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Long-term environmental stability promotes reproducible, depth-structured sediment microbiomes

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Keywords: Inland relict mangroves; Environmental stability, Vertical stratification

Posted Date: February 26th, 2026

DOI: https://doi.org/10.21203/rs.3.rs-8950299/v1

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Additional Declarations: There is **NO** Competing Interest.

Long-term environmental stability promotes reproducible, depth-structured sediment microbiomes

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ABSTRACT
Long-term environmental stability is widely assumed to erode microbial diversity by limiting dispersal and connectivity. However, its effects on sediment microbial organization remain insufficiently characterized. Here, we used rare inland relict mangroves from the San Pedro Mártir River (Mexico), isolated more than 170 km from the coast since the Last Interglacial, as a natural experiment to examine how sustained environmental stability shapes sediment microbiome organization. Using depth-resolved 16S rRNA gene profiling, we showed that inland relict sediments harbor highly cohesive microbial communities characterized by large resident core microbiomes, low compositional dispersion and consistent vertical stratification across sites and sediment depths. Contrary to expectations of diversity loss under isolation, microbial richness is maintained or even accumulates with sediment depth, coupled with coherent taxonomic turnover and inferred functional stratification. In contrast, natural and hydrologically impaired coastal mangroves exhibit high variability, reduced diversity at depth, and erosion of the resident cores. Together, these findings indicate that long-term environmental stability enables predictable, depth-structured modes of microbial community organization, providing a foundational framework for interpreting microbiome variability across dynamic, restored, and disturbed coastal ecosystems.

Keywords: Inland relict mangroves; Environmental stability, Vertical stratification

MAIN
Environmental stability and disturbance are central forces shaping microbial community assembly, yet their long-term effects remain difficult to disentangle in natural ecosystems. In highly dynamic environments, recurrent physicochemical fluctuations and dispersal events can repeatedly restructure microbial communities, obscuring persistent organizational patterns and limiting reproducibility across space and time [1,2]. As a result, it remains unclear whether

sustained environmental stability promotes predictable microbial organization or instead leads to diversity loss through isolation and reduced connectivity [3,4].

Mangrove sediments provide a powerful context to address this question. These ecosystems sustain intense microbial activity that underpins carbon storage, nutrient cycling, and pollutant transformation, tightly linking sediment microbiome organization to ecosystem-scale functioning[5,6]. However, most studies of mangrove microbiomes have focused on coastal systems exposed to strong and rapidly fluctuating environmental gradients, including tidal exchange, salinity oscillations, and variable freshwater inputs[1,2]. Such dynamics disrupt vertical redox stratification and generate high spatial and temporal variability in microbial community composition, complicating efforts to identify consistent and reproducible patterns of microbial assembly.

In contrast, inland mangrove forests represent rare systems in which multiple sources of environmental variability are persistently constrained. Some inland mangroves persist as relicts of past marine incursions associated with sea-level highstands and subsequently became isolated by geological barriers such as karst formations[7,8]. Their long-term hydrological isolation, reduced dispersal connectivity and sustained freshwater influence create environmental regimes characterized by long-term environmental stasis that differ fundamentally from those of tidally dynamic coastal mangroves. By dampening short-term physicochemical fluctuations, these systems approximate long-term stable conditions and offer a natural opportunity to examine how microbial communities organize when disturbance and dispersal are consistently limited.

Although vertical stratification, resident core microbiomes and depth-associated taxonomic turnover have been described in mangrove sediments and other relatively undisturbed environments[3,9], their reproducibility across sites and persistence under long-term isolation remain poorly resolved. In particular, it is unclear whether sustained environmental stability promotes consistent microbial organization across space and sediment depth, or whether previously reported patterns primarily reflect local environmental similarity.

One of the most extreme examples of inland mangroves occurs along the San Pedro Mártir River in Tabasco, Mexico, where stands dominated by *Rhizophora mangle* occur more than 170 km from the coast. These forests occupy karstic riverbanks and fossil lagoons formed during the Last Interglacial period and have remained hydrologically isolated over extended timescales[8]. Calcium-rich substrates, oligotrophic freshwater inputs and limited hydrological connectivity distinguish these inland sites from coastal mangroves of the Yucatán Platform, including Términos Lagoon and Celestún Lagoon (Fig. 1), which experience tidal exchange, salinity variability and, in some cases, hydrological impairment.

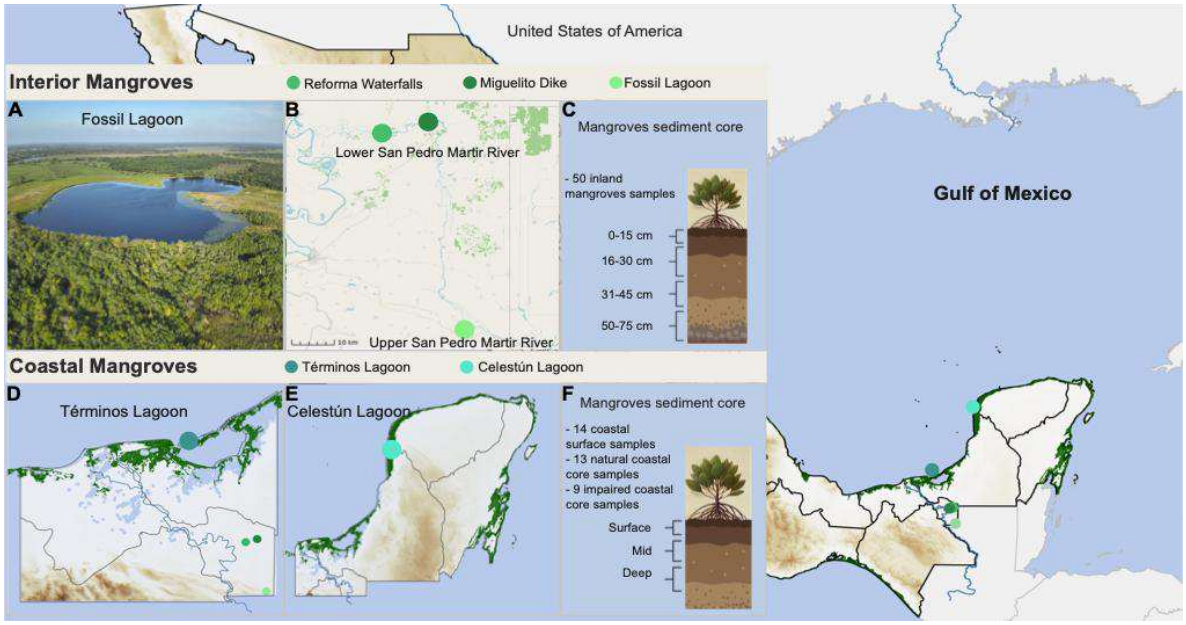


Fig. 1. Geographic distribution of sampling sites from mangroves along the Yucatán Platform, México.

Here, we analyzed depth-resolved sediment microbial communities associated with *R. mangle* in inland relict mangroves of the San Pedro Mártir River and compared them with natural coastal mangroves and a hydrologically impaired coastal mangrove from the Yucatán Platform. Using 16S rRNA gene meta-sequencing, we assessed microbial diversity, community composition, resident core microbiomes and inferred metabolic potential across sediment depth. By contrasting stable, isolated inland systems with dynamic and disturbed coastal environments, we examine how long-term environmental stability shapes the predictability and vertical organization of sediment microbiomes.

RESULTS

Inland and coastal mangroves differ in microbial diversity and community composition

To evaluate whether long-term environmental stability is associated with consistent microbial community organization, we first compared surface sediment microbiomes associated with *R. mangle* from two mangrove types: inland relict and coastal. These were represented by multiple mangrove systems across the Yucatán Platform. Hereafter referred to as types for inland and coastal, and systems for specific geographic locations.

Mangrove system identity strongly structured microbial alpha diversity. Observed richness (q0), exponential Shannon diversity (q1), and inverse Simpson diversity (q2) differed among systems (linear models; all $p < 0.001$), with the model explaining a high proportion of variance (R2 = 0.83, 0.66, 0.78, Supplementary Table S3). Inland relict mangroves exhibited intermediate diversity values with relatively low variability among samples, whereas coastal systems spanned a broad diversity range, from low-diversity assemblages in Términos Lagoon to highly diverse communities in Celestún Lagoon (Fig. 2A, Supplementary Table S3).

Microbial community composition also differed consistently between inland and coastal systems. Ordination based on Bray–Curtis dissimilarities revealed a clear separation between inland relict sediments and coastal sediments (Fig. 2B). Permutational multivariate analysis of variance confirmed significant effects of mangrove type ($R^2 = 0.283$, $p = 0.001$) and system identity ($R^2 = 0.206$, $p = 0.001$). Notably, within-system dispersion was lower in inland relict

mangroves than in coastal systems (PERMDISP, $p < 0.05$), indicating a high degree of compositional consistency among inland samples (Supplementary Table S4).

Differential abundance analysis identified taxonomic groups contributing to the observed compositional divergence (Fig. 2C). Inland relict sediments were enriched in multiple bacterial and archaeal lineages, commonly associated with anaerobic metabolisms and long-term geochemically stable sedimentary environments, including members of Chloroflexi, Desulfobacterota, Planctomycetota, Zixibacteria, Myxococcota, and Crenarchaeota (ANCOM-BC2, adjusted $p < 0.05$). In contrast, coastal mangroves displayed more heterogeneous taxonomic profiles, with no consistent enrichment patterns shared across sites.

Together, these results show that inland relict and coastal mangroves host distinct sediment microbial communities, with inland systems exhibiting greater compositional consistency across samples than coastal environments.

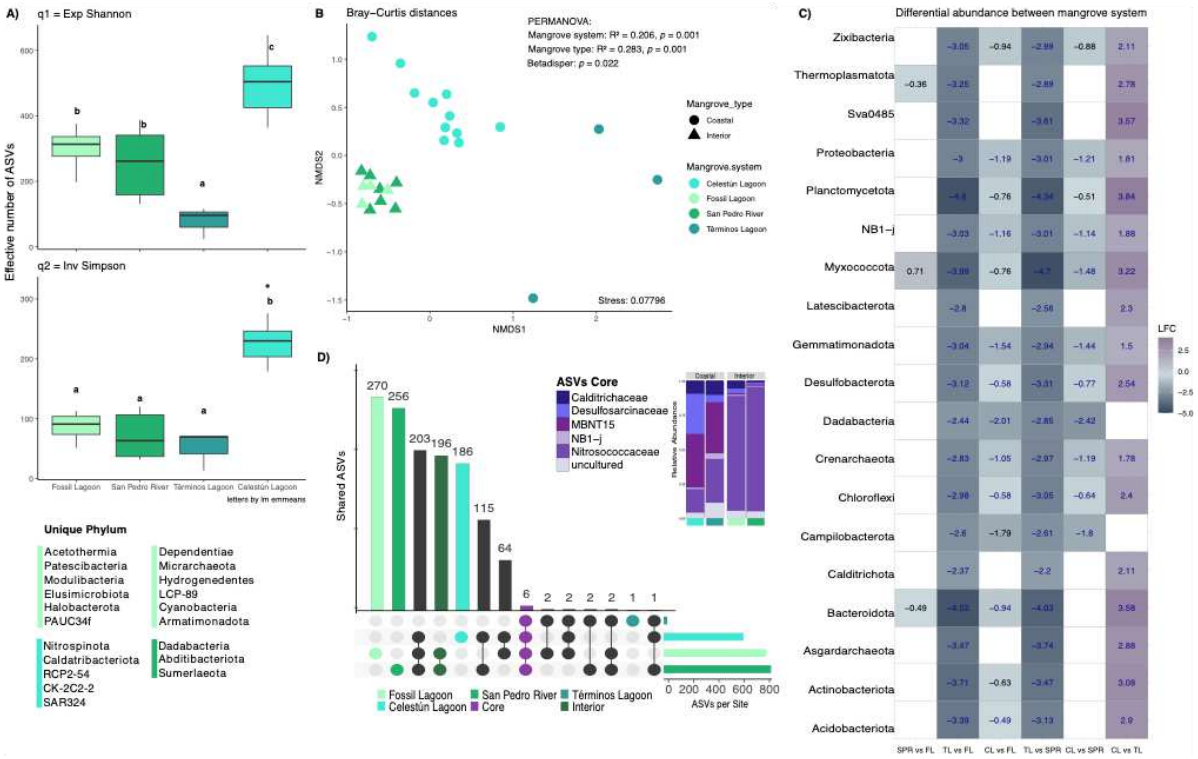


Fig. 2. Microbial community structure and diversity in inland relict and coastal mangroves of the Yucatán Platform. A) Microbial alpha diversity measured by exponential Shannon (q1) and inverse Simpson (q2) indices. Letters indicate significant differences (linear models, $p < 0.001$). B) Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarities. C) Heatmap showing differential abundance (Log Fold Change, LFC) of microbial phyla across systems. D) UpSet plot and bar charts illustrating resident core microbiomes based on ASV prevalence. Stacked bar plots show the relative abundance of core taxa.

Inland relict mangroves harbor large and persistent resident core microbiomes

To assess the persistence of shared microbial taxa, we quantified resident core microbiomes based on ASV prevalence across samples from inland relict and coastal mangroves. Inland

relict supported larger resident core microbiomes than coastal systems (Fig. 2D). Fossil Lagoon and the San Pedro Mártir River shared 196 resident core ASVs, indicating a substantial overlap between geographically isolated inland sites. In contrast, no resident core ASVs were shared between the two coastal systems, consistent with pronounced taxonomic turnover across coastal mangroves of the Yucatán Platform. Across all sampled systems, only six ASVs were shared as a global core microbiome, highlighting limited overlap in resident taxa across the region.

The contribution of resident core taxa to community structure also differed markedly among systems. Inland relict sediments were dominated by a small number of highly prevalent taxa, with members of Nitrosococcaceae accounting for 84–91% of the relative abundance within the resident core microbiome. In contrast, coastal exhibited more diffuse core contributions, with taxa such as MBNT15 and Desulfosarcinaceae varying in prevalence and relative abundance among sites.

Together, these results show that inland relict mangroves maintain large, shared, and dominant resident cores, whereas coastal are characterized by minimal core overlap and higher taxonomic turnover.

Depth-resolved profiles reveal stable vertical organization under long-term environmental stability

We examined whether the contrasts observed between inland and coastal mangroves extended across sediment depth profiles. Using a curated dataset including surface, intermediate, and deep sediment layers from inland relict mangroves and from natural zones of Términos Lagoon, we assessed depth-associated patterns in microbial diversity and community composition.

Microbial alpha diversity was primarily structured by mangrove system identity rather than sediment depth. Linear models accounting for sequencing depth as a covariate indicated that system identity significantly influenced observed richness (q0) and diversity indices (q1, q2; Supplementary Table S5), whereas depth effects were comparatively weak within systems. Notably, while microbial diversity in the coastal system declined and became more variable with depth, inland relict sediments maintained or increased microbial richness in deeper sediment layers (Fig. 3A,B).

Depth-associated patterns in community composition differed markedly between systems. Mangrove system identity explained a substantially larger proportion of compositional variation ($R^2 = 0.334$, $p = 0.001$) than sediment depth alone ($R^2 = 0.042$, $p = 0.012$). The significant interaction between system and depth ($R^2 = 0.083$, $p = 0.004$) indicates that depth-associated organization differs fundamentally between inland and coastal ecosystems. Inland relict sediments exhibited significantly lower multivariate dispersion across depth profiles than coastal sediments (PERMDISP, $F = 47.1$, $p < 0.001$; Fig. 3C; Supplementary Table S6). Quantitative analysis of the average distance to group centroids revealed that inland systems (Fossil Lagoon = 0.291; San Pedro River = 0.346) possess a highly cohesive microbial structure, whereas the coastal system (Términos Lagoon = 0.554) shows nearly twice the compositional variance, reflecting more consistent vertical community organization within inland sites.

Analysis of resident core microbiomes across sediment depth reinforced these trends. Inland relict mangroves retained large resident cores across all sediment layers, comprising 995 ASVs

in Fossil Lagoon and 657 ASVs in the San Pedro Mártir River, reflecting vertically persistent taxa within each site. In contrast, the coastal system showed no resident core ASVs uniquely associated with specific depth layers (Fig. 3D), suggesting limited vertical niche specialization and greater community homogenization across sediment horizons.

Differential abundance analysis identified the taxonomic groups driving this divergence (Fig. 3E). Inland sediments were significantly enriched in lineages associated with complex carbon degradation and anaerobic respiration, including Acidobacteriota, Chloroflexi, Desulfobacterota, Nitrospirota, and Zixibacteria (ANCOM-BC2, LFC > 2, adjusted $p < 0.05$), along with archaeal groups such as Asgardarchaeota and Thermoplasmatota that were prevalent in inland but depleted in coastal sediments.

Together, these results show that under long-term environmental stability, sediment depth functions as a persistent and reproducible organizing axis for microbial communities, whereas depth-associated organization is reduced in dynamic coastal sediments.

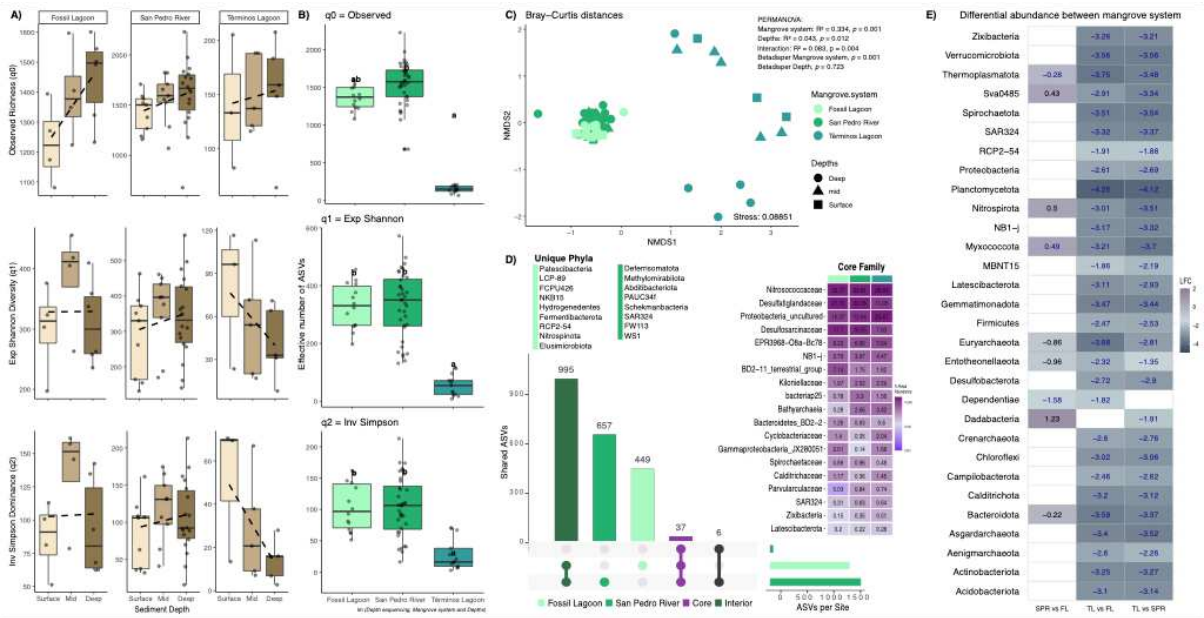


Fig 3. Vertical microbial community structure and diversity across sediment depth profiles. A) Microbial alpha diversity measured by Observed richness (q0) exponential Shannon (q1) and inverse Simpson (q2) indices. A) Across depth horizons in inland relict and coastal mangroves. B) Across system and depth. Letters indicate significant differences (linear models, $p < 0.001$). C) Non-metric multidimensional scaling (NMDS) ordination based on Bray–Curtis dissimilarities. Statistics represent PERMANOVA (R^2 and p-values) for system, depth, and their interaction, alongside multivariate dispersion (Betadisper). D) UpSet plot illustrating ASV intersections across systems and depth layers, and heatmap of relative abundance for resident core families. E) Differential abundance heatmap (ANCOM-BC2) showing Log Fold Change (LFC) of microbial phyla between systems.

Hydrological impairment disrupts microbial community organization

To evaluate how hydrological disturbance affects sediment microbial assembly, we compared inland relict mangroves with a hydrologically impaired coastal mangrove from Términos Lagoon, characterized by restricted tidal exchange and altered hydrological connectivity.

Analyses were restricted to samples collected during the flooding season to minimize the influence of seasonal variability.

Microbial alpha diversity differed consistently between systems. Linear mixed-effects models (LMM), accounting for repeated measures across sediment depth and sequencing depth, showed that inland relict mangroves maintained higher observed richness (q0) and exponential Shannon diversity (q1) than the impaired coastal system ($p < 0.05$; Supplementary Table S7; Fig. 4A). While microbial diversity in inland sediments increased or remained stable across the vertical profile, the impaired coastal site exhibited uniformly low richness across all sediment depths (Fig. 4B).

Community composition also differed markedly between systems. Mangrove system identity explained the largest proportion of compositional variation ($R^2 = 0.379$, $p = 0.001$), exceeding the contribution of sediment depth ($R^2 = 0.050$; Supplementary Table S8). The hydrologically impaired coastal system exhibited significantly higher multivariate dispersion than inland relict sediments (PERMDISP, $p < 0.001$; Fig. 4C), indicating elevated variability and reduced compositional consistency.

Resident core microbiome analysis revealed a pronounced reduction in persistent taxa at the impaired site. While inland relict mangroves supported large resident core microbiomes (up to 1695 ASVs), the impaired coastal system retained a substantially smaller core of 123 ASVs and lacked resident core ASVs uniquely associated with the impaired system (Fig. 4D), indicating the absence of site-specific persistent microbial assemblages. Differential abundance analysis did not identify consistent taxonomic enrichments associated with the impaired system, and several lineages enriched in stable inland sediments were largely depleted.

Together, these results show that hydrological impairment is associated with reduced microbial diversity, increased compositional variability, and erosion of persistent resident microbial assemblages.

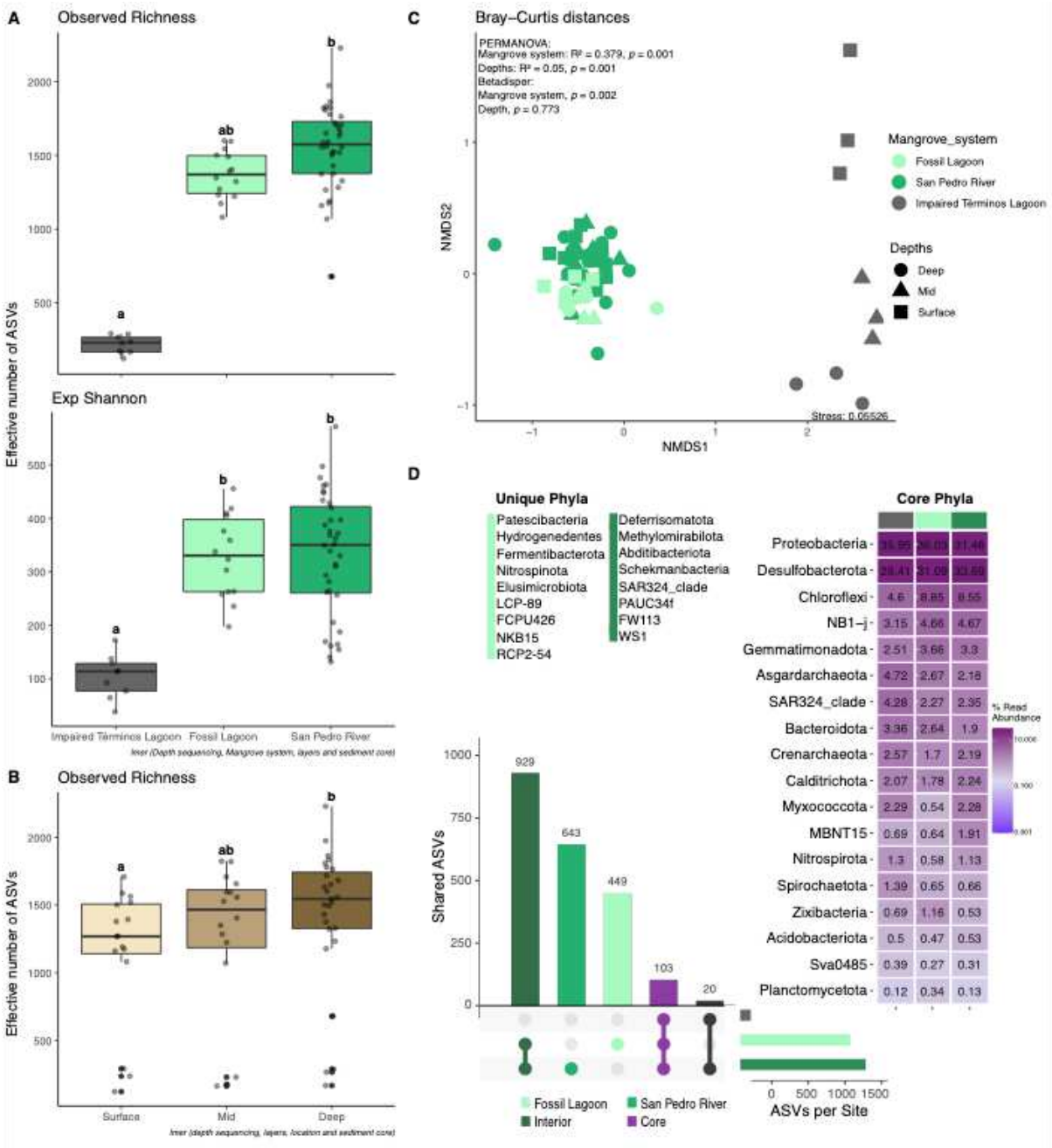


Fig. 4. Microbial diversity and community assembly across inland and coastal mangroves under hydrological impairment. A) Microbial alpha diversity (q0 and q1) in inland relict and impaired coastal mangroves (n = 59 sediment samples). B) Observed richness (q0) across vertical sediment horizons (Surface, Mid, and Deep) in relict and impaired systems. Letters indicate significant differences based on linear mixed-effects models (LMM; $p < 0.05$). C) NMDS ordination of microbial community Bray–Curtis dissimilarities. PERMANOVA (999 permutations) reports that system identity ($R^2 = 0.379$, $p = 0.001$), sediment depth ($R^2 = 0.050$, $p = 0.001$), and their interaction ($R^2 = 0.077$, $p = 0.001$) explain variance. BETADISPER *p*-value for multivariate dispersion between systems is shown ($p < 0.001$). D) UpSet plot illustrating ASV intersections between relict and impaired systems across depth layers. The bar plot shows the total ASVs in the resident core microbiome of each system. The heatmap displays the relative abundance of the resident core phyla.

Inland relict sediments exhibit coherent depth-associated taxonomic and inferred functional stratification

We examined whether the stable vertical organization observed in inland relict mangroves was accompanied by coherent depth-associated taxonomic patterns and inferred metabolic potential. Across inland relict sediments, bacterial lineages dominated community composition at all depths (>98% of sequences), while the relative contribution of archaeal taxa increased with sediment depth (Supplementary Fig. 1). Clear depth-associated shifts in taxonomic composition were observed: Proteobacteria decreased in relative abundance with depth, whereas Chloroflexi increased markedly in deeper sediment layers. Archaeal phyla, including Thermoplasmatota and Crenarchaeota, were preferentially enriched in deeper horizons (Supplementary Fig. 2). Differential abundance analysis further supported this vertical zonation, with surface layers enriched in Nitrospinota and deeper sediments dominated by Zixibacteria, Chloroflexi, and Spirochaetota (ANCOM-BC2, LFC > 2, $p < 0.05$; Fig. 5B).

Within inland sites, sediment depth emerged as the primary axis of community differentiation. Observed richness and Faith's phylogenetic diversity increased with depth, whereas Shannon diversity and evenness showed limited variation across layers (Fig. 5A; Supplementary Table S9). Beta diversity analyses based on weighted UniFrac distances indicated that sediment depth explained a large proportion of compositional variation across inland profiles ($R^2 = 0.346$, $p < 0.001$; Fig. 5C). In contrast, site-level differences were more captured by unweighted UniFrac distances (Fig. 5D; Supplementary Table S10), reflecting differences in taxon presence associated with environmental gradients. Distance-based redundancy analysis explained 61.2% of the compositional variation, with temperature identified as the most significant explanatory variable ($R^2 = 0.209$, $p = 0.016$; Fig. 5E; Supplementary Table S11); notably, Fossil Lagoon exhibited significantly lower temperature and salinity (Kruskal–Wallis, $p < 0.02$). This organization was further supported by a shared resident core of 950 ASVs across all depths and inland sites, reflecting taxa that persist consistently throughout sediment profiles (Fig. 5G).

Functional inference analyses (ANCOM-BC2, $p < 0.05$) revealed coherent depth-associated patterns in predicted metabolic potential. Putative genes associated with oxidative nitrogen transformations were enriched in surface sediments, nitrogen fixation pathways were more prevalent in intermediate layers, and methanogenesis-related genes were enriched in deeper sediments (Fig. 5F). Although these predictions represent inferred functional potential rather than direct measurements of activity, their consistency across inland sites mirrors the taxonomic stratification observed along sediment depth.

Together, these results show that long-term environmental stability is associated with coherent vertical organization of both microbial taxa and inferred functional potential in inland relict mangrove sediments.

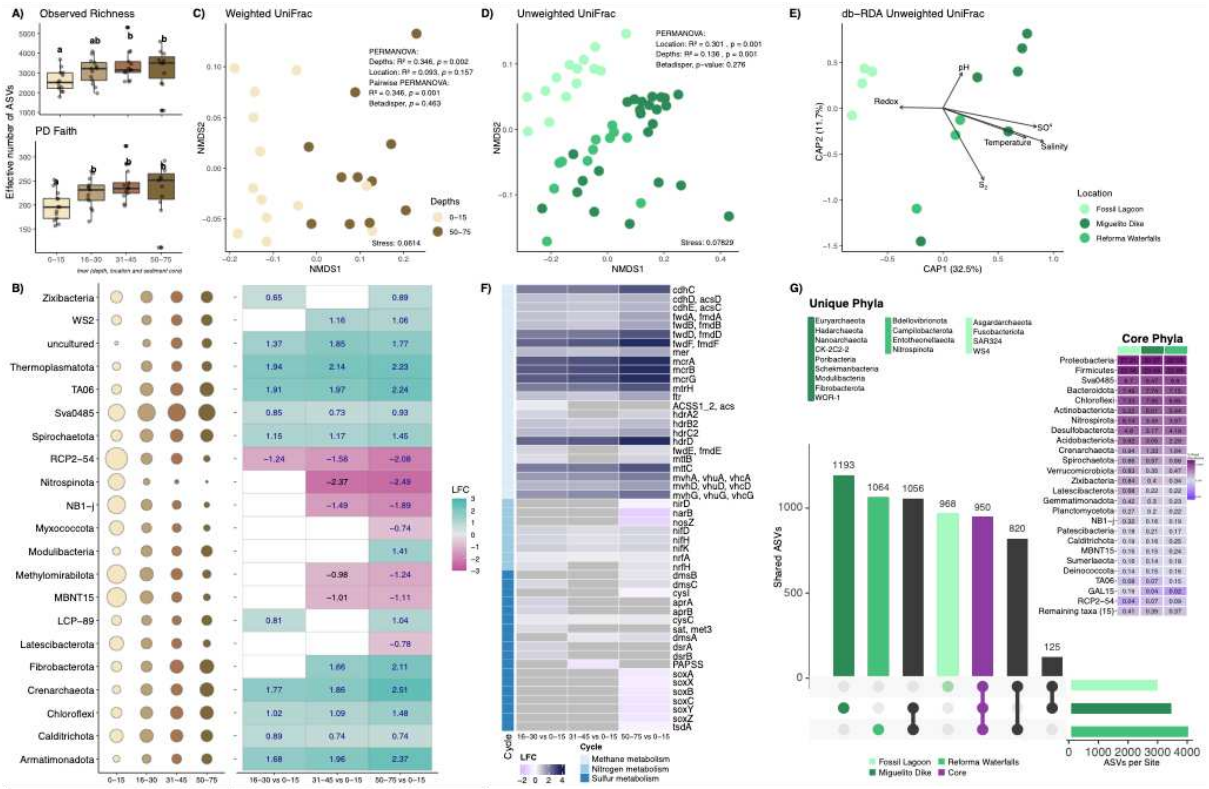


Fig. 5. Vertical taxonomic and functional organization in inland relict mangroves. A) Alpha diversity (q0 and Faith's PD) across sediment depth horizons. Letters indicate significant differences (LLM, $p < 0.05$). B) Differential abundance of microbial phyla across depths (ANCOM-BC2; $p < 0.05$). C) NMDS ordination of weighted UniFrac distances. PERMANOVA (999 permutations) reports variance explained by depth ($R^2 = 0.346$, $p = 0.002$) and location ($R^2 = 0.093$, p = 0.157). D) NMDS ordination of unweighted UniFrac distances showing separation by location ($R^2 = 0.301$, $p = 0.001$) and depth ($R^2 = 0.136$, $p = 0.001$). E) db-RDA of unweighted UniFrac distances of environmental variables. F) Heatmap of differentially abundant inferred functional pathways (ANCOM-BC2; $p < 0.05$) related to methane, nitrogen, and sulfur metabolism. G) UpSet plot of ASV intersections across relic sites. The barplot indicates the total ASVs per site, and the heatmap shows the relative abundance of the resident core phyla.

DISCUSSION

Microbial community assembly in sediments is most often examined in environments dominated by strong physical forcing, frequent disturbance, and high dispersal connectivity [3]. In such systems, hydrological oscillations, salinity fluctuations, and sediment reworking continuously restructure microbial communities, increasing compositional turnover and obscuring persistent organizational patterns [2,10,11]. By focusing on rare inland relict mangroves that have remained geographically and hydrologically isolated since the Last Interglacial (~98 ka) [8], this study leverages a natural experiment to examine how sediment microbial communities organize when environmental variability and dispersal are persistently constrained over geological timescales. In this sense, inland relict mangroves function as biological refugia that preserve modes of sediment microbial organization that have largely disappeared from highly dynamic coastal environments.

Across sites and sediment depths, inland relict mangroves consistently harbored diverse microbial communities characterized by low compositional variability, large resident core

microbiomes, and reproducible vertical structuring. These patterns mirror observations from other environmentally stable sediment systems, including deep subsurface environments [12], stratified lake sediments [13], and undisturbed marine shelves [14], where sustained environmental constancy similarly promotes taxonomic stability, large core assemblages, and predictable depth-structured organization. These features contrasted sharply with natural and hydrologically impaired coastal mangroves, which exhibited greater variability, reduced resident cores, and erosion of depth-associated organization. Our linear models support this observation, with mangrove system identity accounting for a substantial proportion of the variance in alpha diversity (R^2 up to 0.83, $p < 0.001$). These destabilization patterns are consistent with observations from other hydrologically disturbed mangrove systems, where connectivity loss [15], tidal regime alterations [2], and salinity fluctuations [16] similarly disrupt core assemblages and erode vertical stratification. The significantly lower multivariate dispersion observed in inland relicts (distance to centroid = 0.29 vs 0.55 in coastal sites, $p < 0.001$) suggests that under sustained environmental constancy, stable environmental filtering outweighs dispersal driven reassembly. These patterns suggest that long-term environmental stability supports not only the maintenance of microbial diversity but also the emergence of reproducible modes of microbial community organization.

Isolation and reduced dispersal are often assumed to erode microbial diversity by limiting immigration and increasing compositional instability through stochastic processes [3,17]. Our results challenge this assumption. Despite long-term isolation, inland relict mangroves maintained high microbial richness with remarkably low variability among samples, suggesting that under sustained environmental constraints, stable environmental filtering may outweigh dispersal-driven reassembly [18,19]. Rather than reflecting stochastic loss, the persistence of high richness under isolation is consistent with sustained environmental constraints that facilitate niche differentiation and long-term coexistence of specialized sediment lineages, though formal assembly mechanism tests would strengthen this interpretation [18,20]. Large and persistent resident core microbiomes observed in inland relict sediments support this interpretation, with the inland Fossil Lagoon and the San Pedro Mártir River harboring up to 1695 and 1481 core ASVs, respectively. In contrast, the near absence of shared core taxa among coastal systems and the lack of unique resident ASVs in Términos Lagoon underscore how dynamic hydrology and recurrent environmental fluctuations limit the establishment of persistent microbial assemblages [15].

Sediment depth emerged as a central and reproducible axis of microbial organization in inland relict mangroves. Depth-associated taxonomic turnover and redox-driven niche partitioning have been widely documented in undisturbed sediments [9,21,22]. However, in inland relict mangroves, this stratification was highly consistent across sites and accompanied by reduced compositional dispersion, indicating persistent depth-structured communities rather than transient layering repeatedly disrupted by physical reworking. Inland relict sediments maintained or increased microbial richness with depth, contrasting sharply with the depth-associated decline in diversity commonly reported for energy-limited marine sediments [23].

Although functional predictions based on 16S rRNA gene profiles must be interpreted cautiously [24,25], the coherent depth-associated patterns in inferred metabolic potential observed across inland relict sites are consistent. The enrichment of genes associated with oxidative nitrogen transformations in surface sediments, nitrogen fixation in intermediate layers, and methanogenesis-related pathways in deeper horizons aligns with classical thermodynamic expectations for vertically structured sediments [9,26]. These inferred functional patterns across inland sites suggest that long-term environmental stability constrains not only taxonomic

composition, but also the spatial organization of microbial functional potential. Future investigations employing shotgun metagenomics would be valuable to validate these patterns. The decoupling between taxon presence and relative abundance further suggests that long-term stability allows communities to fine-tune abundance distributions along vertical gradients, consistent with thermodynamically structured sediment ecosystems [27,28]. The prominence of temperature as an explanatory variable further underscores the role of thermal stability in groundwater-fed relict systems as a physical constraint that contributes to predictable microbial organization.

These findings position inland relict mangroves as powerful reference systems for understanding how sustained environmental constraints shape the organization of the sediment microbiome. By decoupling long-term stability from the confounding effects of frequent disturbance and dispersal, these systems reveal conditions under which reproducible, depth-structured microbial communities can emerge and persist. More broadly, our results suggest that environmental stability not only preserves microbial diversity but also enables the establishment of predictable modes of microbial community organization across space and depth, providing a framework for interpreting microbial variability in dynamic, restored, and degraded coastal ecosystems. In an era of rapid global change, these relict microbiomes provide a rare baseline of ecological integrity, revealing modes of microbial organization that are increasingly absent from modern coastal systems. As such, they offer a critical reference for assessing the extent of microbial erosion under ongoing environmental disturbance.

Methods

Sampling and experimental design

In this work, we study the microbial diversity associated with *Rhizophora mangle* in inland and coastal mangroves across the Yucatán Platform in Mexico. Sampling locations for inland relict mangroves were selected following the geological and ecological framework established by [8], focusing on the San Pedro Mártir River basin, where these interglacial relict populations persist. To explore the similarities and differences in the microbial community between coastal and inland mangroves, as well as degraded coastal mangroves, public data were taken from sediments isolated in Términos Lagoon (natural coastal core n=13; impaired coastal core n=9) (10.5281/zenodo.14885371) [15], and Celestún Lagoon (n=14) (PRJNA550111) [29] (Fig. 1). These coastal sites were selected as direct contemporary counterparts to the inland relict sites, as both are located on the same geological limestone platform (Yucatán Platform) and share a common evolutionary history during the last interglacial period[8]. Only sediment samples associated with *R. mangle* were included in this analysis to ensure host consistency. To minimize seasonal bias, all samples for cross-system comparisons were restricted to those collected during the flooding season.

The inland relict mangroves are located 170 km from the nearest estuary. Sampling focused on three main localities along the San Pedro Mártir River, following the spatial design described in previous surveys of the area [8]: El Cacahuate (“Fossil Lagoon”, n=14), in the upper part of the river, located near Petén, Guatemala (17°17'59.0" N, 91°05'19.0" W); "Miguelito Dike" (n=24) (17°44'57.03" N, 91° 14.896'O 91°14'51.98" O); and the Reforma Waterfalls (n=12) (17°45'43.42"N, 91°19'30.43"O), in the lower part of the river, in Tenosique, Tabasco (Fig. 1). To ensure independent biological replication, at least two distinct sediment cores were extracted per site, spaced 10-20 m apart. Sediment cores were extracted to a depth of 1 m and stratified into four horizons (0–15, 16–30, 31–45, and 50–75 cm). Samples were transported at 4 °C to the El Carmen station of the Instituto de Ciencias del Mar y Limnología, UNAM, in Ciudad del Carmen, Campeche, and stored at -20 °C until further processing.

Physicochemical parameters
To identify abiotic factors potentially influencing the microbial community structure, interstitial water samples (30 cm depth) were collected at each site using sterile aluminum stakes and surgical syringes. Field measurements included temperature (°C), pH, and redox potential (mV), recorded with a Hanna multiparameter combo tester. Salinity (ppm) was measured using a Hach MP-6 portable meter. Sulfide (S^{2-}, mg/L) and sulfate (SO_4^{2-}, mg/L) concentrations were determined photometrically using Vacu-vials kits (K-9523).

DNA extraction and sample processing
Environmental DNA was extracted from sediment samples using the Fast DNA Spin Kit for Soil (MP Biomedicals, USA) following the manufacturer's instructions. DNA concentration and integrity were assessed using a Nanodrop spectrophotometer and verified by 1% agarose gel electrophoresis stained with GelRed. Amplicons targeting the V3–V4 regions of the 16S rRNA gene were generated using the primers 341F (5′-CCTACGGGNGGCWGCAG-3′) and 805R (5′-GACTACHVGGGTATCTAATCC-3′). Libraries were prepared using the 16S Metagenomic Sequencing Library Preparation protocol for Illumina and indexed using the Nextera XT Index Kit v2 (Illumina). Equimolar pools of libraries were sequenced in a paired-end format on the Illumina MiSeq platform (2 × 300 bp reads) at the Sequencing Unit of the Instituto de Ecología, A.C. (INECOL), México.

Sequence processing, ASV inference, and taxonomic assignment
Raw sequence reads were subjected to quality control using FastQC v0.12.0 [30,] and summary reports were generated using MultiQC v1.12 [31]. Adapter trimming was performed with Cutadapt v4.5 [32], and trimmed reads were imported into R v4.2.0 [33]. To account for distinct error profiles across datasets, error rates were learned independently for each study (Inland, Términos, and Celestún). Denoising and error correction using DADA2 v1.26 [34] performed at 260F–200R sequence truncation according to the best ASVs prediction (Supplementary Tables S1 and S2). Representative ASVs and corresponding frequency tables were imported into QIIME2 v2023.5 [35]. To minimize bias from varying read lengths across datasets, DADA2-inferred ASVs were clustered at 97% identity using VSEARCH within QIIME2 prior to diversity analyses.

Taxonomic classification was performed using the classify-sklearn plugin in QIIME2, based on a naive Bayes model [35], with the SILVA 138 16S rRNA reference database. ASVs identified as eukaryotic, mitochondrial, chloroplast, or present at low abundance (<0.1% of mean sample depth) were excluded [36]. A phylogenetic tree was constructed using MAFFT [37] for sequence alignment and IQ-TREE2 [38] for maximum-likelihood analysis.

Diversity analyses
The ASV frequency table, representative sequences, phylogenetic tree, and metadata table were imported as a phyloseq object using Phyloseq v1.42.0[39]. Alpha diversity metrics, including observed richness (q0), Shannon diversity (q1), Simpson evenness (q2), and Faith's phylogenetic diversity, were calculated using hilldiv v1.5.1 [40].
To evaluate microbial community structure, beta diversity was assessed using Non-metric Multidimensional Scaling (NMDS) based on Bray–Curtis dissimilarity (for interior vs. coastal comparisons) and UniFrac distances (for interior sites). Ordination quality was confirmed via stress values. Statistical significance and homogeneity of multivariate dispersions were assessed using PERMANOVA and betadisper/permutest, respectively, with 999 permutations. Dispersion was quantified as the average distance of individual samples to their group centroids

to serve as a proxy for community cohesion. A Post-hoc pairwise PERMANOVA was also performed. All analyses were conducted using the vegan package v2.6.4 in R [41].

Statistical Modeling of Microbial Diversity

To compare microbial diversity across systems while accounting for variations in sequencing depth and unbalanced sample sizes (n=50 inland vs. n=36 coastal, Fig. 1), we employed linear models (lm) using stats v3.6.2 of R [33] and linear mixed effects models (LMMs) using nlme v3.1-164 [42] instead of utilizing data rarefaction. Diversity metrics (Richness q0, Shannon q1) were modeled as response variables. The fixed effects included the mangrove system and sediment depth. Sequencing depth (total reads per sample) was included as a continuous covariate in the models to statistically control for technical variation in library size. To address the potential for pseudo-replication and account for the hierarchical structure of the data (repeated measures within the same vertical profile), sediment core ID was incorporated as a random effect in models. Model fit was assessed via residual analysis, and significance was determined using ANOVA followed by post hoc Tukey tests, using the nlme v3.1-164 [42] and emmeans [43] packages in R.

Differential abundance

To identify differentially abundant microbes, we applied a microbiome compositional analysis with bias correction (ANCOM-BC2) [44]. This method fits linear mixed models to log-transformed abundances to account for sampling bias. The model included sediment depth and site location as fixed effects, and core identity as a random factor, with Benjamini-Hochberg *p*-value correction.

Core microbiome

Core microbiomes, defined as ASVs present in at least 50% of the samples within each group, were identified. The overlap and uniqueness of core ASVs were visualized using ComplexUpset v1.4.0 [45]. We use the ampvis2 package [46] to illustrate the relative abundance of the core microbiome.

Data availability

Raw sequencing data are available under NCBI Bioproject accession ID PRJNA1243457 (https://dataview.ncbi.nlm.nih.gov/object/PRJNA1243457?reviewer=jqgl2edahnderedhj9guf0e807)

Code availability

Bioinformatic workflows, processed results, and output files from the analytical steps are available in the GitHub repository: https://dianaoaxaca.github.io/InteriorMangroves/

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Acknowledgements
We thank Josefina for her careful measurement of physicochemical parameters in the field and laboratory. We are grateful to Arturo Zaldívar and Julio César Canales for their dedicated assistance during sediment sampling along the San Pedro Mártir River. Their field support was essential to the successful completion of this study. We also acknowledge the technical staff of the Instituto de Ciencias del Mar y Limnología (UNAM) and the El Carmen Station for field and laboratory support.

Founding
This study was supported by grants from the Universidad Nacional Autónoma de México (UNAM) (PAPIIT IA201824) and SECIHTI (CBF2023-2024-1251). FJBO was supported by SECIHTI (Postdoctoral fellowship 2025-2027, EPM-474433).

Ethics declarations

The authors declare no conflict of interest.

Supplementary information

AUTHOR CONTRIBUTIONS

Credit Classification
Conceptualization: VRLM.
Data Curation: HOD, LTY, PCR, BC, and VRLM.
Formal Analysis: HOD, LTY, VRLM.
Funding Acquisition: VRLM, ILE, BC, PCR.
Investigation: VRLM, ILE, OMM, YLT.
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Supervision: VRLM.
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Writing – Original Draft Preparation: DHO, YLT, FBO, and VRLM.
Writing – Review & Editing: VRLM, HOD, FBO, ILE, BC, PCR, LTY.

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