

Centimetre-scale geochemical stratification reorganizes microbial taxonomic and functional profiles in cold-seep sediments

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Abstract

Purpose

Cold-seep sediments contain steep geochemical gradients that can reorganize microbial communities over short vertical distances, yet centimetre-scale coupling between sediment chemistry, microbial taxonomy and functional potential remains poorly resolved. We tested whether fine-scale geochemical stratification is associated with concordant or divergent taxonomic, functional and network responses along a cold-seep sediment profile.

Methods

A 1-m sediment core from the South China Sea was continuously sectioned into 50 samples at 2-cm intervals, integrating sediment geochemistry with shallow shotgun metagenomic sequencing.

Results

Pronounced geochemical stratification coincided with marked microbial turnover. Archaea, including ANME-related lineages, were enriched in upper sediments, whereas Bacteria were enriched in lower sediments. Taxonomic richness decreased significantly with depth, while detected KEGG Orthology (KO) richness increased. Both community and KO compositions tracked the geochemical gradient, transitioning from Ag-, TC- and Sr-associated conditions in upper sediments towards TP-, TS- and Si-associated conditions at depth. Macronutrients explained the largest fraction of variation in KO composition (12%) and coincided with substantial reorganization of C, P and S cycling potential. Network complexity was greater in deeper sediments than in upper sediments, while negative associations and the number of topologically important taxa increased with depth.

Conclusion

Centimetre-scale geochemical stratification was associated with coordinated but non-parallel changes in microbial taxonomy, functional potential and network organization. The opposing trajectories of taxonomic and KO richness indicate that declining taxonomic richness with burial does not necessarily coincide with reduced functional breadth, highlighting the value of high-resolution profiling for resolving microbial-geochemical coupling in cold-seep sediments.

1 Introduction

Cold seeps are highly heterogeneous deep-sea ecosystems sustained by the upward migration of methane and other reduced compounds from subsurface sediments. These reduced substrates support dense chemosynthetic microbial communities and drive tightly coupled biogeochemical transformations

at the sediment-water interface and within the sediment column (Feng et al., 2018; Joye, 2020). Among these processes, sulfate-dependent anaerobic oxidation of methane (AOM), mediated principally by anaerobic methanotrophic archaea (ANME) together with sulfate-reducing bacteria, represents an important biological filter restricting methane transfer from marine sediments to the hydrosphere and atmosphere (Knittel and Boetius, 2009; Ruff et al., 2015). Cold-seep sediments also harbor diverse microorganisms involved in organic-carbon (C) degradation, sulfur (S) oxidation and reduction, and other nutrient transformations (Lyu et al., 2023; Zhong et al., 2025). Because electron donors and acceptors are consumed and regenerated over short spatial distances, cold-seep sediments often contain steep redox and geochemical gradients, creating closely juxtaposed microbial niches (Ruff et al., 2015). Understanding microbial organization along these gradients is therefore essential for resolving how cold-seep microbiomes regulate elemental cycling.

Substantial spatial heterogeneity in cold-seep microbiomes has already been demonstrated, including among geographically separated seeps, contrasting habitats within individual seep systems, and different sediment depths (Ruff et al., 2015; Niu et al., 2017, 2023; Zhai et al., 2022). In the South China Sea, methane, sulfate, sulfide and inorganic carbon have repeatedly been associated with shifts in microbial diversity and community composition, while ANME, sulfate-reducing bacteria and sulfur-oxidizing bacteria show strong habitat- and depth preferences (Sun et al., 2022; Deng et al., 2023). Nevertheless, most existing work has resolved this heterogeneity at relatively coarse spatial scales. Studies of the Formosa and Haima cold seeps have relied largely on 16S rRNA gene sequencing and comparisons among discrete habitats or selected sediment horizons (Sun et al., 2022; Wu et al., 2025). Even a recent vertical investigation of Haima cold-seep sediments used 21 stratified samples across a 2.9-m profile, with sampling intervals increasing from 10 to 20 cm with depth (Zhong et al., 2025). Such designs are valuable for identifying broad vertical zonation, but may overlook microbial transitions occurring over only a few centimetres. This represents an important limitation because oxygen penetration, methane supply, sulfate consumption, mineral transformations and organic-matter availability can vary sharply within centimetre-scale sediment horizons (Joye et al., 2020). A continuously sampled high-resolution profile is therefore needed to determine whether microbial communities change gradually with burial or are reorganized over much finer vertical scales.

A second limitation is that taxonomic patterns alone provide an incomplete representation of microbiome responses to environmental gradients. Microbial taxonomy and function are not necessarily coupled because phylogenetically distinct microorganisms may share metabolic capabilities, while genome-content variation, horizontal gene transfer and ecological adaptation can generate considerable functional differentiation within related taxa (Louca et al., 2016, 2018). Accordingly, taxonomic, KEGG Orthology (KO) and gene profiles can exhibit different biogeographic patterns and environmental sensitivities in marine microbiomes (Louca et al., 2016; Dlugosch et al., 2022). Dlugosch et al. (2022), for example, demonstrated that taxonomic and functional profiles of the Atlantic Ocean microbiome differed in both turnover and environmental controls, emphasizing that functional traits must be considered explicitly when interpreting microbial biogeography. This issue is particularly relevant to cold-seep sediments, where most South China Sea studies have focused on microbial diversity, community

composition or predicted C- and S-cycling functions derived from marker-gene datasets (Zhong et al., 2025). Whether fine-scale burial is accompanied by parallel changes in taxonomic richness and directly detected functional richness therefore remains unclear. Furthermore, comparatively little attention has been given to the simultaneous vertical reorganization of multiple elemental cycles, particularly C, phosphorus (P) and S, despite their potential coupling through microbial nutrient acquisition and energy metabolism.

Beyond composition and functional potential, microbial communities are structured by associations among taxa that can also change with environmental conditions. Co-occurrence network analyses have revealed that methane supply and habitat heterogeneity can reorganize microbial connectivity and potential coexistence patterns in South China Sea cold seeps (Niu et al., 2023; Wu et al., 2025). Keystone taxa occupying highly connected network positions may have disproportionate importance for microbiome organization and ecosystem functioning (Banerjee et al., 2018). However, existing cold-seep network studies have primarily compared horizontal habitats or a limited number of sediment depths; for example, recent vertical work characterized networks at only three representative horizons (Zhong et al., 2025). Consequently, it remains unresolved whether progressive taxonomic impoverishment with sediment burial is accompanied by simpler microbial association networks, or whether increasingly selective subsurface conditions instead promote tighter or more differentiated microbial associations and turnover of keystone taxa.

To address these knowledge gaps, we analyzed a 1-m cold-seep sediment core from the South China Sea that was continuously sectioned at 2-cm resolution into 50 horizons, integrating sediment geochemistry with shallow shotgun metagenomic profiling. We hypothesized that fine-scale geochemical stratification would produce pronounced microbial niche differentiation, but that taxonomic and functional responses would not necessarily vary in parallel. Specifically, we aimed to: (i) resolve centimetre-scale changes in sediment geochemistry and microbial taxonomic and functional composition; (ii) determine whether taxonomic richness and KO functional richness exhibit concordant vertical patterns and identify the geochemical factors associated with their differentiation; and (iii) determine how microbial co-occurrence networks and keystone taxa are reorganized across the sediment profile. By simultaneously resolving environmental, taxonomic, functional and network dimensions at centimetre-scale resolution, this study provides a framework for understanding how cold-seep microbiomes are reorganized during sediment burial.

2 Materials and Methods

2.1 Collection of sediment samples and site description

Deep-sea sediment cores were recovered from the Haima cold seeps region on the northwestern slope of the South China Sea (16°54'N, 110°28'E). Based on the spatial heterogeneity of the cold seep habitats, this specific location is identified as a weak methane seepage area. Unlike the central active seepage zones dominated by microbial mats and strong fluid bubbling, this peripheral habitat is characterized by

the colonization of characteristic deep-sea chemosynthetic macrofauna. Extensive observations in similar weak seepage zones within the Haima seeps indicate that such benthic communities are typically dominated by abundant mussels, clams, and sea anemones (Niu et al., 2023; Wu et al., 2025).

To accurately capture the microscale vertical stratification of benthic microbial communities, a 1-m long sediment core was retrieved utilizing a remotely operated vehicle (ROV) equipped with a push corer. Upon recovery onto the research vessel deck, the intact sediment core was immediately sealed and stored in the dark at 4°C to preserve its structural integrity and minimize the degradation of indigenous microbial communities. Following transportation back to the onshore laboratory, the core was carefully extruded and vertically sectioned at strict 2-cm intervals within an anaerobic glove box, yielding a continuous, high-resolution profile comprising 50 discrete subsamples. Each subsample was thoroughly homogenized and aseptically partitioned: one aliquot was rapidly flash-frozen and stored at – 80°C for subsequent environmental DNA extraction and metagenomic sequencing, while the remaining aliquot was lyophilized for comprehensive solid-phase geochemical characterizations.

2.2 Geochemical analysis

A total of 24 solid-phase geochemical indicators were quantified for each of the 50 sediment subsamples. These parameters were classified into five distinct functional categories: 1) macronutrients including total carbon (TC), total nitrogen (TN), total phosphorus (TP) and total sulfur (TS). 2) metabolic metals including nickel (Ni), zinc (Zn), copper (Cu), vanadium (V), chromium (Cr), manganese (Mn), iron (Fe), sodium (Na), magnesium (Mg), potassium (K), calcium (Ca), which encompass metals vital for microbial life (Morel and Price, 2003; Glass and Orphan, 2012). 3) biogenic Indicators including strontium (Sr) and barium (Ba), which are highly reliable proxies for biological productivity and biogenic sedimentation (Tribovillard et al., 2006; Paytan and Griffith, 2007). 4) terrigenous Indicators including aluminum (Al), silicon (Si), and titanium (Ti), which are highly conservative, refractory elements comprising aluminosilicate minerals (Tribovillard et al., 2006; Calvert and Pedersen, 2007). 5) toxic trace elements including silver (Ag), thallium (Tl), lithium (Li) and boron (B), of which these specific elements exert severe ecotoxicological stress on benthic communities with increasing concentrations (Aral and Vecchio, 2008; Luoma and Rainbow, 2008).

Prior to geochemical determination, all sediment aliquots were lyophilized (freeze-dried) for 48 hours, pulverized thoroughly in a pre-cleaned agate mortar, and passed through a 200-mesh stainless steel sieve to ensure complete homogenization.

For the determination of TN, TS, and TC, the powdered samples were analyzed directly utilizing an automated CHNS elemental analyzer (Vario EL cube, Elementar, Germany). To quantify the concentrations of TP and the remaining 20 major, trace, and toxic elements, a standardized multi-acid digestion protocol was applied. Approximately 50 mg of the sieved sediment was digested in closed Teflon vessels using a concentrated, analytical-grade acid matrix (HNO₃-HF-HClO₄) on a heating block until total dissolution was achieved. The digests were evaporated to incipient dryness and reconstituted in 2% (v/v) HNO₃. High-abundance major elements and structural indicators (Na, Mg, K, Ca, Fe, Al, Si, Ti,

P) were measured using Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES). Trace, ultra-trace, and toxic elements (Ni, Zn, Cu, V, Cr, Mn, Sr, Ba, Tl, Li, Ag, B) were precisely quantified via Inductively Coupled Plasma Mass Spectrometry (ICP-MS).

2.3 DNA extraction, shallow metagenomic sequencing, and bioinformatic analysis

Total community DNA was extracted from freeze-dried sediment subsamples using the FastDNA™ SPIN Kit for Soil (MP Biomedicals, Solon, OH, USA) according to the manufacturer's instructions. DNA concentration and purity were assessed using a NanoDrop 2000 spectrophotometer and a Qubit 3.0 fluorometer (Thermo Fisher Scientific, Waltham, MA, USA). Metagenomic libraries with an average insert size of approximately 350 bp were constructed and sequenced on the Illumina NovaSeq 6000 platform to generate 150-bp paired-end reads. To achieve a balance between taxonomic and functional resolution and sequencing cost across the 50 high-resolution sediment intervals, shotgun metagenomic sequencing was standardized to approximately 1 Gb of clean data per sample. Recent benchmarking studies have demonstrated that shallow shotgun metagenomic sequencing provides robust species-level taxonomic profiling and reliable pathway-level functional characterization in well-characterized microbial communities (Xu et al., 2021; Tremblay et al., 2022; Treichel et al., 2026), although it is less suitable for de novo genome reconstruction, strain-level analyses, or exhaustive recovery of low-abundance functional genes. Accordingly, the sequencing strategy adopted here was designed to provide sufficient resolution for investigating depth-dependent changes in microbial community composition and major biogeochemical functions across the cold seep sediment core while maintaining cost-efficiency for high-resolution vertical sampling.

Following quality control to remove adapter sequences, low-quality bases and potential contaminants, high-quality reads were used for downstream taxonomic and functional analyses. Taxonomic profiling was performed using MetaPhlAn4, which estimates the relative abundance of microbial taxa by mapping sequencing reads to a curated database of clade-specific marker genes rather than to complete reference genomes, thereby minimizing false taxonomic assignments while enabling accurate species-level profiling in complex microbial communities. Functional annotation was subsequently performed by assigning predicted gene families to Kyoto Encyclopedia of Genes and Genomes (KEGG) Orthology (KO) identifiers, which were further reconstructed into metabolic pathways associated with C, P, S and other biogeochemical cycles. This analytical framework provides a robust basis for characterizing depth-dependent functional differentiation of microbial communities within the cold seep sediment column while remaining consistent with the current methodological recommendations for shallow shotgun metagenomics (Treichel et al., 2026).

2.4 Statistical analysis

All statistical analyses were performed in R software (v 4.1.1). Linear regression was used to assess depth-dependent changes in sediment geochemical variables and the relative abundances of functional genes or pathways, with P values adjusted for multiple comparisons using the Benjamini-Hochberg false

discovery rate (FDR) procedure. Non-metric multidimensional scaling (NMDS) based on Bray-Curtis dissimilarities was performed using the *vegan* package to visualize depth-related variation in microbial community compositions. Permutational multivariate analysis of variance (PERMANOVA) was subsequently conducted to evaluate the effects of sediment depth on community composition, using the 'adonis2' function in *vegan* package (Anderson, 2001).

To identify microbial taxa characterizing different sediment depths, the 50 sediment samples were divided into three approximately equal depth sections, hereafter referred to as the upper, middle and lower layers. Linear discriminant analysis effect size (LEfSe) was performed using the *microeco* package, and taxa with linear discriminant analysis (LDA) scores > 4 were considered depth-associated biomarkers (Segata et al., 2011; Liu et al., 2021). Redundancy analysis (RDA) was conducted using the *vegan* package to assess relationships of geochemical variables with microbial community and KO functional compositions. Prior to RDA, both microbial community and KO abundance matrices were Hellinger-transformed (Legendre and Gallagher, 2001). Multicollinearity among environmental variables was assessed using variance inflation factors (VIF), and variables with VIF > 10 were sequentially excluded. The significance of individual environmental variables was evaluated by Monte Carlo permutation tests. Variance partitioning analysis (VPA) was further performed using the *rdacca.hp* package to quantify the relative contributions of five groups of geochemical variables (Lai et al., 2022), including macronutrients, metabolic metals, biogenic indicators, terrigenous indicators and toxic trace elements, to microbial community and KO functional compositions.

Microbial co-occurrence networks were separately constructed for three different sediment layers using the SPIEC-EASI method implemented in the *SpiecEasi* package, which infers sparse microbial association networks while accounting for the compositional nature of sequencing data (Kurtz et al., 2015). Only species with the relative abundances $\geq 0.1\%$ were selected for network analysis. Network topological properties were calculated using the *igraph* package. The topological roles of individual taxa were further characterized according to within-module connectivity (Z_i) and among-module connectivity (P_i), with taxa classified as peripherals ($Z_i \leq 2.5$, $P_i \leq 0.62$), connectors ($Z_i \leq 2.5$, $P_i > 0.62$), module hubs ($Z_i > 2.5$, $P_i \leq 0.62$), or network hubs ($Z_i > 2.5$, $P_i > 0.62$). Connectors, module hubs and network hubs were considered keystone taxa (Banerjee et al., 2016). Venn analysis was then used to compare shared and unique keystone taxa among the three depth layers, using *venn* package.

3 Results

3.1 Depth-dependent variations in sediment geochemical characteristics

Sediment geochemical characteristics exhibited pronounced depth-dependent patterns (Fig. 1). Among the macronutrients, TS and TP increased significantly with sediment depth, whereas TC showed a significant decreasing trend; no significant relationship was detected for TN. Most metabolic metals, including Ni, Zn, Cu, V, Cr, Mn, Fe, Mg and K, increased significantly with depth, whereas Na and Ca

showed no significant trends. Among the biogenic indicators, Ba increased significantly with depth, while Sr remained relatively unchanged. All three terrigenous indicators (Si, Ti and Al) exhibited significant positive relationships with depth. For toxic trace elements, Li and B increased significantly, whereas Ag decreased significantly and TI showed no significant depth-related variation (Fig. 1). Overall, 19 of the 24 measured geochemical variables exhibited significant depth-dependent trends, indicating marked geochemical stratification throughout the sediment profile.

3.2 Vertical variations in microbial taxonomic and functional profiles

Metagenomic profiling revealed pronounced changes in microbial community composition along the sediment profile (Fig. 2). Bacteria dominated the microbial communities throughout the core, whereas Archaea accounted for a larger proportion in the upper sediments and generally declined with increasing depth; Fungi represented a relatively minor component (Fig. 2A). At the phylum level, bacterial communities were predominantly composed of Pseudomonadota, together with Thermodesulfobacteriota, Bacteroidota, Bacillota and Actinomycetota, whereas archaeal and fungal communities were dominated by Euryarchaeota and Ascomycota, respectively. NMDS plots further revealed significant depth-dependent shifts in overall microbial community composition (PERMANOVA, $R^2 = 0.44$, $p < 0.001$), as well as in bacterial ($R^2 = 0.40$, $p < 0.001$), archaeal ($R^2 = 0.45$, $p < 0.001$) and fungal communities ($R^2 = 0.20$, $p < 0.001$; Fig. 2B).

Consistent with these compositional shifts, LEfSe analysis identified distinct microbial biomarkers among the upper, middle and lower sediment layers (LDA > 4; Fig. 3A). Archaea were significantly enriched in the upper sediments, as indicated by k__Archaea and several affiliated lineages, including Euryarchaeota, Methanomicrobia, Methanosarcinales and ANME-2c, whereas Bacteria were significantly enriched in the lower sediments, together with Pseudomonadota, Gammaproteobacteria, Thiotrichales, Hydrogenovibrio and Kangiellaceae-related taxa. The middle sediments were primarily characterized by Bacteroidota-associated and Marinobacteraceae-related taxa. Meanwhile, taxonomic-profile richness decreased significantly with sediment depth ($r = -0.58$, $p < 0.001$), whereas KO-profile richness showed the opposite pattern and increased significantly with depth ($r = 0.39$, $p = 0.006$; Fig. 3B-C).

3.3 Geochemical associations with microbial communities, functions, and elemental cycling

Sediment geochemical characteristics were closely associated with the depth-dependent differentiation of both microbial community and KO functional composition (Fig. 4A-B). The upper sediments were primarily associated with Ag, TC and Sr, whereas deeper sediments were more strongly associated with TP, TS and Si, indicating a clear shift in the geochemical conditions accompanying changes in microbial taxonomic and functional composition. Monte Carlo permutation tests further confirmed significant effects of most of these variables. Variation partitioning analysis showed that the five groups of

geochemical variables collectively explained 44.8% and 41.2% of the variation in microbial community and KO functional composition, respectively, with macronutrients contributing the largest proportion (Fig. 4C-D).

Consistent with these geochemical-functional associations, genes involved in C, P and S cycling exhibited pronounced depth-dependent changes (Fig. 5). Overall, a substantial proportion of C-, P- and S-cycling functions became more abundant toward deeper sediments, although individual pathways showed contrasting responses. For C cycling, functions associated with the Calvin-Benson-Bassham cycle, glycolysis and several transformations involving pyruvate, acetate and acetyl-CoA generally increased with depth, whereas the reductive TCA cycle, Wood-Ljungdahl pathway and several methane-related transformations tended to decrease (Fig. 5A). P cycling showed particularly widespread increases at greater depths, involving genes related to organic P mineralization, inorganic P solubilization, phosphate transport and polyphosphate turnover (Fig. 5B). S cycling likewise exhibited marked vertical differentiation, with several sulfate-reduction and thiosulfate-oxidation functions changing significantly along the sediment profile (Fig. 5C).

3.4 Depth-dependent shifts in microbial co-occurrence patterns

Microbial co-occurrence networks exhibited substantial changes in complexity and association patterns among sediment layers (Fig. 6A). The upper-layer network contained 562 nodes and 3,010 edges, of which 86% were positive and 14% were negative. Network complexity increased markedly in the middle layer, reaching 757 nodes and 26,027 edges, before declining in the lower layer to 748 nodes and 15,511 edges. Nevertheless, the lower-layer network remained considerably more connected than that of the upper layer. Meanwhile, the proportion of positive associations progressively decreased from 86% in the upper layer to 76% in the middle layer and 64% in the lower layer, whereas negative associations increased correspondingly from 14% to 24% and 36%, respectively (Fig. 6A). Zi-Pi analysis identified 15, 42 and 45 keystone taxa in the upper, middle and lower sediments, respectively, mainly comprising module hubs and connectors (Fig. 6B; Table S1). Keystone composition showed pronounced turnover among depths, with only one taxon, *Desulfatiglandales* bacterium, shared across all three sediment layers (Fig. 7A). Its relative abundance decreased significantly with increasing depth ($r = -0.86$, $p < 0.001$; Fig. 7B), but was positively correlated with overall microbial taxonomic richness ($r = 0.73$, $p < 0.001$; Fig. 7C).

4 Discussion

Cold-seep sediments are characterized by steep gradients in electron donors and acceptors, mineral phases and organic substrates, which generate vertically compressed microbial niches (Suess, 2014; Ruff et al., 2015). Previous studies in the South China Sea have demonstrated substantial microbial differentiation among seep habitats and sediment depths, with methane, sulfate, sulfide and inorganic carbon emerging as important environmental correlates of community structure (Sun et al., 2022; Wu et

al., 2025; Zhong et al., 2025). However, the 2-cm resolution used here reveals that this environmental heterogeneity is accompanied not only by taxonomic turnover, but also by contrasting patterns of functional richness, C-P-S metabolism and microbial association networks. These coordinated responses suggest that centimetre-scale geochemical stratification is an important dimension of microbial niche organization within cold-seep sediments.

4.1 Fine-scale geochemical stratification is accompanied by pronounced microbial turnover

The strong depth dependence of sediment chemistry provides an environmental context for the observed microbial differentiation. In particular, upper sediments were more closely associated with Ag, TC and Sr, whereas TP, TS and Si were increasingly associated with deeper sediments (Fig. 4). The corresponding RDA patterns for taxonomic and KO functional composition were remarkably similar, suggesting that the vertical geochemical transition was reflected at both levels of microbiome organization. The importance of environmental filtering in cold-seep microbiomes is well established (Sun et al., 2022; Deng et al., 2023). Globally distributed methane seeps harbor characteristic microbial assemblages, yet the relative abundance of major functional groups varies considerably with local environmental conditions and sediment depth (Ruff et al., 2015). Likewise, studies of the Formosa and Haima cold seeps in the South China Sea have identified methane, sulfate, sulfide, dissolved inorganic carbon and other geochemical properties as major correlates of microbial distribution (Sun et al., 2022; Wu et al., 2025; Zhong et al., 2025). Nevertheless, individual RDA vectors should not automatically be interpreted as direct physiological controls. In particular, Ag, Sr and Si can also record mineralogical, lithogenic or diagenetic changes and may therefore act partly as indicators of broader sediment conditions (Paytan and Griffith, 2007; Aral and Vecchio, 2008). Their strong associations with microbial profiles may reflect correlated environmental gradients rather than direct selection by the elements themselves. The more important ecological result is consequently the coordinated transition between distinct upper- and lower-sediment geochemical regimes, which was accompanied by substantial microbial turnover.

This fine-scale geochemical differentiation coincided with a clear taxonomic transition (Fig. 3A). Archaea, including Euryarchaeota, Methanomicrobia, Methanosarcinales and ANME-2c-related taxa, were preferentially enriched in the upper part of the profile, whereas Bacteria and several Pseudomonadota/Gammaproteobacteria-related lineages were enriched in deeper sediments. ANME and sulfate-reducing bacteria are characteristic components of methane-seep sediments because sulfate-dependent anaerobic oxidation of methane constitutes one of the major energy-yielding processes in these ecosystems (Knittel and Boetius, 2009; Egger et al., 2018). Previous South China Sea studies similarly found pronounced depth- or habitat-specific distributions of ANME and sulfur-metabolizing bacteria (Sun et al., 2022). Thus, the vertical replacement observed here likely reflects changing energetic and substrate niches along the sediment profile rather than sediment depth per se.

4.2 Declining taxonomic richness is accompanied by increasing functional richness

One of the most notable findings was the contrasting response of taxonomic and KO richness to sediment depth (Fig. 3B). Taxonomic richness decreased significantly, consistent with the general tendency for energy availability, organic-matter reactivity and microbial growth rates to decline with burial in marine sediments (Jørgensen and Marshall, 2016). A recent vertical investigation of Haima cold-seep sediments likewise reported declining microbial α -diversity with increasing depth (Zhong et al., 2015). In contrast, however, detected KO richness increased significantly across our 1-m profile. Thus, progressive taxonomic filtering did not translate into a corresponding loss of functional potential. Indeed, taxonomic and functional diversity need not respond in parallel. Functional redundancy permits phylogenetically different microorganisms to carry similar metabolic capabilities, whereas horizontal gene transfer, genome-content variation and niche-specific gene retention can generate functional variation that is not predicted directly from taxonomy (Louca et al., 2016). Global ocean metagenomics has demonstrated that taxonomic, KO and gene profiles can exhibit distinct biogeographic patterns and respond differently to environmental conditions (Dlugosch et al., 2022). The contrast observed here therefore suggests that deeper sediments may select a smaller set of taxa carrying a comparatively broad repertoire of detectable metabolic functions.

The increasing predominance of Bacteria toward deeper sediments may contribute to this pattern, particularly because several bacterial groups inhabiting marine subsurface sediments possess diverse capacities for organic-matter transformation and anaerobic metabolism (Hoshino et al., 2020; Dong et al., 2023). However, the increase in KO richness should not simply be attributed to a greater abundance of Bacteria. Functional redundancy among taxa, genome-level variation and differences in reference-database representation can all contribute to the observed pattern (Louca et al., 2016, 2018). More importantly, KO richness describes the breadth of detected functional categories, rather than microbial activity or realized biogeochemical rates. This pattern is particularly interesting when considered across spatial scales. A recent global analysis of P-cycling genes in cold-seep sediments found strong depth stratification but an overall decline in P-functional richness from surface (< 1 m) to deep (> 10 m) sediments (Zhang et al., 2025). Our results describe a much narrower 0–1 m interval at 2-cm resolution and show the opposite trend for the whole KO repertoire. Together, these observations suggest that functional diversity may not change monotonically with sediment burial: fine-scale diversification within the upper metre may precede the eventual loss of metabolic capacity characteristic of the deeper energy-limited biosphere.

4.3 Macronutrient gradients are linked to the reorganization of C, P and S cycling potential

The VPA results provide additional support for a close coupling between geochemical stratification and microbial function (Fig. 4C-4D). Among the five geochemical groups examined, macronutrients accounted for the largest individual fraction of variation in both microbial community and KO functional

composition. This result is consistent with the strong RDA associations of C-, P- and S-related variables and provides a mechanistic context for the marked vertical differentiation of C, P and S cycling genes. C and S metabolisms are intrinsically coupled in cold-seep sediments (Zhong et al., 2025). Upward methane flux supplies reducing power, while downward sulfate penetration supports sulfate-dependent AOM, which acts as an important microbial filter limiting methane escape from marine sediments (Knittel and Boetius, 2009; Egger et al., 2018). Our data revealed depth-dependent changes not only in methane-related functions, but also in C fixation and central C transformations, with several functions associated with the Calvin-Benson-Bassham cycle, glycolysis and pyruvate/acetate metabolism becoming more abundant at depth (Fig. 5). S pathways likewise showed substantial vertical differentiation. Rather than indicating uniformly greater C or S cycling at depth, these results imply reorganization among alternative metabolic strategies as substrate availability and geochemical conditions change.

The pronounced restructuring of P metabolism is particularly noteworthy because microbial P cycling has historically received less attention in cold-seep research than methane and S metabolism. Marine microorganisms regulate P acquisition, mineralization, transport and storage through diverse metabolic pathways (Karl, 2014), and recent work has revealed unexpectedly extensive P-cycling potential in global deep-sea cold-seep microbiomes (Zhang et al., 2025). That study identified widespread genes involved in organic-P mineralization, inorganic-P solubilization, phosphate transport and PhoR–PhoB regulation and demonstrated that p-cycling genes are strongly depth-stratified in cold-seep sediments (Zhang et al., 2025). Our observation that TP increased with depth while numerous genes related to P mobilization, transport and polyphosphate turnover also increased therefore adds a fine-scale dimension to this emerging picture. Importantly, increasing TP does not necessarily imply increasing biological P availability. Total sedimentary P encompasses mineral-bound, organic and other pools with widely differing accessibility, microbial investment in phosphatases, transport systems or P-solubilization pathways can therefore remain important even in P-rich sediments (Karl et al., 2014). The close association between P gradients and KO composition in our study suggests that P availability and transformation may represent an underappreciated component of cold-seep microbial niche differentiation.

4.4 Increasing network complexity is accompanied by strong turnover of keystone taxa

Microbial association networks also underwent pronounced reorganization with sediment depth (Fig. 6A). Although taxonomic richness declined, the number of network connections increased strongly from the upper to middle sediments and remained relatively high in the lower sediments. At the same time, negative associations increased from 14% in the upper layer to 36% in the lower layer. Previous work at the Haima cold seep similarly demonstrated that environmental heterogeneity can generate distinct microbial coexistence networks and that network architecture varies with methane-related geochemical conditions (Wu et al., 2025). Zhong et al. (2025) also found particularly high microbial network complexity in deep cold-seep sediments. The increasing prevalence of negative associations

may indicate stronger niche differentiation, resource partitioning or mutually exclusive environmental preferences as sediment conditions become more restrictive (Koffel et al., 2021; Fang et al., 2024). It should not, however, be equated directly with biological competition. Even compositionally robust approaches such as SPIEC-EASI infer conditional statistical associations rather than experimentally demonstrated interactions (Kurtz et al., 2015). Thus, the network results are best interpreted as evidence for increasingly differentiated community association structure with depth.

A particularly striking result was the strong turnover of topologically important taxa. The number of keystone taxa increased from 15 in the upper layer to 42 and 45 in the middle and lower layers (Fig. 6B), respectively, yet only *Desulfatiglandales bacterium* was retained across all three depth zones (Fig. 7A). Such turnover suggests that different geochemical niches are supported by different structural components of the microbial network, consistent with the concept that keystone taxa can influence microbiome organization despite not necessarily being the most abundant community members (Banerjee et al., 2018). Keystone taxa and their specialized metabolic traits have also been linked to microbiome stability and ecosystem functioning in experimental microbial systems (Wagg et al., 2019; Xun et al., 2021). The persistence of *Desulfatiglandales bacterium* is intriguing in a cold-seep context. Members of *Desulfatiglandales* occur widely in anoxic marine sediments, and genome-resolved studies have demonstrated capacities for sulfate-associated metabolism and anaerobic degradation of aromatic hydrocarbons in at least some lineages (Zehnle et al., 2023). Nevertheless, substantial metabolic heterogeneity exists within the order, and the ecological function of the unclassified *Desulfatiglandales* taxon detected here cannot be inferred confidently from taxonomy alone. Its abundance declined strongly with depth while remaining positively correlated with overall microbial richness, suggesting that it may track sediment conditions that support a richer community. Taken together, the network results reveal a second form of taxonomic-ecological decoupling: decreasing richness was accompanied by more elaborate association networks and a larger set of topologically important taxa. This suggests that microbial community simplification with sediment depth does not necessarily imply simplification of community organization (Zhong et al., 2025).

5 Conclusion

This study revealed that that centimetre-scale geochemical stratification within cold-seep sediments is accompanied by coordinated but non-parallel changes in microbial taxonomy, functional potential and association structure. The decline in taxonomic richness contrasted with increasing KO richness and increasingly complex microbial networks, while macronutrient gradients were closely associated with C, P and S functional differentiation. Meanwhile, despite strong keystone turnover, *Desulfatiglandales bacterium* persisted across all depth zones, and its abundance was positively associated with microbial richness. Collectively, these findings demonstrate that sediment burial does not simply lead to progressive microbial impoverishment. Rather, declining taxonomic richness was accompanied by an increasingly differentiated detected functional repertoire and association structure, highlighting the importance of integrating taxonomic, functional and ecological-network dimensions when resolving microbial-geochemical coupling in cold-seep sediments. The 2-cm sampling resolution further highlights

that substantial microbiome differentiation can occur over very short vertical distances that may be obscured by conventional coarse-depth sampling. Nevertheless, because shallow metagenomics provides incomplete recovery of low-abundance genes and is unsuitable for comprehensive genome- or strain-resolved analyses, the functional patterns reported here should be interpreted primarily as comparative differences in metabolic potential. In addition, observations from a single core principally represent within-core vertical organization. Future studies combining replicated high-resolution cores with deeper metagenomics, metatranscriptomics and process-based measurements would help verify the activity and ecological roles of the identified functional pathways and keystone microorganisms.

Declarations

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Declaration of Interest Statement

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Figures

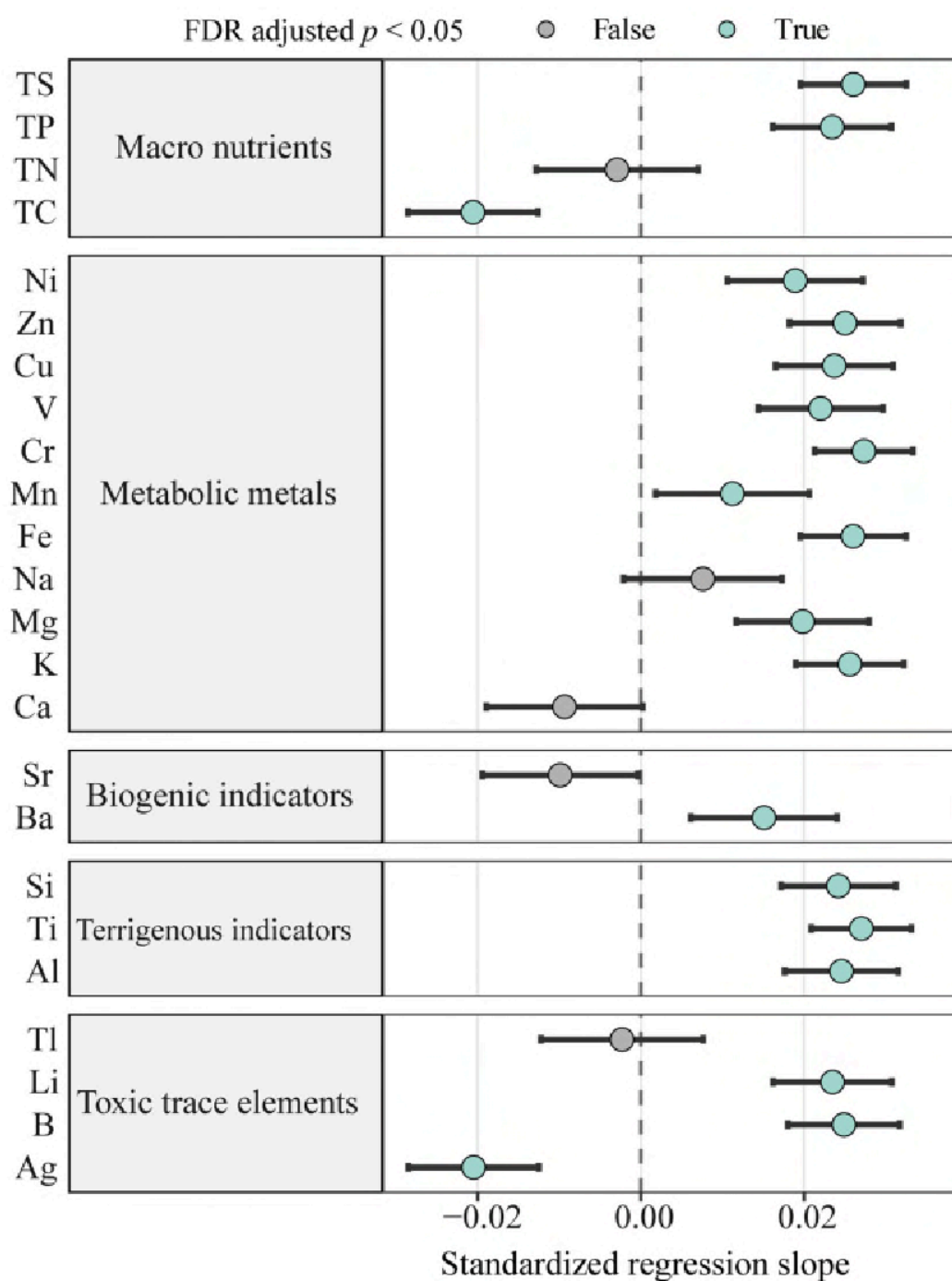


Figure 1

Depth-dependent variations in sediment geochemical characteristics. Standardized regression slopes showing relationships between sediment depth and five categories of geochemical variables, including macronutrients, metabolic metals, biogenic indicators, terrigenous indicators and toxic trace elements. Positive and negative slopes indicate increasing and decreasing trends with depth, respectively. Cyan and grey circles indicate significant and non-significant relationships, respectively, after false discovery

rate (FDR) adjustment ($p < 0.05$). Horizontal bars indicate the confidence intervals of standardized regression slopes. TS, total sulfur; TP, total phosphorus; TN, total nitrogen; TC, total carbon.

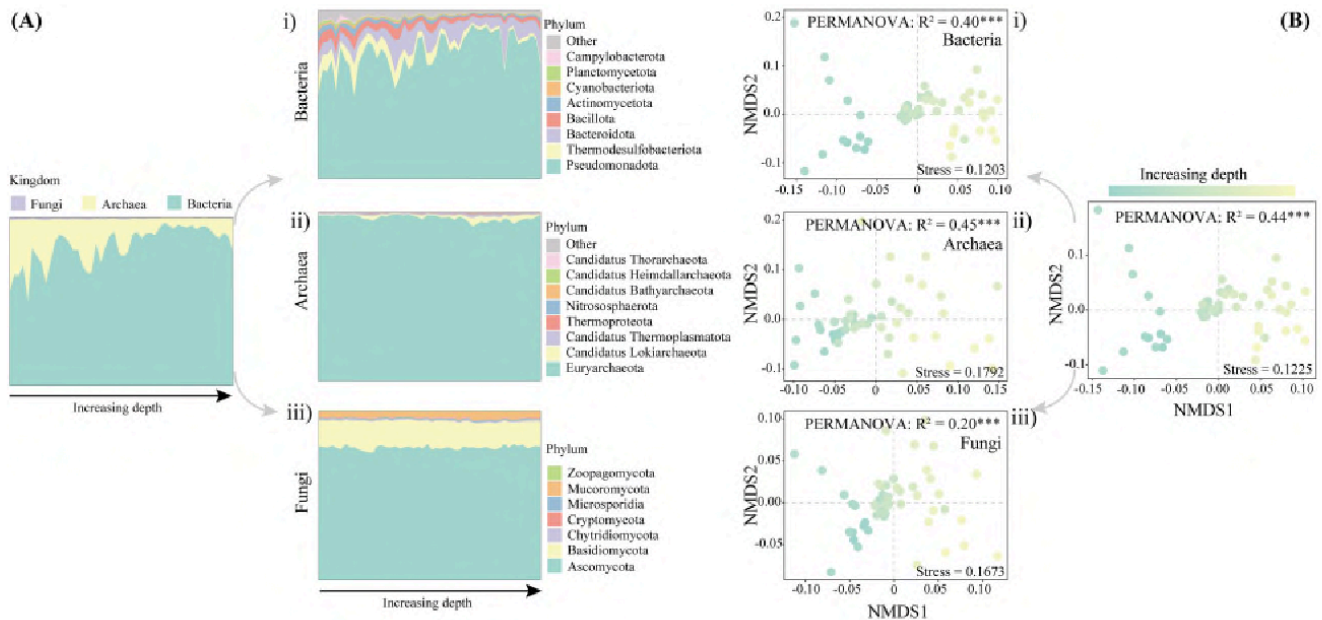


Figure 2

Microbial community composition along the sediment depth gradient. (A) Relative abundance of microbial communities at the kingdom level and phylum-level composition of (i) Bacteria, (ii) Archaea and (iii) Fungi. (B) Non-metric multidimensional scaling (NMDS) ordinations showing depth-dependent variation in the overall microbial community and in (i) bacterial, (ii) archaeal and (iii) fungal communities. The color gradient from green to yellow indicates increasing sediment depth. PERMANOVA R^2 values and significance levels are shown within the corresponding panels. $***$, $p < 0.001$.

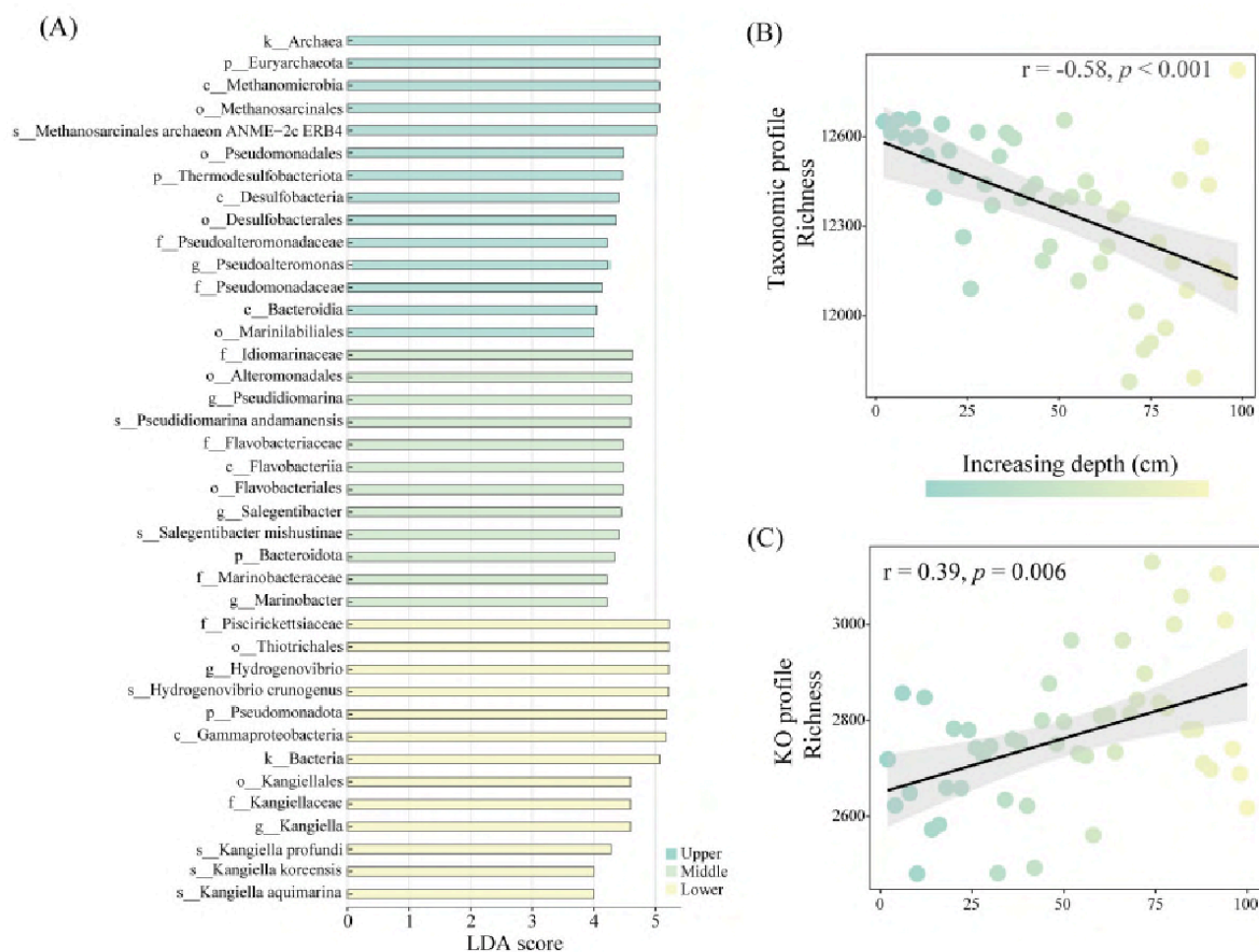


Figure 3

Depth-associated microbial biomarkers and changes in taxonomic and functional richness. (A) LefSe analysis showing microbial taxa significantly associated with the upper, middle and lower sediment layers. Only taxa with linear discriminant analysis (LDA) scores > 4 are shown. Colors indicate the corresponding sediment-depth groups. Relationships between sediment depth and taxonomic-profile richness (B) and KO-profile richness (C). Solid lines represent fitted regressions and grey shading indicates the associated confidence intervals. Taxonomic prefixes k_, p_, c_, o_, f_, g_ and s_ denote kingdom, phylum, class, order, family, genus and species, respectively. KO, KEGG Orthology.

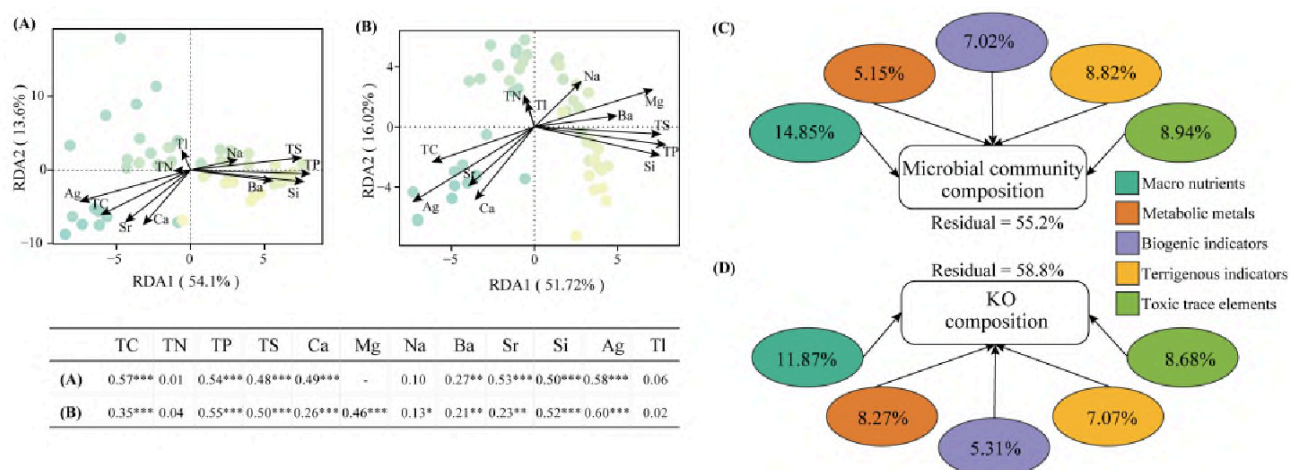


Figure 4

Effects of sediment geochemical characteristics on microbial community and functional composition. Redundancy analysis (RDA) showing relationships of geochemical variables with microbial community composition (A) and KO functional composition (B). Arrows indicate environmental vectors, and the color gradient of samples indicates increasing sediment depth. Results of Monte Carlo permutation tests for individual geochemical variables are shown below the RDA plots. *, $p < 0.05$; **, $p < 0.01$; ***, $p < 0.001$. Variation partitioning analysis (VPA) showing the relative contributions of macronutrients, metabolic metals, biogenic indicators, terrigenous indicators and toxic trace elements to microbial community composition (C) and KO functional composition (D). Percentages indicate the variation attributed to each group of geochemical variables, with residual variation shown below the corresponding response variable.

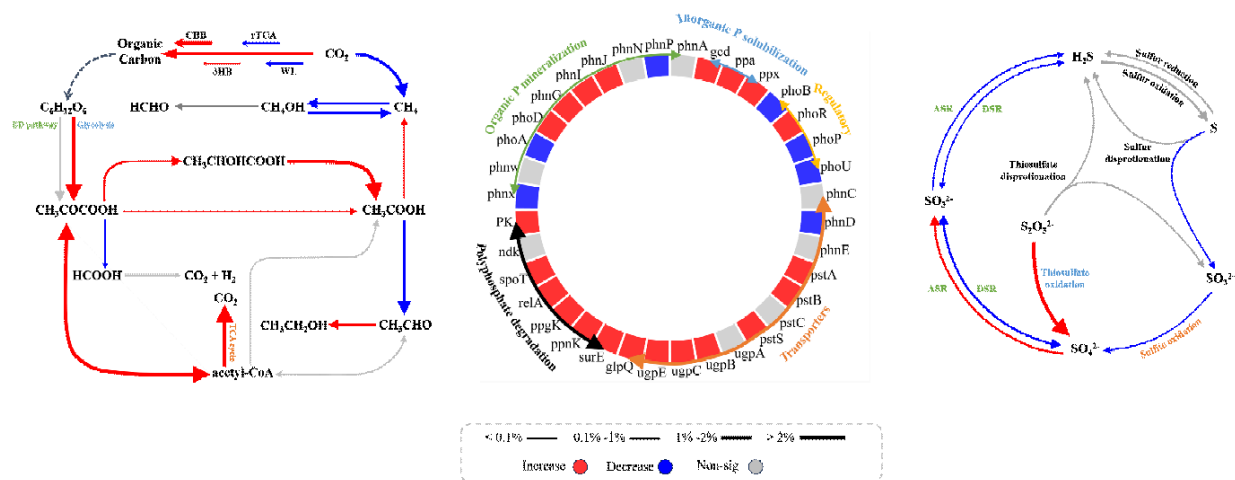


Figure 5

Cycling processes of elements at different depths in sediments. Carbon (A), phosphorus (B) and sulfur (C) cycling processes inferred from metagenomic functional profiles. Arrows indicate metabolic transformations in the carbon and sulfur cycles, whereas sectors in the phosphorus-cycle diagram represent genes associated with organic phosphorus mineralization, inorganic phosphorus solubilization, phosphate regulation, phosphate transport and polyphosphate degradation. Red, blue and grey indicate significant increases, significant decreases and non-significant changes with increasing sediment depth, respectively. Line width represents the total relative abundance of the corresponding metabolic pathway. CBB, Calvin-Benson-Bassham cycle; rTCA, reductive tricarboxylic acid cycle; WL, Wood-Ljungdahl pathway; 3HB, 3-hydroxypropionate bicycle; ED, Entner-Doudoroff pathway; TCA, tricarboxylic acid cycle; ASSR, assimilatory sulfate reduction; DSR, dissimilatory sulfate reduction.

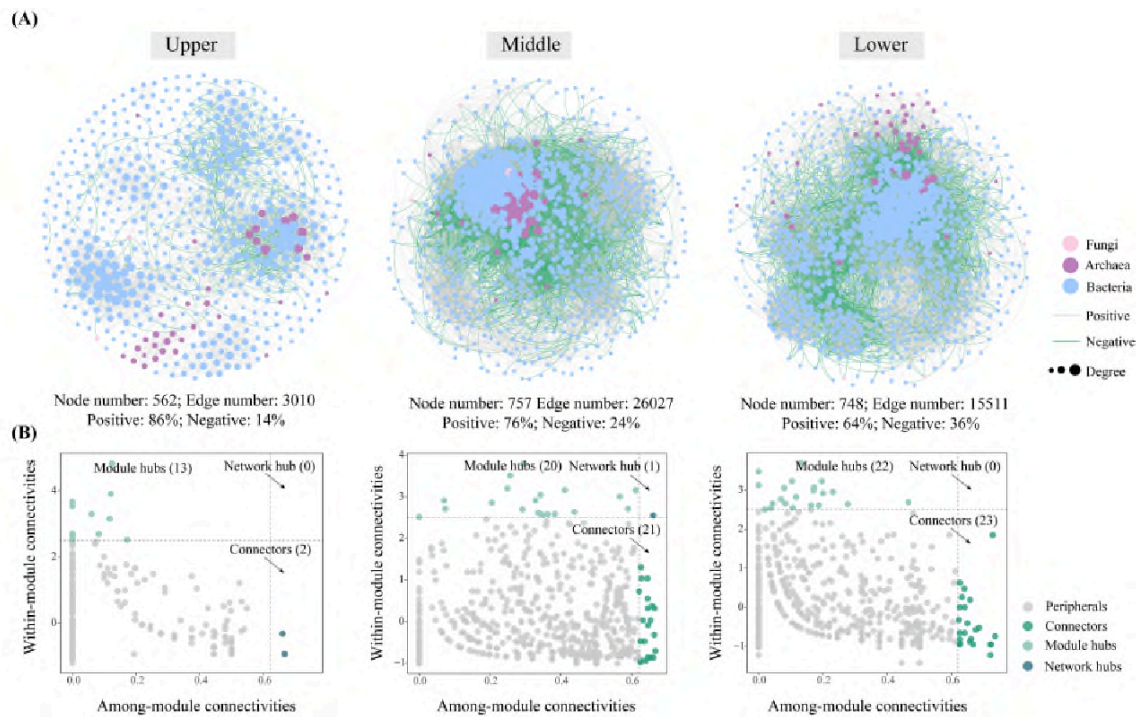


Figure 6

Depth-dependent changes in microbial co-occurrence networks and topological roles of microbial taxa. (A) Co-occurrence networks of microbial communities in the upper, middle and lower sediment layers, arranged from left to right with increasing depth. Nodes represent microbial taxa and are colored according to kingdom (Bacteria, Archaea and Fungi); node size is proportional to degree. Grey and green edges indicate positive and negative associations, respectively. Node number, edge number and percentages of positive and negative associations are shown below each network. (B) Zi-Pi plots showing the topological roles of microbial taxa in the corresponding networks. Taxa are classified as peripherals, connectors, module hubs and network hubs according to their within-module connectivity (Z_i) and among-module connectivity (P_i). Numbers in parentheses indicate the number of taxa within each topological category.

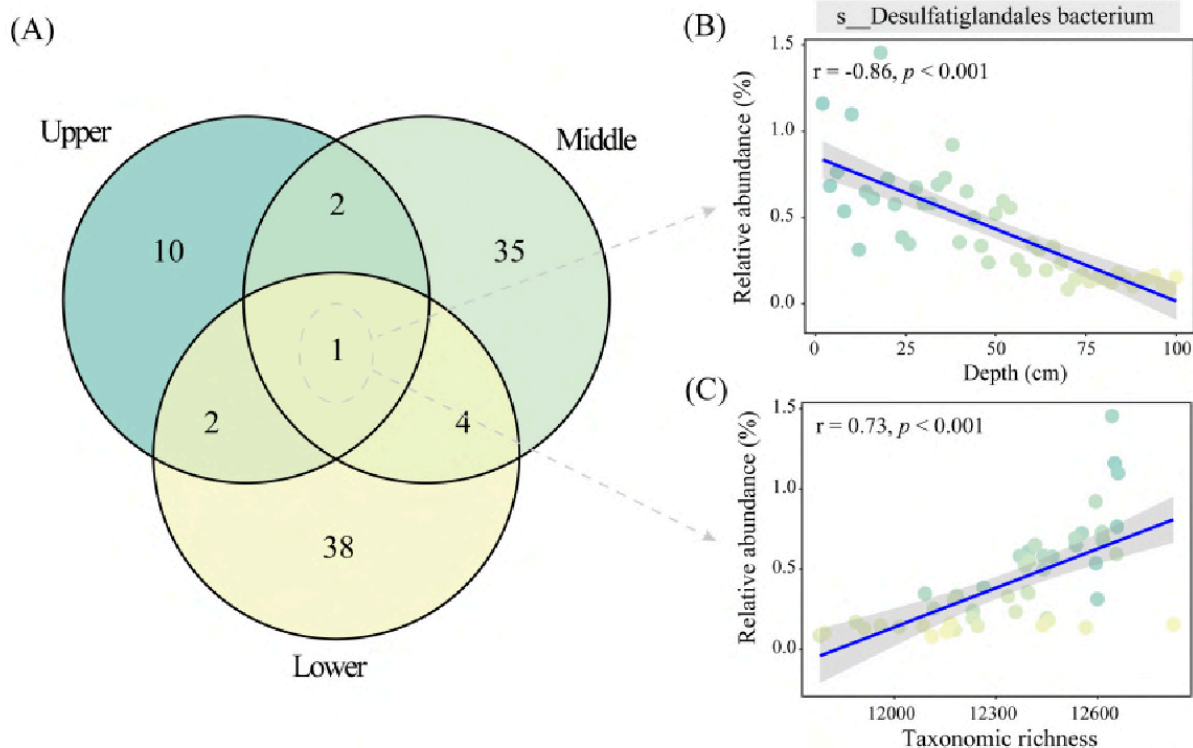


Figure 7

Depth-dependent turnover of microbial keystone taxa and variation in the shared keystone species based on co-occurrence network. (A) Venn diagram showing unique and shared keystone taxa among the upper, middle and lower sediment layers. Numbers indicate the number of keystone taxa unique to or shared among depth groups. Relationships between the relative abundance of *Desulfatiglandales bacterium*, the only keystone taxon shared across all three sediment layers, and sediment depth (B) and microbial taxonomic richness (C). Solid lines represent fitted linear regressions and grey shading indicates 95% confidence intervals. The color gradient indicates increasing sediment depth.

Supplementary Files

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