Plasmid Miniprep kit (Invitrogen). Automated sequencing of the purified plasmid was performed with the vector-specific primer MPF (Jungblut and Neilan 2009), PRISM BigDye Terminator v3.1 and an Applied Biosystems ABI 3730 Capillary Sequencer at the Ramaciotti Centre for Gene Function Analysis, UNSW.

Sequencing of nifH-containing plasmids was undertaken until a sufficient number of sequences were obtained, as determined by rarefaction analysis at 97 % similarity of non-unique nucleotide sequences. Translated amino acid sequences were aligned using ClustalW. The alignment was visually inspected, and non-overlapping terminal regions were truncated, with the resulting alignment representing 117 amino acid positions. Non-unique amino acid sequences were removed from the alignment and 23 reference sequences corresponding to *nifH* genes from five phyla introduced for comparison. The *Leptolyngbya boryana* FrxC protein (BAA00565) was used as the out-group. Phylogenetic tree reconstruction was performed using PhyML version 3.0 with the LG model of amino acid substitution and a bootstrap re-sampling value of 1,000. Translated amino acid sequences submitted to GenBank were made available under accession numbers JQ012734-JQ012740.

Results

Morphological characterisation

Microscopic inspection of the bloom material indicated that greater than 80 % of the total biomass was comprised of *L. robusta* filaments (Rejmánková et al. 2011). Trichomes were encapsulated in a persistent polysaccharide sheath, which often extended beyond the terminal end of the trichome. Large aggregations were observed throughout the bloom material in close association with the *L. robusta* filaments (Fig. 1a, b). DAPI-induced fluorescence of the *L. robusta* filaments provided evidence of bacteria associated with the sheath, particularly at the terminal ends of filaments (Fig. 1c).

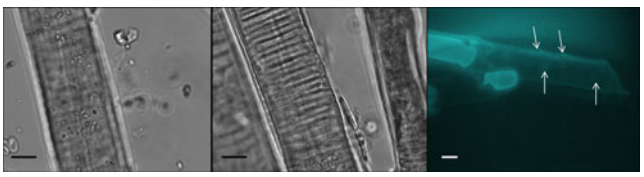

Fig. 1 Bright field (**a**, **b**) and fluorescence micrographs (**c**) of *L. robusta*-dominated microbial assemblage. DAPI fluorescence was visualised with a U-MNUA2 (excitation 360–370 nm, emission 420–460 nm) mirror unit. *Arrows* have been provided to highlight putative bacterial association with the *L. robusta* polysaccharide sheath. *Scale bar* in each image indicates 10 μm

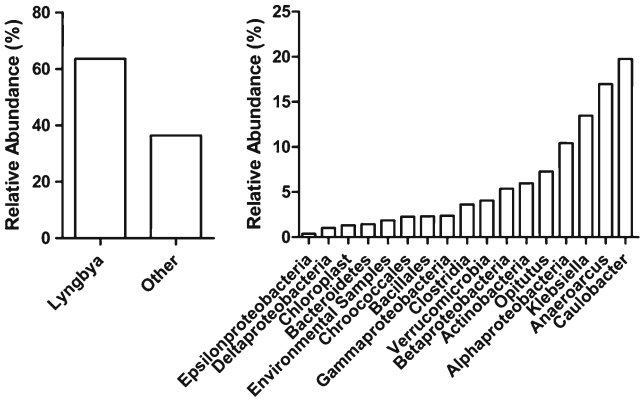

Fig. 2 The relative abundance of the eubacterial population within the (**a**) total bloom sample and (**b**) the non-*Lyngbya* population as determined by 16S rRNA amplicon pyrosequencing

Bacterial community analysis

Amplicon sequencing of 16S rRNA genes in the sample produced a total of 7,949 high-quality reads of at least 279 bp in length, representing 1,333 unique sequences. Clustering of sequences with a 97 % similarity resulted in 250 unique OTUs. Rarefaction analysis indicated that sampling of the community was not performed to completion, with a terminal frequency of greater than one unique OTU being identified every 100 sequences (as sampled over the last 500 sequences).

Sequences assigned to the taxon *Lyngbya* were clearly dominant, representing 63.61 % of the total bacterial population (Fig. 2). The remaining 36.39 % of sequences consisted of taxa from the phyla Proteobacteria, Firmicutes, Cyanobacteria, Actinobacteria, Bacteroidetes and Verrucomicrobia (Fig. 2a). Proteobacteria represented 19.23 % of the total bacterial population and 52.84 % of the non-*Lyngbya* population and included representatives from all five proteobacterial classes. The genera *Caulobacter* (19.75 %) and *Klebsiella* (13.48 %) were the most dominant representatives of the Proteobacteria (Fig. 2b). Cyanobacterial sequences identified as representatives of the genera *Synechococcus*, accounted for 2.29 % of the non-*Lyngbya* population. The Firmicutes genus *Anaeroarcus* (16.98 %), the Verrucomicrobia genus *Opitutus* (7.28 %) and the Actinobacteria (5.96 %) were also prevalent (Fig. 2b).

Nitrogenase (nifH) phylogeny

A total of 25 *nifH* sequences, each at least 359 bp in length, were obtained by the sequencing of extracted plasmids from within a clone library. Rarefaction analysis of *nifH* nucleotide sequences, grouped on the basis of 97 % similarity,

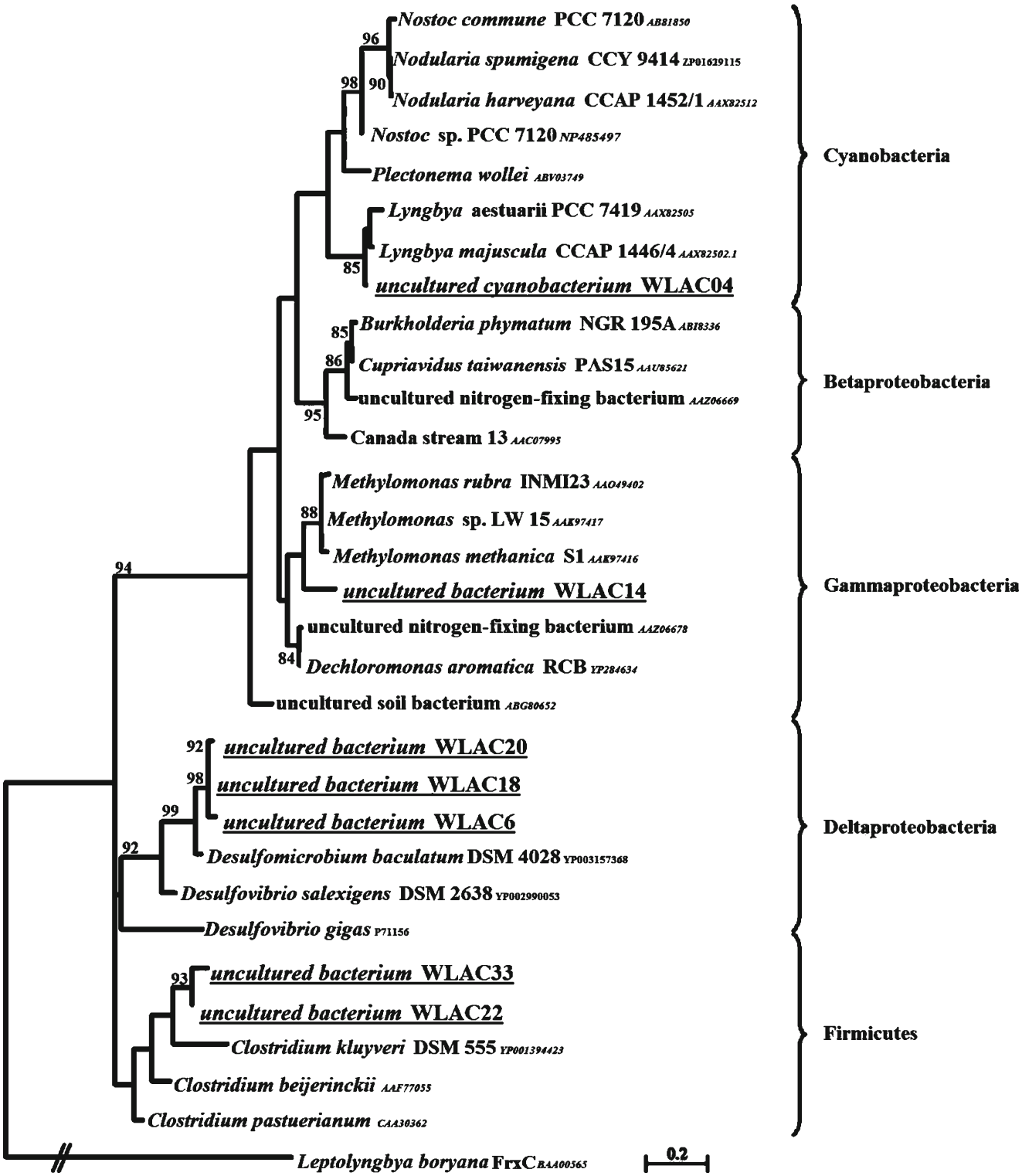

Fig. 3 Maximum likelihood phylogeny of *nifH* amino acid sequences containing 23 sequences obtained from GenBank and seven sequences (*underlined*) derived from a 329-bp gene product amplified from the *L. robusta*-dominated assemblage. The *L. boryana* FrxC amino acid sequence was included as the out-group. Sequences obtained from the *L. robusta*-dominated assemblage were deposited in the GenBank database under accession numbers JQ012734-JQ012740. *Scale bar length* represents 0.2 amino acid substitutions per site

indicated that the sample had been sequenced to extinction, with an average of one unique sequence obtained from the last 20 rounds of sampling. Clustering of nucleotide sequences based again on 97 % similarity resulted in the generation

of four distinct OTUs. Removal of non-unique amino acid sequences indicated the presence of seven unique nitrogenase genes across the four OTUs. Maximum likelihood phylogenetic inference revealed that each of the four OTUs corresponded to the organisms, *Lyngbya*, *Methylomonas*, *Desulfovibrio* and *Clostridium* (Fig. 3). Heterogeneity between amino acid sequences was observed for the *Desulfovibrio* and *Clostridium* OTUs with three and two unique sequences obtained, respectively. In relation to abundance of the total 25 sequences obtained, 13 were from *Desulfovibrio*; five each corresponded to *Lyngbya* and *Methylomonas*, and two were most closely related to known *Clostridium* sequences.

Discussion

As expected from microscopic examination, microbial community composition of the Lake Atitlan bloom was dominated by *Lyngbya* sequences with a high similarity to those reported from other freshwater or terrestrial environments (Engene et al. 2010). However, the relative abundance of cyanobacterial sequences within the bloom population was very high in contrast to those observed in cyanobacterial blooms comprised of other genera (Eiler and Bertilsson 2004; Wilhelm et al. 2011). The occurrence of *Synechococcus* sequences at low abundance in these samples indicated the presence of picophytoplankton, commonly observed in freshwater bodies. Periphytic forms of *Synechococcus* have been reported associated with artificial substrates, macrophytes and microbial mats (Ferris et al. 1996; Becker et al. 2004). Whilst this may suggest a possible mechanistic association between the *Lyngbya* and *Synechococcus* picoplankton communities, no direct attachment was observed under bright field or fluorescence microscopy. *Microcystis* cf. *botrys*, noted as a common species within Lake Atitlan (Rejmánková et al. 2011), was not observed either microscopically or by molecular methods, including microcystin gene PCR (data not shown), during the bloom event. It was also noted that C, N and P content at the time of the bloom resulted in a low N/P molar ratio indicating nitrogen limitation (Rejmánková et al. 2011). This nitrogen limitation is likely to limit the proliferation of toxic *Microcystis* spp. which lack nitrogen-fixing mechanisms (Wilhelm et al. 2011).

The occurrence of the *Lyngbya* bloom at a low N/P ratio was shown, in a previous study, to correlate with diurnal patterns of nitrogenase activity indicative of cyanobacteria-dependent nitrogen fixation (Zehr et al. 1995; Steppe and Paerl 2005). Translated amino acid sequences of *nifH* genes obtained from the total bloom DNA suggested the presence of four potential nitrogen-fixing organisms: *Lyngbya*, *Methylomonas*, *Clostridium* and *Desulfovibrio*, within the sample. A single *nifH* gene that grouped exclusively with other cyanobacteria sequences was most similar to homologous sequences in *Lyngbya* sp. PCC 7419 and *Lyngbya* sp. CCAP 1446/4. The true nature of nitrogen fixation among strains, within the genus *Lyngbya*, is contentious due its polyphyletic nature (Engene et al. 2010). Recently, the complete genome sequence of a marine *Lyngbya majuscula* (*Moorea producens*) isolate revealed the absence of a *nif* gene operon, corresponding to a lack of nitrogenase activity (Jones et al. 2011). Amongst the freshwater *Lyngbya*, evidence of nitrogen fixation has been reported either by physiological or molecular methods (Paerl et al. 1991; Suda et al. 1998; Leitao et al. 2005). In the context of the newly revised phylogeny of *Lyngbya*, which establishes a number of new taxonomic groupings, *L. robusta* is the only species of *Lyngbya* that has been demonstrated to be capable of nitrogen fixation, by both physiological and molecular methods (Rejmánková et al. 2011).

The heterotrophic bacterial population consisted of the major phyla associated with freshwater bodies, including Actinobacteria, Bacteroides, Proteobacteria (α, β and γ subgroups) and Firmicutes. The heterotrophic population associated with the *L. robusta* bloom material was in contrast to those associated with freshwater blooms comprising *Microcystis*, *Aphanizomenon* and *Anabaena* (Wobus et al. 2003; Eiler and Bertilsson 2004; Oh et al. 2011; Wilhelm et al. 2011). Variations within and amongst individual water bodies are expected due to the influence of nutrient availability, pH, temperature and geography. The abundance of taxa, specifically the Firmicutes genera, Anaeroarcus and Bacillus, as well as Actinobacteria and β-Proteobacteria, was outside those ranges typically observed for cyanobacteria-dominated water bodies. Elevated proportions, well below those observed here, of Firmicutes genera, have been observed in some instances where cyanobacteria are present, corresponding specifically to increased levels of dissolved ammonia (Wobus et al. 2003; Wilhelm et al. 2011). Whilst the ability of some Firmicutes genera to tolerate and thrive in high levels of NH_3 (Caskey and Tiedje 1980; Slobodkin and Verstraete 1993) is likely the reason for the elevated levels reported in this and other instances (Wobus et al. 2003; Wilhelm et al. 2011), the ability of polysaccharide mucilage may allow for association with the *Lyngbya* biomass, resulting in the vastly increased abundance observed within this study. Observations of interactions between cyanotoxins and the associated microbial community may account for some of the variability observed amongst the Actinobacteria and β-Proteobacteria (Wilhelm et al. 2011). Trace levels of cylindrospermopsin and saxitoxin were reported within the lake (Rejmánková et al. 2011); however, genetic analysis confirmed that the *Lyngbya* was not responsible for these (Komarek et al. unpublished). The absence of microcystins in Lake Atitlan and, hence, microcystin-degrading bacteria (Rapala et al. 2005;

Ho et al. 2007; Manage et al. 2009) may account the abundances of these phyla decreasing to levels typical of oligotrophic freshwater lakes (Wobus et al. 2003).

Heterotrophic associations between eubacteria and cyanobacteria have been reported previously, with particular reference to cyanobacteria producing a sheath or mucilage (Paerl 1996; Reid et al. 2000). Microorganisms identified from the *L. robusta* bloom, implicated in forming heterotrophic associations with cyanobacteria, include Actinobacteria (Albertano and Urzi 1999), Proteobacteria (Zehr et al. 1995; Abed et al. 2007), Verrucomicrobia (Sánchez et al. 2005; Stevenson and Waterbury 2006; Abed et al. 2007; Wilhelm et al. 2011) and Bacteroidetes (Sánchez et al. 2005; Abed et al. 2007). In addition to those members of the Firmicutes previously highlighted, *Opitutus* represents the strongest argument for a permanent heterotrophic association within oxygen-depleted zones of the *L. robusta* bloom material. Known as an obligate anaerobe, *Opitutus* has been previously associated with nitrogen fixation under strict anaerobic conditions in rice fields (Chin et al. 2001). Whether or not *Opitutus* and *Lyngbya* form a facultative symbiotic relationship that influences nitrogen fixation, as is the case for other cyanobacteria (Paerl et al. 1989; Steppe et al. 1996; Sandh et al. 2012), warrants further study.

Nitrogenase genes were identified in the *L. robusta*-dominated bloom that belonged to members of the Proteobacterial classes Gammaproteobacteria and Deltaproteobacteria, along with several Firmicutes phyla. The Firmicutes, in particular *Clostridium*, have been implicated in primary nitrogen fixation during the winter months in marine cyanobacterial mats (Zehr et al. 1995). Notably in the *L. robusta* bloom, with the exception of the *L. robusta* itself, the organisms capable of nitrogen fixation were present at very low levels. Nitrogenase genes corresponding to the genus *Klebsiella*, detected at high levels by 16S rRNA gene analysis, were not observed in the clone libraries, contrary to previous reports of its nitrogenase activity (Houmard et al. 1980). Associations between anaerobic heterotrophs and cyanobacteria may provide a source of nitrogen fixation for filamentous cyanobacteria that lack sufficient nitrogenase activity (Steppe et al. 1996; Steppe and Paerl 2005).

Cyanobacterial diazotrophy in the absence of heterocysts is contentious for a number of taxa due to the complex nature of cyanobacterial assemblages and blooms. Heterotrophic bacteria in these systems are capable of primary and secondary nitrogen fixation. In addition, heterotrophic bacteria may exclude oxygen, enhancing cyanobacterial nitrogen fixation at the site of association. Correlations between identified heterotrophic bacteria, the presence of nitrogenase genes, oxygen sensitivity and other processes increase our understanding of these systems. Within the *L. robusta* bloom in Lake Atitlan, the dominant cyanobacterium appears to be providing both primary carbon autotrophy and nitrogen fixation in a diurnal cycle, with heterotrophic bacteria capable of persisting under these conditions, providing a putative role in enhancing anoxic nitrogenase activity.

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