

Microbial mediation of cryptic methane cycle in semiclosed marine water column

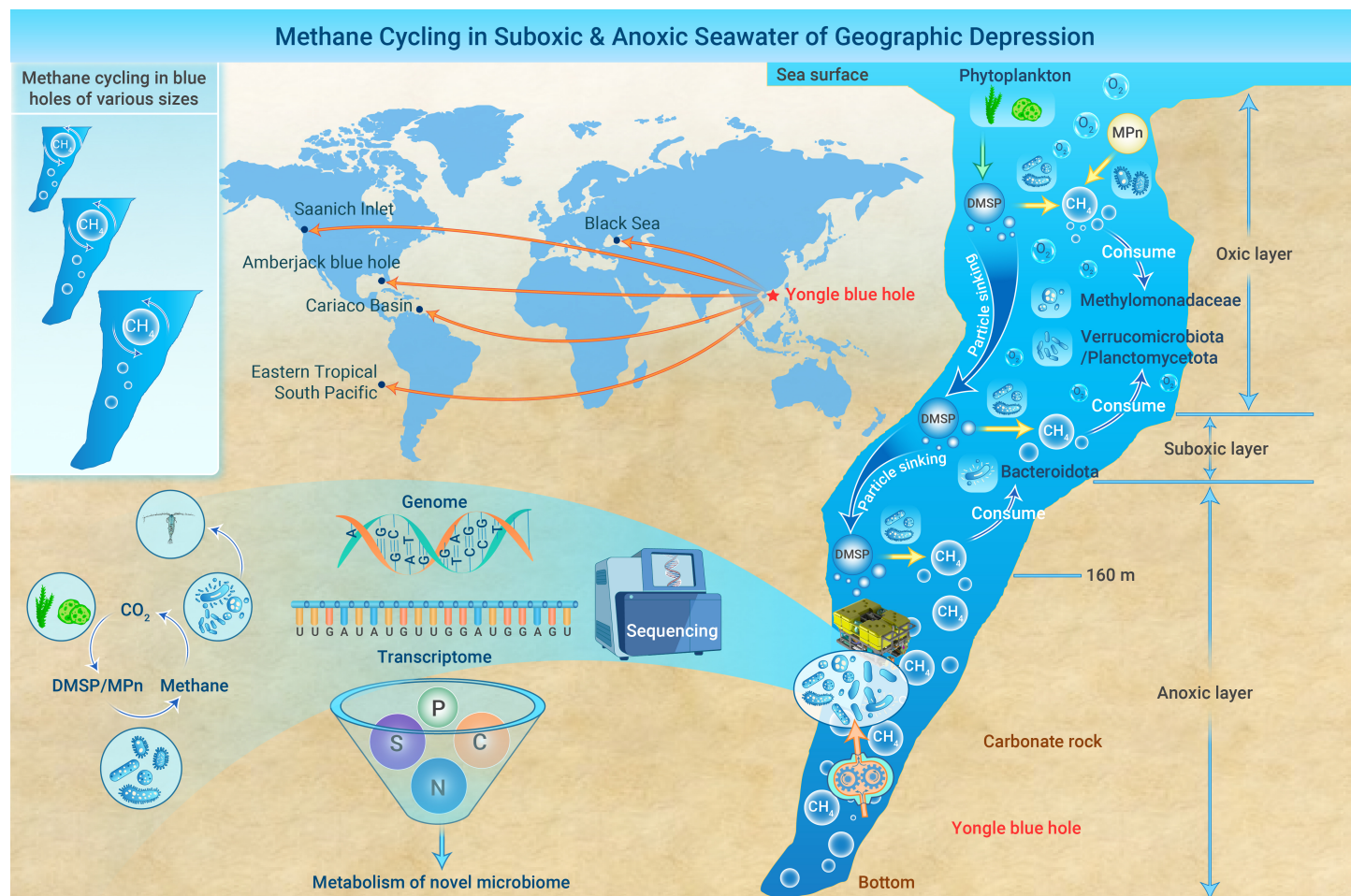
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GRAPHICAL ABSTRACT



PUBLIC SUMMARY

- Yongle blue hole exhibits remarkable microbial diversity and novel metabolic pathways.
- The identification of microbial cleavage of MPn and DMSP as key drivers of methane production.
- A new group is probably attributable to ~77% drop of methane concentration in anoxic water.

Microbial mediation of cryptic methane cycle in semiclosed marine water column

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The continuous expansion of oxygen minimum zones (OMZs) has promoted methane emissions, and the origin and fate of methane in semi-enclosed OMZs are poorly understood. In this study, we sampled 21 water layers across a 300-m depth of the Yongle blue hole (YBH) located in the South China Sea for metagenomics and metatranscriptomics work, coupled with data from global anoxic/suboxic water columns. The 16S rDNA reads in the metagenomes indicate high percentages of unclassified prokaryotes (on average 38%) and high microbiome novelty scores in anoxic layers of YBH, which are significantly higher than other semiclosed oxygen minimum zones. Analyses of 318 draft genomes and functional genes indicate that the methane source of YBH probably resulted from microbial cleavage of methylphosphonate (MPn) and dimethylsulfoniopropionate (DMSP). Methane oxidation that prevents methane emission from YBH was probably conducted by a new group of aerobic methanotrophic Planctomycetota, Bacteroidota, and Verrucomicrobiota in suboxic and anoxic environments of YBH, in addition to Methylococcales in oxic layers. The Bacteroidota solely contribute to ~77% of methane decline from the peak at 180 m depth. Our research casts light on the cryptic methane cycle mediated by the novel microbiome that controls the release of greenhouse gases from marine geographic depressions exemplified by YBH, offering valuable insights into mitigating climate change effects in marine environments.

INTRODUCTION

Expanding areas of oceanic methane accumulation include anoxic basins and fjords, as well as coastal upwelling systems, especially those characterized by anoxic water, known as oxygen minimum zones (OMZs).¹ Among these regions, marine blue holes, as semi-enclosed OMZs, have become crucial sites for studying methane cycling due to their unique geological structures and environmental conditions. In marine blue holes, the anoxic layers formed by the degradation of organic carbon input from the ocean surface are stabler due to relatively limited water exchange,^{2,3} resulting in unique ecosystems. In such conditions, microbial processes are the main source of the potent greenhouse gas methane to the atmosphere. However, it is currently unclear to what extent the methane flux from anoxic water is mitigated by microbial oxidation, and the microorganisms and metabolic pathways involved are still elusive. Nevertheless, given the predicted expansion of anoxic waters in the ocean, it is imperative to understand these processes.

At present, there are many known blue holes such as Bahamas Dean's Blue Hole,⁴ Egypt Dahab Blue Hole,⁵ and Belize Great Blue Hole,⁶ and many smaller and shallower others mostly composed of carbonate bedrock.⁷ The Yongle blue hole (YBH, also known as the Dragon Hole) in the Xisha islands of the South China Sea (SCS) is known to be the deepest marine blue hole with a depth of 300.89 m.⁸ The stable anoxic water column of over 200 m thickness contains a large amount of methane, ammonia, and hydrogen sulfide. A large number of sulfur oxidation and nitrification genes have been detected in the oxic and suboxic layers of YBH.⁹ Several previous studies have revealed microbial communities of YBH that harbor a large number of anaerobic microorganisms related to methane, nitrate, and hydrogen sulfide metabolisms, providing an important reference model for the microbial survival mode, especially microorganisms utilizing hydrogen sulfide or hydro-

gen for reducing and fixing carbon dioxide.¹⁰⁻¹³ The distribution of these genes and microorganisms basically corresponds to the vertical environmental changes in the water layers of YBH.¹⁰ In addition, it has been reported that the vertical transformation of microbial lifestyle in YBH from photoautotrophic and chemoautotrophic to mixotrophic has a complex carbon metabolism pattern.¹³ However, the ecological functions and metabolic activities of microorganisms in different water layers have not been studied using genomes of the major inhabitants and their *in situ* gene transcriptional profiles, which makes it difficult to understand the microbiome origin, taxonomic status, and metabolic potential of these microbial communities.

In addition, the origin of methane in YBH has not yet been determined. Typical methanogenic archaea, such as Methanomicrobiales and Methanosarcinales,⁹ were not found in the water column of YBH. The source of methane in the ocean is generally believed to originate from anaerobic sediments;¹⁴ however, in oxygenated seawater, the bacterial cleavage of dimethylsulfoniopropionate (DMSP) and methylphosphonate (MPn) has been observed.^{15,16} This discovery provides a possible explanation for the origin of methane in YBH. YBH is located in a tropical marine area where marine surface photosynthetic algae are abundant.¹⁷ These algae, primarily comprising the classes Dinophyceae (dinoflagellates) and Prymnesiophyceae (coccolithophores), directly biosynthesize DMSP, constantly delivering DMSP and its derivatives downward to the bottom of the YBH.¹⁸ This hypothetical source of methane in the water column of YBH and other geographic depressions remains to be examined with comprehensive work on the water column. In anoxic seawater, different microorganisms may be responsible for linking methane and nitrogen cycles. For instance, members of NC10 produce intracellular oxygen through the dismutation of nitric oxide (NO) from nitrite reduction to oxidize methane aerobically.¹⁹ The "*Candidatus* Methanoperedens nitroreducens" from the ANME-2d clade is known to couple anaerobic methane oxidation with nitrate reduction.²⁰ In YBH, the relationship between methane metabolism and nitrogen cycling in anoxic zones has not been reported yet.

In order to address the issue of methane sources and associated element cycles mediated by potentially novel niche-specific microorganisms, we collected 21 water samples in YBH. By analyzing the metagenomes and metatranscriptomes of prokaryotic microorganisms, we reported 318 metagenome-assembled genomes (MAGs) to demonstrate their distribution across different layers of YBH. Based on functional gene analysis, we investigated the methane producers and identified novel groups of methane microbial consumers in both anoxic and suboxic layers within YBH. Additionally, we described the carbon, nitrogen, and sulfur-related element cycles within YBH based on the distribution of functional genes. This study exhibits a unique YBH ecosystem and casts light on the potential impacts of similar natural processes occurring in global marine depressions on the intensifying climate change.

MATERIALS AND METHODS

Sample collection

All the samples were collected from YBH in the South China Sea (16°31'30"N; 111°46'05"E) (Figure 1A). During two research cruises carried out in April and September of 2021, water samples were collected at 21 different depths within YBH using the ISMIF apparatus.²¹ In the April cruise,

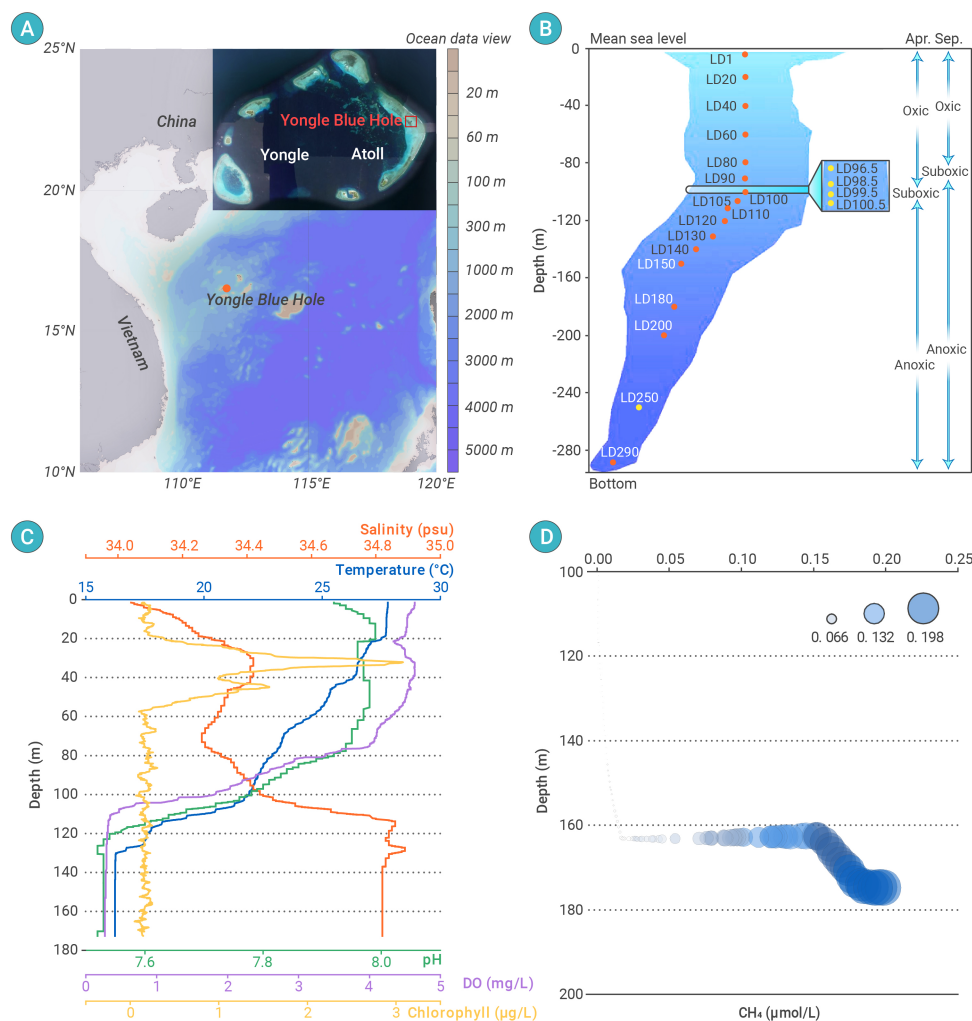


Figure 1. Sampling sites and environmental parameters in the YBH. (A) Location of the YBH in the South China Sea. (B) Vertical distribution of the sampling sites (detailed information is provided in Table S1). (C) Profiles of hydrological parameters including seawater temperature, pH, DO, salinity, and chlorophyll in the YBH. (D) CH₄ profile inside the YBH in April 2021.

Qubit™ 2.0 Fluorometer (Invitrogen, Carlsbad, CA, USA). The DNA libraries were prepared as mentioned in our previous study.²² The RNA extracts were treated with DNase I (Tiangen, Beijing, China) as instructed by the manufacturer to ensure the removal of DNA. PCR was performed using the universal primers 341F (5'-CCTAYGGGRBGCASCAG-3') and 802R (5'-TACNVGGGTATCTAATCC-3') with the RNA extracts as a template, using 2xTaq PCR Mix (Tiangen, Beijing, China) to detect DNA residues. These reactions were performed at 94°C for 3 min, followed by 35 cycles of 94°C (30 s), 55°C (30 s), and 72°C (1 min). Then, the metatranscriptome libraries were constructed using the VAHTS® Universal V8 RNA-seq Library Prep Kit for Illumina (Vazyme, Nanjing, China). The DNA/cDNA libraries were sequenced using an Illumina Novaseq 6000 platform (2×150 bp).

RESULTS

Environmental parameters of the YBH

In two cruises carried out in April and September of 2021, a total of 21 samples were obtained from the water column of YBH by the *in situ* microbial filtration and fixation (ISMIFF) sampler (Figures 1A-B, Table S1).²⁴ The temperature, pH, and dissolved oxygen (DO) readings

decreased with increasing water depth, while salinity was in the opposite trend (Figures 1C-D). The suboxic layer, with DO concentration ranging from 0–2 mg/L,²⁵ was observed at a depth range of 98–110 m in April and 75–101 m in September, respectively (Figures 1C & S1A). Particularly, the methane concentration increased sharply at a depth of ~160 m and peaked at ~180 m with a concentration of about 0.19 μM (Figure 1D). The total organic carbon (TOC) showed an overall decreasing trend with depth (Figure S1B). Nitrite (NO₂⁻), nitrate (NO₃⁻), and ammonium (NH₄⁺), all have large fluctuations below 100 m (Figure S1C). H₂S is close to zero between 0 m and 100 m, similar to the water outside the blue hole. With increasing depth, the concentration of H₂S increased rapidly from 120 m to a maximum of 0.68 mg/L. Sulfate (SO₄²⁻) increased sharply after 75 m, followed by notable fluctuations (Figure S1D). The total water volume in YBH was estimated as 1,062,840 m³ and was hereby filled up with 1.07 kg methane and 1.55 t O₂. Between 110–180 m, the anoxic water volume was estimated as 344,647 m³, with a total methane content of 0.23 kg.

Microbial community structure and microbiome novelty in YBH water column

The 21 samples were subjected to metagenomic sequencing, resulting in a dataset of 162.48 billion base pairs (Gbp). Among these samples, we generated a total of 23.61 Gbp reads for 11 metatranscriptomes (Table S2). The 16S rDNA fragments (16S miTags) were extracted from the clean reads of the metagenomic dataset (Table S3), and the classification results have been partially demonstrated in our recent study.¹⁰ The proportion of 16S miTags with <97% similarity to the Tara oceans database was 46.70 ± 21.80%, significantly higher compared to the samples collected from most of the other sites (Wilcoxon test, *p* < 0.05) (Figure 2A). Similarly, compared to the Silva database (Silva 138.1),²⁶ the proportion of 16S miTags with <97% similarity was, on

16 samples were collected in the depth range of 1–290 m, while in the September cruise, five samples were collected at depths of 96.5–100.5 m and 250 m (Figure 1B, Table S1). The microorganisms were collected as previously described using the ISMIFF apparatus.²² Temperature, salinity, and dissolved oxygen (DO) were measured using an SBE 37SMP-ODO conductivity-temperature-depth (CTD) profiler and DO sensor (Sea-Bird, Bellevue, WA, USA). Methane concentrations in the water column were determined using a methane sensor (METS, Franatech, Germany), and pH was measured using a pH sensor (AMT Analysenmesstechnik GmbH, Rostock, Germany). Water samples were collected at 13 different depths from 0 m to 170 m using Niskin sampling bottles (Sea-Bird, Bellevue, WA, USA) and were analyzed for sulfide, nitrite, ammonia, and sulfate concentrations using a HACH DR900 apparatus (HACH, Loveland, CO, USA). The Niskin water samples were filtered through a 0.22-μm pore size polycarbonate membrane (Millipore, Massachusetts, USA) and preserved with chloroform. Some Niskin water samples were analyzed for TOC using a TOC analyzer (Elementary, Hanau, Germany). Due to the shift of the YBH vertical axis, deployment of Niskin bottles and sensors in >180 m layers is difficult. The three-dimensional topography of YBH was obtained by a remotely operated vehicle (ROV) equipped with two-dimensional sonar scanning. The total water volume of YBH was calculated using Python vtk visual simulation modelling,²³ and the total amount of methane and DO in the water column of the cave was calculated based on their concentrations at different layers.

Nucleic acid extraction and sequencing

The preserved 0.22-μm pore size membranes were pulverized using liquid nitrogen, and total genomic DNA and RNA were extracted using the DNA/RNA co-extraction kit (Tiangen, Beijing, China) following the manufacturer's instructions. DNA and RNA concentrations were measured using a

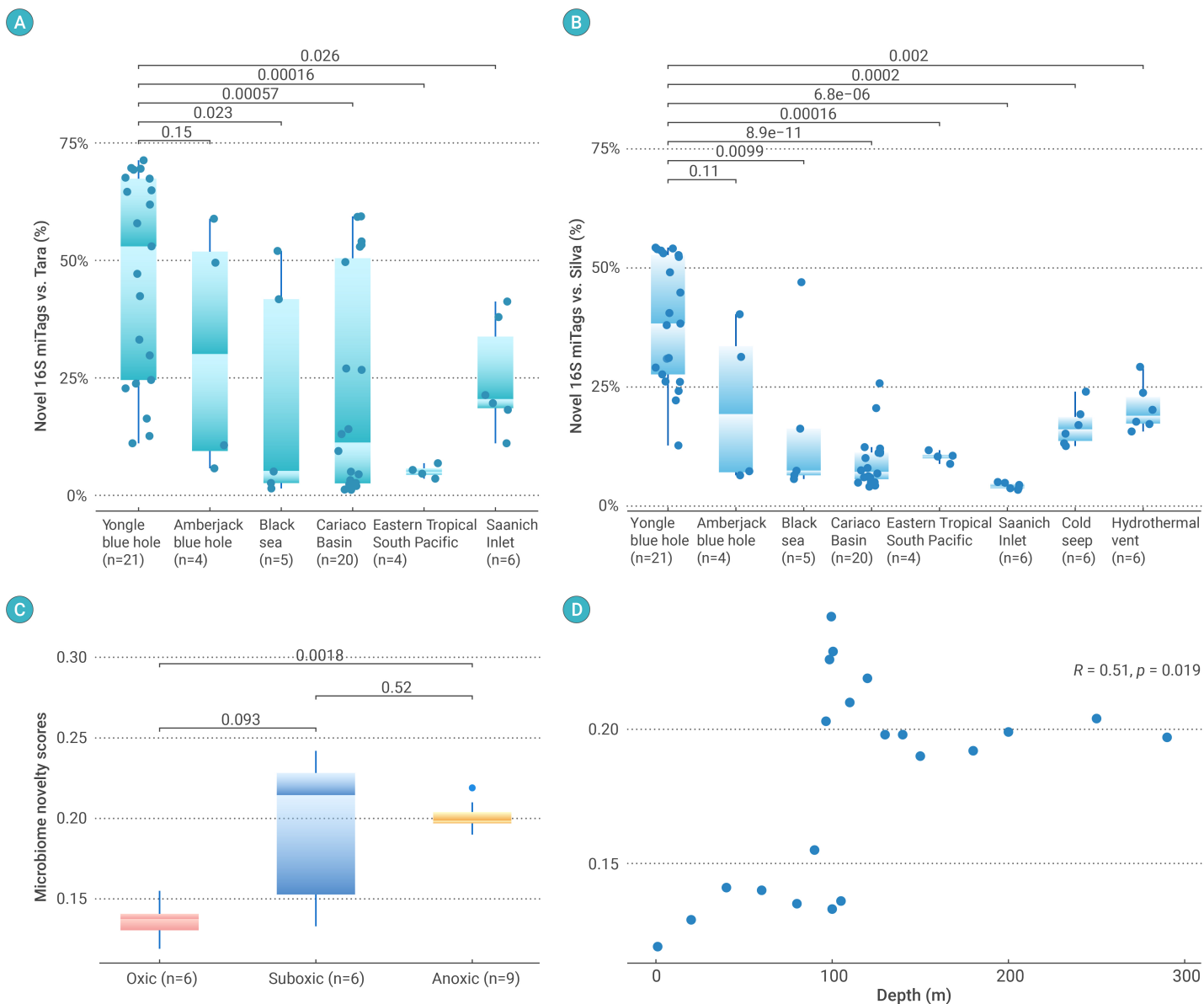


Figure 2. Overview of microbiome novelty in YBH water column (A) The average percentage of novel 16S miTags (<97% similarity to Tara oceans database) in the YBH metagenomes (this study), and the reference datasets from public databases for several marine water columns with anoxic/suboxic layers (Table S4) (p values estimated using the two-sided Wilcoxon test). (B) The average percentage of novel 16S miTags (<97% similarity to the Silva database) in the metagenomes of YBH water column (this study) and reference datasets from public databases for several marine water columns with anoxic/suboxic layers (p values estimated using the two-sided Wilcoxon test). (C) MNS at different depths of the sampling sites (p values were calculated using the two-sided t-test). (D) MNS across the water column of the YBH water column (p values estimated using the two-sided Wilcoxon test).

average, $37.67 \pm 14.07\%$, which was significantly higher than samples collected from the reference water columns, as well as those from deep-sea hydrothermal (Mid-Atlantic Ridge and Guaymas Basin, $20.64 \pm 5.08\%$) and cold seep (Gulf of Mexico, $16.89 \pm 4.28\%$) sediment metagenomes (Wilcoxon test, $p < 0.05$), but with no significant difference compared to the Amberjack blue hole ($21.35 \pm 17.09\%$, $p > 0.05$) (Figure 2B, Table S4). The percentage of novel 16S miTags (relative to the Silva database) in the YBH water column drastically increased in the suboxic and anoxic zones (Figure S2). We also estimated the Microbiome Novelty Score (MNS) for all samples as a proxy of microbiome novelty (Figures 2C-D). The estimated MNS for all the samples was > 0.12 , which is the cutoff for novel microbiomes.²⁷ Compared to the oxic samples, the MNS values of samples from the anoxic zone were significantly higher (Wilcoxon test, $p < 0.01$) (Figure 2C).

Reconstruction, characterization, and transcriptional activity of YBH genomes

A total of 364 metagenome-assembled genomes (MAGs) (>50%

completeness and <10% contamination) were binned from 259,567 contigs ($\geq 2,000$ bp) that were assembled from 21 metagenomes. Among these, 318 non-redundant MAGs (<97% average nucleotide identity, Table S2) were filtered for taxonomic classification. The MAGs were classified into five archaeal and 29 bacterial phyla (Figure 3A, Table S5). Phylogenomic analysis revealed that all the MAGs were clustered with 222 reference genomes, primarily sourced from the Amberjack Hole, the Black Sea, the Saanich Inlet, cold seeps, hot springs, hydrothermal vents, and the Fennoscandian Shield of Sweden, and those originated from diverse water sources (Figures 3A & S3, Table S6). The quality-controlled reads from the 21 metagenomes showed high mapping rates to the MAGs (average 48.26%, Table S2), with coverage exceeding 50% for 13 samples, indicating a good representation of the MAGs in the recovered microbiomes.

The MAGs exhibited distinct relative abundances in the layers with different oxygen gradients (Figure 3B, Table S7), which supports the niche specificity of microbial communities in YBH. The top 10 most abundant phyla are: Desulfobacterota, Cloacimonadota, Marinisomata, Proteobacteria, Omni-

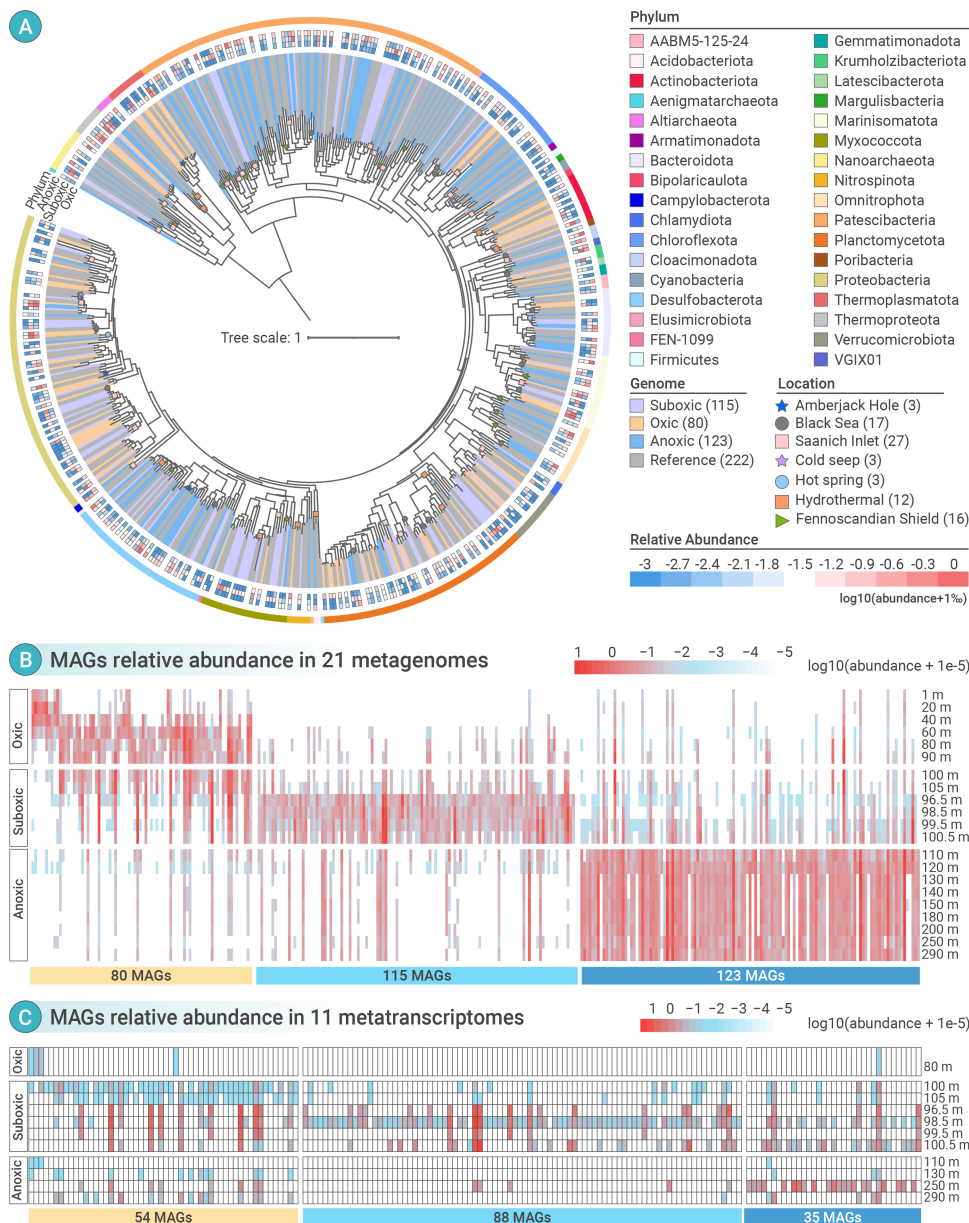


Figure 3. Phylogeny, taxonomy, distribution, and transcriptional level of reconstructed MAGs. (A) Maximum-likelihood phylogenomic tree of 43 concatenated conserved proteins identified from the 318 MAGs (orange, purple, and blue clades represent the non-redundant MAGs of oxic, suboxic, and anoxic layers, respectively) and 222 reference genomes (gray clades) from GTDB.²⁸ (B) Relative abundance (%mapped reads) of the MAGs in the 21 YBH metagenomes (detailed information is provided in Table S7). (C) Relative abundance of the transcribed MAGs in the 11 metatranscriptomes. The bottom strip indicates the number of transcribed MAGs in each zone. The relative abundance values were log-transformed using the log₁₀(abundance + 1e-5).

gene (*pepM*),²⁹ and those associated with MPN degradation, such as the *phn* gene cluster, showed certain abundances in different water layers (Figures 4 & S5). The alpha-D-ribose 1-methylphosphonate 5-phosphate C-P lyase gene (*phnJ*), a methane-producing gene,³⁰ was abundantly present below 40 m (Figure 4B). Furthermore, the key gene *phnJ* was identified in five MAGs belonging to Proteobacteria (Oxic-bin53, Oxic-bin102, Suboxic-bin128, and Anoxic-bin42) and Desulfobacterota (Suboxic-bin56) (Figure 4A, Table S9).

We also detected several genes (*dmdABCD*, *tmm*, *mddA*) related to DMSP demethylation, which generates methanethiol (MeSH) and dimethylsulfide (DMS) for further methane production (Figures 4B & S5A). This reaction has been frequently verified in the oxic surface waters.³¹ The related genes (*dmdAB*, *tmm*, *mddA*) exhibited certain abundances in different layers of YBH and were transcriptionally active (Figures 4B-C). In light of the MAGs that harbor these genes, 97 MAGs (30.48%) belonging to 14 phyla are responsible for DMSP demethylation and the production of DMS (Table S10), which is regarded as a major substrate for methane generation in marine water.³² In addition, the *mtsA* gene encoding methylthiol: coenzyme M methyltransferase is present in seven MAGs (Oxic-bin23, Anoxic-bin111, Suboxic-bin59,

Suboxic-bin53, Suboxic-bin133, Suboxic-bin131, Anoxic-bin84), belonging to Actinobacteriota, Chloroflexota, Planctomycetota, Desulfobacterota, Poribacteria and AABM5-125-24 (Figure 4, Table S11). The methyltransferase catalyzes the methyl transfer from DMS to coenzyme-M forming methyl-CoM, a key component of methane production.^{33,34} All the collected metagenomes for global suboxic and anoxic waters contain the same DMSP metabolism genes as YBH, mainly DMS oxidation genes and DMSP demethylation genes (Figure S5B).

Considering the descending methane concentration in the <160-m depth water of YBH (Figure 1D), we then investigated microbial consumption of methane in the blue hole. We identified a methane-oxidizing aerobic bacterium *Methylococcales* (Oxic-bin92) that possesses methane oxidation-related *pmoABC* genes. These genes were only abundant above 105 m (April of 2021), indicating their restricted ability to utilize methane in the oxic zone to produce methanol.³⁵ Additionally, using the PmoA protein sequences in the public databases, we annotated putative genes encoding PmoA-like proteins in the YBH suboxic and anoxic zones. These proteins exhibited a conserved domain (Pfam 14100) (Table S12) and were encoded by six MAGs belonging to Planctomycetota (Suboxic-bin54), Bacteroidota (Suboxic-bin57 and Anoxic-bin38), and Verrucomicrobiota (Suboxic-bin106, Suboxic-bin70, and Suboxic-bin94). Among them, only one MAG (Anoxic-bin38 for Bacteroidota) was obtained from metagenomes of the anoxic zone, while the rest originated

trophota, Thermoproteota, Patescibacteria, Nanoarchaeota, Krumholzibacteriota, Thermoplasmata. Among them, Thermoproteota exhibited the highest relative abundance in oxic layers, Proteobacteria dominated in suboxic layers, and Desulfobacterota thrived largely in anoxic layers. Additionally, 177 MAGs showed transcriptional activity across the 11 metatranscriptomes (Figure 3C). In oxic layers, Planctomycetota exhibited the highest relative abundance in the metatranscriptomes. However, in suboxic and anoxic layers, Proteobacteria dominated in terms of relative abundance. Principal coordinates analysis (PCoA) confirmed the separation of microbial communities among the layers with different oxygen levels (Figure S4A). Redundancy analysis (RDA) revealed the relationships between microbial community composition and environmental parameters, which explains 68.28% of the variation in community structures (Figure S4B). NO₃⁻ concentration was the factor that determined the microbial communities of the suboxic zone, while the communities in anoxic zone were highly related to methane and H₂S.

Genome-centric methane production and consumption

To investigate the ecological roles of prokaryotes in the YBH water column, a total of 883,658 genes were predicted from the 318 MAGs and were functionally annotated (Tables S5 & S8). The key gene *mcrA* involved in methane metabolism was not found in the metagenomes and MAGs. Instead, genes involved in phosphonate biosynthesis, such as phosphoenolpyruvate mutase

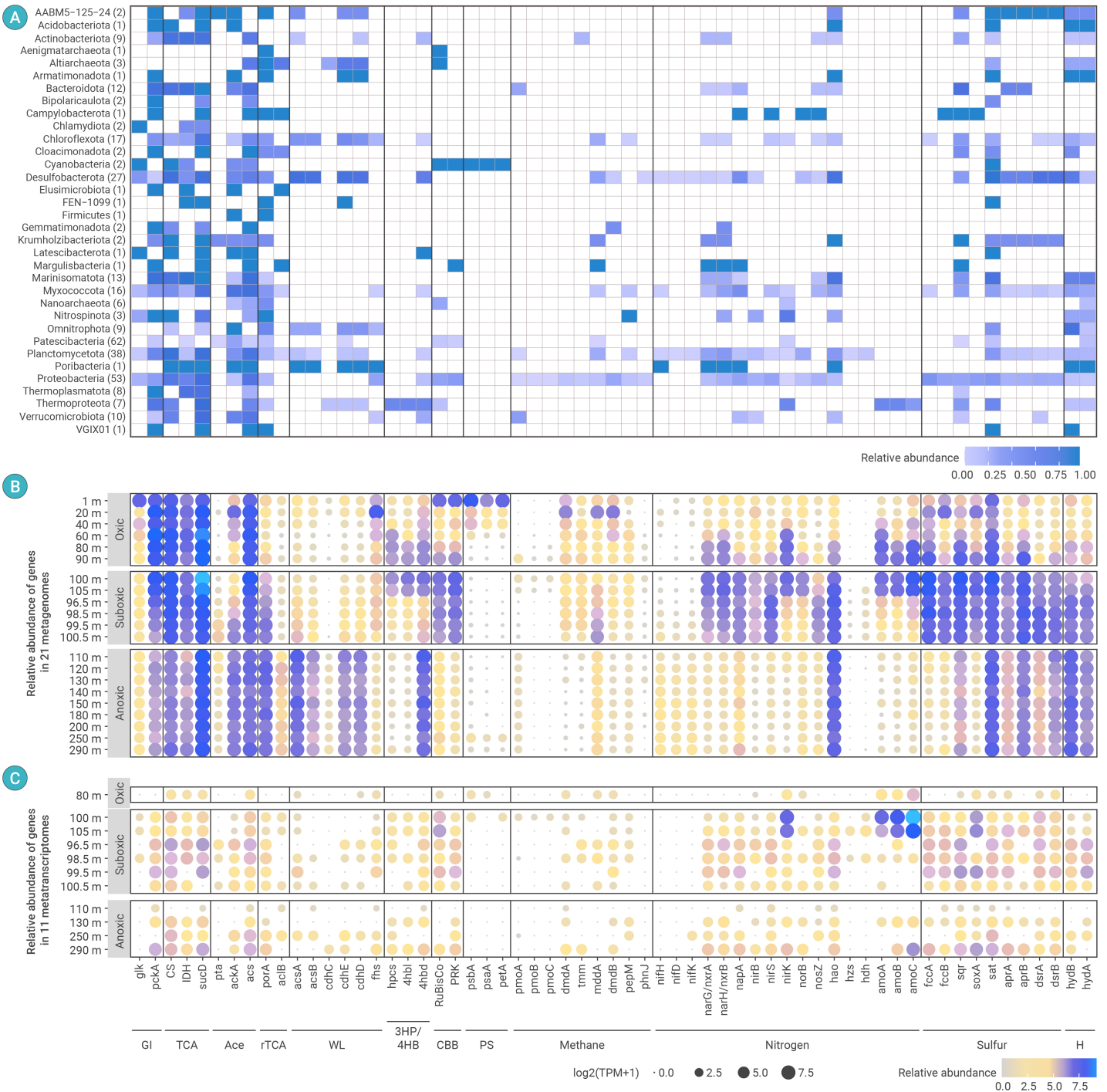


Figure 4. Relative abundance of functional genes in MAGs, metagenomes, and metatranscriptomes (A) Heatmap illustrating relative abundances of metabolic genes across phyla identified in the YBH water column. The number of genomes per phylum is shown in parentheses. (B) Heatmap showing TPM values of genes in the 21 metagenomes. (C) Heatmap for TPM values of gene transcripts in the 11 metatranscriptomes. GI: glycolysis; TCA: tricarboxylic acid cycle; rTCA: reductive tricarboxylic acid cycle; Ace: acetate metabolism; WL: Wood–Ljungdahl pathway; 3HP/4HB: 3-hydroxypropionate/4-hydroxybutyrate cycle; CBB: Calvin cycle; PS: Photosystem; Methane: methane metabolism; Nitrogen: nitrogen metabolism; Sulfur: sulfur metabolism; H: hydrogen metabolism. TPM: transcripts per million reads. Details of the functional genes are shown in Table S8.

from the suboxic zone. We conducted a phylogenetic analysis of their deduced PmoA-like proteins, which showed that the PmoA-like proteins of these bacteria from suboxic and anoxic zones were clustered together but were far away from the homologs from Proteobacteria (represented by Methylococcales in the oxic layers) (Figure 5A). The mixture of PmoA proteins from different phyla in the same clades also indicates the occurrence of horizontal gene transfer between the anaerobic and low-oxygen tolerant organisms.³⁶ Based on protein structure prediction, the PmoA protein in this study is structurally similar to that of atypical methane-oxidizing Verrucomicrobiota bacteria identified in global oceans (Figures 5B & S6).³⁷ The neighboring

12 genes of the Verrucomicrobiota *pmoA* are all hypothetical at present. These MAGs containing PmoA-like proteins were considered to have the potential for methane oxidation, so we defined them as MOB-like MAGs.

For the metabolic network predicted in the MOB-like MAGs, the microbes might oxidize methane to methanol, followed by further transformation to formaldehyde except for the Anoxic-bin38. The Methylococcales (Oxic-bin92) have a complete methane oxidation pathway for potential conversion of methane into CO₂ (Figure 5C, Tables S11 & S13). In the suboxic and anoxic zones, formaldehyde was probably metabolized into formic acid by Planctomycetota (Suboxic-bin54) and Verrucomicrobiota (Suboxic-bin70), which is

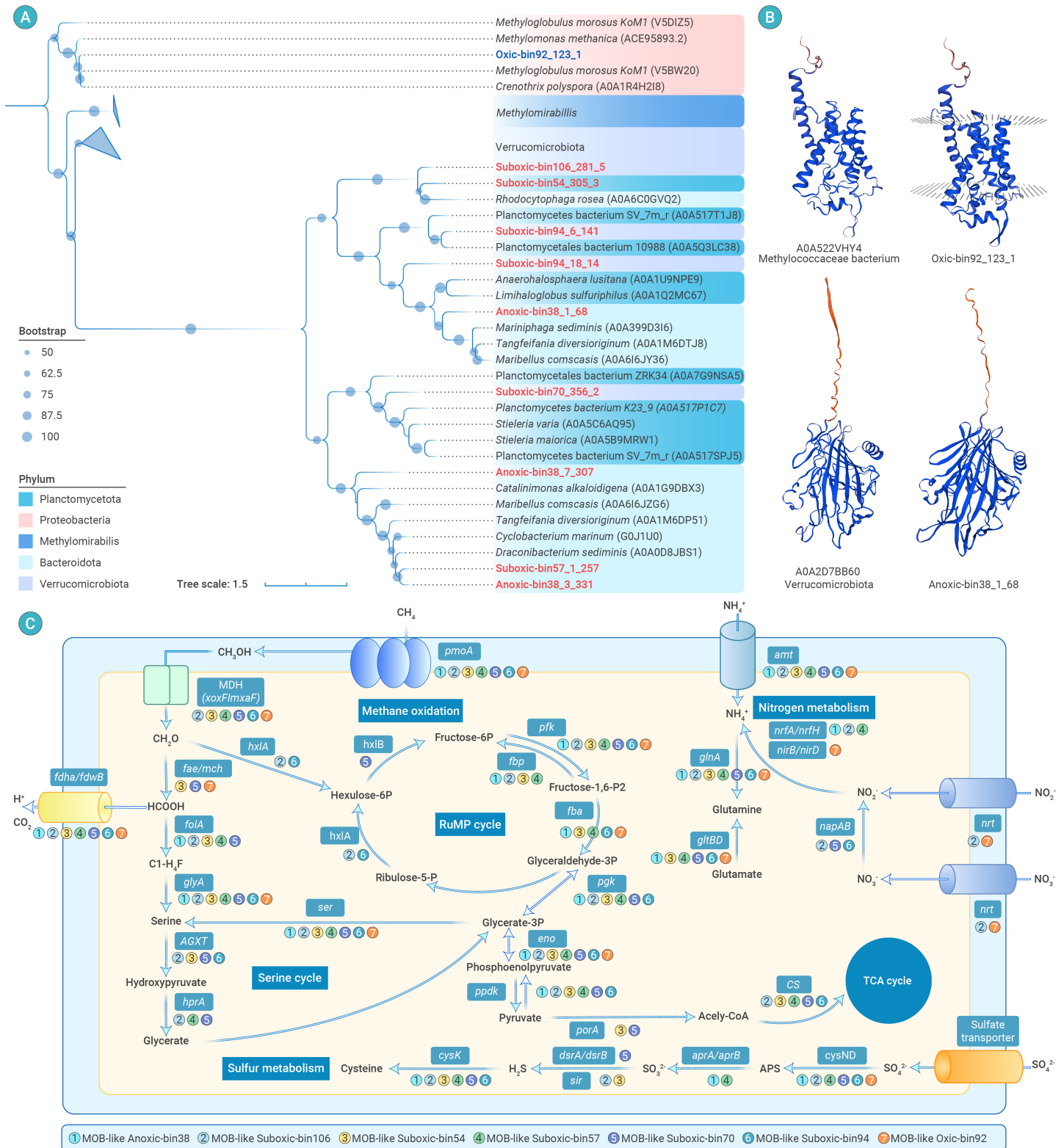


Figure 5. Identification of potential methane microbial consumers in YBH (A) Maximum-likelihood phylogenetic tree of the PmoA-like proteins identified in the seven recovered MAGs (red), with published PmoA proteins (black). Bootstrap values (1,000 replicates) $\geq 50\%$ are marked with a blue circle. Background colors indicate the proteins from different phyla. (B) Three-dimensional structures of the PmoA proteins. (C) Schematic presentation of the metabolic reconfiguration of the putative methane-oxidizing bacteria in YBH. The three-dimensional structure of the proteins was predicted using Swiss-model with GMQE > 0.9 and sequence identity $> 70\%$. The reference proteins (A0A522VHY4 and A0A2D7BB60) are downloaded from AlphaFoldDB.

then used by the species represented by Verrucomicrobiota (Suboxic-bin106 and Suboxic-bin94) via the RuMP (ribulose monophosphate) pathway for biosynthesis of organic carbons. Formic acid might then be integrated into the serine cycle as indicated by the coding capacity of five other MAGs except for the Suboxic-bin94. The species represented by Suboxic-bin106 and Oxic-

bin92 were possibly able to transport nitrate and nitrite for denitrification.

Overcoming nitrogen limitation with microbially mediated nitrogen reduction and fixation

With a high rate of methane consumption over the 160-m layer, nitrogen is

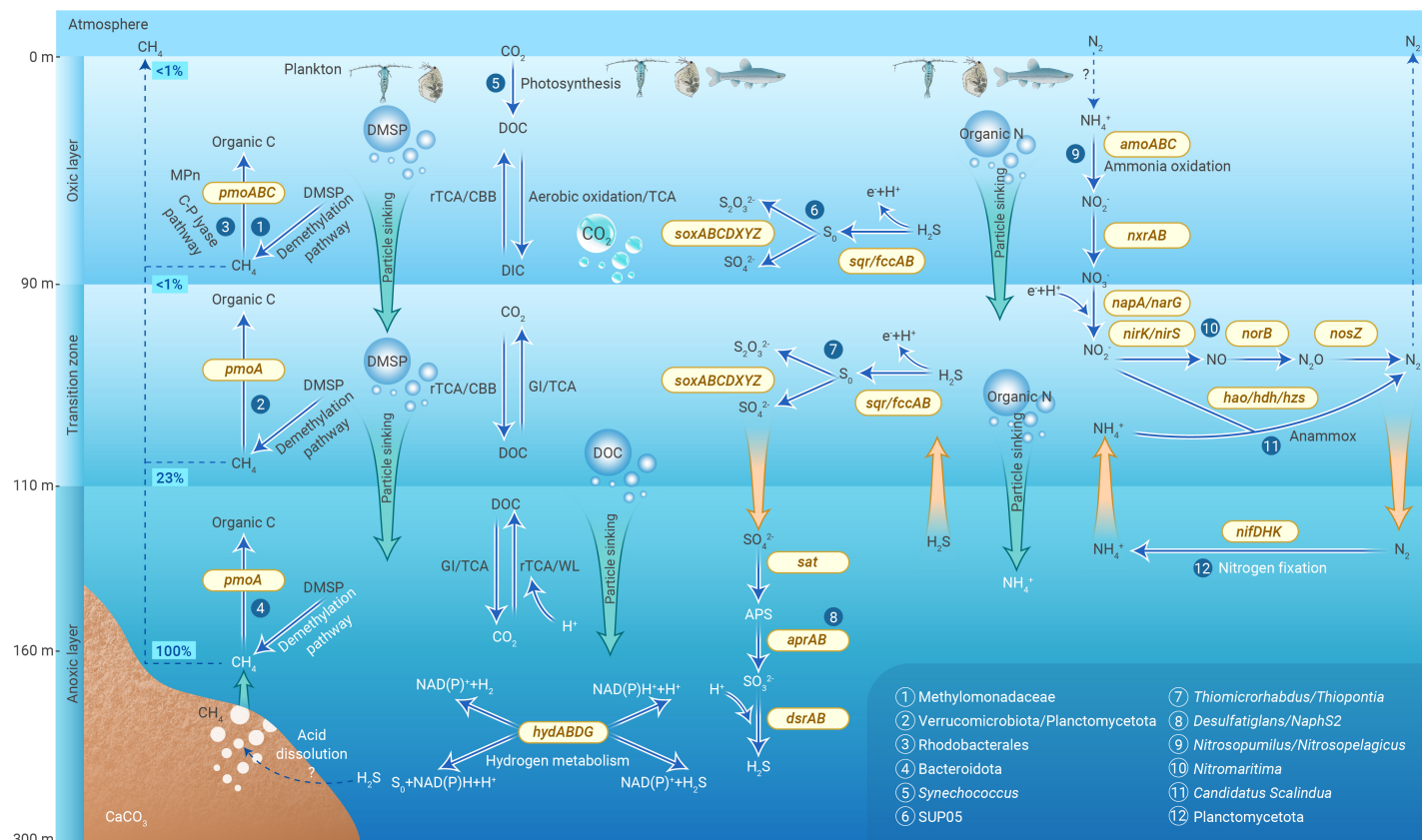


Figure 6. Schematic representation of potential carbon, nitrogen, and sulfur cycles in YBH water column The species in the lower right corner represent the major players in the network. The oxic-anoxic interface was designated for April of 2021. Source data are listed in Table S8.

probably a limiting factor for microbial growth and metabolism (Figures 1D & S1C).³⁸ Cyanobacterial populations have long been regarded as the primary providers of bioavailable nitrogen in the marine environment,³⁹ while the set of genes *nifHDK* for nitrogen fixation were also present in two MAGs belonging to Desulfobacterota (Suboxic-bin81) and Planctomycetota (Anoxic-bin77) (Figures 4A & S7). Among them, the NifD protein encoded by Anoxic-bin77 obtained from the anoxic zone showed a closer evolutionary relationship to nitrogen-fixing populations of Planctomycetota in the surface ocean (HBD-09_PSW_MAG and HBD-08_PSW_MAG) (Figure S7A).⁴⁰ However, their NifH proteins were not within the same branch (Figure S7B), suggesting that these NifH subunits of nitrogen fixation enzymes might have undergone divergent evolution to adapt to different oxygen levels. Furthermore, both of the MAGs contain the minimum gene set for nitrogen fixation (*nifH*, *nifD*, *nifK*, *nifE*, *nifN*, and *nifB*),⁴¹ implying their potential ability to participate in nitrogen fixation (Figure S7C).

Due to the weak vertical water mixture in YBH, there is limited nitrogen input from the surface waters, and the main sources of nitrogen available for fixation in the deep layers remain mysterious. In the suboxic zone, four MAGs representing "*Candidatus Scalindua*", anaerobic ammonia-oxidizing (anammox) bacteria, were discovered (Figure S8A), which represented a dominant inhabitant in the suboxic zone. Phylogenetic analysis of the anammox-related proteins, hydrazine synthase (Hzs) and hydroxylamine hydrogenases (Hdh), from these MAGs revealed that all the essential proteins were clustered with the homologs from known Planctomycetota groups (Figures S8B-C). The Hdh proteins of Suboxic-bin129 and Suboxic-bin35 were more closely related to "*Candidatus Brocadii*" rather than "*Ca. Scalindua*". In the oxic zone where methane concentration was close to zero, ammonia-oxidizing archaea (AOA) *Nitrosopumilus* (Oxic-bin62) and *Nitrosopelagicus* (Oxic-bin68 and Oxic-bin94) from the Thermoproteota phylum were responsible for chemoautotrophic growth and nitrogen oxides production. The AOA exhibited the highest abundance among the prokaryotes in the suboxic layer and showed higher transcriptional activity of the ammonia oxidation-related *amoABC* genes (Figure 4C). The AOA *Nitrosopumilus* (Anoxic-bin7) in anoxic layer of

YBH was classified as *Nitrosopumilus maritimus* SCM1, which is phylogenetically closely related to the relative capable of nitrogen and oxygen production in anoxic condition (Figure S9).⁴²

Microbial CO₂ fixation probably occurs across the YBH water column

Aside from the methane cycle in YBH, CO₂ fixation was probably also conducted by prokaryotes in YBH. In the oxic zone, Cyanobacteria performed aerobic carbon fixation through photosynthesis and the Calvin-Benson-Bassham (CBB) pathway, providing readily available organic carbon for other underlying microorganisms (Figure 4, Table S8). In the suboxic and anoxic zones, carbon fixation was expected to be primarily performed through the reductive tricarboxylic acid (rTCA) and Wood-Ljungdahl (WL) pathways (Figure 4B). The complete set of genes for rTCA is present in Campylobacterota MAGs (Figure 4A). The WL pathway was present only in the suboxic and anoxic layers and was mainly carried by the MAGs representing Poribacteria, Chloroflexota and Omnitrophota (Figure 4A). The 3-hydroxypropionate/4-hydroxybutyrate (3HP/4HB) cycle identified in the MAGs of various regions, was mainly hosted by *Nitrosopumilus* (Thermoproteota) (Figure 4). The carbon and sulfur cycles were coupled in the deep YBH as demonstrated previously.⁸ In the anoxic water, sulfate-reducing bacteria (SRB) affiliated with Desulfobacterota have been identified as the dominant group (on average 17.83%),¹⁰ which agrees with high relative abundances of the genes related to sulfate reduction (*dsrAB*) in the anoxic zone (Figures 4B-C). The results are consistent with the sharply increased concentration of H₂S at ~120 m (Figure S1D). On the other hand, sulfide produced by the SRB might be used by sulfur-oxidizing bacteria (*Thiomicrobium*, *Thiopontia*, and SUP05),¹⁰ consistent with the richness of the genes associated with sulfur oxidation (*fccA* and *sqr*) and thiosulfate oxidation (*soxA*) in the oxic and suboxic zones (Figure 4, Table S8). Based on the abundance and transcriptomic expression of carbon, nitrogen, and sulfur metabolism-related functional genes mentioned above (Figure 4, Tables S8 & S11), we constructed a schematic diagram illustrating the material cycles within the water column of YBH (Figure 6). Metabolism-related dominant species were annotated according

to the microbial niches in different layers to reveal the element recycling patterns.

DISCUSSION

In this study, we performed genome mining and predicted *in-situ* metabolic activities to classify prokaryotic microorganisms from different layers of the YBH, mainly revealing the microbial-mediated methane cycle in the YBH. The prokaryotes within YBH exhibited high novelty, especially in the anoxic zone, probably due to the accelerated evolution of anaerobic microorganisms in response to the nutrient-depleted, semi-closed environment in the special topological shape of YBH.⁴³ Previous studies have identified numerous novel species in the Amberjack blue hole.³ However, only three MAGs of YBH showed phylogenetically affinity to those of the Amberjack, much less than the Saanich Inlet and the Black Sea, which likely stemmed from the lack of a thick and stable anoxic layer in the Amberjack blue hole. This suggests that these novel microorganisms might have entered YBH from other marine anoxic regions and re-established their colonization in YBH following niche-specific evolution.⁴⁴

In the YBH water column, our results indicate existence of a complex network composed of carbon, nitrogen, and sulfur metabolic processes that were microbially mediated by the novel microbiomes (Figure 6). In this network, photosynthetic eukaryotic organisms such as algae and prokaryotic microorganisms like Cyanobacteria in the surface zone carry out photosynthesis, absorbing atmospheric CO₂ and converting it into organic carbon for other microbial inhabitants in the water column. In oxic layers, chemoautotrophy is primarily carried out by AOA species (*Nitrosopumilus* and *Nitrosopelagicus*) and SOB (SUP05), while in suboxic layers, it is primarily conducted by different SOB species (*Thiopontia* and *Thiomicrothrix*). In anoxic layers, dark carbon fixation is primarily performed by a diverse array of microorganisms, including representatives of Poribacteria, Chloroflexota, and Omnitrophota. The descending organic particles are remineralized to inorganic materials such as ammonia, CO₂, and methane at different depths of YBH.

Microbial methane production was predicted to occur at different oxygen gradients, with methane production being accomplished through the degradation of methylphosphonate (MPn) and dimethylsulfoniopropionate (DMSP) based on our "omics" work (Figure S10). Specifically, MPn degradation was only indicated to occur between 40–80 m of YBH, where phosphate concentration was undetectable and gradually increased in underlying layers⁴⁵ coupled with suppression of MPn microbial utilization. Only under conditions of inorganic phosphate limitation in water column, MPn serve as a phosphorus source for microbial growth, releasing methane as a byproduct.⁴⁶ In the oxygenated surface waters, our YBH metagenomes contained abundant genes related to DMSP metabolism, suggesting that DMSP was synthesized by phytoplankton. It has been recently discovered that DMSP can produce methane through degradation in oxic seawater.^{47,48} Algae in shallow water areas are the main producers of DMSP.^{18,49} It has been observed that a large number of diatoms are present at approximately 20 m in YBH,¹⁷ indicating their potential contribution to high DMSP levels. During summer algal blooms, more DMSP is delivered to the deep waters. In suboxic and anoxic zones, DMSP may be associated with particulate matter descending from the upper to the bottom layer.^{29,50} Microorganisms containing DMSP metabolism-related genes in the anoxic layers (Table S10) may metabolize DMSP, leading to the release of methane, which might result in further methane accumulation in the anoxic layers. The clear associations between CH₄ and DMSP suggest that DMSP may be a potential substrate of CH₄, not only in oxic surface seawater but also in deep water.⁴⁸ In natural seawater, most DMSP undergoes demethylation, and a small proportion is cleaved into DMS and acrylic esters.⁵¹ Furthermore, the encoding catalytic enzyme gene *mddA*, responsible for the conversion of MeSH to DMS,⁵² exhibits a high abundance in various water layers. Under anoxic conditions, DMS production is enhanced while its consumption is inhibited, resulting in intensified DMS emissions from the ocean into the atmosphere.^{29,53} This implies that the seawater in YBH may also contribute to DMS emissions, thereby participating in the global sulfur cycle and having a great impact on climate change. Further investigation of DMS gas flux is needed to verify this hypothesis. DMSP demethylation can also occur in anoxic seawater, but the rate is

notably lower compared to oxygenated seawater.⁵³ DMSP-dependent methane production has not been reported in anoxic seawater but has been observed exclusively in anoxic sediments.¹⁴ Although the gene *mcrA* that ultimately functions in methane generation has not yet been found, the presence of the *mtsA* gene in our related MAGs suggests the potential of methane production by these DMSP utilizing bacteria.³³ This enigmatic pathway may explain the methane source in anoxic YBH water bodies. Furthermore, our findings demonstrate the widespread presence of genes associated with DMSP metabolism in other open oceans, semiclosed water bodies, and blue holes, indicating the universality of the DMSP degradation pathway for methane production and accumulation in OMZ water. However, we could not show the DMSP concentrations across the YBH water column in this study.

Although methane production might occur in all water layers of YBH, the sharp increase in methane was only detectable below 160 m depth. This indicates anaerobic microbial consumption of methane in the overlying water column above 160 m. However, known methanotrophs such as NC10²⁰ and anaerobic methanotrophic archaea (ANME)⁵⁴ were not present in the communities based on our YBH metagenomes and 16S miTags. Instead, potential methanotrophic bacteria with the *pmoA* genes were identified in several MAGs, regardless of the lack of *pmoB* and *pmoC* genes. This is similar to the *pmoA*-bearing Verrucomicrobia found in an oxygen-limited bioreactor involved in methane oxidation with a probably novel pathway.⁵⁵ This can indicate that the hypothetical PmoA proteins and associated unknown genes in YBH may also perform methane oxidation under the suboxic and anoxic zones.

The electron acceptor is possibly trace amounts of oxygen that are generated by the AOA in the anoxic region in the collaborative process between *N. maritimus* SCM1 (AOA) and nitrite-oxidizing bacteria (NOB).⁴² The presence of AOA in the anoxic zone (Anoxic-bin7) and the close phylogenetic relationship to the known oxygen-producing lineage⁴² support this hypothesis; however, the gene involved in oxygen production was not identified in the MAG of this study. Alternatively, the methane-oxidizing bacteria produce oxygen for methane oxidation by disproportionating NO independently, similar to NC10 as methane-driven oxygenic denitrifiers.¹⁹ However, the key gene *nodF*⁶⁵ was also not found in the MAGs of these putative methane-oxidizing bacteria (Figure 5B). Cultivation of the novel microbes in YBH will help to answer the question.

The generated methane is predominantly consumed by the methane-oxidizing bacteria, resulting in the minimal release of this greenhouse gas to atmosphere. Initial investigations on methane flux at the water-vapor interface indicate that YBH acts as a natural source of methane, with released methane ranging from 12.3 to 17.4 μmol m⁻² d⁻¹.^{9,57} Based on the stabilization of methane concentration beyond 180 m, in conjunction with the methane concentration profile, we estimate that microbially mediated methane consumption by the Bacteroidota represented by Anoxic-bin38 solely contributed approximately 77% of the methane decline in the anoxic zone. Furthermore, the total cell count of the Bacteroidota MOB in this zone was estimated to be 2.14 × 10¹⁵ cells. Additionally, the other MOBs in the suboxic zone were responsible for ~22% of the methane concentration decline (Figure 6). Our estimates of total dissolved gases indicate that YBH is a huge methane sink, which is also true for other marine geographic depressions. Considering different rates of accumulation and consumption, methane emission from YBH varies in different seasons. This is related to oxygen supply in the photic zone and consumption by oxidation processes of reduced substrates in YBH. As such, the depth of the anoxic-oxic interface of YBH is an ideal indicator of methane emission here.⁵⁸ Therefore, systematic quantification of microbial ecological effects in YBH and similar depressions with different sizes and a similar topology is essential for understanding the carbon cycle in the marine water column and the potential greenhouse effect.

The presence of abundant sulfate-reducing bacteria in the anoxic zone utilizes sulfate to produce hydrogen sulfide, forming a stable sulfide layer in the approximately >140-m layers of the cave. As such, we speculate that the embedded methane in pores of the carbonate bedrock of YBH might be released due to acid erosion by H₂S in a high concentration,⁸ but further confirmation is required. Hydrogen sulfide overflows into the suboxic and upper anoxic zones, where it might be consumed by sulfur-oxidizing bacteria

that perform denitrification and CO₂ fixation (Figure 6). From the oxic to anoxic zone, a variety of prokaryotes might function at the carbon cycle.

This study utilized ribosomal fragments from metagenomic and metatranscriptomic data to conduct a comparative analysis with global databases, assessing microbial novelty. Based on the expression activity of genes related to element cycling, it revealed the microbially-mediated methane cycling in YBH with genomics and transcriptomics evidence. By combining geographical measurements with biogeochemical data, the study was able to more accurately assess the ecological role of microbial communities in methane cycling, particularly their contribution to methane consumption. This holds promising application value for estimating global greenhouse gas emissions and developing emission reduction strategies.

CONCLUSION

In this study, we investigated a range of prokaryotic microorganisms adapted to extreme suboxic and anoxic environments and demonstrated their involvement in carbon, nitrogen, sulfur, and other element cycles in YBH water column. We also revealed the cryptic methane cycle mediated by the unique microbial community of YBH with genomics and transcriptomics evidence, highlighting the crucial role of MPn and DMSP degradation in methane production, as well as the methane-consuming activity of anaerobic methanotrophic bacteria. These findings provide an important theoretical basis for the future cultivation and isolation of microorganisms that mediate methane cycle in YBH. More importantly, these provide valuable insights into the potential sources of methane in coastal areas and marine carbonate reef formations similar to YBH, and offer valuable perspectives on mitigating the climate change effects in marine environments.

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AUTHOR CONTRIBUTIONS

H.Z. and Y.W. conceived and designed the study. H.Z. conducted bioinformatic analyses and results visualization. H.Z., T.W. and J.Z. analyzed data and summarized the results. T.W. and Q.L. collected the samples and performed the experiments. L.H. and L.F. provided assistance for sample and environmental data collection. H.Z. drafted the manuscript. L.H. and Y.W. critically revised the manuscript. All authors contributed to and approved the manuscript.

DECLARATION OF INTERESTS

The authors declare no competing interests.

DATA AND CODE AVAILABILITY

The assembly data of metagenome and metatranscriptome sequencing data, MAG sequences, and the 16S miTags generated in this study have been deposited in the NCBI database under BioProject PRJNA900714.

SUPPLEMENTAL INFORMATION

It can be found online at <https://doi.org/10.59717/j.xinn-geo.2024.100082>

LEAD CONTACT WEBSITE

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