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# Methane Anaerobic Oxidation on Carbonate in Cold Seep Environments with High-Pressure Enrichment Cultivation

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**ABSTRACT**

The process of methane anaerobic oxidation coupled with sulfate reduction promotes alkaline conditions and the formation of carbonate minerals in cold seep environments. The existing studies on microbial communities inhabiting carbonate rock surfaces in cold seep environments primarily revolve around in situ investigations. In this study, high-pressure enrichment cultivation was conducted using methane as the sole carbon source, and periodic monitoring of community dynamics was performed. The results revealed a selective increase in the abundance of the ANME-2c group, which is a typical type of microorganism in methane utilization, anaerobic oxidation, and the synthesis of other nutrients. Additionally, temperature was identified as a crucial factor influencing microbial populations and carbonate processes. Higher temperatures stimulated microbial metabolism, resulting in the production of acidic substances and extracellular hydrolytic enzymes, potentially leading to carbonate dissolution. Conversely, lower temperatures had minimal impact. These findings reveal the significance of methane metabolism and temperature in microbial community dynamics and carbonate kinetics in deep-sea environment.

**Keywords:** Cold seep, Anaerobic oxidation of methane, Carbonate, prokaryotic communities

## 1. INTRODUCTION

The fluxes of oceanic methane to the atmosphere are reduced by the sulfate-dependent anaerobic oxidation of methane (AOM), which is the primary methane sink in the ocean [1]. AOM driven by sulfate generates two units of alkalinity (Alk) per unit of dissolved inorganic carbon (DIC), thereby increasing the saturation state of sedimentary pore water relative to calcium carbonate. $CH_4 + SO_4^{2-} \leftrightarrow HCO_3^- + HS^- + H_2O$, Alk = + 2, ΔDIC = + 1. $2HCO_3^- + Ca^{2+} \leftrightarrow CaCO_3$ (s) + $CO_2$ (aq) + $H_2O$, ΔAlk = − 2, ΔDIC = − 1. Based on conventional reaction equations, this AOM process can be inferred to promote the precipitation of authigenic carbonate. Approximately 10-20% of the methane oxidized by AOM in cold-seep environments is incorporated into authigenic deposits [2, 3]. It is estimated that these deposits account for 11-15% of carbonate accumulation in continental shelf sediments annually (11-15 Tmol yr-1) [4].

Authigenic carbonate deposits on the seafloor serve as a geologically stable phase for carbon sequestration. They are commonly associated with seep environments and AOM, exhibiting variations in form, size, and mineralogical characteristics. Examples include colluvium [5], nodules [6], massive chemosynthetic structures extending into the water column [7], and extensive bedding covering hundreds of square meter [8]. Authigenic carbonates and nodules are frequently observed within sedimentary columns or at the sediment/water interface [9]. However, modern and ancient methane seep carbonates often display signs of seafloor dissolution, such as pits, holes, and micro-etching on exposed surfaces. These features have been attributed to aerobic methane oxidation, sulfur oxidation, or both [10]. Altered surfaces are commonly found alongside bacterial mats that contain sulfur-

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oxidizing bacteria at the sediment/water interface. Authigenic carbonates and nodules not only provide habitats for microbial assemblages but also host metabolically active methanotrophic populations that play a significant role in methane oxidation within seepage areas [11].

In this study, we conducted research on carbonate samples obtained from cold seep environments in the South China Sea. To explore the microbial community composition on the surfaces of these carbonate, as well as to determine whether they support the enrichment of anaerobic methane-oxidizing bacteria, we simulated in-situ pressure conditions in the laboratory to encourage the growth of microorganisms. Additionally, we investigated the dynamics of microbial communities at different temperature.

## 2. MATERIAL AND METHODS

### *2.1 Material and methods*

Carbonatite samples were collected from a cold seep in the South China Sea and preserved under low-temperature conditions. These samples were then mixed with an artificial medium and transferred to a high-pressure-resistant culture bioreactor. The air in the bioreactor was removed using a vacuum pump and flushed with nitrogen gas three times. Methane was added to pressurize the bioreactor to 11 MPa. The bioreactor was divided into two groups and incubated at constant temperatures of 4 ℃ and 30 ℃ respectively, with each sample undergoing three parallel experiments. The medium used for cultivation consisted of various components such as NaCl, $MgCl^{2-}\cdot H_2O$, $CaCl^{2-}\cdot 2H_2O$, $Na_2SO_4$, $NH_4Cl$, $KH_2PO_4$, KCl, bicarbonate solution, vitamin mixture solution, trace element solution, thiamine solution, and vitamin B12 solution, with the pH adjusted to 6.8 using sulfuric acid. Throughout the experiment, periodic sampling was conducted under constant pressure to monitor changes in the microbial community. DNA extraction from the samples was performed using Magnetic Bead-Based Soil Genomic DNA Extraction Kit purchased from TIANGEN company, followed by PCR amplification of the extracted DNA using universal primers targeting the 16S rRNA V3~V4 region. The resulting PCR products were purified and sent for sequencing by PersonalBio company.

### *2.2 Data analysis*

Sequencing data processing was conducted using the QIIME2 software package, encompassing a series of essential steps. These steps involved the pairing of sequencing ends, trimming for high-quality reads, assessing and eliminating chimeric sequences, clustering ASVs, and discarding rare ASVs with relative abundance thresholds below 0.01%. The taxonomic classification of representative sequences was performed based on the training set generated from Silva 183 data, ensuring accurate annotations. Alpha diversity calculations and PCoA analysis based on Bray-Curtis dissimilarity distances between samples were performed using the vegan package within the R environment. Differential species abundance between groups was assessed utilizing the DESeq2 package. Visual representation of species composition proportions in samples, as well as other graphical illustrations, was achieved through the utilization of ggplot2.

## *3. RESULTS*

According to the results of 16S rRNA sequencing, the microbial community on the carbonate surfaces of the in-situ samples was predominantly composed of methane anaerobic oxidation archaea (ANME-2c and ANME-1b). Following enrichment cultivation within the conditions of high pressure and adequate methane supply, there was an increase in the proportion of ANME-2c group, which remained dominant, while the relative abundance of ANME-1b decreased (Fig 1). Additionally, certain samples exhibited an increased abundance of *Saltibacter* genus and Nitrincolaceae family, which are associated with nitrogen metabolism.

To explore the inter-group differences caused by temperature and cultivation duration, we employed DESeq2 for species-level differential analysis of samples obtained under different cultivation temperatures and durations. Temperature primarily affected the microbial species composition during the enrichment process, particularly the bacterial. For example, the abundance of *Psychrobacter* and *Polycyclovorans* significantly increased, while *Marinobacter* and *Tepidicaulis* significantly decreased (Fig 2A). Compared to the initial stage, the microbial composition after 22 days of cultivation exhibited a significant increase in ASV numbers within the *Idiomarina*, *Kapabacteriales*, and ANME-1a groups, while the ASV numbers within the ANME-2c and Clostridium significantly decreased (Fig 2B). Based on the previous stacked column chart, there was an increasing trend in the proportion of ANME groups, but according to statistical results, this increase was not significant.

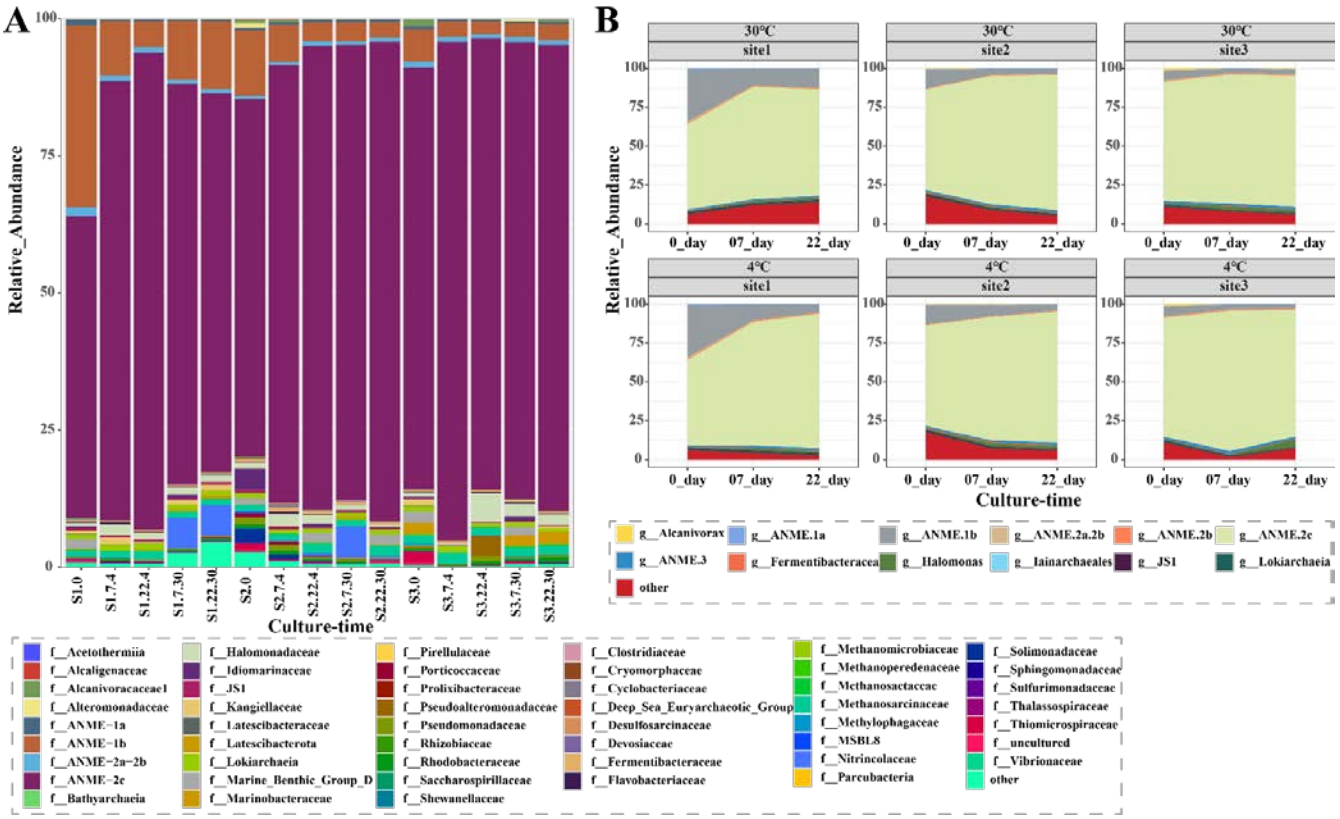


Fig 1 The temporal variations of microbial communities. Fig 1A presents a stacked bar chart depicting the relative abundance proportions at family, with the horizontal axis representing the cultivation time and different colors representing different family-level. Fig 1B demonstrates the temporal changes in dominant genera within the samples, different colors represent different genus-level.

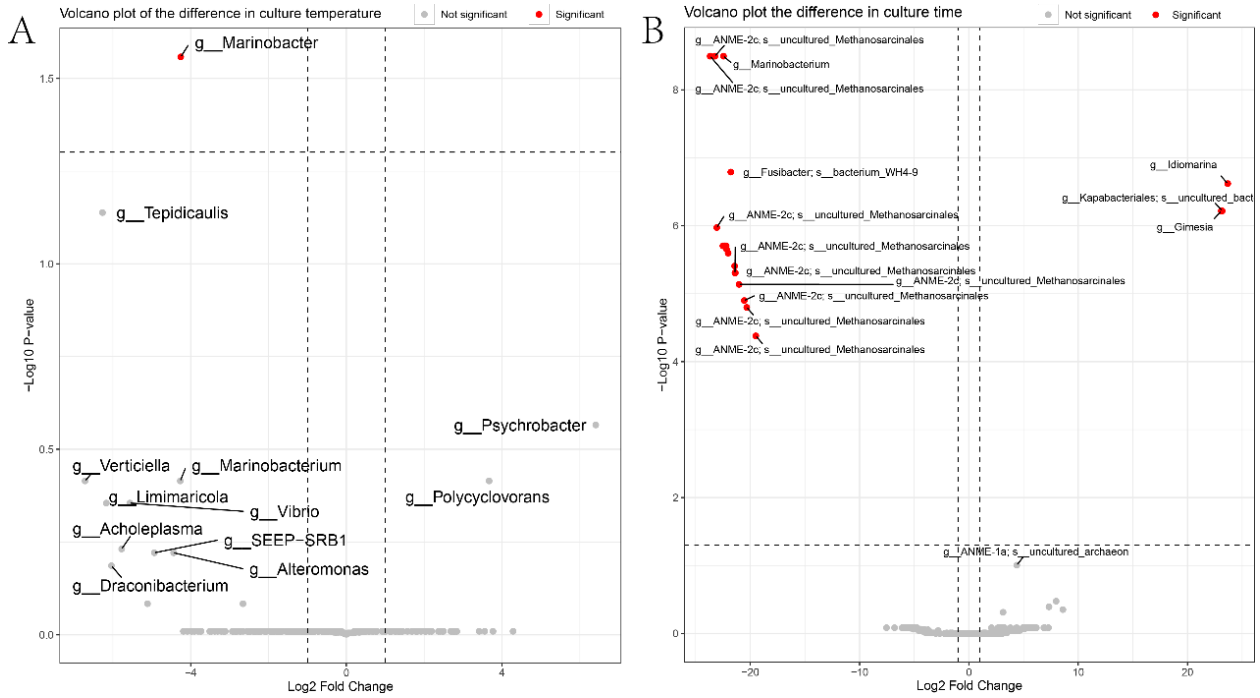


Fig 2. Volcano plots depicting significant differences in species between groups. Fig 2A represents intergroup differences at different cultivation times, Fig 2B represents intergroup differences at different temperatures.

## *4. DISCUSSION*

During the enrichment cultivation process, methane as the sole carbon source in the entire cultivation system selectively increased the abundance of ANME (anaerobic methane oxidation) groups. This is because the ANME groups contain genes involved in methane anaerobic oxidation, which can convert methane into carbon sources such as formic acid and methanol that contain methyl groups. These carbon sources can be further utilized by microorganisms for the synthesis of other nutrients. The process of methane anaerobic oxidation may be accompanied by the reduction of sulfate within the system, generating $HCO_3^-$ and $HS^-$ that shift the pH of the reaction system towards alkaline conditions. In an alkaline environment, the reaction between $HCO_3^-$ and $Ca^{2+}$ promotes the formation of carbonate minerals. The confirmation of this hypothesis requires further investigation through subsequent measurements to examine the changes in sulfate and calcium ion concentrations. In addition to the variations in ANME groups, an increase in the abundance of Nitrincolaceae was observed. On one hand, Nitrincolaceae may participate in the coupling of methane anaerobic oxidation with nitrate. On the other hand, Nitrincolaceae possesses most of the genes required for the synthesis of vitamin B12. In bacteria, a key reaction dependent on cobalamin (vitamin B12) is the synthesis of methionine from homocysteine, as the enzyme methionine synthase requires cobalamin. Therefore, the lack of this vitamin may hinder DNA synthesis, methionine regeneration, and result in the accumulation of homocysteine. The ability of Nitrincolaceae to synthesize vitamin B12 may be crucial for the majority of symbiotic dependency on it [12].

In the in vitro cultivation process under different environmental conditions, temperature has been identified as a primary parameter influencing the AOM rates of ANME-1 and ANME-2 consortia, in contrast to variations in sulfate concentration, pH, and salinity [13]. ANME-2/-3 archaea of marine origin are typically found in low-temperature environments. ANME-2 archaea primarily inhabit marine cold seeps and meso- to psychrophilic regions with temperatures ranging from 4 to 20°C, while ANME-3 archaea are predominantly associated with mud volcanoes and cold seeps. In comparison, ANME-1 archaea may exhibit compatibility with a broader range of temperatures. In this experiment, it was also observed that the ANME-2 consortium had a competitive advantage when cultured in low-temperature environments [14]. In addition to the shifts in ANME populations, temperature significantly affected the abundance of Psychrobacter and Tepidicaulis, which might be involved in acetate metabolism at different temperatures [15], thereby influencing the aceticlastic methanogenesis pathway. Temperature exerts not only an impact on microbial community abundance but also potentially affects the formation and dissolution of carbonate. At a temperature of 30℃, microbial metabolism exhibits heightened activity, leading to the production of metabolites, including organic acids that acidify the environment and certain extracellular enzymes with decomposition capabilities. Under the influence of microbial metabolism, carbonate may undergo subtle dissolution. Conversely, within a low-temperature environment of 4℃, only a fraction of psychrophilic microorganisms remains viable, resulting in a minor effect on carbonate.

## 5. CONCLUSIONS

In summary, the enrichment cultivation process using methane as the sole carbon source selectively increased the abundance of ANME groups. These ANME groups possess genes involved in methane anaerobic oxidation, facilitating the conversion of methane into carbon sources that can be utilized by microorganisms for the synthesis of other nutrients. Additionally, the process of methane anaerobic oxidation may lead to the reduction of sulfate, resulting in the generation of substances that promote alkaline conditions and the formation of carbonate minerals. Further investigation is required to confirm this hypothesis by measuring changes in sulfate and calcium ion concentrations. Furthermore, the temperature was identified as a crucial factor influencing the rates and competitiveness of specific microbial populations, such as ANME-2 archaea and species like *Psychrobacter* and *Tepidicaulis*. Microbial metabolism at higher temperatures stimulates the production of organic acids and enzymes, potentially leading to the dissolution of carbonate, while lower temperatures have a minor effect on carbonate due to the limited viability of psychrophilic microorganisms. These findings emphasize the significance of temperature in both microbial community dynamics and carbonate processes.

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**REFERENCE**

[1] J.J. Marlow, J.A. Steele, W. Ziebis, A.R. Thurber, L.A. Levin, V.J. Orphan, Carbonate-hosted methanotrophy represents an unrecognized methane sink in the deep sea, Nat Commun, 5 (2014) 5094.

[2] T.H. Naehr, P. Eichhubl, V.J. Orphan, M. Hovland, C.K. Paull, W. Ussler, T.D. Lorenson, H.G. Greene, Authigenic carbonate formation at hydrocarbon seeps in continental margin sediments: A comparative study, Deep Sea Research Part II: Topical Studies in Oceanography, 54 (2007) 1268-1291.

[3] X. Sun, A.V. Turchyn, Significant contribution of authigenic carbonate to marine carbon burial, Nature Geoscience, 7 (2014) 201-204.

[4] S.A. Akam, R.B. Coffin, H.A.N. Abdulla, T.W. Lyons, Dissolved Inorganic Carbon Pump in Methane-Charged Shallow Marine Sediments: State of the Art and New Model Perspectives, Frontiers in Marine Science, 7 (2020) 206.

[5] N.O. Jørgensen, Methane-derived carbonate cementation of marine sediments from the Kattegat, Denmark: Geochemical and geological evidence, Marine Geology, 103 (1992) 1-13.

[6] W. Ussler, C.K. Paull, Rates of anaerobic oxidation of methane and authigenic carbonate mineralization in methane-rich deep-sea sediments inferred from models and geochemical profiles, Earth and Planetary Science Letters, 266 (2008) 271-287.

[7] S.B. Gulin, G.G. Polikarpov, V.N. Egorov, The age of microbial carbonate structures grown at methane seeps in the Black Sea with an implication of dating of the seeping methane, Marine Chemistry, 84 (2003) 67-72.

[8] A. Boetius, E. Suess, Hydrate Ridge: a natural laboratory for the study of microbial life fueled by methane from near-surface gas hydrates, Chemical Geology, 205 (2004) 291-310.

[9] A. Haas, J. Peckmann, M. Elvert, H. Sahling, G. Bohrmann, Patterns of carbonate authigenesis at the Kouilou pockmarks on the Congo deep-sea fan, Marine Geology, 268 (2010) 129-136.

[10] W.-J. Cai, F. Chen, E.N. Powell, S.E. Walker, K.M. Parsons-Hubbard, G.M. Staff, Y. Wang, K.A. Ashton-Alcox, W.R. Callender, C.E. Brett, Preferential dissolution of carbonate shells driven by petroleum seep activity in the Gulf of Mexico, Earth and Planetary Science Letters, 248 (2006) 227-243.

[11] J.J. Marlow, J.A. Steele, W. Ziebis, A.R. Thurber, L.A. Levin, V.J. Orphan, Carbonate-hosted methanotrophy represents an unrecognized methane sink in the deep sea, Nature Communications, 5 (2014) 5094.

[12] T. Zvi-Kedem, S. Vintila, M. Kleiner, D. Tchernov, M. Rubin-Blum, Metabolic handoffs between multiple symbionts may benefit the deep-sea bathymodioline mussels, ISME Commun, 3 (2023) 48.

[13] K. Nauhaus, T. Treude, A. Boetius, M. Krüger, Environmental regulation of the anaerobic oxidation of methane: a comparison of ANME-I and ANME-II communities, Environ Microbiol, 7 (2005) 98-106.

[14] A. Boetius, T. Holler, K. Knittel, J. Felden, F. Wenzhöfer, The Seabed as Natural Laboratory: Lessons From Uncultivated Methanotrophs, in: S.S. Epstein (Ed.) Uncultivated Microorganisms, Springer Berlin Heidelberg, Berlin, Heidelberg, 2009, pp. 293-316.

[15] E.M. Prem, B. Stres, P. Illmer, A.O. Wagner, Microbial community dynamics in mesophilic and thermophilic batch reactors under methanogenic, phenyl acid-forming conditions, Biotechnol Biofuels, 13 (2020) 81.