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In situ cultivation of deep-sea water with bicarbonate fueled a different microbial community

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Abstract

Some deep-sea microbes may incorporate inorganic carbon to reduce CO_2 emission to upper layer and atmosphere. How the microbial inhabitants can be affected under addition of bicarbonate has not been studied using *in situ* fixed and lysed samples. In this study, we cultivated 40 L natural bottom water at ~1 000 m depth with a final concentration of 0.1 mmol/L bicarbonate for 40 min and applied multiple *in situ* nucleic acids collection (MISNAC) apparatus for nucleic acids extraction from the cultivation. Our classification result of the cultivation sample showed a distinct microbial community structure, compared with the samples obtained by Niskin bottle and six working units of MISNAC. Except for notable enrichment of *Alteromonas*, we detected prevalence of *Asprobacter*, *Ilumatobacter* and Saccharimonadales in the cultivation. Deep-sea lineages of Euryarchaeota, SAR406, SAR202 and SAR324 were almost completely absent from the cultivation and Niskin samples. This study revealed the dominant microbes affected by bicarbonate addition and Niskin sampling, which suggested rapid responses of deep-sea microbes to the environmental changes.

Key words: carbon fixation, MISNAC, CO_2, *Asprobacter*, community structure

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1 Introduction

The extra emission of CO_2 since industrial revolution is raising mounting concerns on global climate change and disastrous impact on marine ecosystem. Although deep-sea microorganisms were proposed to be critical in assimilation and sequestration of CO_2, deposit of extra CO_2 to deep-sea zone can lead to ecological detrimental impacts (Seibel and Walsh, 2003; Worden et al., 2015). Therefore, it is still controversial for the approach to geological sequestration of CO_2 for storage in the deep ocean.

The oligotrophic deep zone of marine water column is lack of labile organics due to high consumption rate in upper water column (Calleja et al., 2019). As the major autotrophic inhabitants, ammonia-oxidizing archaea (AOA) and nitrite oxidizing bacteria carry out the majority of labile organic carbon production for the deep-sea ecosystem (Zhang et al., 2020). In 2005, *in situ* carbon fixation conducted in 4 000 m depth in the Atlantic Ocean revealed 5- to 10-fold higher carbon fixation rate by Bacteria than by Archaea (Varela et al., 2011). Nitrospirae were estimated to fix 15%–45% of deep ocean CO_2 using catalyzed reporter deposition fluorescence *in situ* hybridization (CARD-FISH) method (Pachiadaki et al., 2017). These studies confirm the linkage of nitrification and carbon fixation in deep waters for labile carbon supply (Wang et al., 2019b; Zhang et al., 2020). Nevertheless, heterotrophic bacteria such as *Alteromonas* could also incorporate ^{14}C-labeled bicarbonate through anaplerotic reactions as demonstrated by metaproteomics in the bottom of the Mediterranean Sea (Yakimov et al., 2014). It was then estimated that bicarbonate assimilation was 396–873 mg/(m^2·d) (according to carbon) to support a microbial consortium composed mainly of ammonia-oxidizing *Nitrosopumilus* and heterotrophic Proteobacteria (La Cono et al., 2018). However, this *in situ* cultivation study was performed in Niskin bottles where the nutrients and oxygen were depleted gradually due to isolation from the natural water. The microbial communities in Niskin samples were found to be different from those in the samples obtained by *in situ* filtration and fixation apparatus (ISMIFF) (Wang et al., 2019a). Aside from environmental alterations by addition of inorganic carbon, dissolved oxygen and trace minerals would descent and ^{14}C-labeled organic carbons had probably been circulated among the organisms in hours of cultivation (La Cono et al., 2018). *In situ* conditions of deep-sea cold seep and hydrothermal vent may be simulated in bioreactors and the CO_2 fixation rate of dwelling microbes can be estimated for their contribution of CO_2 emission reduction (McNichol et al., 2018; Leprich et al., 2021).

Multiple *in situ* nucleic acids collection (MISNAC) apparatus was recently invented to obtain nucleic acids from *in situ* filtered and lysed microbes (Wei et al., 2020b). It may be used for collection of microbes from *in situ* cultivation tank and subsequent genomic DNA and RNA extraction. This is expected to avoid the impact of temperature and pressure changes on microbial com-

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munity structural alteration in a Niskin bottle during a deep-sea water sampling. Water samples collected by Niskin bottles were widely used in the current marine microbial research. Not only temperature and pressure of the deep-sea samples cannot be maintained, but also the exposure to air during water filtration will affect the microbial activity. In this study, we used MISNAC to obtain nucleic acids from *in situ* cultivation with bicarbonate in the South China Sea (SCS). Our result showed microbial structural shift in the cultivation and Niskin samples, compared with MISNAC samples. *Altermonas*, rather than nitrifying bacteria and AOA, was enriched in the cultivation along with several genera that have been identified in CO_2 assimilating bioreactors, suggesting the deep-sea low-abundant and rare species were potential CO_2 assimilators in the dark ocean.

2 Data and methods

2.1 *In situ cultivation and water sample collection*

Three deep-sea deployments of "Phoenix" lander equipped with MISNAC were conducted in May, 2019. The sampling started 30 min after the landing of the lander, and video record showed clearance of turbidity plume by bottom water current. All the sampling tools and sensors were at least one meter over the seafloor. Six working units of MISNAC in "Phoenix" deployment No. 21 (hereafter FH21-5 to FH21-10) were used to filter bottom waters for about 1 h and *in situ* collect nucleic acids at sites in depth of about 1 000 m in the SCS (17°31.337′N, 110°27.522′E) (Table 1 and Fig. S1). Before the start of the fourth working unit, a plastic bag was filled up with 40 L sea water at speed of 500 mL/min for 80 min by an ISMIFF. The bag had been carefully cleaned with 1 L 70% ethanol and subsequently distilled water to avoid contamination before the experiment. Approximate 40 mL 100 mmol/L $NaHCO_3$ was injected into the bag for cultivation of 40 min. Such $NaHCO_3$ concentration would not result in precipitation at 4℃. The seventh working unit was initiated after the cultivation to filter the microbes from the cultivation in the plastic bag on a 0.22 μm membrane (Millipore, Bedford, MA, USA) in the filtration chamber, followed by cell lysis, nucleic acids precipitation and collection controlled by MISNAC (Fig. 1). After the lander was recalled on board, the polymer columns of seven working units were immediately collected and washed with distilled water by centrifuge at 4 000 r/min (Xiangrui, Changsha, China). The quality and quantity of the nucleic acids were measured by Qubit 2.0 fluorometer before storage of the extractions in −20℃ on board until further processing. A 10 L Niskin bottle was used to collect bottom waters at the same sites in "Phoenix" deployments No. 18 and No. 22 (FH18-N; FH22-N). Ten liter water samples were filtered through 0.22 μm membrane (Millipore, Bedford, MA, USA) immediately in the laboratory of the vessel. The membranes were stored in −20℃.

A CO_2 sensor (Pro-Oceanus, Bridgewater, NS, Canada) and conductivity, temperature and depth instrument (Sea-Bird, Bellevue, WA, USA) carried by the lander were used to monitor environmental changes during the deployments.

2.2 *High-throughput sequencing and analyses of 16S rRNA gene amplicons*

Genomic DNA of Niskin samples was extracted from the filtration membranes using MO BIO Powersoil DNA isolation kit (Qiagen, Carlsbad, CA, USA). The quality and quantity of the extractions were checked by Qubit 2.0 fluorometer. RNA in the nucleic acids collected by MISNAC was digested with RNaseA (TaKaRa, Dalian, China) with a final concentration of 0.1 ng/μL for 1 h in 37℃ incubation. The V3–V4 regions were targeted to amplify 16S rRNA gene fragments with a set of universal primers 341F (5′-CCTAYGGGRBGCASCAG-3′) and 802R (5′-TACNVGGGTATCTAATCC-3′) (Wang and Qian, 2009) using 1 ng DNA as template. The PCR conditions of 16S rRNA amplification were as following: initial denaturation at 98℃ for 10 s; 30 cycles of denaturation at 98℃ for 10 s, annealing at 50℃ for 15 s, and extension at 72℃ for 30 s; and a final extension at 72℃ for 5 min. The amplicons were used for library preparation with TruSeq Nano DNA LT kit (Illumina, San Diego, USA) and were then sequenced on an Illumina Miseq platform for 2×300 bp paired-end sequencing (Illumina, San Diego, USA).

The sequencing data of 16S rRNA gene amplicons were evaluated using FASTQC (v0.11.8) (http://www.bioinformatics.babraham.ac.uk/projects/fastqc/) and merged by join_paired_ends.py integrated in QIIME 1 workflow (Caporaso et al., 2010). The adaptors and low-quality reads were removed by NGS QC Toolkit (v2.3.3) (Patel and Jain, 2012). The sub-libraries for barcoded amplicon reads were trained by emp in QIIME 1 to standardize the original files into a format for QIIME 2 processing (Bolyen et al., 2019). The deblur method was employed for accusation process. All the data were merged using merge model in QIIME 2. Amplicon sequence variants (ASVs) were selected at 97% similarity between the qualified reads. Taxonomic classification of the ASVs was accomplished with Vsearch v2.15.0 (Rognes et al., 2016) at 97% similarity against with SILVA database (132) (Quast et al., 2013). Principal Coordinates Analysis (PCoA) of the microbial communities at genus level was performed using vegan in R package (Dixon, 2003). The raw data of amplicon reads were deposited in NCBI database under accession number of PRJNA-734086.

2.3 *Construction of phylogenetic tree*

The representative sequence of an ASV was used for construction of a maximum-likelihood phylogenetic tree. Reference

Table 1. Information of sampling methods and sites

Sample	Method	Deployment	Depth/m	Latitude	Longitude	Time
FH18-N	Niskin	FH18	1 069	17°31.203′N	110°25.880′E	2019. 5. 21 12:30
FH22-N	Niskin	FH22	1 048	17°31.337′N	110°27.522′E	2019. 5. 24 17:30
FH21-5	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 22 22:00–23:00
FH21-6	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 22 23:30–1:30
FH21-7	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 23 2:00–3:30
FH21-8	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 23 4:00–5:00
FH21-9	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 23 5:30–6:30
FH21-10	MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 23 7:00–8:00
FH21-C	Cultivation+MISNAC	FH21	1 030	17°30.729′N	110°25.880′E	2019. 5. 23 8:00–10:00

Note: MISNAC means multiple *in situ* nucleic acids collection.

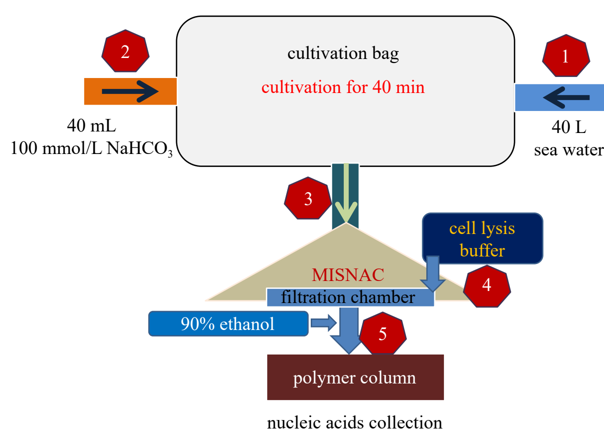

Fig. 1. Diagram illustrating deep-sea *in situ* cultivation of microbes with bicarbonate. The cultivation was started with pumping about 40 L deep-sea water into a plastic bag, followed by injection of 40 mL 100 mmol/L $NaHCO_3$. After 40 min, the water was filtered thought a working unit of multiple *in situ* nucleic acids collection (MISNAC). The microbes on the membrane in the filtration chamber were broken by lysis buffer. The nucleic acids in cell lysate were precipitated by ethanol and collected by a polymer column.

16S rRNA sequences of the closest relatives were downloaded from NCBI. All the sequences were aligned with MAFFT (v.7.407) (Katoh and Standley, 2013) and optimized with trimAl (v.1.4) (Capella-Gutiérrez et al., 2009). RAxML (v.8.2.12) (Stamatakis, 2014) was used to build a 16S rRNA phylogenetic tree (GTRGAMMA model) with bootstrap values based on 1 000 replicates. The phylogenetic tree was visualized by iTOL (Letunic and Bork, 2007).

3 Results and discussion

3.1 *Environment and in situ cultivation*

The temperature of the sampling site and bicarbonate cultivation was about 4.4℃ and dissolved CO_2 was $(984±169)×10^{-6}$ ((22.36±3.84) μmol/L) of the deep-sea sites in May of 2019. The depth of the site for MISNAC sampling and cultivation was 1 030 m. Genomic DNA of the deep-sea microbes was collected by MISNAC and Niskin bottle, separately. Six working units (FH21-5 to FH21-10) were used to collect nucleic acids from 10:00 pm of May 22 to 8:00 am of 23, each lasting one hour to filter about 30 L water. Two samplings by Niskin bottle (FH18-N and FH22-N) were carried out on May 21 and 24, respectively. *In situ* cultivation with addition of $NaHCO_3$ at a final concentration of 0.1 mmol/L was conducted within a 40 L plastic bag (FH21-C), followed by *in situ* nucleic acids extraction in the last working unit of MISNAC (Fig. 1). A part of the HCO_3^- would be precipitated by Ca^{2+} and Mg^{2+} ions in the natural sea water (Li and Tsui, 1971), while the extra HCO_3^- would change the equilibrium of CO_2 and HCO_3^-. In this study, we could not calculate the exact increase of CO_2 level in the cultivation bag, since a CO_2 sensor could not be set up in the cultivation bag. Considering the average dissolved CO_2 (~0.22 μmol/L) in the deep waters, the addition of 0.1 mmol/L HCO_3^- (if fully transformed to CO_2) might result in four-fold increment of CO_2 concentration in the cultivation.

3.2 *Microbial community in the deep-sea cultivation site*

Using Illumina high-throughput sequencing of 16S rRNA gene amplicons, we obtained 19 478 qualified amplicon reads for the nine samples (Table S1). For the cultivation sample FH21-C, 2 673 qualified reads were grouped into 245 ASVs. Rarefaction curves of the observed ASVs showed that the sequencing depth is not sufficient to capture all the species in the samples (Fig. S2), but the detection of microbial communities was not affected by the sequencing depth (see below). Chao1 as an estimate of biodiversity factor showed the lowest biodiversity of the cultivation sample (Table S1), suggesting the bicarbonate cultivation simplified the microbial consortia. The different samples (from FH21-5 to FH21-10) collected by working units of MISNAC demonstrated differences in diversity estimates, suggesting variations of microbial communities driven by deep-sea environmental changes such as oxygen and sinking organic matter flux. The microbial communities of this study differed notably from those in the samples collected by MISNAC in June–July of 2018 (Wei et al., 2020b). Particularly, the percentage of Proteobacteria and Euryarchaeota increased, while Nanoarchaeota and Omnitrophicaeota were also absent from the communities in this study, compared with the previous study conducted at the same area (Wei et al., 2020b). Our result indicates strong dynamics of deep-sea microbial communities in the deep-sea zones of the SCS.

The classification of the amplicon reads of the present study demonstrated the highest relative abundance of Proteobacteria in all the samples (Fig. S3 and Table S2). For the MISNAC samples, there were also high relative abundances of Euryarchaeota, Chloroflexi and Bacteroidetes, which were almost absent in the Niskin (FH18-N and FH22-N) and cultivation (FH21-C) samples. Variations in community structures were also exhibited among the MISNAC samples. Decrease of Proteobacteria and increase of Actinobacteria and Planctomycetes were obvious between PH21-6 and PH21-7, suggesting a microbial compositional change occurred within early morning time periods. Despite the small number of sequencing reads (<1 000) for PH21-5 and PH21-8, the microbial community structures of the two samples resembled those of other MISNAC samples.

At genus level, taxonomic classification showed enrichment of *Alteromonas*, *Hirschia*, *Nocardioides*, *Ilumatobacter* and *Saccharimonadales*. Most of these bacterial genera were also abundant in one of Niskin samples except for *Hirschia*. In contrast, some common marine genera such as SAR202, SAR406, Marine group II, and SAR324 in the MISNAC samples were dramatically low in microbial communities of the Niskin and cultivation samples (Fig. 2 and Table S3). These differences in community structures were further illustrated by PCoA, which showed clustering of MISNAC samples and a large separation distance between the Niskin and cultivation samples in 2-D plotting of axes 1 and 2 (Fig. 3). Rapid growth of autotrophic microbes stimulated by bicarbonate addition might result in lower proportion of the SAR202, SAR406 and SAR324 in the community. Limited accession to detrital organic matter and lowering oxygen for respiration for the degradation probably prohibited metabolism and growth of heterotrophic SAR202, SAR406 and SAR324 (Sheik et al., 2014; Huang and Wang, 2020; Wei et al., 2020a) in the Niskin and cultivation bag. The two Niskin samples were obtained at different time points and lander deployments (Table 1), which may partially explain the discrepancy in community structure. The genera of *Alteromonas* and *Pseudoalteromonas* accounted for 79.50% of the PN22-N sample, which agrees with the predominance of *Alteromonas* in some samples of marine water column (Quaiser et al., 2011; Li et al., 2019) and *in situ* ^{14}C-labeled bicarbonate cultivation in Niskin bottle (Yakimov et al., 2014). This is, however, due to a possible impact of environmental changes during the Niskin sampling and filtration onboard on

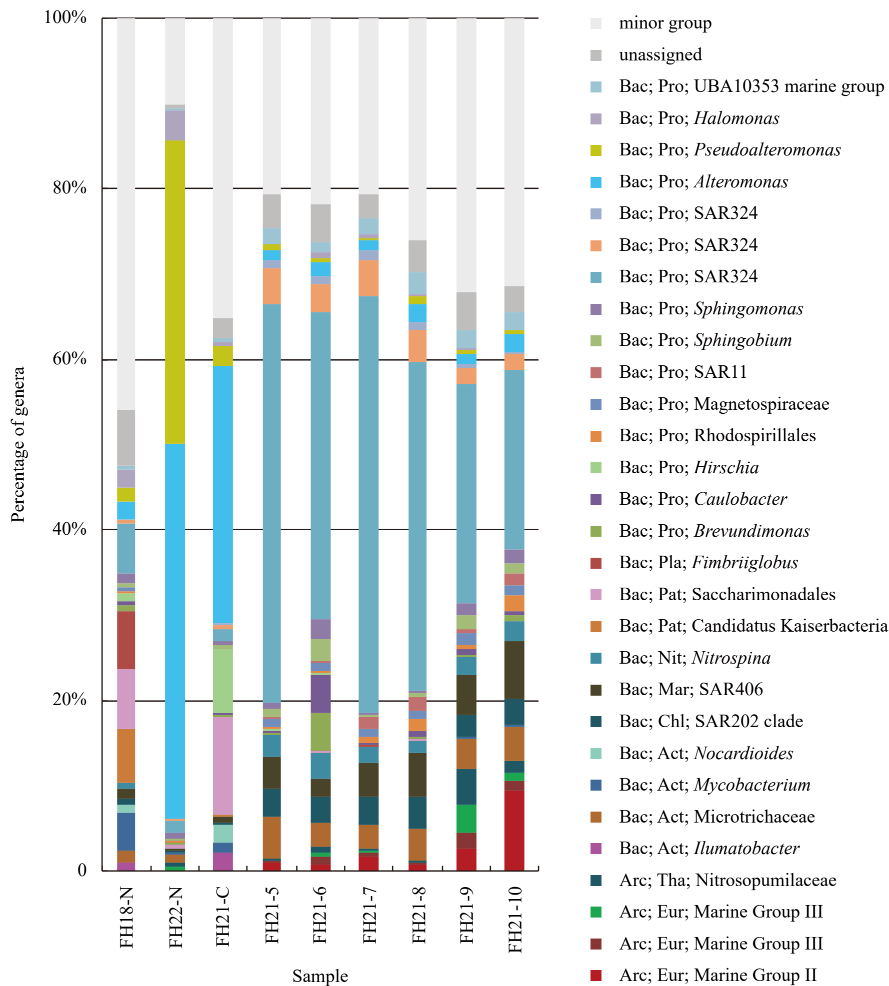

Fig. 2. Microbial structures at genus level. Taxonomic classification of amplicon sequence variants at 97% similarity was performed by comparing with references sequences of SILVA database (www.arb-silva.de/). The samples were referred to Table 1. The percentage and taxonomic names of the genera were shown in Table S2.

the microbial community. The closest relatives of the *Alteromonas* in the NCBI were *Alteromonas macleodii* (identity 98.68%; 99% length coverage) and *Alteromonas marina* (identity 98.68%; 99% length coverage). A previous study showed a deep-sea *Alteromonas* ecotype was adaptive to microaerophilic conditions (Ivars-Martinez et al., 2008). In this study, we did not monitor the changes of dissolved oxygen concentration in the Niskin bottle and cultivation bag, and therefore cannot preclude the possible causal effect of low oxygen on the prevalence of *Altermonas* in the Niskin and cultivation samples. The percentage of *Ilumatobacter* in the cultivation sample and FH18-N was 2.05% and 0.82%, respectively. The representative sequence of the most abundant *Ilumatobacter* ASV was 99.30% similar to the 16S rRNA gene of *Ilumatobacter fluminis* isolated from coastal sediment with an unknown ecological function (Matsumoto et al., 2009). The ASV representing Saccharimonadales of Patescibacteria could not be further classified in SILVA database and was abundant in both the cultivation and FH18-N (11.47% and 6.99%, respectively). However, a parasitic bacterium "*Candidatus* Mycosynbacter amalyticus" (96.92% similar to the representative sequence of the ASVs for Saccharimonadales) was recently identified in a bioreactor and had been used to control bacteria responsible for foam formation (Batinovic et al., 2021). An uncultured strain (GenBank accession LT856719: 99% identity), a relative of the Saccharimonadales bacteria in our cultivation, enables to convert CO_2 and organic substrates into useful chemicals in a bioreactor. There were also other Saccharimonadales relatives detected in deep sea zones. As these Saccharimonadales have been applied to industrial biosynthesis with CO_2, we propose that the Saccharimonadales bacteria in our study were probably fueled by addition of bicarbonate for the cultivation experiment (Batinovic et al., 2021). In Niskin bottle, respiration of some heterotrophic microbes using organic matter in deep water might have produced CO_2 to nourish Saccharimonadales in FH18-N. On the other hand, considering their possible parasitic lifestyle, the Saccharimonadales bacteria in our cultivation were likely prevalent along with enrichment of other bicarbonate-stimulated bacteria. In the MISNAC samples, Saccharimonadales occupied 0.06%–0.18% of the MISNAC samples, indicating at least 64-fold enrichment by the *in situ* cultivation. This remarkable discrepancy in microbial community structures between the Niskin and MISNAC samples raises the issue regarding reliability of deep-sea metagenomics depending on traditional sampling methods. For deep-sea hydrothermal vents, *in situ* sampling and filtration were preferred to display spatial and temporal variations of microbial inhabitants (Perner et al., 2009, 2013). Although deep-sea waters are not as extreme as hydrothermal vents with anoxic condition and high temperature, the present studies also showed temporal differences in microbial communities among MISNAC samples and between MISNAC and

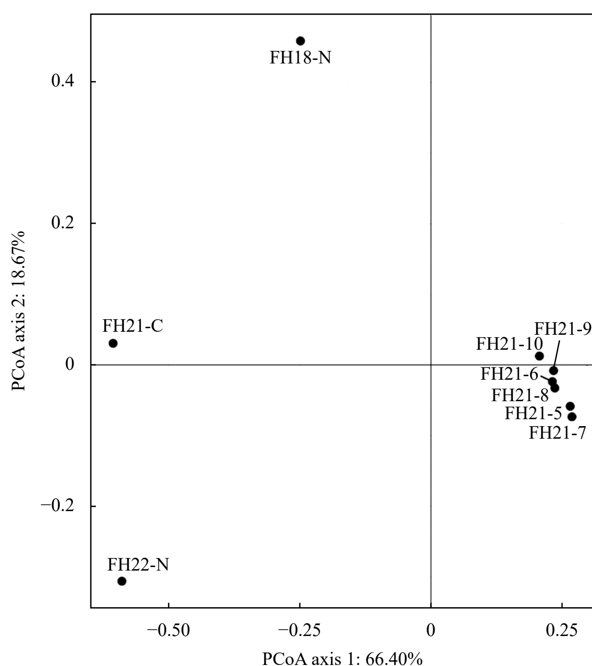

Fig. 3. Principal Coordinates Analysis (PCoA) analysis of microbial communities. The PCoA analysis was performed using the microbial communities at genus level. The samples were referred to Table 1.

Niskin samples. To capture the temporal variations of the microbial communities and determine the driving forces require future efforts for long-term *in situ* detection in deep ocean.

3.3 *Phylogenetic position of Hirschia-like species*

The relative abundance of *Hirschia* was 7.64%, much higher than that in other samples (0.9% in FH18-N and 0–0.07% in MISNAC samples) (Fig. 2). This suggests the *Hirschia* species was most likely activated and enriched by bicarbonate addition. However, the representative 16S rRNA amplicon of *Hirschia* ASV was only 91.98% to that of known species *Hirschia baltica.* To confirm the taxonomic assignment, we constructed a phylogenetic tree using the representative sequence of the *Hirschia* ASV and reference 16S rRNA sequences to determine its phylogenetic position. The tree topology exhibited that the *Hirschia* ASV was far from *Hirschia* species (Fig. 4) and stayed in an independent branch consisting of *Asprobacter aquaticus* and uncultured strains from forest soil, sludge, plant root and marine sediment. Although most of the strains within the *Asprobacter* clade were not isolated from marine environments, the most adjacent sequences were originated from marine hot spring and Baltic Sea sediment. Moreover, the ASV displayed a considerable phylogenetic distance with the *Asprobacter* relatives, indicating that the *Asprobacter* bacteria in this study had not been widely detected in deep waters, perhaps due to their low relative abundance in natural deep marine zones. The only cultivated species of the clade is *A. aquaticus.* High similarity (98.11%) of our *Asprobacter* ASV to that of *A. aquaticus* suggests that the *Asprobacter* of this study was probably a new strain of *A. aquaticus* (Edgar, 2018). *Asprobacter* as a novel genus in *Hyphomonadaceae* was proposed recently with respect to cultivation and characterization of *A. aquaticus* (Jin et al., 2017). However, currently our knowledge on *A. aquaticus* was restricted to its cell membrane component and aerobic respiration. How the *A. aquaticus* was stimulated by the bicarbonate incubation remains a question.

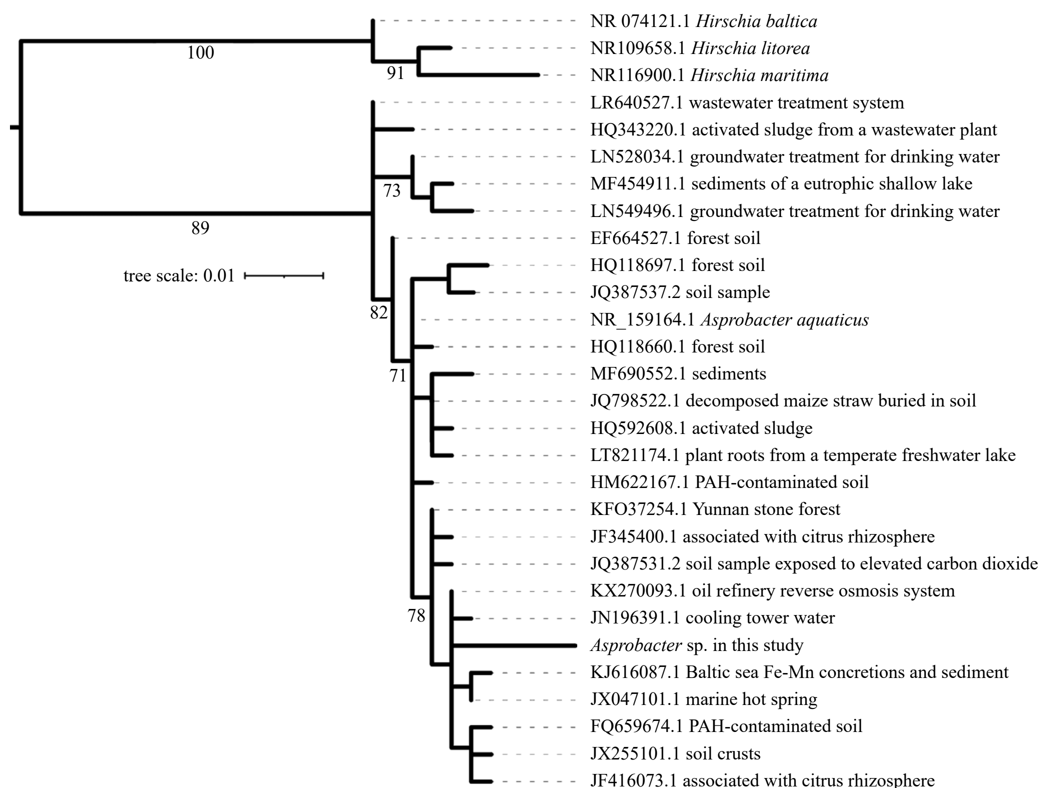

Fig. 4. Phylogenetic inference of *Asprobacter* sp. The phylogenetic tree was constructed with aligned 16S rRNA fragments using maximum-likelihood algorithm. The bootstrap values larger than 90 were shown on the branches. Species name or isolate source was next to accession number of the sequence in NCBI.

4 Conclusions

In this study, we demonstrated a distinct microbial community in the cultivation of bottom sea water with bicarbonate. *In situ* filtration and nucleic acids extraction avoided further compositional change of microbial community during upwards lifting and onboard filtration. The enrichment of rare species in deep-sea water indicates that the potential of CO_2 assimilation by microbial inhabitants was enormous. The rapid alteration of the *in situ* community suggests a rapid response of the microbes to environmental change. The present study only provides the result from one cultivation, and more *in situ* cultivation experiments will be performed to exbihit the changes of microbial structures across different time periods under different CO_2 concentrations in future. Metagenomics and cultivation of the cultivation in laboratory will allow to obtain the genomes and cultured strains of the deep-sea microbes for future work on their industrial applications using waste gases.

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Supplementary information:

Fig. S1. Sampling and *in situ* experimental sites. “Phoenix” lander was deployed on the continental slope at about 1 000 m depth in the western South China Sea. The three deployments FH18, FH21, and FH22 were adjacent to each other.

Fig. S2. Rarefaction curve of observed ASVs. The samples were described in Table 1.

Fig. S3. Microbial community structure at phylum level. The percentage of the phyla were shown in Table S2. The samples were referred to Table 1.

Table S1. Biodiversity estimates based on 16S rRNA gene amplicons sequencing.

Table S2. Relative abundance of orders in microbial communities.

Table S3. Relative abundance of phyla in microbial communities.

The supplementary information is available online at https://10.1007/s13131-021-1959-z and www.aosocean.com. The supplementary information is published as submitted, without typesetting or editing. The responsibility for scientific accuracy and content remains entirely with the authors.