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A **a** 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 sequenc-

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

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Escherichia coli

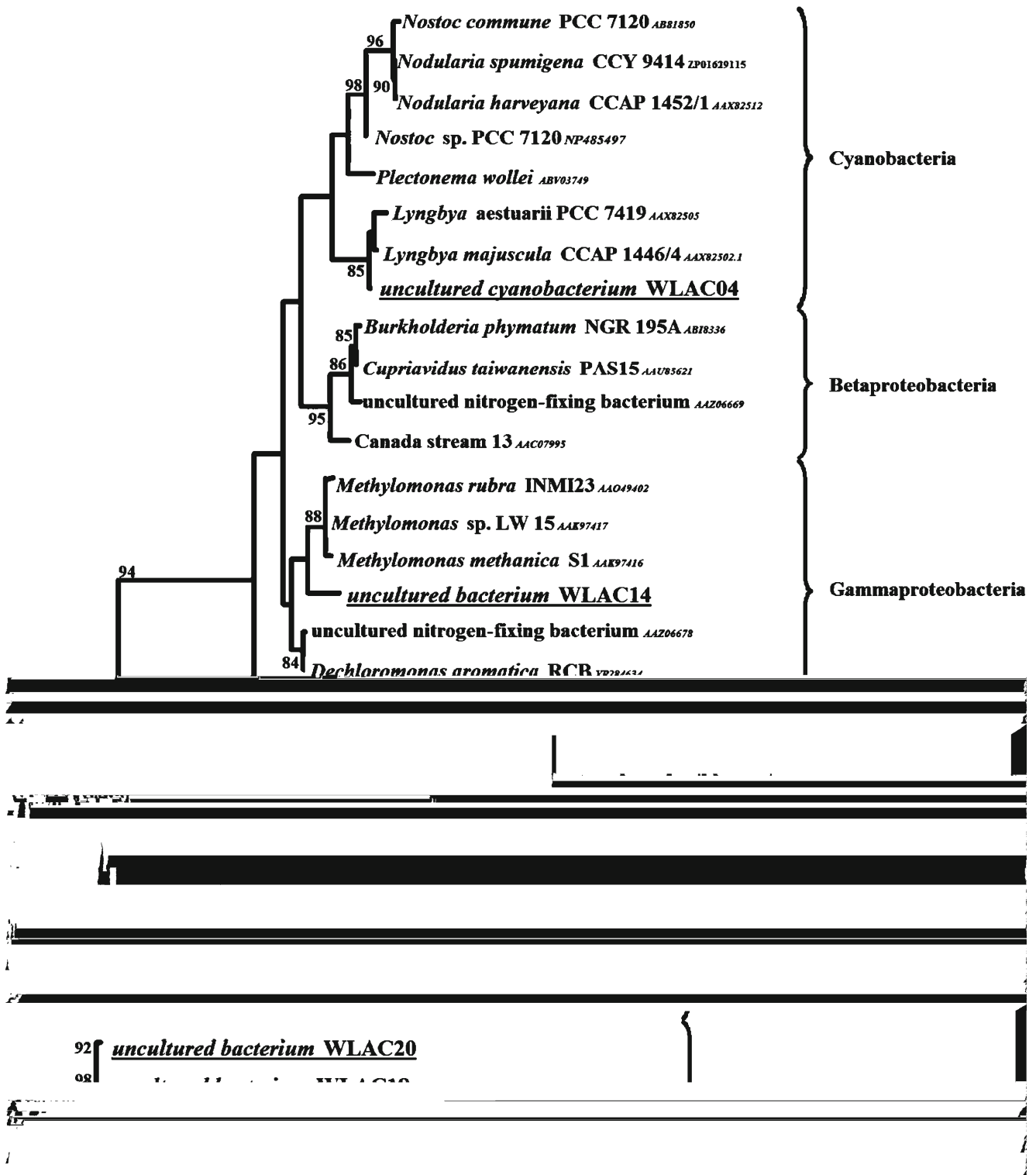


Fig. 3 Maximum likelihood phylogeny of *nifH* amino acid sequences containing 23 sequences obtained from GenBank and seven sequences (underlined) derived from a 329-bp gene product amplified from the *L. robusta*-dominated assemblage. The *L. boryana* FrxC amino acid

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

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, *Opiritatus* represents the strongest argument for a permanent heterotrophic association within oxygen-depleted zones of the *L. robusta* bloom material. Known as an obligate anaerobe, *Opiritatus* has been previously associated with nitrogen fixation under strict anaerobic conditions in rice fields (Chin et al. 2001). Whether or not *Opiritatus* 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 09.992Nu5(i)02(og)15.5(en)-32611a

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