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Benthic Bacterial Diversity in Submerged Sinkhole Ecosystems▽†

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Physicochemical characterization, automated ribosomal intergenic spacer analysis (ARISA) community profiling, and 16S rRNA gene sequencing approaches were used to study bacterial communities inhabiting submerged Lake Huron sinkholes inundated with hypoxic, sulfate-rich groundwater. Photosynthetic cyanobacterial mats on the sediment surface were dominated by *Phormidium autumnale*, while deeper, organically rich sediments contained diverse and active bacterial communities.

Groundwater intrusion is becoming recognized as an important source of nutrients, contaminants, and trace elements in aquatic ecosystems (7). Recent reports regarding marine habitats suggest that groundwater influences nitrogen inputs (29) and may have a significant impact on nutrient dynamics over seasonal (27) and longer (25) time scales. To date, investigations of groundwater effects have focused primarily on marine habitats. To better understand the impact of groundwater intrusion into freshwater habitats, we have been studying submerged sinkholes in the Laurentian Great Lakes.

Sinkholes typically develop in areas of terrestrial karst when underground caverns collapse (36). We recently discovered submerged sinkholes that occur beneath the surface of Lake Huron in water up to a depth of 93 m (9, 41). These unique habitats are formed by groundwater dissolution of Paleozoic limestone and marine evaporite sediments in the Michigan Basin (16). Some sinkholes actively release cold, dense groundwater through underwater vents onto the lake floor. The venting groundwater has a lower pH (~7.1), higher specific conductivity (~2.3 $\text{mS} \cdot \text{cm}^{-1}$, due to high levels of dissolved sulfate [>1,000 $\text{mg} \cdot \text{liter}^{-1}$], carbonate, and chloride ions), and lower concentrations of dissolved oxygen (<0.4 $\text{mg} \cdot \text{liter}^{-1}$) and nitrate than Lake Huron water (2, 3, 41, 42). The intrusion of cold, hypoxic, sulfate-rich groundwater greatly alters the local lake habitat and has a significant impact on the sediment microbial community. Sediments at nearby control sites are sandy, but submerged sinkhole sediments (with carbon accounting for 5 to 35% of the sediment dry weight) are rich in organic matter originating from phytoplankton in the overlying water column (S. C. Nold, M. J. Bellecourt, B. A. Biddanda, S. C. Kendall, S. A. Ruberg, and J. V. Klump, submitted for publication). In sunlit sinkholes, organically rich sediments are covered by purple-pigmented cyanobacterial mats—vertically stratified communities composed of interdependent layers of phototrophic, chemotrophic, and heterotrophic microorganisms (35).

While the Laurentian Great Lakes are important freshwater resources, there are remarkably few molecular surveys of their microbial communities and no surveys of such communities in submerged sinkhole habitats. Here, we describe the physicochemical conditions and microbial inhabitants in the sediments of two submerged sinkholes near Alpena, MI (3) (see Fig. S1 in the supplemental material). El Cajon sinkhole, located in Misery Bay, is a small, shallow (<1-m-deep), near-shore spring that receives full sunlight. In contrast, the deeper (23-m) Middle Island sinkhole experiences ~5% surface irradiance and affects a large (>1-ha) area of the lake floor. This study characterizes microbial communities that are unique within the Laurentian Great Lakes and are composed largely of novel, uncultivated microorganisms, some of which may represent new lineages. We detected primarily cyanobacteria similar to Antarctic *Phormidium autumnale* strains that likely grow as sulfide-oxidizing autotrophs. We also detected proteobacterial lineages and members of the *Bacteroidetes*, *Firmicutes*, and *Chloroflexi* that likely participate in the sulfur and carbon cycles, consistent with physicochemical gradients present in the habitat.

Briefly (methods are described in detail in the supplemental material), pore water chemistry data were obtained from sections of extruded sediment cores. Profiles of bacterial community composition were obtained using the automated ribosomal intergenic spacer analysis (ARISA) technique (15, 58). We used PCR to amplify the entire 16S rRNA gene and the intervening transcribed spacer (ITS) region between the 16S and 23S rRNA genes from DNA extracted from sinkhole sediment and cyanobacterial mat samples. Clone libraries were constructed, and full-length sequence data were obtained.

Cyanobacterial mat samples collected from rocks at El Cajon Spring and Middle Island sinkhole displayed a thin (<0.5-cm-thick) layer of purple filamentous cyanobacteria with minimal accumulation of organic material beneath. Middle Island sediment samples contained a similar surface mat but displayed distinct vertical layering of microbial species overlying thick (>2-m), organically rich sediments (Fig. 1). Sediment pore water profiles suggest that sulfate is the dominant electron acceptor in the habitat, as the water column is hypoxic (41) and NO_3^--N concentrations were just above detectable levels (Fig. 1). A reproducible decline in sulfate concentrations

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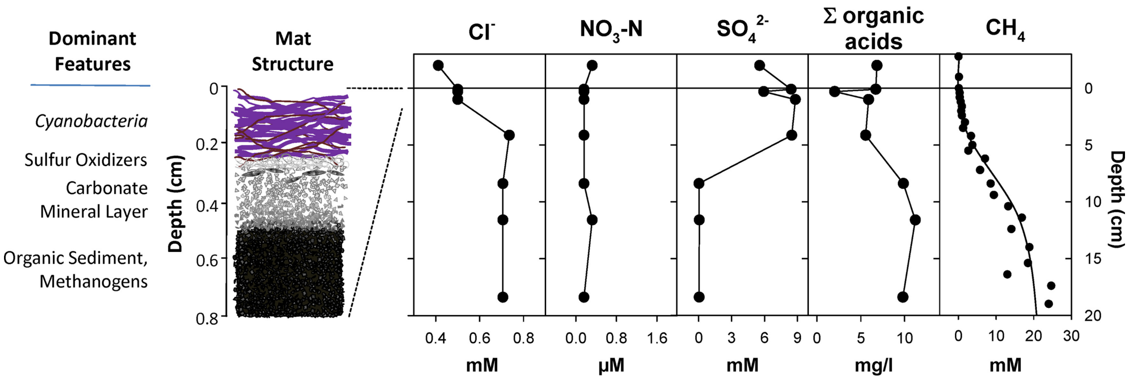

FIG. 1. Schematic drawing of sediment architecture and corresponding pore water chemistry profiles for organically rich Middle Island sinkhole sediments.

within the carbonate-rich mineral layer (0.3 to 0.5 cm) was detected (Fig. 1). The simultaneous removal of sulfate and organic acids in this layer provides evidence of sulfate reduction. The accumulation of carbonate-rich minerals further supports sulfidogenesis since sulfate-reducing bacteria (SRB) can form carbonate layers in marine stromatolites (26, 40). SRB are important in the mineralization of organic carbon in marine sediments (24), but low sulfate concentrations in freshwater limit sulfidogenesis in typical Great Lakes sediments (48). Sinkhole sediments, however, are continuously supplied with sulfate by groundwater advection (41), allowing an active sulfur cycle. In addition to sulfate reduction, sulfur oxidation likely occurs, as white filamentous bacteria morphologically similar to *Beggiatoa* or *Epsilonproteobacteria* species were observed just below the cyanobacterial layer (Fig. 1) (14). Cyanobacteria may also participate in the sulfur cycle, growing via anoxygenic photosynthesis using SRB-produced sulfide as an electron donor (8, 45). At depths of >7 cm, sulfate was absent from the sediment, allowing methanogenesis to occur (52) and resulting in the accumulation of methane at sediment depths of >5 cm (Fig. 1). The depletion of methane and organic acids at depths of <5 cm (Fig. 1) indicates the occurrence of anaerobic methane oxidation. This process is frequently observed in concert with sulfate reduction due to a syntrophic association between methane-consuming *Archaea* and SRB based on interspecies electron transfer (33, 49, 51).

Cyanobacterial mat samples from the two sites exhibited similar ARISA community profiles that were distinct from those of sediment samples (Fig. 2). Mat communities were dominated by sequences with an ITS length of 837 bp, characteristic of cyanobacterial gene sequences (see Table S1 in the supplemental material). Mat clone libraries were also dominated by cyanobacterial sequences (Fig. 3; see Table S2 in the supplemental material). Due to the long ITS regions in cyanobacteria, PCR amplification results may be biased against cyanobacteria (1, 22), underestimating the actual abundance of these organisms in the surface mat community. Most cyanobacterial sequences were similar to sequences from *P. autumnale* and its relatives (see Table S1 in the supplemental material). *Phormidium* strains have been found in Australia, the Bahamas, Greece, Antarctica, and Yellowstone National Park (5, 11, 54) and are capable of anoxygenic photosynthesis using sulfide as an electron donor (17). Clone TS-36-39 is closely related (98% similar) to a *P. autumnale* strain cultivated from permanently ice-covered Antarctic lakes (10, 46, 47). The high degree of similarity between Lake Huron and Antarctic strains is intriguing and may provide insights into the distributional ecology of cyanobacterial species, especially those adapted to cold, sulfidic groundwater environments.

Compared to mat samples, sediment samples obtained from the 0- to 2-cm-depth interval displayed increased levels of clone diversity with broad representation of many lineages (Fig. 3; see Table S2 in the supplemental material). While the vast majority of the most closely related sequences in the GenBank database were from uncultivated environmental organisms whose ecosystem functions are unknown (see Table S1 in the supplemental material), some comparisons may help us understand community activity (34). Previous studies of sulfidic environments have focused on terrestrial landscapes, namely, caves (e.g., those described in references 14 and 39) and surface springs (e.g., those described in references 12, 28, and 38). The reports of these studies identify novel bacterial sequences, especially from the *Epsilonproteobacteria*, that may function to oxidize sulfur compounds and hasten the dissolu-

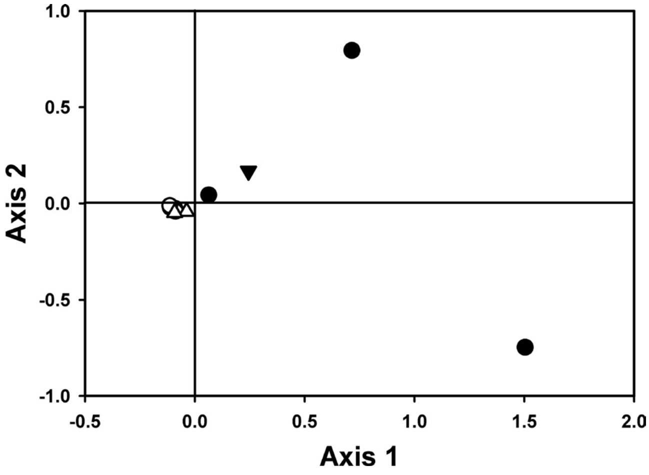

FIG. 2. Correspondence analysis ordination for ARISA community profiles from Lake Huron sinkhole mat and sediment environments. ●, Middle Island mat; ○, Middle Island sediment; ▼, El Cajon mat; △, El Cajon sediment.

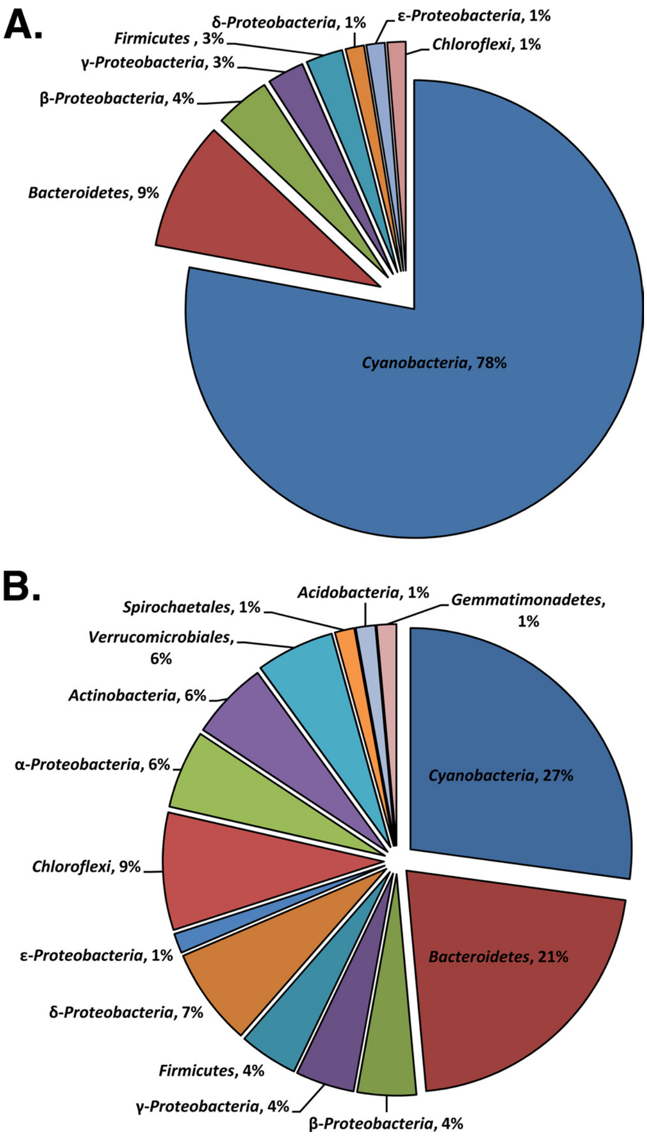

FIG. 3. Relative abundances of 16S rRNA gene clone types retrieved from Lake Huron sinkhole environments. (A) Composite analysis of cyanobacterial mat samples retrieved from El Cajon and Middle Island sinkholes; (B) analysis of an integrated sediment sample from a depth of 0 to 2 cm in the Middle Island sinkhole.

tion of carbonate-bearing rocks (6). The *Epsilonproteobacteria* we detected were similar to others from groundwater group I, sulfur-oxidizing organisms found in cold sulfidic springs that have been implicated in speleogenesis (13). Other sinkhole microorganisms likely participate in the sulfur cycle, especially *Gamma-* and *Deltaproteobacteria*, whose closest relatives belong to genera capable of sulfur metabolism, and *Cyanobacteria*, which use sulfide as an electron donor during anoxygenic photosynthesis. Clones CS-42-12 and AS-39-1 are related to obligately fermentative *Clostridium* strains of the *Firmicutes* (56). Their sinkhole counterparts may also act as heterotrophs. None of the retrieved clones were identical to known SRB, but *Deltaproteobacteria* and some Gram-positive strains are known to reduce sulfate (4, 39a). Other phyla we detected (*Verrucomicrobia*, *Actinobacteria*, and *Bacteroidetes*) are common in aquatic habitats (18, 31, 32, 53, 59), but the ecological functions performed by these organisms are less well understood.

Sinkhole sediments were more diverse and displayed higher degrees of species evenness than overlying mat communities (see Table S2 in the supplemental material). Species diversity rarefaction curves reflect these differences (see Fig. S2 in the supplemental material). Rarefaction analysis likely underestimates overall diversity (21, 23), but after the acknowledgment of biases due to methodological differences, it does allow us to examine relative differences between habitats. Species diversity within sinkhole sediments is similar to that in marine sediments (43) and that of bacterioplankton collected from near-shore sites in Lake Michigan (30). The degree of sinkhole sediment diversity is still lower than the degree of microbial diversity found in typical farm soils (50). Surface cyanobacterial mats display a relatively low level of species diversity, similar to that in oligotrophic seawater (43) and that of the phytoplankton community found in a humic lake (19).

Studies of bacterial diversity in the Laurentian Great Lakes include descriptions of bacterioplankton (20, 30, 57) and picoplankton (37) communities. An oligonucleotide probe study found that picoplankton communities in all the Great Lakes except Lake Superior were similar; in Lake Superior, a more unique epilimnetic community was found (37). There are two descriptions of Great Lakes community composition based on 16S rRNA clone library techniques. In one study, near-shore Lake Michigan bacterioplankton communities were found to contain primarily *Betaproteobacteria* (30) but also included other common freshwater organisms. Another study investigating the chemocline of hypoxic waters in Lake Erie found communities dominated by *Synechococcus* and *Cyanobium* (57). While existing Great Lakes clone libraries do display differences between lakes and between epilimnetic and hypolimnetic waters, representation of phylogenetic groups in these libraries does not differ significantly from that in libraries from other freshwater habitats of equivalent trophic status (32, 59). In contrast, Lake Huron sinkhole environments differ significantly from the open lake, and our clone libraries reflect the uniqueness of these habitats. Sinkhole sediments were dominated by cyanobacteria but also contained a unique combination of phylogenetic groups that included an abundance of *Bacteroidetes* and *Chloroflexi* clones in addition to the more typical *Proteobacteria*, *Actinobacteria*, and *Verrucomicrobiales*. Freshwater sediments generally contain greater proportions of *Deltaproteobacteria* and *Acidobacteria* than bacterioplankton communities (44), but only one *Acidobacteria* clone was detected in our library (see Table S2 in the supplemental material). Overall, the relative abundances of phylogenetic clusters within sinkhole clone libraries appear to be unique within the Laurentian Great Lakes. While most of our clones were closely related to sequences previously retrieved from aquatic habitats, including aquifers and freshwater sediments (see Table S1 in the supplemental material), some clones (e.g., *Chloroflexi* clones AM-45-7, TS-45-5, and AS-39-5; *Firmicutes* clone AS-39-1; and *Verrucomicrobiales* clone CS-42-3) were only distantly related. Such sequences may represent new lineages of groundwater-adapted strains.

Nucleotide sequence accession numbers. The 16S rRNA gene and ITS region sequences have been deposited in the

GenBank database under accession numbers GQ406144 to GQ406203.

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