Methanogenesis in oxygenated soils is a substantial fraction of wetland methane emissions

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The current paradigm, widely incorporated in soil biogeochemical models, is that microbial methanogenesis can only occur in anoxic habitats. In contrast, here we show clear geochemical and biological evidence for methane production in well-oxygenated soils of a freshwater wetland. A comparison of oxic to anoxic soils reveals up to ten times greater methane production and nine times more methanogenesis activity in oxygenated soils. Metagenomic and metatranscriptomic sequencing recover the first near-complete genomes for a novel methanogen species, and show acetoclastic production from this organism was the dominant methanogenesis pathway in oxygenated soils. This organism, Candidatus Methanothrix paradoxum, is prevalent across methane emitting ecosystems, suggesting a global significance. Moreover, in this wetland, we estimate that up to 80% of methane fluxes could be attributed to methanogenesis in oxygenated soils. Together, our findings challenge a widely held assumption about methanogenesis, with significant ramifications for global methane estimates and Earth system modeling.
Modeling and biological studies investigating methane flux from wetlands discount microbial methane production in surface, oxic soils. The basis of this assumption is that critical methanogen enzymes are inactivated by oxygen and methanogens are poor competitors with other microorganisms for shared substrates. Because of the assumed physiological constraint that oxygen has on methanogens, global terrestrial biogeochemical models limit soil methane production in the presence of dissolved oxygen (DO).

Recent reports present an alternative view that in some ecosystems methanogenesis also occurs in oxic environments, known as the methane paradox. In freshwater lakes, isotopic and molecular biology techniques provided evidence for the presence and activity of methanogens in well-oxygenated portions of the water column. Similarly, isotopic signatures in oxygenated soils and activity measurements from soil laboratory enrichments have provided intriguing evidence for methanogenesis in soils with up to 19% oxygen. Despite this mounting, indirect evidence, comprehensive genomic investigations that link methanogens to methane production in any oxic habitat in situ are lacking.

Here we analyze observations from the Old Woman Creek (OWC) National Estuarine Research Reserve, a freshwater wetland at the shore of Lake Erie in Ohio. In this study, we experimentally assess biological methane production and emission in freshwater wetland soils across multiple spatial and temporal gradients. The results presented here provide the first ecosystem-scale demonstration of methane production in bulk-oxic soils, its microbial drivers, and the global significance of this currently underappreciated process.

Results
Methanogens are most active in oxic surface soils. To account for differences associated with distinct ecological sites in the wetland (ecosites), we sampled soils beneath three land coverage types: emergent vegetation (plant); periodically exposed mud flats (mud); and continuously submerged under open water (water) (Supplementary Fig. 1). Seasonal variability, especially the effects from photosynthesis and climate, was accounted for by sampling the three ecosites in summer (peak primary production) and late fall before freezing (senescence), while differences in vertical oxygen distributions were examined in 5 cm intervals up to 35 cm deep (Supplementary Data 1).

All ecosites were net methane emitting during both summer and fall sampling seasons (Supplementary Fig 2A). In summer, regardless of ecosite (plant, mud, water), the porewater DO profiles were similar; for instance, depths shallower than 10 cm were always oxic while soils deeper than 25 cm were always anoxic (Fig. 1, Supplementary Data 1). The in situ porewater dialysis samplers (peepers) measured the greatest methane concentrations in oxic, surface porewaters in the four summer months sampled (June–Sept). For mud and water ecosites, we paired these concentration measurements with direct surface flux measurements from static chambers, and used a dynamic diffusion model to calculate the net methane source (production and destruction) rate at each layer within the soil column (Supplementary Note 1).

FIGURE 1 Methane concentrations and production rates across soil depths. a Porewater dialysis peepers provide 2.8 cm resolved depth methane measurements. b, d Monthly in situ porewater dissolved methane concentrations in mud and water-covered soils with data collected from June (blue), July (yellow), August (red), and September (purple). Black dashed lines depict the 95% confidence interval for location of the oxic to anoxic transition. c, e The calculated net methane volumetric fluxes in soils columns from mud and water ecosites show seasonal methane production (orange and red) in oxic soils (Supplementary Note 1).
These findings demonstrate that the methane paradox occurs in wetland soils and provides the first evidence for the extent to which it operates over spatial and temporal gradients.

In order to measure methanogenesis activity from these surface and deep soils, we quantified methyl-coenzyme reductase subunit A (mcrA) gene transcripts, a key functional gene for inferring methanogenesis activity\(^1\). On average, across all ecosites and seasons, oxic soils contained nine times more mcrA transcripts and twice the methane concentration per gram of wet soil than anoxic soils (Fig. 2, Supplementary Data 1). Methanogen activity was positively correlated to porewater DOC and acetate concentrations, but not to other soluble methanogenic substrates like formate, methylamines, and methanol (Supplementary Fig. 2B and Supplementary Data 1). Taken together, these findings suggest that methanogens utilizing acetate may be responsible for sustaining the methane paradox in these soils.

**Candidatus Methanothrix paradoxum is active in oxic soils.**

Paired metagenomic and metatranscriptomic sequencing provided the first holistic insight into the methanogens active in oxic environments. From metagenomic sequencing we reconstructed six (two estimated to be > 90% complete) genomes from oxic soils that represent a new species of methanogenic Archaea. Based on whole genome comparisons and phylogenetic analyses (e.g., 16S rRNA, concatenated ribosomal protein, and mcrA) (Supplementary Fig. 3) these genomes clearly represent a new species within the genus *Methanothrix* (formerly *Methanosaeta*). Based on these analyses this new species was phylogenetically most closely related to *M. concilii*, a methanogen species widely distributed in anoxic terrestrial methanogenic environments, such as flooded rice paddy soils and lake sediments\(^12, 13\). Comparative genomic analyses between these wetland genomes to four genomes from cultivated *Methanothrix* demonstrated the *Candidatus Methanothrix paradoxum* genomes expanded the *Methanothrix* pangenome by 27%, with 467 genes uniquely encoded in our wetland genotypes. Of these unique genes, the majority (55%) lacked any functional annotation information (Supplementary Fig. 3C). Here we propose the name *Candidatus Methanothrix paradoxum*, after the implied role for this organism in the soil methane paradox (Supplementary Figs. 4–6 and Supplementary Note 2).

From our metatranscriptomic analyses, we conclude methanogenesis in oxic soils is conducted primarily via a canonical acetoclastic pathway (Supplementary Note 3 and Supplementary Data 2). Transcripts from these genomes were in the top 3% of all community-wide metatranscripts and accounted for on average 84% of the mcrA transcripts in surface soils (Fig. 3a, Supplementary Fig. 7, and Supplementary Data 3). In addition to the methanogenesis pathway, genes for protein synthesis and energy generation were consistently and highly expressed in both seasons and ecosites (Fig. 3b and Supplementary Fig. 8), signifying active

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**Fig. 2** The relationship between soil dissolved oxygen concentration and methanogenic activity with depth and ecosites from Summer. **a** Schematic of the three ecosites examined in this study with methane emissions shown in colored boxes and depicted by red lines (Supplementary Fig. 2A). **b** Dissolved oxygen concentrations (black boxes), transcripts for mcrA (colored bars), and porewater methane concentrations (red triangles) in soils. Error bars reflect s.e. (mcrA) and s.d. (oxygen); n = 3
methanogenesis by this organism stably occurs in these oxic wetland soils.

Prior laboratory investigations have shown that methanogens in pure culture or from soil mesocosms upregulate antioxidant mechanisms to attenuate oxygen toxicity\textsuperscript{14-16}. Consistent with those reports, \textit{Candidatus} Methanothrix paradoxum genomes encode known oxygen detoxification genes including those for stabilizing free radicals, reducing toxic reactive oxygen species, and repairing oxidative disulfide damage (Supplementary Note 3 and Supplementary Data 2). However, these genes were not unique to our wetland genomes, present in similar abundances and metabolisms in bulk oxygenated soils (e.g., for denitrification and iron reduction) and particle-associated models explain methanogenesis in oxic lake waters\textsuperscript{6, 7}.

![Diagram](Image)

**Fig. 3** \textit{Candidatus} Methanothrix paradoxum genes transcribed in oxic soils. a Taxonomic assignment and relative abundance of \textit{mcrA} transcripts in surface soils assigned to \textit{Candidatus} Methanothrix paradoxum (black), \textit{Methanoregula} (dark gray), and other methanogens (light gray). b The relationship between the 100 most transcribed genes (by log\textsubscript{10} FPKM) in each season, with gene functional categories denoted in color and key steps of the methanogenesis pathway highlighted. c Gene expression levels for selected genes from (b), across all samples with color legend used from (b) (black line, boxes, and whiskers represent the median, quartiles, and minimum/maximum of the log\textsubscript{10} FPKM values). For comparison, oxygen detoxification genes are not consistently transcribed at detectable levels.

Analysis of the methane paradox literature revealed several possible explanations for methane production in oxic habitats. In our data, we failed to find metabolite or molecular evidence supporting methane production from microbial decomposition of methylated compounds\textsuperscript{19} or by protozoan endosymbionts\textsuperscript{20} (Supplementary Discussion). Instead, the data convincingly demonstrate methane production by canonical methanogenic archaea as the driver of methane in these oxic soils. Together, these findings demonstrate that known oxygen detoxification mechanisms used by other methanogens in the laboratory are not a requirement to sustain methanogenesis in these oxic wetland soils.

Accounting for the black queen hypothesis\textsuperscript{18}, we did consider that oxygen tolerance could be provided to \textit{Ca}. Methanothrix paradoxum by other members in the soil community. In our metatranscriptomes, we recovered transcripts for a catalase gene and several superoxide dismutase genes belonging to non-methanotrophic Gammaproteobacteria; however, only one of these transcripts was detected in as many as 5 of the 12 samples, and at very low abundances. Importantly, none of these transcripts were highly abundant in our data set, nor correlated to methanogen activity, suggesting that the ability for methanogens to compensate for oxygen toxicity is not likely to originate from other community members. Together, these findings demonstrate that known oxygen detoxification mechanisms used by other methanogens in the laboratory are not a requirement to sustain methanogenesis in these oxic wetland soils.
Candidatus Methanothrix paradoxum is globally distributed.

To assess the contribution of Candidatus Methanothrix paradoxum to methanogenesis in this wetland and beyond, we mined our data and public databases for highly similar (>99%) 16S rRNA gene sequences. In this wetland, this methanogen species is cosmopolitan, recovered from 97% of soil samples collected from various depths, ecosites, and time points over 3 years. Moreover, as we previously reported, these methanogens are dominant members of the oxic soil community and unlike other methanogens show a strong enrichment in the top 5 cm of soil (Supplementary Fig. 9 and Supplementary Note 4). Candidatus Methanothrix paradoxum is also globally distributed, detected in 102 locations across 4 continents spanning a range of habitats including rice paddy, wetland, and peatland soils (Supplementary Data 4). From these analyses, we infer Candidatus Methanothrix paradoxum is well adapted to diverse hydric soils and sediments.

Our analysis of ribosomal 16S rRNA genes from previous methane paradox publications revealed that Candidatus Methanothrix paradoxum was detected and often acknowledged as a critical member in 10 studies where the methane paradox was previously reported (Supplementary Note 4 and Supplementary Fig. 10). For instance, many of the reported Methanoseta sequences in oxic lake waters share >99% 16S rRNA gene identity with Candidatus Methanothrix paradoxum (Supplementary Fig. 10). We posit that perhaps the increased activity of Candidatus Methanothrix paradoxum over other acetoclastic methanogens in oxic soils may be due to its competitive substrate acquisition under low acetate concentrations (<1 mM) found in our (Supplementary Data 1) and others’ soils. Similarly, a recent report on the importance of acetoclastic methanogenesis to the methane paradox in lakes also alluded to low acetate concentrations in oxic surface waters as a potential contributor.

Oxic soil methanogenesis contributes to the methane budget.

To understand the importance of methanogenesis in oxic soils, we estimated the contribution of this process to the total methane budget in this wetland using simplifying assumptions (Supplementary Note 5). We first decomposed the eddy covariance flux signal into its ecosystem-level contributors. We then applied a diffusion model of porewater dissolved CH₄ concentrations to determine the location of microbial CH₄ activity within soil columns. We overlaid the microbial activity profile with the DO concentration profile to determine microbial activity in the oxic layers. Previously, Bogard et al. used a scaling method to demonstrate methanogenesis in the oxic portion of the water column contributed to 20% of lake-wide emissions. Using a similar approach, when integrating over the course of this study, we estimated that between 40 and 90% of methane emitted originated in oxic soil layers (Fig. 4).

This study provides the first genome-resolved view of the methane paradox in any ecosystem and identifies the important contribution of a newly defined and globally distributed methanogen species, Candidatus Methanothrix paradoxum. We provide clear evidence for the extent of methanogenesis in oxic soils from multiple seasons and ecosites, and show this process is a significant contributor to overall wetland methane emissions. These findings have important ramifications for global biogeochemical models, as current simulations downregulate methanogenesis in surface soil layers due to oxygen concentrations, potentially greatly underestimating methane emissions. It is therefore critical to refine global biogeochemical models to account for methanogenesis in bulk-oxic surface soils, more accurately predicting net wetland methane emissions and their effects on climate.

Methods

Field sampling. The field location, OWC National Estuarine Research Reserve (41° 22′ N 82° 30′ W), is a 573-acre freshwater wetland and reserve located on the southern point of Lake Erie near Huron, Ohio. This site is co-operated by the National Oceanic and Atmospheric Administration (NOAA) and Ohio Department of Natural Resources. This is one of 28 coastal (only two are in the Great Lakes region) NOAA designated sentinel research sites. The site consists of a permanently flooded channel surrounded by marsh, mud flats, and forested upland habitat. We collected soil cores from three (~2 m²) ecologically differentiated sites (ecosites): plant, mud, and open. Four or more water-saturated soil cores were collected per ecosite to a depth of 35 cm (width 7 cm) using a modified Mooring...
System soil corer. Cores were kept on ice in the field until processing in the laboratory (no more than 2 h), where soils were immediately hydraulically extracted23, sub-sampling (0.5–2 cm) for each anion. To more directly probe soil methane concentrations to microbiological soil data, in situ methane concentrations were calculated as described previously27 following immediate transfer to 4 °C for transport and measurement on a Shimadzu GC-2014 gas chromatograph.

Soil porewater geochemical analyses. Soil total carbon and porewater dissolved organic carbon (DOC) were analyzed via Shimadzu TOC-L with SSM-5000A solid sample combustion unit attachment using methods previously described28. Concentrations of soil and porewater acetate, nitrate, nitrite, and sulfate were determined via ion chromatography. For soils, 5 g of soil was mixed with 5 mL of MilliQ water (1.1 v/v), filtered with a 0.2 μm filter, and quantified using a Dionex ICS-2100 Ion Chromatography System with an AS18 column with standard curves prepared for each anion. To more directly probe soil methane concentrations to microbiological soil data, in situ methane concentrations were calculated as described previously27 following immediate transfer to 4 °C for transport and measurement on a Shimadzu GC-2014 gas chromatograph.

Soil porewater was extracted using methods and infrastructure previously described in detail from this wetland24, 25. Porewaters were then sent to the Pacific Northwest National Laboratory and metabolites were identified by proton nuclear magnetic resonance (1H NMR) as described previously28. Metabolomic responses were characterized using the ENSM, 800 MHz and 600 MHz NMR spectrometers equipped with cryogenically cooled triple resonance probes for their high sensitivity and quantitative determination via 1H NMR metabolite libraries (presently ~1000 metabolites). The two-dimensional (2D) NMR metabolomics methods including 1H–13C correlation experiments (heteronuclear single quantum coherence), and connectivity experiments total correlation spectroscopy and correlation spectroscopy on a subset of samples (< 8) to enhance metabolite identification. Geochemical and metabolite data were analyzed in relationship to methane emissions activity by linear correlations determined via Pearson's correlation (p < 0.05).

Collecting dissolved gases and greenhouse gas emissions. Surface emissions were measured by non-steady-state chambers, with floating chambers used for measurements over open water. Chambers were measured in duplicate in each ecosystem and season24 and sampling was coordinated to peeper measurement times. Additional greenhouse gas emissions were collected with an eddy covariance and meteorological station (3 m tall tower, site-wide footprint). We have previously shown that both chambers and eddy covariance measurements provide congruent measurements24. Porewater dialysis samplers (peepers) were used to sample for dissolved CH4, CO2, and N2O below ground monthly, with a vertical resolution of 2.8 cm, measured concentrations outside. Gas concentrations in the peeper samples were quantified both chambers and peepers followed protocols previously described24. Both chamber and peeper measurements were taken simultaneously, once a month during the 2015 growing season. Temperature probes and co-located oxygen-level measurements (via a PreSens Fibox 4 handheld oxygen meter) provided vertical detail near each peeper measurement location. The oxygen horizon was determined by fitting a reverse exponential curve to all DO data collected per packet per month. The horizon was taken as where the curve crossed 20 (μmol O2 per kg H2O)52. To determine the upper and lower bounds of the horizon, we identified the soil depths at which the 2.5th and 97.5th confidence intervals of the exponential fit crossed the same 20 (μmol O2 per kg H2O) threshold. Theoxic horizon and confidence bounds were interpolated linearly between measurement periods.

Transport and production model. A numerical model was used to combine chamber and peeper measurements to determine the rates of methane production/oxidation in different layers of the wetland soil. A diffusion model was separately created for mud and open ecosites (not plants), due to the complexity of including plant transport and roots. We discretized Fick's 2nd Law (Eq. 1) in one dimension using an implicit backwards Euler method to account for diffuse transport within the soil column. A production/oxidation term was included to account for the implied biological activity.

\[
\frac{dc(t)}{dt} = \frac{d}{dz} \left( D(z) \frac{dc(z)}{dz} \right) + R(z)
\]

Here, C is the soil porewater concentration of methane, z is the vertical position in the soil column, D is the temperature-dependent diffusion coefficient, t is the time in days, and R is a methane sink/source (generation/oxidation) term. The temperature profile was determined through measurements made with nearby soil temperature probes. A Neumann no-flux boundary condition was prescribed at the bottom of the soil column. We used a known flux top boundary condition (implemented by discretizing Fick's 1st Law) which was prescribed based on time-interpolated chamber measurements. Each month’s measured concentration profile was used to model the next month’s first using an ignorant guess of R (determined by solving over the above with a month-long time step). We then refined the time step to 0.1 days and used a Markov Chain Monte Carlo Metropolis Hastings (MCMC-MH)33,34 approach with 40,000 repetitions to alter the value of R along the vertical column in order to minimize the error between the modeled future methane concentration profile and its measured value. We took the average of the 10% best performing MCMC runs as the optimal activity. Uncertainty was quantified as 1 s.d. of the 4000 selected runs. This simplistic model interprets observational concentration data as production/oxidation with no assumptions about the oxic conditions of the soil, providing a unique way of observing the data.

Eddy covariance collection and data processing. Eddy covariance data were collected from July to October 2015. The flux calculation approach was fully outlined previously35,36. Briefly, a 3D rotation was applied to wind observations to force the vertical and cross wind components gathered from the sonic anemometer (CSAT3, Campbell Scientific, Logan, UT, USA) to average to 0 for each half-hour.27. To correct for the separation of the sensors, the time series of concentration measurements were shifted in time using the maximal-covariance approach38. CO2, CH4, and N2O below ground monthly, with a vertical resolution of 2.8 cm, measured concentrations outside. Gas concentrations in the peeper samples were quantified both chambers and peepers followed protocols previously described24. Both chamber and peeper measurements were taken simultaneously, once a month during the 2015 growing season. Temperature probes and co-located oxygen-level measurements (via a PreSens Fibox 4 handheld oxygen meter) provided vertical detail near each peeper measurement location. The oxygen horizon was determined by fitting a reverse exponential curve to all DO data collected per packet per month. The horizon was taken as where the curve crossed 20 (μmol O2 per kg H2O)52. To determine the upper and lower bounds of the horizon, we identified the soil depths at which the 2.5th and 97.5th confidence intervals of the exponential fit crossed the same 20 (μmol O2 per kg H2O) threshold. Theoxic horizon and confidence bounds were interpolated linearly between measurement periods.

\[
F_{\text{meas}} = F_{\text{avg}} + F_{\text{avg}} + F_{\text{avg}} + F_{\text{meas}}
\]

Site-level methane budget. To determine the site-level methane budget and the oxic production contribution, we used an expanded version of the fixed frame eddy covariance scaling methodology developed for mud and open ecosites24, 25. Briefly, this method combines eddy covariance data with a footprint method and ecosite flux measurements collected using the chamber method to decompose the eddy covariance flux signal into its contributing parts by ecosite. We used the Detto footprint method44, which is a 2D expanded version of the Hsieh model45. We used monthly varying displacement height and roughness lengths to represent the Typha spp. growing around the tower. There were four relevant ecosites with footprint contributions to the eddy covariance tower: open water, Typha spp., Nelumbo spp., and mud flat.

\[
f_{\text{meas}} = f_{\text{mud}} + f_{\text{mud}} + f_{\text{mud}} + f_{\text{mud}}
\]

Where F of each ecosite indicates the relative flux strength of that ecosite at the landscape level (different than that provided by the chamber measurements), and e is the footprint contribution of that patch per day. Chamber flux data determined the relative flux strengths of each ecosite compared to open water fluxes. This provided a solvable system of equations for the relative flux strength contributing to the eddy covariance tower (F) with a daily temporal resolution.

\[
m_{1} = f_{\text{mud}} + f_{\text{mud}} + f_{\text{mud}} + f_{\text{mud}}
\]

We interpolated each ecosite’s percent of production in theoxic layers to daily values over the course of the study. We scaled these values to the site level by integrating spatially over the site-level footprint and temporally over all times we were able to model methane production for (Fig. 4).
where $i$ is the site-level percent area contribution of each ecosite and $p$ is the percent generation in theoxic zone (determined by the diffusion model) of each ecosite.

**Metagenomic analyses.** Genomic DNA was extracted from triplicate 0.5 g of soil using a MoBio PowerSoil DNA Isolation Kit following the manufacturer's protocol. DNA from three representative Fall and Summer surface soil samples (Plant, Mud, and Water) were sequenced at The Ohio State University and The Joint Genome Institute using an Illumina Library creation kit (KAPA Biosystems) with solid-phase reversible immobilization size selection. The quantified libraries were then prepared for sequencing on the Illumina HiSeq 2500 sequencing platform utilizing a TrueSeq Rapid paired-end cluster kit, v4. We obtained 304 Gbp of metagenomic sequencing (Supplementary Table 1). Sequence assembly generated ~3.8 Gbp of contiguous sequences $> 5$ kb from the six surface soil metagenome samples. Fastq files were generated from Illumina MiSeq, trimmed from both $3^\prime$ and $5^\prime$ ends using Sickle, then each sample was assembled individually using IDBA-UD with default parameters as previously described$^{36, 46}$. Scaffold coverage was calculated by mapping reads back to the assemblies using Bowtie2$^{47}$. Genes on scaffolds $> 5$ kb were annotated as described previously$^{48, 49}$, by predicting open reading frames (ORF) using MetaProgid$^{50}$. Called genes were compared using USEARCH$^{51}$ to KEGG, UniRef90, InterProScan$^{52}$ with single and reverse best hit (RBH) matches $> 60$ bits reported. The collection of annotations for a protein were ranked: Reciprocal best BLAST hits (RBH) with a score $> 350$ given the highest (A) rank, followed by reciprocal best blast hit to Uniref with a bit score $> 350$ (B rank), blast hits to KEGG with a bit score $> 60$ (C rank), and UniRef90 with a bit score $> 60$ (C rank). The next rank represents proteins that only had InterProScan matches (D rank). The lowest (E) rank comprises the hypothetical proteins, with only a prediction from Prodigal but a bit score $< 60$. Assembled scaffolds were binned into genomes based on GC coverage, and taxonomy by using the Bowtie2 and mapping to the KEGG database and compared all genes via all vs. all blast, orthoMCL clustering and sqlite db, HTSDB tools. A cluster is defined as having bidirectional best hits based on a percent identity cutoff $> 90$.

**Transcriptomic analyses.** Metatranscripts were performed on three cores sampled in two ecosites (plant and mud) during two seasons (Fall and Summer) $(n = 12)$, as these soils demonstrated the highest surface methanogenesis activity. Total RNA was extracted from soils previously analyzed by metagenomically$^{53}$ or metatranscriptomically$^{54}$ by using the MoBio PowerSoil DNA Isolation Kit and DNA Elution Kit following the manufacturer's instructions. For metatranscriptomics, RNA was processed according to JGI established protocols. Briefly, ribosomal RNA (rRNA) was removed using the Ribo-Zero RNA Removal Kit. Stranded complementary cDNA (cDNA) libraries were generated using the Illumina Truseq Stranded RNA LT kit. The rRNA-depleted RNA was fragmented and reverse transcribed using random hexamers and SSII (Invitrogen) followed by second-strand synthesis. The fragmented cDNA was treated with end-pair, A-tailing, adapter ligation, and 8 cycles of PCR. We obtained 462 Gbp of metatranscriptomic sequencing (Supplementary Table 1). The resulting ~150 bp nucleotide sequences were trimmed as described above (see metagenomic section) and separately mapped via Bowtie2$^{55}$ to three databases (the metagenomic scaffolds $> 5$ kb, mcrA and mcrA-rev primer, balanced with a slight deviation in reaction mix including 1 μl of cDNA, 10 μl 2X SoAdvanced Universal SYBR Green Supermix reaction buffer, 0.5 μM of each mls and mcrA-rev primer, balanced with 20 μl total volume with PCR grade H2O. Serial diluted mcrA genes amplified from M. acetoaceticum DNA were used as standards. Statistical differences in mcrA transcript copy number between depths were evaluated via ANOVA (DF = 17, $p < 0.05$).

**Phylogenetic analyses.** Single gene and concatenated gene analyses were performed as described previously$^{56}$. Reference data sets for the 15 ribosomal proteins chosen as single-copy phylogenetic markers (Rpl2, 3, 4, 5, 6, 14, 15, 18, 22, and 24 and RpS 3, 8, 10, 17, and 19), small subunit ribosomal protein 3 (rps3), and mcrA were created using sequences mined from the NCBI and Joint Genome Institute Integrated Microbial Genomes/Microbiome (JGI-IMG/M) databases (December 2016). Each individual protein data set was aligned using MUSCLE 3.8.31 and then manually curated to remove end gaps$^{57}$. For amino acid phylogenetic analyses (S3 and concatenated ribosomal trees), we used ProtPipeliner, a python script developed in-house for generation of phylogenetic trees (https://github.com/lmsolden/protpipeliner). The pipeline runs as follows: alignments are curated with minimal editing by GBLOCKS$^{58}$, and model selection conducted via ProtTest 3.4$^{59}$. A maximum likelihood phylogeny for the concatenated alignment was constructed using RAxML version 8.3.1 under the LG model of evolution with 100 bootstrap replicates$^{60}$ and visualized in iTOL$^{61}$. For the mcrA nucleotide tree, a similar analysis was used but except under the GTRCAT model.

The V4 portion of 16S rRNA genes was sequenced at Argonne National Laboratory at the Next Generation Sequencing facility with a single lane of Illumina MiSeq using $2 	imes 251$ bp paired-end reads and analyzed as described previously$^{44}$. The full-length 16S rRNA sequence recovered from the Ca. Methanothrix paradoxum M1 genome was used to recover a single Methanothrix OTU from the V4 data set that was $> 99\%$ similar to the metagenomic recovered sequence. Additionally, the 16S rRNA fragment sequence from the M1 genome was searched against GenBank using BLASTN (e-value $1e^{-10}$, 100,000 alignments, 100,000 descriptions). Hits ($> 500$ bp) of at least $99\%$ identity were retained.

**Data availability.** Amplicon sequencing and metatranscriptomic data have been deposited in the sequence read archive under Bioproject PRJNA338276. The draft Candidatus Methanothrix paradoxon genome has been deposited in NCBI Genbank under accession numbers SAMN05908753, SAMN05908754, SAMN05908755, SAMN05908752, SAMN05908749, and SAMN05908846, with corresponding transcriptomic data also released. Flux data have been released to AmeriFlux, site ID US-OWC (https://ameriflux.llnl.gov/sites/siteinfo/US-OWC) with additional meteorological and data available via National Estuarine Research Reserve System Centralized Data Management Office (http://cdm.boaruch.scd.edu/#/landing.cfm).

FASTA files used to construct all phylogenetic analyses in this manuscript are included as Supplementary Data 6–10. Necessary scripts and analyses to perform metagenome assembly, EMIRGE, annotation, and single-copy genes can be accessed from github (https://github.com/TheWeightonLab/methanogenic_molecules). Code and additional repositories can be found at https://github.com/TheWeightonLab/methanogenic_molecules_emirge; https://github.com/TheWeightonLab/methanome_assembly; https://bitbucket.org/berkeleylab/metabat.

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