

Bacterial and methanogenic archaeal communities associated with *Avicennia germinans* in restored mangrove sites from the Yucatán Peninsula

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Abstract

Mangrove ecosystems are hotspots of microbial diversity, with bacterial and archaeal communities playing crucial roles in biogeochemical and nutrient cycles. Among these processes, methanogenesis is enhanced by anaerobic conditions typically induced by flooding and high organic matter accumulation. Despite extensive mangrove coverage on the Yucatán Peninsula, microbial communities in these sediments remain underexplored. This study aims to analyze, through 16S rRNA and *mcrA* gene sequencing, the structure and composition of microbial communities, particularly methanogenic archaea, in sediments associated with *Avicennia germinans* in restored sites with high (PH), medium (YM), and low (PL) mangrove recovery. While alpha diversity was consistent across sites, environmental variables—particularly total phosphorus (TP), total nitrogen (TN), total carbon (TC), sand, and silt content—varied significantly. Microbial community structure exhibited strong site-specific differences ($R^2=0.96$, $p = 0.004$), primarily associated with TP, total carbon (TC), and sand content. LEfSe analysis showed 20 differentially abundant genera in the three sites. Analysis of *mcrA* gene sequences indicated a dominance of methylotrophic methanogens of the Methanosarcinales order in the three sites. Nevertheless, the PH site also exhibited hydrogenotrophic (Methanobacteriales), acetoclastic (Methanotrichales), and hydrogen-dependent methylotrophic (Ca. Methanomethylales) sequences. Finally, two clusters of unassigned *mcrA* sequences, distantly related to methylotrophic groups, and one cluster, distantly related to a hydrogenotrophic group, were retrieved from this study, suggesting the presence of environmental clusters exclusive to the region. This study contributes to the comprehension of methanogenic communities in mangroves and provides a baseline for future research on methane emissions in mangroves of the Yucatán Peninsula.

Introduction

Mangroves are wetland ecosystems rich in carbon and highly productive (Gao et al. 2021). They can store long-term carbon in the form of biomass above- and below-ground, playing an essential role in climate change mitigation (Cameron et al. 2019). Additionally, mangroves sequester a large amount of carbon in the soil due to their anaerobic conditions and high rates of organic matter accumulation. However, these conditions also promote the production of greenhouse gases such as methane, which can be released back into the atmosphere (Arai et al. 2021). Methane is a greenhouse gas with a global warming potential about 25 times that of CO_2 over a 100-year period (Zheng et al. 2018). This gas is synthesized and consumed in these ecosystems through microbial activity (Upadhyay et al. 2020).

Methanogenic archaea are a group of microorganisms that inhabit strictly anaerobic environments and obtain energy by reducing C1 and C2 compounds, including CO_2 , formate, acetate, methanol, ethanol, methyl amines, and methyl sulfides, to produce methane, a process called methanogenesis (Evans et al. 2019). This gas is the final product of the anaerobic decomposition of organic matter, and its synthesis is catalyzed by a series of enzymes, with methyl coenzyme M reductase (MCR) being key, as it reduces methyl coenzyme M to methane. The gene that encodes the α -subunit of this enzyme (*mcrA*) is used as a molecular marker for studying methanogenic archaeal communities. Depending on the substrate they

use, these microorganisms are classified as hydrogenotrophic (those that use hydrogen), acetoclastic (those that use acetate), methylotrophic (those that use methylated compounds), and H_2 -dependent methylotrophic (those that use H_2 and methylated compounds) (Evans et al. 2019). In natural freshwater wetlands, hydrogenotrophic and acetoclastic methanogenic archaea tend to dominate. In contrast, in coastal wetlands, due to the great abundance of sulfate-reducing organisms and competition with methanogenic archaea for substrates such as hydrogen and acetate, methylotrophic methanogenic archaea are more abundant because they use a non-competitive substrate (Arai et al. 2021).

It has been reported that mangrove degradation increases greenhouse gas emissions by altering microbial community diversity and the metabolic pathways they support (Padhy et al. 2022). Furthermore, nutrient inputs from anthropogenic activities promote anaerobic conditions and increase the availability of substrates for methanogenesis. It has been estimated that mangroves affected by anthropogenic activities have methane emissions up to 14 times higher than those of undisturbed mangroves (Zheng et al. 2018).

Although wetlands are among the main natural sources of atmospheric methane, there is significant uncertainty about the magnitude of methane emissions from mangrove sediments and their contribution to climate change. Furthermore, few studies have focused on identifying the abundance, structure, and composition of methanogenic communities in these sediments (Shiau & Chiu 2020).

Mexico accounts for 6% of the world's total mangroves, ranking fourth after Indonesia, Australia, and Brazil. Fifty-five percent of Mexico's mangrove extension is in the Yucatán Peninsula (Osorio-Olvera et al. 2023). Previous studies of microorganisms in mangroves from the Yucatán Peninsula are scarce and have focused on 16S rRNA gene sequencing, finding mainly groups with metabolisms such as sulfate-reduction, nitrate-reduction, denitrification, H_2 oxidation, and sulfur oxidation, while methanogenic archaea have not been deeply surveyed with specific gene markers (Navarrete-Euan et al. 2021; Gómez-Acata et al. 2023; Esguerra-Rodríguez et al. 2024). Therefore, information on the diversity of microorganisms and methanogens in mangrove sediments from the Yucatán Peninsula remains to be further addressed. This can be useful within greenhouse gas mitigation strategies and the conservation of these ecosystems. This study aimed to analyze the structure and composition of microbial communities, particularly methanogenic archaea, in sediments associated with *Avicennia germinans* in a coastal lagoon of the Yucatán Peninsula, Mexico, with previous mangrove restoration actions. We expect a high diversity of bacteria and methanogenic archaea in the study sites, with differences in composition and structure related to the environmental characteristics at each site.

Materials and Methods

Study site and sampling

Chelem Lagoon is located on the northeast coast of the Yucatán Peninsula. It has three climatic seasons: dry (March-May), rainy (June-October), and *nortes* (north winds) (November-February). The soil

is karstic, superficial, porous, and thin, with high permeability; therefore, there are no surface water currents, but instead an extensive underground aquifer discharges water to the coast (Cinco-Castro & Herrera-Silveira 2020). Vegetation in and around Chelem Lagoon consists of a scrub mangrove forest dominated by *Avicennia germinans* and *Rhizophora mangle* that surrounds most of the lagoon (Chuang et al. 2017).

Progreso and Yucalpetén are areas within the Chelem Lagoon where different natural and anthropogenic pressures have affected the mangrove ecosystem. In response, ecological restoration actions have been carried out in these areas since 2010. The restoration efforts included rehabilitating the natural tidal channels, constructing 2,118 m of new channels, removing debris, and conducting topographic leveling with dispersion centers, depositing the material resulting from the channels in the deeper sites identified on a topographic map (Teutli-Hernández et al. 2020). Since the restoration actions, the mangrove species *A. germinans* has established naturally, and the ecosystem has recovered differently.

Three sampling sites were selected: a site with high mangrove recovery in Progreso (PH), a site with medium mangrove recovery in Yucalpetén (YM), and a site with low mangrove recovery in Progreso (PL) (Fig. 1).

In October 2022, during the rainy season, we collected surface sediment associated with *A. germinans* pneumatophores. At each sampling site, triplicate samples were collected and immediately homogenized, with one aliquot placed in 15 mL sterile plastic tubes containing RNA later for molecular analysis and another in sealed plastic bags for chemical analyses. All samples were stored at 4°C for later laboratory analysis. To quantify surface water temperature, salinity, redox potential, and dissolved oxygen, we used a portable multi-parameter analyzer (YSI-85, YSI Inc., USA) on-site.

Physicochemical characterization

Sediment samples were placed on trays to dry in the laboratory. They were sieved using a fine mesh. Subsequently, the organic matter content (OM) in sediments was determined using the Jackson method (Jackson 1964). Texture analysis was performed using the Bouyoucos Hydrometer method to quantify sand, silt, and clay content (Bouyoucos 1927). The determination of total phosphorus (TP) was carried out according to the method of Strickland & Parsons (Strickland & Parsons 1972). The total carbon (TC) and nitrogen (TN) contents were determined by the dry combustion method, in an elemental autoanalyzer (Flash EA-1112).

DNA extraction and gene sequencing

Sediment samples of 0.25 g were used for DNA extractions using the Powersoil DNA isolation kit (Mobio Labs Inc.) following the manufacturer's protocol. The quality of the extracted DNA was verified by electrophoresis on 1% agarose gels, and the final concentrations were quantified with Qubit® 3.0 from Life Technologies (HS dsDNA). 16S rRNA and *mcrA* genes were amplified to analyze the microbial and

the methanogenic archaeal communities, respectively. For the 16S rRNA gene, primers 515F-Y (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGTGYCAGCMGCCGCGGTAA-3') and 926R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGAGACAGCCGYCAATTYMTTTRAGTTT-3') (Parada et al. 2016) were used, and for *mcrA* gene, primers mlas-mod-F (5'-TCGTCGGCAGCGTCAGATGTGTATAAGAGACAGGGYGGTGTMGGDTTCACMCARTA-3') and mcrA-rev-R (5'-GTCTCGTGGGCTCGGAGATGTGTATAAGGACAGCGTTCATBGCAGTAGTTVGGRTAGT-3') (Angel et al. 2012) were used. Both primer pairs contained adaptors for sequencing on an Illumina MiSeq platform.

Polymerase chain reactions were performed in triplicate for each sediment sample and visualized on a 1% agarose gel. For the 16S rRNA gene, 20 µl reactions were carried out with 10 µl of Phusion Flash High-Fidelity Master Mix (Thermo Scientific, Waltham, MA, USA), 0.5 µl of each primer at 10 µM, 7 µl of PCR-grade water, and 2 µl of template DNA. PCR conditions involved an initial denaturing step at 95°C for 2 min, followed by 25 cycles of 95°C for 45 s, 52°C for 45 s as alignment temperature, and 68°C for 90 s as elongation, followed by a final extension at 68°C for 5 min (Parada et al. 2016). For the *mcrA* gene 20 µl reactions were carried out with 10 µl of Phusion Flash High-Fidelity Master Mix (Thermo Scientific, Waltham, MA, USA), 2.5 µl of each primer at 10 µM, 4 µl of PCR-grade water, and 1 µl of DNA. PCR conditions involved an initial denaturing step at 95°C for 5 min, followed by 5 touchdown cycles of 95°C for 30 s, 60 – 55°C for 45 s as alignment temperature, decreasing 1°C in each cycle and 72°C for 1 min as elongation, followed by 35 cycles of 95°C for 30 s, 54.5°C for 30 s and 72°C for 1 min and finally, a final extension at 72°C for 5 min (Angel et al. 2012).

PCR products were indexed using Nextera XT Index Kit v2 (Illumina, San Diego, CA, USA). The gene amplicon libraries were prepared according to Illumina's 16S rRNA gene Metagenomic Sequencing Library Preparation protocol and sequenced with an Illumina MiSeq instrument at Cinvestav Mérida in a 2x250-bp paired-end run. The datasets generated in this study are available in the online repository of the National Center for Biotechnology Information. The data are available under the BioProject number PRJNA1242378.

Bioinformatic and statistical analysis

The demultiplexed sequences obtained from Illumina sequencing were imported into the QIIME2 (Quantitative Insights Into Microbial Ecology) workflow. We used the DADA2 plugin (Divisive Amplicon Denoising Algorithm 2) for quality filtering, trimming, and denoising, with the “consensus” method for chimera removal. Further analyses were performed with a range of 4,055–5,457 reads for 16S rRNA and 1,596–19,305 for *mcrA* across the samples. For 16S rRNA gene, the genetic database SILVA 138 was used for ASVs (Amplicon Sequence Variants) assignment, while for *mcrA*, a database of the *mcrA* protein sequences was built using all entries from UniProt (UniProt Consortium 2021) for EC:2.8.4.1, and a BLASTX v.2.9.0 + analysis of the high-quality and non-chimeric amplicons was computed, setting the parameters to report the top five matches. Sequences without hits were discarded from the upstream analysis.

The R environment was used to remove unassigned sequences with the phyloseq and MetagMisc packages (McMurdie & Holmes 2013; Mikryukov 2023). Subsequently, the reads were standardized to the lowest sequencing depth, and the MicrobiotaProcess package (Xu et al. 2023) was used to make rarefaction curves and relative abundance graphs. To analyze the environmental variables and α -diversity metrics the dunn.test package (Dinno 2024) was used to perform the Kruskal-Wallis test, with post-hoc pairwise comparisons using Dunn's test. A distance-based redundancy analysis (dbRDA) was carried out to determine differences in beta diversity (Bray-Curtis distances). The linear discriminant analysis effect size (LEfSe) was performed at a 95% confidence level ($\alpha = 0.05$) to identify significant taxa for each site using the microeco and ggplot2 packages (Wickham 2016; Liu et al. 2021).

Phylogenetic tree construction

A phylogenetic tree was created using unassigned sequences of the *mcrA* gene. Sequences corresponding to different methanogenic orders from NCBI and UniProt were used as references. The Aliview software was used to translate the sequences into their corresponding aminoacid sequences and then align the sequences in FASTA format. The MEGA7 software was used to build the phylogenetic tree using the maximum likelihood method under the Tamura-Nei model (Tamura & Nei 1993). The tree's robustness was evaluated using Bootstrap with 1,000 resamples of the data. Finally, the iTOL tool was used to visualize and export the tree.

Results

Environmental characterization

Superficial water from the sampling sites exhibited temperatures above 30°C. All sampling sites presented hypersaline conditions, the highest being 43.69 UPS at the YM site. The dissolved oxygen was lower at the YM site (5.91 mg/l) and higher at the PL site (8.16 mg/l). All sites showed different redox potential, with a mostly reduced environment at the YM site (-101.6 mV), and a mostly oxidized environment at the PH and PL sites (20.9 and 37.1 mV, respectively) (Table S1).

Analysis of superficial sediments revealed significant differences in TN, TP, TC, sand, and silt content among sites ($p < 0.05$). The YM site exhibited significantly lower TN but higher TP than the PH and PL sites. The PH site was characterized by the highest TC content. Sediment texture also varied notably: PL had the highest sand content and the lowest silt content (Table 1). All three analyzed sites exhibited high sand content and low clay content. However, on average, the PH site had a clayey sand texture, the YM site had a silty sand texture, and the PL site had a sandy texture.

Table 1

Environmental measurements of sediment samples and alpha diversity metrics of the microbial communities based on the 16S rRNA gene sequence survey.

Site	TN(%)	TP ($\mu\text{mol/g}$)	TC (%)	OM (%)	Sand (%)	Silt (%)	Clay (%)	Richness (#ASVs)	Shannon index
PH	1.42 $\pm$ 0.44 ^a	3.58 $\pm$ 1.02 ^a	16.56 $\pm$ 0.65 ^a	8.95 $\pm$ 3.15 ^a	63.81 $\pm$ 13.61 ^a	21.12 $\pm$ 14.72 ^a	15.07 $\pm$ 1.54 ^a	327.33 $\pm$ 2.52 ^a	5.34 $\pm$ 0.1 ^a
YM	0.83 $\pm$ 0.12 ^b	4.81 $\pm$ 0.23 ^b	13.99 $\pm$ 0.9 ^b	7.04 $\pm$ 0.66 ^a	57.17 $\pm$ 2.02 ^a	28.87 $\pm$ 2.31 ^a	13.97 $\pm$ 1.14 ^a	309.67 $\pm$ 37.07 ^a	5.22 $\pm$ 0.14 ^a
PL	1.32 $\pm$ 0.14 ^a	2.57 $\pm$ 0.23 ^a	14.5 $\pm$ 1.36 ^b	9.89 $\pm$ 0.31 ^a	76.48 $\pm$ 2.09 ^b	9.76 $\pm$ 2.05 ^b	13.77 $\pm$ 1.01 ^a	349 $\pm$ 30.05 ^a	5.45 $\pm$ 0.06 ^a

TN = Total nitrogen, TP = Total phosphorus, TC = Total carbon, OM = Organic matter. ASV = Amplicon Sequence Variant. PH = Progreso (high mangrove recovery), YM = Yucalpetén (medium mangrove recovery), PL = Progreso (low mangrove recovery). Data are presented as mean $\pm$ standard deviation (n = 6 for environmental variables; n = 3 for alpha diversity metrics). Different superscript letters indicate statistically significant differences among sites ($p < 0.05$).

Microbial communities

Rarefaction curves based on the 16S rRNA gene sequences gradually plateaued, indicating that the sequencing depth in this study provided sufficient information on alpha diversity and richness within the samples (Fig. S1). A total of 1837 ASVs were retrieved, from which 89% corresponded to Bacteria and 11% to Archaea. At lower taxonomical levels, 53 phyla, 104 classes, 185 orders, 230 families, 273 genera, and 20 species were identified. Alpha diversity metrics showed no significant differences among sites ($p > 0.05$). Both ASV richness (327–349 ASVs) and Shannon diversity index (5.22–5.45) were similar across all locations, indicating a high microbial diversity in the three sites (Table 1).

Regarding beta diversity, dbRDA analysis showed that the first axis explained 39.3% of the variation, while the second axis explained 23.4%. The variables that explained the most variability in the microbial communities were TP ($R^2=0.77$, $p = 0.019$), TC ($R^2=0.62$, $p = 0.062$), and sand content ($R^2=0.60$, $p = 0.092$) (Fig. 2). Sites explained in a statistically significant way the variation in the structure of the microbial communities ($R^2=0.9623$, $p = 0.004$), based on the goodness of fit test. In terms of composition, the most abundant phyla across all sites were Bacteroidota (24–54.1%), Desulfobacterota (7.7–17.1%), and Spirochaetota (2.7–16.9%); while the most abundant archaeal phyla were Nanoarchaeota (0.3–2.7%) and Halobacterota (0.2–2.5%). The most abundant bacterial classes for all

sites were Bacteroidia (18.72–32.97%), Rhodothermia (3.77–30.58%), Desulfobacteria (3.3–14.08%), and Spirochaetia (2.69–14.72%); while the most abundant archaeal classes were Nanoarchaeia (0.34–2.74%) and Halobacteria (0.2–2.49%) (Fig. S2).

LEfSe analysis revealed significant differences in microbial community composition across sites, identifying 9 differentially abundant classes (Fig. S3) and 20 genera. One class and 2 genera were enriched in the PH site, 7 classes and 15 genera in the PL site, and one class and 3 genera in the YM site. A minimum LDA score of 3.21 and a maximum of 3.97 was recorded. The most distinctive taxa included *Calorithrix* and *Spirochaeta*, both enriched in the PL site, and *Tangfeifania*, enriched in the YM site (Fig. 3).

Methanogenic archaeal communities

Rarefaction curves based on the *mcrA* gene sequences gradually plateaued, indicating that the sequencing depth in this study provided sufficient information on alpha diversity and richness within the samples (Fig. S4). A total of 224 ASVs were recorded, of which only 11 ASVs were shared between the three sampling sites. The YM site had the highest number of ASVs, while the PL site had the lowest (Fig. S5). The ASVs were grouped into 3 phyla, 5 classes, 6 orders, 5 families, and 10 genera. In terms of taxonomic composition, the most abundant phylum was Euryarchaeota (20.4–79.1%), which was present in all samples. The phylum Candidatus-Thermoplasmatota (0.06–7.2%) was also found in 5 of the samples and all three sites, whereas the phylum Candidatus-Verstraetearchaeota (0.69%) was found only in one sample from the PH site. At the order level, the most abundant was Methanosarcinales, present in all samples, followed by Methanomassilicoccales, present in all three sites but not in all samples. The orders Methanotrichales, Methanobacteriales, and Candidatus-Methanomethylales were present only in one sample from the PH site. The order Methanomicrobiales was only present in one sample from the PH site and one from the YM site (Fig. 4). The most abundant genus was *Methanolobus*, present in all samples, followed by *Methanohalobium*, present only at the YM site. The genus *Methanothrix* was present only at the PH site. Other genera, such as *Methanobacterium*, *Methanosarcina*, and *Candidatus-Methanosuratincola*, were found only at the PH site. Meanwhile, the genera *Methanosalsum* and *Methanococcoides* were found only at the YM site. The genera *Methanohalophilus* and *Methanomicrobium* were found at both sites (Fig. S6). However, it was observed that 20.9–79.6% of the sequences were not assigned to any phylum (Fig. S6).

Phylogenetic associations of methanogenic archaea

To assess the phylogenetic relationships from the unassigned *mcrA* gene sequences, a phylogenetic tree was built using McrA reference sequences from NCBI and UniProt, containing all methanogenic orders and some sequences from methanotrophic archaea (Fig. 5). Two main clusters were formed, containing most of the detected sequences but being distantly related to methylotrophic halophile genera, such as *Methanohalobium evestigatum* and *Methanohalophilus halophilus*. One of these clusters contained sequences that were the most abundant and detected at the three sites (ASVs 1, 2, 3,

4, 5, 6, 7, 8, 9, 10, and 13); these sequences were assigned up to kingdom level (Archaea) and were more abundant at the PL site. The second cluster contained sequences mainly present at the YM site (ASVs 17, 16, 19, 20, 23, 26, 27, 31, 35, 36, and 38). The ASVs 67, 68, 69, and 76 –which were present only at the YM site at low abundance– were closely related to *Methanohalobium evestigatum* and *Methanohalophilus halophilus*.

Another cluster was formed by ASVs 15, 25, 28, 32, 34, 37, 44, 45, 49, and 50, and it was closely related to the hydrogenotrophic genus *Methanobacterium bryantii* (Fig. 5). These sequences were mainly present in the PH site. Another cluster was distantly related to this genus (ASVs 11, 12, 14, 18, 21, 22, 24, 29, 30, 33, 39, and 42) and contained sequences present primarily in the PL site. ASV64 was present at the PH site in low abundance and was closely related to other environmental sequences and the methylotrophic genus *Methanosalsum zhilinae*.

Discussion

This study analyzed bacterial and methanogenic archaeal communities in sediments associated with *A. germinans* at restored mangrove sites with different recovery levels. Differences in microbial community structure were observed between the sampling sites, which may be related to the extent of mangrove recovery and the sites' physicochemical characteristics. The restoration strategies on the sites focused on hydrology and did not involve reforestation, allowing *A. germinans* to develop naturally. As a result, recovery has been uneven, with some areas showing more substantial plant growth and others exhibiting limited growth and colonization.

At the local level, several factors can influence the establishment of mangroves; one is microtopography. Minor changes in topography lead to shifts in hydrology and its associated variables, such as salinity and redox potential, and these changes are sufficient to impact the growth of plant communities in coastal wetlands (Flores-Verdugo et al. 2007). Monitoring of these restoration sites has shown that the area where the PH site is located has a lower topographic elevation than the YM and PL sites (Teutli-Hernández et al. 2020), allowing for greater root extension and better seedling establishment for colonization. Additionally, this site is closer to a conserved mangrove area dominated by *R. mangle*, which may also provide more resources and greater stability for the plants.

This variation in mangrove recovery can influence microbial community composition, as plant establishment creates unique physical and chemical conditions that affect soil anaerobiosis, organic matter accumulation, nutrient availability, and primary productivity (Flores-Verdugo et al. 2007). It has been reported that mangroves experiencing prolonged drought periods –which occurred at these sites due to disrupted hydrological flow–undergo an increase in interstitial salinity, a decrease in redox potential, and an increase in sulfide concentrations (Zaldivar-Jimenez et al. 2010).

Influence of mangrove recovery on microbial communities

High microbial diversity was found in the study sites (Table 1), which was previously expected since mangroves provide nutrients and optimal conditions for microbial colonization. The low redox potentials, salinity levels, large reserves of organic matter, and nutrient recycling rates in these ecosystems facilitate the abundance of microbial resources, making them hotspots for microbial diversity (Palit et al. 2022). The structure of the microbial communities presented significant differences (Fig. 2), which could be a response to the mangrove's degree of recovery. It has been observed that the structure of microbial communities in mangroves is highly complex due to the infinitely large number of niches that microorganisms can inhabit (Ghizelini et al. 2012). Several studies have shown that microbial communities respond significantly to mangrove status, such as conserved, degraded, or restored, where mostly, conserved sites show a higher diversity than degraded ones. In these studies, a similar pattern is observed, where the most abundant groups are shared across recovery conditions, while differences are observed in less abundant taxa (Ma et al. 2021; Costa et al. 2023; Esguerra-Rodríguez et al. 2024). The results also suggested that mangrove recovery influenced the structure of microbial communities. In our study, the site with the lowest mangrove recovery (PL) showed the highest diversity in average, which in other studies has been related to human contamination, which provides an input of organic and inorganic compounds that can serve as substrates for opportunistic bacteria (Fernandes et al. 2015).

The variables that contributed the most to the differences in the microbial structure between sites were TP, TC, and sand content (Fig. 2). The YM site showed the highest average percentage of TP, potentially indicating anthropogenic contamination or bird nesting activities. Due to the karst conditions of the Yucatán Peninsula, the nutrient contributions, such as nitrogen and phosphorus, are mainly internal, with limited external input from bird excreta (guano) and groundwater discharges. Without the contribution of nutrients from river discharge, the mangroves in these sites are characterized by being limited in phosphorus (Twilley et al. 2018). The PH site showed the highest average of TC percentage. In karst environments, a significant portion of TC is likely inorganic, derived from calcareous organisms and carbonate dissolution processes (Twilley et al. 2018; Saderne et al. 2019;). A low OM content was observed at all sites, suggesting a limited availability of indigenous organic material to support microbial processes, including methanogenesis. However, mangrove growth can benefit the accumulation of organic matter in the sediments. Soil's texture, with a high sand content at the sampling sites, accelerates OM degradation, further limiting its accumulation (Zhou et al. 2019). The PL site, with the highest sand content and lowest silt and clay percentages, may be particularly susceptible to rapid OM degradation and greenhouse gas release. To better assess the effect of mangrove recovery on both the environmental conditions and the microbial communities, future studies should consider a control site, such as sediments associated with *A. germinans* from a pristine site.

LEfSe analysis revealed differentially abundant genera at each site (Fig. 3). The PH site exhibited an enrichment of the sulfate-reducing bacterium *Desulfotignum*, which belongs to the phylum Desulfobacterota. This genus has demonstrated the capability to degrade hydrocarbons, and its abundance has been observed to increase with nitrate content (Wang et al. 2016). The PL site showed enrichment of *Calorithrix* and *Spirochaeta*. *Calorithrix*, a member of the phylum Calditerrichota, comprises organoheterotrophic, anaerobic, thermophilic bacteria (Marshall et al. 2017), while *Spirochaeta* are

anaerobic or facultative chemoorganotrophic bacteria (Zhou et al. 2017). The YM site was enriched with *Tangfeifania*, a member of the phylum Bacteroidota, facultatively anaerobic chemoorganotrophs exclusive to saline environments, as well as the sulfate-reducing bacterium *Desulfopila*, a member of the phylum Desulfobacterota (Gittel et al. 2010). All these genera had abundances below 1%; therefore, microorganisms belonging to the rare biosphere in these sites contributed to differences in the structure of the microbial communities. The rare biosphere plays a key role in mangrove biochemical cycles; it accounts for the majority of microbial turnover during mangrove degradation and recovery (Costa et al. 2023). These groups should be further studied to see if they can act as bioindicators of mangrove recovery, contributing to the monitoring and restoration of these ecosystems.

Methanogenic archaeal communities and phylogenetic associations

To our knowledge, this work presented the first description of methanogenic archaeal communities inhabiting the mangrove sediments of the Yucatán Peninsula using the *mcrA* gene marker (Fig. 4). Methanosarcinales dominated all sampling sites. This order encompasses methylotrophic, acetoclastic, and hydrogenotrophic methanogenic archaea, as well as methanotrophic archaea from the ANME-2 and ANME-3 groups (Jing et al. 2016). Methanomassilicoccales were the second most abundant group at the three sampling sites; members of this order carry out a hydrogen-dependent methylotrophic metabolism (Kallistova et al. 2020). At lower taxonomic levels, 10 genera were found (Fig. S6). The most abundant at all sampling sites was *Methanlobus*, a finding consistent with previous reports in mangrove sediments (Jing et al. 2016; Yu et al. 2020; Padhy et al. 2022). This genus has been reported as a moderately halophilic organism with methylotrophic metabolism and has shown a positive correlation with TC and TN (Kallistova et al. 2020; Yu et al. 2020). As far as we know, it is the first time that the genera *Methanohalobium*, *Ca.-Methanosuratincola*, and *Methanomicrobium* have been reported in mangrove sediments.

All sites exhibited methanogenic groups with methylotrophic and hydrogen-dependent methylotrophic metabolisms. However, the PH site was the only one with all methanogenic metabolisms, including acetoclastic (Methanotrichales) and hydrogenotrophic (Methanobacterales and Methanomicrobiales) groups. This suggests that mangrove recovery and the physicochemical and environmental changes associated with this restoration could have a positive impact on the diversity of methanogenic archaea. The YM site, which had the highest salinity, was characterized by halophilic genera such as *Methanohalobium*, *Methanosalsum*, and *Methanococcoides*; all three with a methylotrophic metabolism (Kallistova et al. 2020). The PL site had the lowest metabolic diversity, since no hydrogenotrophic or acetoclastic groups were observed. This limited diversity at the PL site may be attributed to a lower availability of substrates and an increased competition with sulfate-reducing bacteria, inhibiting the growth of other but methylotrophic methanogens at this site. Other studies have reported a significantly lower microbial diversity in degraded mangrove sediments, with a decrease in taxa responsible for sulfur, nitrogen, and carbon cycles compared to preserved mangroves and those in recovery (Costa et al. 2023). Interestingly, the methanogenic community at the PL site was dominated by unassigned environmental

sequences (Fig. 4). These uncharacterized lineages could represent the primary methane producers at this site, suggesting a pronounced shift in the rare biosphere in response to the mangrove's recovery conditions.

As expected, the predominant methanogenic metabolism at the sampling sites was methylotrophic. This is consistent with reports that methylotrophic methanogenic archaea are the main contributors to methane production in sulfate-rich sites (Sela-Adler et al. 2017; Xiao et al. 2018; Li et al. 2020). The methylated compounds used as substrate by these methanogenic archaea are generated in marine sediments from osmolytes of bacteria, algae, phytoplankton, and some plants (Liu & Whitman 2008). However, this work provides evidence that in hypersaline environments, groups of methanogenic archaea with hydrogenotrophic and acetoclastic metabolism can coexist despite competition with sulfate-reducing bacteria, a phenomenon also reported in other hypersaline environments in the country, such as the microbial mats in Guerrero Negro, Baja California Norte (García-Maldonado et al. 2023).

Previous studies report that mangroves harbor a high diversity of unique bacterial and archaeal populations, and several novel lineages have been reported from these ecosystems (Baskaran et al. 2023). Moreover, in methanogenic archaea studies it is common to find many unassigned groups due to the lack of environmental sequences of the *mcrA* gene in databases, because in most ecosystems these microorganisms are part of the rare biosphere, and because many groups are difficult to be cultured, making their study at a phylogenetic level challenging (Liu & Whitman 2008; García-Maldonado et al. 2023; Jia et al. 2023). In the phylogenetic analysis (Fig. 5), we identified three clusters that did not closely associate with any of the reference sequences, and two of them were distantly related to the methylotrophic groups *Methanohalobium* and *Methanohalophilus* and included ASVs with high abundances at all sites. The most parsimonious explanation is that these clusters also belong to the methylotrophic metabolism, which was the most abundant among the assigned taxa and is expected in these ecosystems (Sela-Adler et al. 2017). However, based on the phylogenetic distances, it is probable that they correspond to an environmental cluster specific to this region. Some other sequences were associated with the hydrogenotrophic group *Methanobacterium bryantii*, suggesting that this metabolism is also present at the PL site. Acetoclastic metabolism was not represented in these sequences. The association of ASVs exclusively at the YM site with halophile genera reaffirms the inference that at this site, high salinity favored the growth of groups of halophilic methanogenic archaea (Kallistova et al. 2020). On the other hand, we discarded the possibility of anaerobic methanotrophic archaea in the study sites, since none of the *McrA*-ASVs were associated with these groups. This study provides the basis for reconstructing the genomes of the unassigned ASVs in future studies to better understand their ecological roles and evolutionary histories within these unique ecosystems.

Conclusions

To our knowledge, this study provides the first description of methanogenic archaeal communities inhabiting mangrove sediments of restored sites in the Yucatán Peninsula, Mexico, using the *mcrA* gene as a molecular marker. Our findings indicated that differences in mangrove recovery and the

physicochemical characteristics associated with them can influence the composition and structure of microbial communities, including methanogenic archaea. Microbial communities in sediments associated with *Avicennia germinans* showed structural differences that may be linked to the degree of mangrove recovery. Notably, microorganisms from the rare biosphere, including *Desulfotignum*, *Calorithrix*, *Spirochaeta*, *Tangfeifania*, and *Desulfopila*, contributed to these variations. The main environmental variables explaining microbial community variability were TP, TC, and sand content.

As hypothesized, these mangrove ecosystems harbor a high diversity of methanogenic archaea. Our findings revealed that these communities are dominated by Methanosarcinales. Still, they have site-specific differences: the PH site exhibited all four methanogenic metabolisms, the YM site was notable for its halophilic genera, and methylotrophic groups exclusively dominated the PL site. This suggests that greater mangrove recovery positively influences methanogenic community diversity, with more mature systems supporting a broader array of metabolic pathways. Significantly, our work contributes to the understanding of these understudied ecosystems by identifying a substantial proportion of unassigned *mcrA* sequences. This indicates the presence of a region-specific environmental cluster, distantly related to methylotrophic and hydrogenotrophic genera. Future research should focus on characterizing these unclassified methanogens to fully elucidate their ecological roles and metabolic potential in Yucatan mangroves.

Our findings contribute to understanding microbial community dynamics in mangrove sediments and highlight the importance of further research on methanogenic archaeal communities that contribute to methane production in these environments.

Declarations

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Data availability

Data have been deposited into NCBI under BioProject accession number PRJNA1242378 and it can be downloaded with the next link: <https://www.ncbi.nlm.nih.gov/bioproject/PRJNA1242378/>.

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Figures

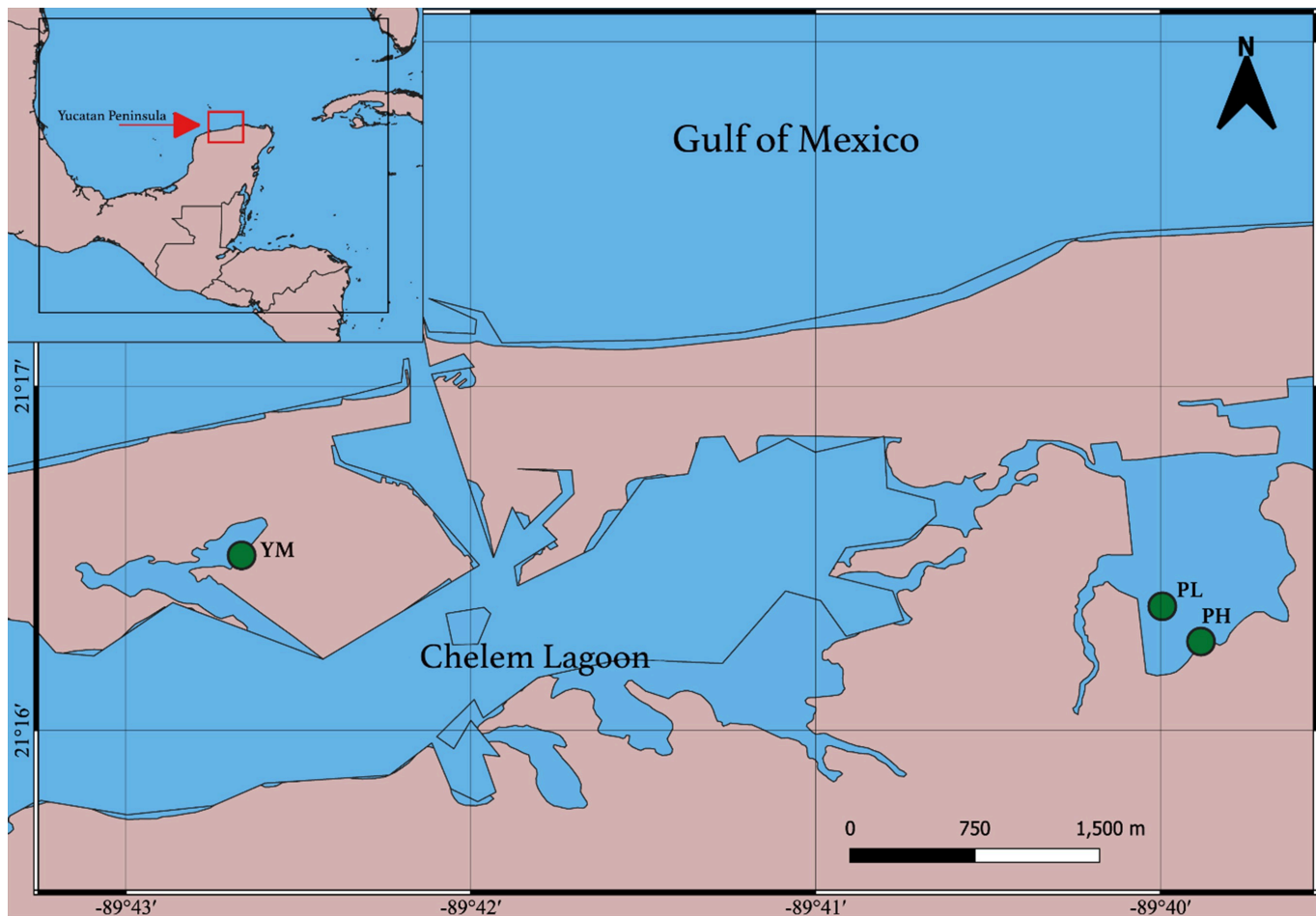


Figure 1

Location of the study with the three sampling sites. PH= Progreso (high mangrove recovery), YM= Yucalpetén (medium mangrove recovery), PL= Progreso (low mangrove recovery). Map made with QGIS 3.22.5

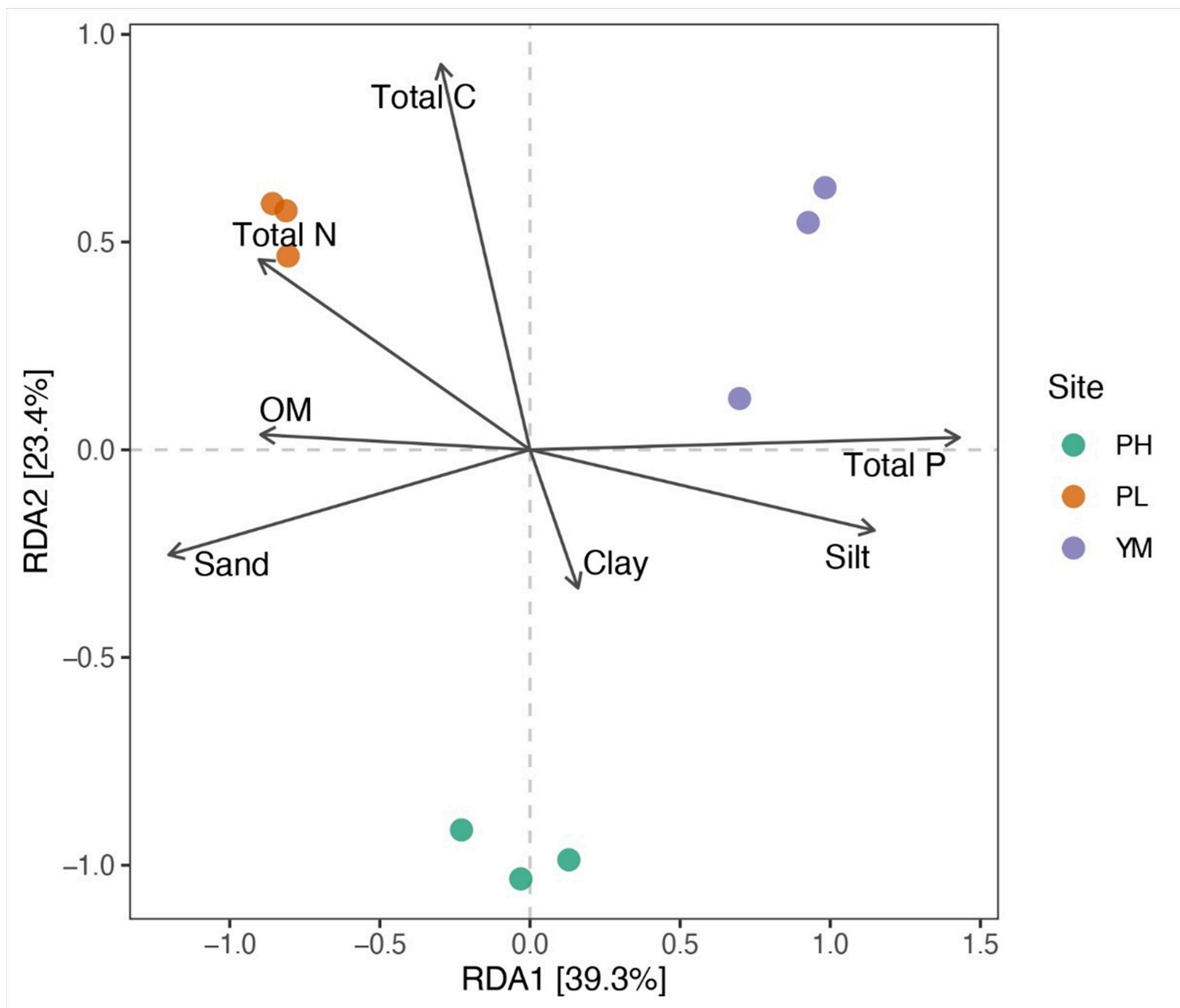


Figure 2

Distance-based Redundancy Analysis (dbRDA) using the Bray-Curtis index based on the 16S rRNA gene sequences and associated with the physicochemical characteristics. Sites: PH= Progreso (high mangrove recovery), YM= Yucalpetén (medium mangrove recovery), PL= Progreso (low mangrove recovery)

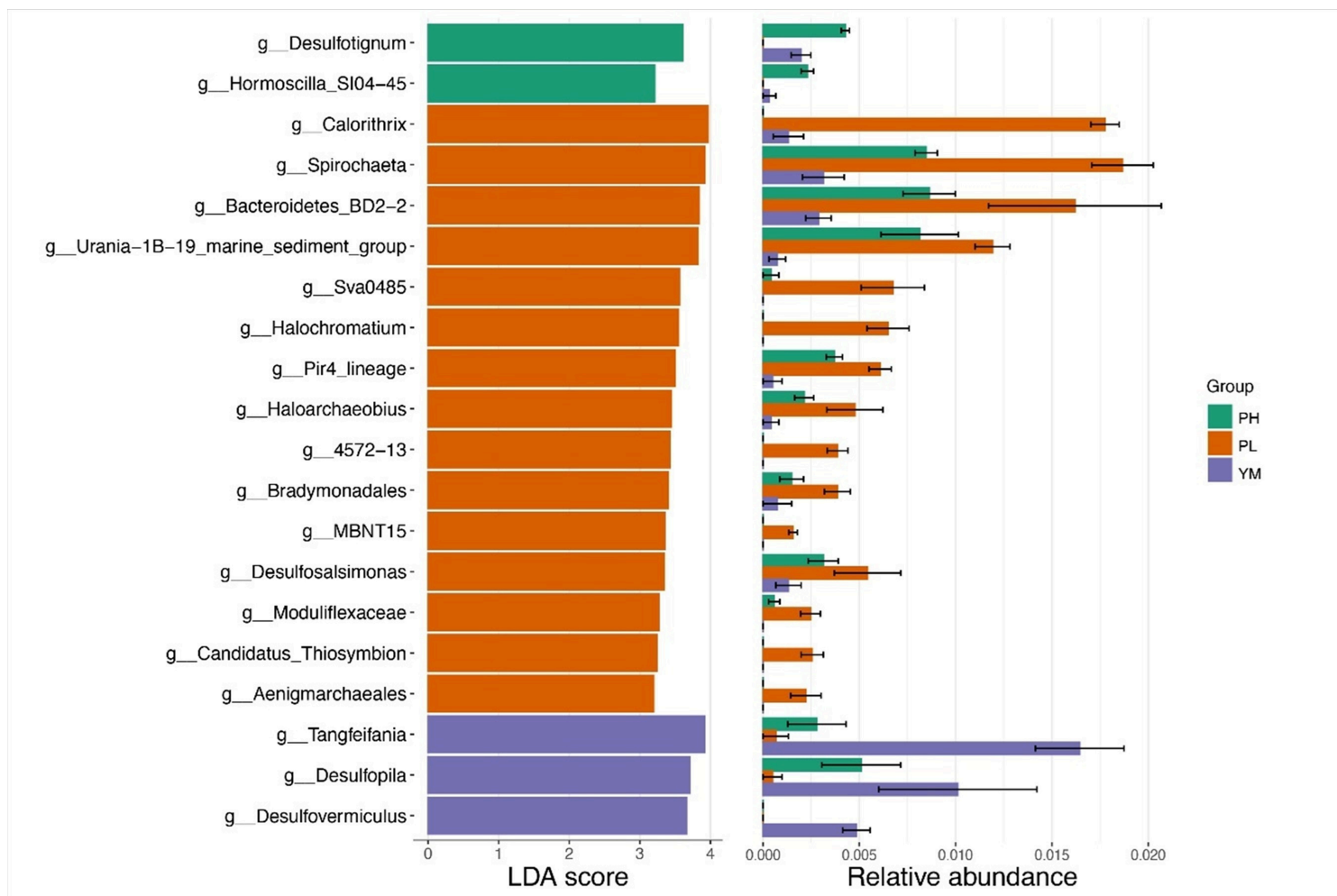


Figure 3

LDA values and relative abundances of the differentially abundant prokaryotic genera at each site. Groups: PH= Progreso (high mangrove recovery), YM= Yucalpetén (medium mangrove recovery), PL= Progreso (low mangrove recovery)

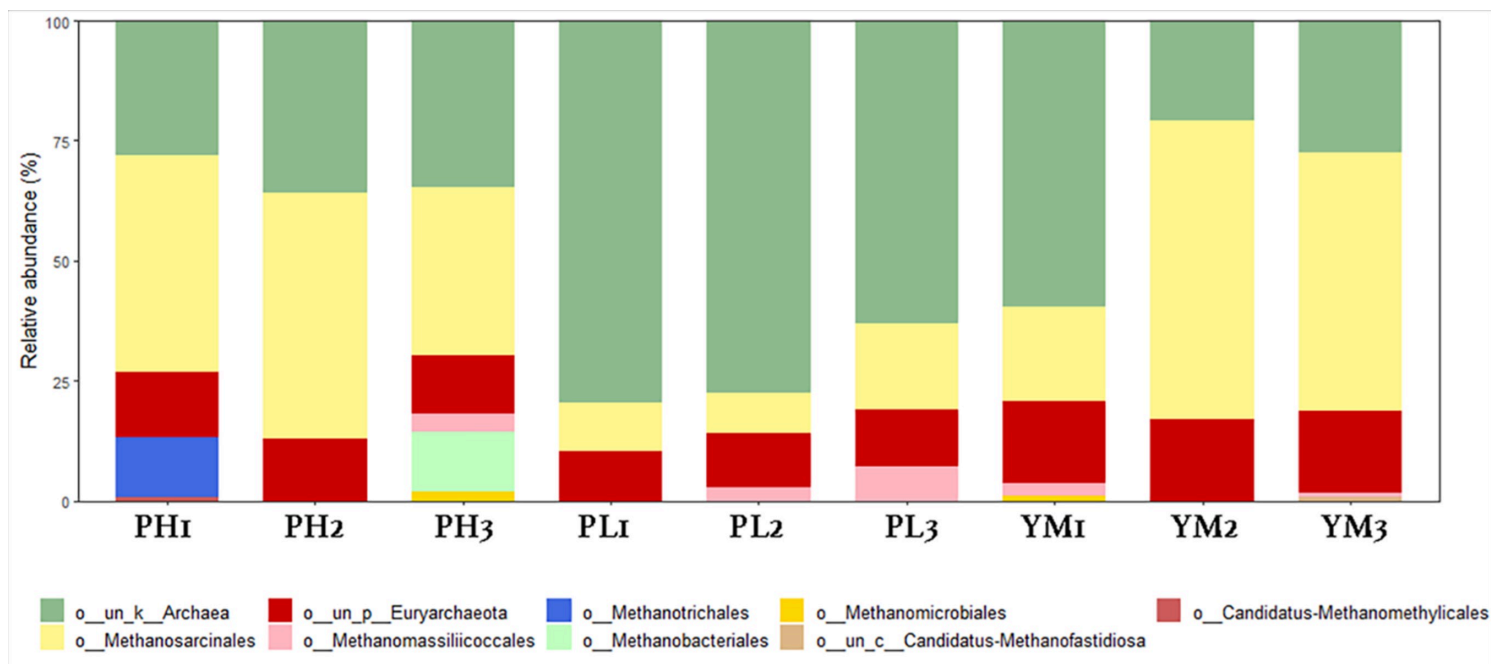


Figure 4

Relative abundances (%) at the level of order of methanogenic archaeal communities based on the *mcrA* gene sequences. Triplicate data are shown for each sampling site. PH= Progreso (high mangrove recovery), YM= Yucalpetén (medium mangrove recovery), PL= Progreso (low mangrove recovery)

