Diversity and composition of cave methanotrophic communities

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ABSTRACT Methane oxidizing microorganisms (methanotrophs) are a major sink for the greenhouse gas methane (CH₄), and have been investigated in several environments. Recent studies show that CH₄ consumption in caves is pervasive and is a result of active methanotrophy. However, little is known about what controls the distribution and abundance of methanotrophs in caves. We sampled 42 sediments from 21 caves in North America to elucidate the factors shaping cave methanotroph communities. We hypothesized that cave methanotroph communities should be related to cave-air CH₄ concentrations and exhibit dispersal-limited biogeographical patterns due to the insular nature of caves. Using 16S rRNA sequencing, we recovered methanotrophs from 88 % of samples collected, including locations in caves where CH₄ concentrations were at or below detection limits (≤ 0.3 ppmv). Methanotrophs from the Methylocystaceae (Type II) were the dominant methanotrophs as has been seen in other environments with low CH₄ concentrations. Despite being insular ecosystems, we found that the composition of methanotrophs did not vary with distance, both within and among caves. Instead, we found evidence for a core microbiome, perhaps suggesting that high-affinity methanotrophs are not dispersal limited. Additionally, we observed that the relative abundance of methanotrophs was positively related the proportion of gravel in cave sediments and the relative abundance of methylotrophs. Last, we found that the relative abundance of methanotrophs was inversely correlated with cave-air CH₄ concentrations. Our results suggest that methanotrophs in caves have influences on cave biogeochemistry beyond CH₄ oxidation and that high-affinity methanotrophs may disperse easily into caves.

IMPORTANCE Recent observations have shown that the atmospheric greenhouse gas methane (CH₄) is consumed by microorganisms (methanotrophs) in caves at rates comparable to CH₄ oxidation in surface soils. Caves are abundant in karst landscapes that comprise 14 % of Earth's land surface area, and therefore may be acting as a substantial CH₄ sink. A detailed ecological understanding of the forces that shape methanotrophic communities in caves is lacking. We sampled cave sediments to better understand the community composition and structure of cave methanotrophs. Our results show that the relative abundance of methanotrophs was negatively correlated with CH₄ concentrations in cave air and that methanotrophic communities were similar to each other over distances of 10s of m to 100s of km.

KEYWORDS Biogeography; Cave; Greenhouse Gas; Karst; Methane; Methanotroph;

INTRODUCTION

Methane (CH₄) is a greenhouse gas in Earth's atmosphere, an energy source for humanity, and has been observed in the atmosphere of Mars (1–3). Thus, enhancing the knowledge of the pathways that add and remove CH₄ to and from Earth's atmosphere has implications for several fields. Two major processes are responsible for the removal of CH₄ from Earth's atmosphere. Oxidation by hydroxyl radicals accounts for 90 % of atmospheric-CH₄ removal and methane-consuming microorganisms (i.e., methanotrophs) in surface soils account for an additional 5 % (4). Despite the influence that methanotrophs have on regulating Earth's climate, gaps in our understanding of these organisms, like how

they respond to changing CH₄ concentrations or how they disperse through the environment, remain (2, 3).

Rocks that host caves are widespread on Earth, have been observed on other planets, and are emerging as a global CH₄ sink (7–10) (Figure 1). The relative importance of deterministic and stochastic factors responsible for shaping the methanotroph community in caves is unknown (7, 9, 11–14). Observations of atmospheric CH₄ consumption in caves suggest that high-affinity or Type II methanotrophs may be present in cave environments. These organisms have broad phylogenetic affiliations (15–20), i.e. are known from the Methylocystaceae, as well as several groups in clades affiliated with upland soils (upland soil cluster methanotrophs). They tend to be the most prominent members of the methanotrophic community in environments with low CH₄ concentrations and high O₂ concentrations. They are less common in environments with high CH₄ and low O₂ concentrations, like lakes or geological seeps, which are dominated by methanotrophs from the Methylococcales (Type I methanotrophs) (21, 22).

The factors that control the distribution and abundance of methanotrophs in caves are unknown. Previous work has shown that methanotrophs are frequently related to CH₄ concentrations, sediment texture, and other microorganisms, but it is unknown how these factors influence methanotroph communities in caves. Type I and Type II methanotrophs are known to exhibit niche partitioning between high and low CH₄ subenvironments respectively (22, 23). However, it is unknown if Type II methanotrophs show similar deterministic changes in community composition with atmospheric to subatmospheric CH₄ concentration gradients. Methanotrophs have also been shown to be more abundant in clay-rich soils even though these soils may limit the diffusion of oxygen (24–26). Prior

work has documented associations between methanotroph and methylotroph abundances in aquatic environments (18), and that methanotrophs interact with other microbial species through volatile compounds in co-cultures (27).

Almost nothing is known about the importance of biogeography in structuring high-affinity methanotroph communities. Previous observations have shown that methanotrophs from Hawai'i exhibited close taxonomic affiliations with Arctic methanotrophs (5). This suggests that these organisms may be able to readily disperse through the environment. However, biota in caves are typically unique due to their isolation from other environments (28, 29). Thus, it may be possible that dispersal limitation is important in structuring high-affinity methanotrophic communities in caves.

We sampled 21 caves in North America, analyzed the 16S rRNA gene, and developed the following hypotheses to examine the factors regulating the community composition of cave methanotrophs (Figure 1). First, we hypothesized that their community structure should mirror the structure of methanotrophic communities in other low CH₄ environments, and that methanotroph abundance should be correlated with cave-air CH₄ concentrations. Additionally, we hypothesized that methanotrophs should show relationships to sediment texture and other microorganisms in the environment. Finally, we hypothesized that high-affinity methanotroph communities in caves should be dispersal-limited due to the insular nature of caves.

RESULTS

Methanotroph community structure. Methanotrophs were recovered in 88 % of the cave-sediment samples including locations where the CH₄ concentrations were at or below our instrumental detection limits of ≤ 0.3 ppmv (Supplemental Table 1). In samples where methanotrophs were recovered, their relative abundances ranged from 1.1×10^{-5} to 2.2×10^{-2} ($Q_1 = 5.4 \times 10^{-4}$, $Q_2 = 2.3 \times 10^{-3}$, $Q_3 = 4.7 \times 10^{-3}$). Cave methanotrophic communities were dominated by the Methylocystaceae, but members of the Methylococcales were also present (Figure 2). The median abundance of the Methylocystaceae at each site was 96 % of the total methanotrophic community. Members of the Methylocystaceae accounted for 51 unique operational taxonomic units (OTUs). Unknown OTUs at the genus level accounted for 94 % of the OTUs and virtually all (99.99 %) of the observed sequences from the Methylocystaceae. The Methylococcales were more diverse than the Methylocystaceae with 69 unique OTUs but only contributed 997 DNA sequences. 21 OTUs within the Methylococcales were identified from two undescribed families and accounted for 45 % of the total observed Methylococcales.

Methanotrophs from other clades constituted much less of the observed diversity. Seven OTUs were observed from the genus *Methylibium* (beta Proteobacteria: Comamonadaceae), and one DNA sequence from a member of the genus *Methylocella* (Beijerinckiaceae) was observed. Two OTUs were found from the family Acidimethylosilex (Verrucomicrobia) in Cave 2 in Indiana. We did not recover members from the candidate phylum NC10, or members of the USC methanotrophs.

Environmental influences on community structure. The CH₄ concentration in cave air was correlated with the relative abundance of methanotrophs. The relative abundances of both the Methylocystaceae ($\log_{10}(\text{RA}) = -0.5 \pm 0.3 \times [\text{CH}_4] - 2.4 \pm 0.4$, $r^2 =$

0.24, $p = 1 \times 10^{-3}$) and Methylococcales ($\log_{10}(\text{RA}) = -0.5 \pm 0.2 \times [\text{CH}_4] - 3.6 \pm 0.3$), $r^2 = 0.41$, $p = 1 \times 10^{-4}$) increased with decreasing CH_4 concentration (Figure 2). An analysis of covariance (ANCOVA) test revealed that there was no significant difference between the slopes of the two clades (ANCOVA, $F = 1.6$, $p = 0.21$).

We investigated the influence of the amount of CH_4 , gravel, sand, silt, and clay on methanotroph community composition using canonical correspondence analysis. As a whole, the composition of the methanotrophic community was not related to concentrations of CH_4 , or the volumetric proportions of gravel, sand, silt, and clay at the sample locations. However, the community composition of the Methylococcales was related to CH_4 concentrations in cave air (Canonical Correspondence Analysis, $p = 0.001$) and the amount of clay at the sample location ($p = 0.008$) (Figure 4). OTUs within the total methanotrophic community and within Methylocystaceae were not related to any of the measured environmental parameters.

We tested if the proportions of gravel, sand, silt, and clay influenced the relative abundance of methanotrophs using Spearman's rank correlation tests. Methanotrophs from the Methylocystaceae, and thus of methanotrophic communities overall, were correlated with the volumetric abundance of gravel in a sample (Spearman's rank correlation, Methylocystaceae, $\rho = 0.39$, $S = 10231$, $p = 0.02$) (Figure 3). The relative abundance of methanotrophs from the Methylocystaceae was not related to any other sediment size fraction present in the samples. Furthermore, the relative abundance of the Methylococcales was not related to any sediment size fraction (Spearman's rank correlations, vs. Gravel $p = 0.7$; vs. Sand $p = 0.12$; vs. Silt $p = 0.76$; vs. Clay $p = 0.90$).

We examined potential interactions between methanotrophs and other microorganisms by testing if the relative abundance of methanotrophs was related to the abundance of methylotrophs in caves. The relative abundance of methanotrophs was positively correlated to the relative abundance of methylotrophs in the sampled cave sediments (Spearman's rank correlation, $\rho = 0.71$, $S = 3849$, $p = 1 \times 10^{-7}$) (Figure 5). The relative abundance of methylotrophs was not, however, related to CH₄ concentrations in cave air (Spearman's rank correlation, $\rho = -0.25$, $S = 8914$, $p = 0.15$).

Biogeographical influences on community structure. We tested the ability of methanotrophs to disperse in the environment by measuring how the methanotroph community changed with measures of geographic distance. Cave methanotroph communities did not show significant relationships to measures of geographic distance. The relative abundance of methanotrophs was not correlated with the distance from a cave entrance (Spearman's rank correlation, $\rho = 0.33$, $S = 2432$, $p = 0.08$). Furthermore, distance-decay analysis—a measure of how the spatial distance between two samples affects their similarity—of the total methanotrophic community assemblage was not statistically significant (Mantel test, $r = 0.08$, $p = 0.14$).

We also tested methanotroph dispersal by determining if a core group of methanotrophs was present in the samples. We defined a core community as taxa present in at least 60 % of samples. If no group of methanotrophs could consistently be found among caves, this may suggest that methanotrophs are dispersal limited. We observed a core methanotroph biome of 3 OTUs. All 3 OTUs were from unidentified genera in the Methylocystaceae. Together these taxa were present in 88 % of samples, and one OTU

was present in 86 % of all the samples. The core methanotroph biome accounted for 59 % of the obtained methanotroph sequences.

DISCUSSION

Methanotroph community structure in caves. Methanotrophs are widespread in cave environments. The dominance of the Methylocystaceae in cave methanotroph communities is consistent with observations that have been made in other low CH₄ environments. For example, high-affinity methanotrophs are observed to be dominant in aerated riparian environments, Arctic soils, and soils associated with basaltic rocks (5, 23, 30, 31). Additionally, the relative abundance of methanotrophs observed in the sampled cave sediments is similar to the relative abundance of methanotrophs in Arctic soils (0.026 to 0.589 %) (32), and from caves in Vietnam (9). However, we did not recover any USC methanotrophs as have been observed in some soils that consume atmospheric CH₄ (16, 33). The lack of USC methanotrophs in our study contrasts with the methanotrophic community structure present in a cave from Australia where USC methanotrophs comprised 2 to 12 % of the community (10) and observations of USC- α methanotrophs in several caves (19). Additionally, our results are somewhat inconsistent with observations of methanotrophic communities from soils overlying caves in Australia where *Methylocella* sp. and *Crenothrix* sp. comprised 12 to 15 % of the community (12).

The cave sediments in our study also contained relatively rare methanotrophs, but the overall diversity of these taxa exceeded that of the dominant taxa. Minor contributors to the methanotroph community consisted of the Methylococcales, as well as methanotrophs from the Verrucomicrobia. The diversity of methanotrophs identified in our

study caves contrasts with the relatively depauperate assemblage found in a Spanish cave, which reportedly consisted of only three species, *Methylocapsa aurea*, *Methylomicrobium album*, and *Methylococcus capsulatus* (34). Our results suggest that CH₄ oxidation in caves may be carried out by a relatively diverse yet uncharacterized methanotrophic bacteria.

Environmental influences on methanotroph communities. The deterministic relationships between cave methanotrophs and their environment may be best understood from the perspective of metabolism. The process of life is fundamentally centered on obtaining energy from the surrounding environment and methanotrophs are no exception. Their distributions in caves appear to result from the fact that methanotrophs rely on both CH₄ and oxygen to derive energy.

The relative abundance of methanotrophs was inversely related to CH₄ concentrations in cave air. This pattern was documented for two major groups of methanotrophs, the Methylocystaceae and the Methylococcales. While the relative abundance of methanotrophs is sometimes known to positively track CH₄ concentrations (35), methanotrophs have been observed to be negatively correlated with CH₄ concentrations in some environments including glacial termini, geologic seeps, and a cave (10, 22, 30, 36). One explanation for the inverse CH₄-methanotroph relationship is that methanotrophs are actively driving down CH₄ concentrations in locations where they make up a large fraction of the total microbial community. Another possibility is that methanotroph abundance in caves may be positively related to CH₄ fluxes, but our observed pattern is a result of seasonality in cave-air flow. Many caves are known to exhibit faster air flow when the external atmospheric temperature is lower than the

temperature of cave air. This leads to higher CH₄ concentrations in caves during the winter (13, 37). Our samples were acquired during summer, so it is possible that our sites experience higher CH₄ concentrations during the winter and thus greater CH₄ fluxes on an annual basis which may explain the observed negative relationship.

The permeability of cave sediments also appeared to influence the methanotroph community. The relative abundance of methanotrophs in caves was positively related to the amount of gravel in the samples. This may be due to the fact that cave sediments with greater portions of larger clasts, like gravel, may decrease the water-holding capacity while increasing the permeability of cave sediments and the diffusion of gasses (38). As the proportion of larger clasts in a matrix increases, the permeability of the sediment tends to increase (38). This gives methanotrophs greater access to CH₄ and O₂ which are crucial for their metabolism. Previous research observed that methanotrophs tend to be more abundant in silt and clay fractions of soils because these fractions made up the bulk of the soils themselves (24). This previous study did not characterize larger sediment sizes, nor did it examine clay floccules that may serve to enhance permeability.

The abundance of clay particles influenced the community composition of the Methylococcales (a less dominant group of methanotrophs in this study) as did the CH₄ concentration of cave air. These relationships suggest that members of the Methylococcales may be partitioning themselves across different niches with regard to CH₄ and O₂ availability. The Methylococcales are typically associated with environments that have low O₂ and high CH₄ concentrations. Members of Methylococcales may therefore be responding to dysoxic to hypoxic environments in cave sediments that result from tightly packed clay-sized particles and/or extra-atmospheric CH₄ sources (11, 13, 14).

The community gradient across CH₄ and clay particle size may represent the transition from potentially unknown members in the Methylococcales that consume CH₄ at low (i.e. atmospheric) concentrations to those more tolerant of lower O₂ concentrations.

Organisms alter the physical and chemical characteristics of the environment and thus may influence the distributions of other species in the environment. The positive correlation between methylotrophs and methanotrophs is consistent with observations of metabolic interactions between these functional groups of bacteria. For example, Methylotrophs are known to consume methanol that is produced by methanotrophs (39). Co-occurrences between members of Methylococcales and Methylophilaceae (methylotrophic) have been observed in environmental samples, and so has the transfer of methanol from methanotrophs to methylotrophs (40, 41). A similar process may be occurring in caves. Alternatively, methylotrophs may simply be responding to the presence of other organic compounds in cave air besides CH₄. In addition to consuming methanol, methylotrophs are known to consume methyl halides, like methyl chloride and methyl bromide which are present as trace gases in the atmosphere (42). These gases would be advected into cave air in the same fashion as atmospheric CH₄ (43).

Biogeography of methanotroph communities. Methanotrophic communities did not show statistically significant relationships with local or regional assessments of geographic distance. The composition of methanotrophic communities close to cave entrances was not different compared to those in more interior locations. This pattern has several possible explanations, but two seem to be the most probable. The methanotrophic community in caves is dominated by Type II methanotrophs and these methanotrophs do best under environmental conditions of low CH₄ concentrations and high O₂

concentrations. The change from about 2 to < 0.3 ppmv CH₄ in cave air may not impose strong selective pressure on Type II methanotrophs. Additionally, the lack of a pattern in methanotrophic community composition may be a result of the seasonality present in cave air (44, 45). Thus, CH₄ concentrations in cave air may be near the atmospheric concentration at one point in the year and shift to sub-atmospheric concentrations at other times. This could cause location to be a poor predictor of the methanotrophic assemblage due to microbial physiological states like dormancy (46).

Likewise, cave methanotrophic communities were not spatially autocorrelated at the regional scale. The lack of a distance-decay relationship in methanotrophic communities from the studied caves may indicate that the scale at which distance-decay relationships occur in these communities is larger than the regional scale of 500 km in our study. This may suggest that dispersal is high among methanotrophs.

We found a core group of three OTUs that were present in the studied caves. This suggests that high-affinity methanotrophs are able to move about the environment relatively easily. The fact that these methanotrophs accounted for 59 % of the observed methanotrophs suggests these organisms are reproducing in these environments as well. Previous observations are also indicative of high-dispersal capabilities for these organisms. For example, methanotrophs from a newly formed soil in Hawai'i showed close taxonomic affiliations with Arctic methanotrophs (5). Additionally, microbes are known to travel intercontinental distances on windblown dust (47), and perhaps windblown dust may carry methanotrophs into caves. Our results suggest that in cases where microorganisms derive part of their energetic and nutritional requirements from air, that barriers to dispersal into

other habitable subenvironments, like recurring slope lineae or caves on Mars (8, 48), may be decreased.

CONCLUSIONS

The structure of cave methanotroph communities appears to be best understood in terms of how cave subenvironments provide methanotrophs with access to CH₄ and oxygen. Cave methanotrophic communities showed relationships with cave-air CH₄ concentrations and the abundance of gravel-sized clasts in cave sediments. The CH₄ concentrations of cave air and the abundance of gravel both influence how much CH₄ and O₂ methanotrophs have access to. CH₄ is typically at low (i.e., atmospheric) concentrations in caves and as such, Methanotrophs from the Methylocystaceae were numerically dominant. This community structure mirrors that of other atmospheric CH₄ oxidizing communities (9, 32). Further, the community composition of the Methylococcales showed responses to CH₄ concentrations and the abundance of clay in cave sediments that could represent a transition from uncultivated members in the Methylococcales that consume CH₄ at low (i.e. atmospheric) concentrations to those more tolerant of lower O₂ concentrations.

Our results suggest that methanotrophs in caves have secondary influences on the cave-microbial community structure. The abundance of known methylotrophic organisms is correlated with the abundance of methanotrophs. This suggests that molecules produced along the methane oxidation pathway, like methanol, are leaked from methanotrophic cells

allowing for the growth of methylophils. Our findings suggest that CH₄ may be an overlooked energy source for cave communities.

Methanotrophs did not show relationships with measures of geographic distance in this study. This study focused on environments that are thought to present atmospheric to subatmospheric CH₄ concentrations year-round and in sediments that are forming from the breakdown of similar rocks. The similarity of the environments may be one reason for the observed lack a relationship with measures of distance. This result also suggests that the dispersal barrier for high-affinity methanotrophs from cave to cave may be low.

MATERIALS AND METHODS

Microbial Sampling. We sampled microbial communities from caves along the western front of the Appalachian fold and thrust belt, in intracratonic settings of the USA, and in the Sierra Madre of Mexico. We obtained 42 sediment samples from caves along transects from cave entrances to interiors. Samples were scraped from ~0.1 m² large areas of cave walls and floors using a spatula that had been sterilized with 70 volume % ethanol in water (49). Samples were stored on ice until they could be transferred to a –80 °C freezer.

Environmental Parameters. We measured multiple environmental parameters to assess factors that potentially influence the composition of cave methanotrophic communities. We measured CH₄ concentrations *in-situ* with Fourier Transform Infrared Spectroscopy (FTIR) (Gasmet DX4030 – Milton Keynes, United Kingdom), or in the laboratory with FTIR gas-chromatography (Varian – Agilent Technologies, Palo Alto,

California) using discrete air samples collected in the field. Some of the CH₄ concentrations listed in this study have been previously published (14, 50), and new data were collected according to methods reported in the same publications. In cases where cave maps were available, the distance from the nearest entrance to the sampling location was calculated along the length of the cave passages. Cave sediment grain-size distributions were measured with a Malvern Mastersizer 3000 (Malvern Instruments Inc., Westborough Massachusetts). Raw data from the Mastersizer were converted to % gravel, % sand, % silt, and % clay sized particles by volume using the GRADISTAT software package (51). Metadata used for statistical analyses are reported in Supplemental Table 2.

Molecular Techniques. Genomic DNA was extracted from cave sediment samples with a MoBio PowerSoil™ extraction kit (MoBio, Carlsbad, California USA). About 10 ng of extracted DNA was used as a template for amplification by polymerase chain reaction (PCR). The V4 region of the 16S rRNA gene was amplified using 515F and 806R with barcoded primers designed to work with the Illumina MiSeq platform (52). DNA amplification was performed using 50 µL reactions from each sample. PCR reactions were carried out by a 3 min denaturing step at 94 °C, followed by 30 cycles of 94 °C for 45 s, 50 °C for 60 s, and 72 °C for 90 s. A final 10-minute extension was carried out at 72 °C. Quality of the PCR amplifications was checked by gel electrophoresis. Amplified DNA was cleaned using a commercial kit (Beckman Coulter Agencourt AMPure XP, Indianapolis, Indiana, USA). Cleaned DNA from the reactions was pooled to a final concentration of 20 ng per sample.

We sequenced the cleaned PCR amplicons using Illumina MiSeq technology (Illumina Reagent Kit v2, 500 reaction kit) at the Center for Bioinformatics and Genomics

at Indiana University. Data quality and unique sequences obtained from the PCR amplifications were analyzed using mothur (53). DNA sequence data were aligned using the Needleman algorithm. Sequences matching chimeras were removed using UCHIME (54). Sequences that matched chloroplasts, Archaea, and other non-bacterial sequences were also removed. OTUs were created by binning the data at 97 % sequence similarity. OTUs were identified using a SILVA reference database (version 128).

Statistical Analyses. The R computing environment was used for all quantitative analyses (55). We used 16S rRNA gene sequences to assess diversity and composition of the methanotroph community. Sequences related to known methanotrophs and methylotrophs were subset from the larger 16S rRNA dataset using the “subset_taxa” function in the *phyloseq* software package to create OTU abundance tables and for only the taxa of interest for a particular sample (56). Relationships between methylotrophs and CH₄ concentrations were examined due to the similarities of their metabolic pathways with methanotrophs. We defined the methanotrophic community as members of the Methylocystaceae, Methylococcales, Methylocella, USC- α , and USC- γ (17, 18). We defined the methylotrophic community as members of the Methylophilaceae and Methylobacteriaceae.

We assessed the relationships between methanotrophs and environmental conditions through a series of statistical analyses. Raw abundance data were transformed into fractional abundance data to avoid variations in the number of reads between samples. Community dissimilarity between samples was assessed with Bray-Curtis dissimilarity. Dissimilarity measures were only calculated if the number of OTUs in a phylogenetic grouping was greater than 100 OTUs to avoid statistical problems arising from small

sample size. The effect of CH₄ concentrations on the methanotrophic community was assessed using ANCOVA and Mantel tests. We tested the effect of sediment grain size on cave methanotrophic communities using canonical correspondence analysis that was performed in the R package *vegan* (57).

The spatial autocorrelation of methanotrophic communities was tested by performing distance-decay analyses. Distance-decay is a spatial-autocorrelation pattern that describes the decreasing similarity of biological communities with increases in geographic distance (58). We performed distance-decay analysis in R by first removing data from Cave 38 (Cueva de Villa Luz) since it was roughly 2000 km away from the nearest samples. We then tested for a distance-decay pattern using a Mantel test in the package *vegan* (57).

We characterized the methanotroph core microbiome, which is defined as a suite of members shared in an environment (59). We calculated the core microbiome based on a cutoff of 60 %, a value that is in the middle of ranges that have used when calculating core microbiomes in other ecosystems including corals and humans (60, 61). We also chose this value because it is larger than the majority of samples, but accounts for some unpredictability in an organism's distribution.

Data and software availability. All code and data used in this study can be found in a public GitHub repository (<https://github.com/websterkgd/CaveMethanotrophs>).

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Figure 1: (A) Locations of karst at the global scale. Inserts show the occurrence of karst (B) in the eastern United States (C) and in Mexico. Numbers represent the locations of sampled caves in this study. Karst land cover data were obtained from (7, 62).

Figure 2: Relative abundances of methanotrophic community members plotted against the CH₄ concentration at each sample location. Members of the Methylocystaceae, typically associated with high-affinity methanotrophs, were more abundant than members from the Methylococcales, typically associated with low-affinity methanotrophs.

Figure 3. The relative abundance of methanotrophs from the Methylocystaceae plotted against the volumetric proportion of gravel in a sample (Spearman's rank correlation, $p = 0.02$).

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629 **Figure 4:** Canonical correspondence analysis of the community structure of the
630 Methylococcales in caves. Community structure of this group was significantly related to
631 clay content and CH₄ concentrations.

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634 **Figure 5:** Relative abundance of methyloprophs plotted against the relative abundance of

635 methanotrophs from sampled caves (Spearman's rank correlation, $p = 1 \times 10^{-7}$).