Unique bacterial communities and lifestyles in deep ocean blue holes: Insights from the Yongle Blue Hole (South China Sea)

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Deep ocean blue holes possess steep physicochemical gradients, especially low
dissolved oxygen concentration, which shape the extraordinary microbial
communities. However, the environmental responses of microorganisms with
different lifestyles and knowledge of culturable microorganisms in the blue holes
are still unknown. Here, we investigated the bacterial community structure with
different lifestyles of the world's deepest blue hole - the Yongle Blue Hole (YBH) in
the South China Sea using both culture-dependent and -independent methods.
YBH can be divided by oxygen content into an oxic zone, a suboxic zone and two
anoxic zones. The abundance of bacteria, archaea, genes *dsrB* and *soxB* were all
higher in the free-living (FL) lifestyle than in the particle-associated (PA) lifestyle,
yet the diversity and richness of PA bacteria were higher than that of FL bacteria.
More *Gammaproteobacteria* and less *Alphaproteobacteria*, *Chloroflexi* and
Nitrospinae were observed within the FL fraction than within the PA fraction.
The relative abundance of sulfur-oxidizing bacteria (SOB) was dominant between
100-140 m (anoxic zone I) in YBH, with a maximum of 90.0% (140 m FL fraction).
The SOB in YBH were mainly colorless sulfur bacteria and purple non-sulfur
bacteria, of which *Thiomicrorhabdus* and *Sulfurimonas* were the main
representatives. In addition, a total of 294 bacterial strains were isolated on a
variety of media and culture conditions, and 22.2% (18/81) of anaerobic strains
were identified as potential novel species. Our study reveals a distinction between
FL and PA bacteria in YBH. It contributes to further understanding of the bacterial
community in deep ocean blue holes, and provides bacterial resources for
subsequent studies on their adaptation to extreme marine environments.

Introduction

A marine blue hole is a submerged marine cave, generally considered to be an underwater carbonate karst system with a large horizontal scale, found in all the world's ocean waters (Smart et al., 1988; Mylroie et al., 1995; Schwabe, 1999; Bishop et al., 2015). Most of the marine blue holes in the world are offshore and are the product of repeated global sea level rise and fall (Mylroie et al., 1995; Becking et al., 2011; Smith et al., 2011). The relatively isolated internal environment causes physical-chemical jumps in temperature, salinity, dissolved oxygen and hydrogen sulfide, and stable anoxic environments (Seymour et al., 2007; Iliffe and Kornicker, 2009; Pohlman, 2011). The blue hole is named for its dark blue color, which contrasts with the lighter colored waters surrounding it (Mylroie et al., 1995). The world's proven ocean blue holes include Yongle Blue hole (300.89 m) (Xie et al., 2019), Bahamas Dean's Blue Hole (202.08 m) (Wilson, 1994), Egypt Dahab Blue Hole (131.06 m) (Naumann et al., 2015) and Belize Great Blue Hole (124.05 m) (Schmitt et al., 2020).

The Yongle Blue Hole (YBH, also known as the Dragon Hole) is located between the reefs of Jinqing Island and Shiyu Island in Yongle Atoll, Xisha Islands, Sansha City. YBH is 300.89 m in depth and about 130 m in diameter (Xie et al., 2019), and its formation may date back to the Miocene (Li et al., 2018a; Han et al., 2020). Due to the barrier effect of the pycnocline, the vertical exchange of dissolved oxygen is blocked, which in turn leads to a sharp decrease in the dissolved oxygen content at about 80-100 m (Bi et al., 2018; Xie et al., 2019; Yao et al., 2020). Below the depth of 100 m, there is an anoxic state in YBH since it is semi-enclosed. In such an environment, the water body transforms from oxic to hypoxia and then to anoxic at a depth of nearly 200 m (Bi et al., 2018; Yao et al., 2020). Such unique and rare oxygen conditions provide an excellent natural laboratory for biogeochemical studies (Xie et al., 2019). The biological community structure and potential functions of YBH have been investigated, including the phytoplankton (Liu et al., 2019; Fu et al., 2020; Ge et al., 2020), zooplankton (Chen et al., 2018; Li et al., 2020b), archaeal (He et al., 2019) and bacterial components (He et al., 2019; He et al., 2020; Li et al., 2020a; Zhang et al., 2021), and most research focused on the diversity and function of bacteria.

Previous studies (Zhang et al., 2021) divided YBH into three zones according to the oxygen concentration, including the oxic zone (0-80 m), the hypoxic zone (80-100 m) and the anoxic zone (100-300 m). Although the water depth in YBH is only 300 m, several studies found multiple dramatic changes in the bacterial community structure from the surface to the bottom waters based on the high-throughput sequencing of 16S rRNA gene amplicon (He et al., 2019; He et al., 2020; Li et al., 2020a; Zhang et al., 2021). For the oxic zone, in addition to the overwhelmingly dominant *Pseudomonadota* (*Proteobacteria*), a relatively high abundance of *Bacteroidetes* was also found, with over 20% at 0 m and 20-50 m (Zhang et al., 2021). In the hypoxic zone, the abrupt increased in abundances of bacterial, archaeal and anammox 16S rRNA genes, and genes *nirS* and *dsrB* were found by qPCR, possibly due to the presence of more phytoplankton in this zone (He et al., 2019). Moreover, genes associated with carbon (methane for example), nitrogen and sulfur cycles appeared enriched in the hypoxic zone by metagenome

sequencing (He et al., 2020). In the anoxic zone, bacteria belonging to *Pseudomonadota* were dominant, especially at depths of 130-170 m and 270 m where the relative abundance even reached 97.0% (Zhang et al., 2021). At the genus level, *Thiomicrospira* and *Arcobacter* contributed significantly to the microbial community in the anoxic zone of YBH, with abundances of up to 96.1% and 31.5% at 270 m and 130-150 m, respectively (Zhang et al., 2021). Dissolved oxygen (DO), temperature, salinity, pH, sulfur and nutrient concentrations were considered to be the main environmental factors affecting the microbial community within YBH (He et al., 2019; He et al., 2020). Current results indicated that microbial communities in YBH shifted along depths and the environment in YBH shaped the unique microbial communities. In addition, many studies reported that free-living (FL) and particle-associated (PA) communities showed different responses to various environmental factors, resulting in diverse microbial communities (DeLong et al., 1993; Tarn et al., 2016; Mestre et al., 2017; Liu et al., 2020b). However, little is known about the microbial communities of FL and PA lifestyles in YBH. Therefore, it is hypothesized that microbial communities of FL and PA lifestyles may show different distribution patterns in YBH, and there are still a lot of unclassified microorganisms in YBH that could be explored using various culture conditions.

In this study, we comprehensively revealed the structure of the bacterial community within YBH at a fine scale combining cultivation-independent methods with traditional cultivation-dependent methods. On the one hand, high-throughput sequencing and quantitative PCR were used to study the composition and abundance of the bacterial communities of FL and PA lifestyles. On the other hand, a variety of media and cultural conditions were used for isolating diverse bacteria including sulfur oxidizing bacteria and anaerobic bacteria.

Methods and materials

Sample collection

Water samples were collected in mid-March 2017 with YBH (Yao et al., 2020). Briefly, due to the shallow water around YBH, the research vessel (Qiong Qionghai 03138) had to be moored in the lagoon at Yongle Atoll during the survey, approximately 1.7 km from YBH. There was a buoyant platform built on the water surface of YBH, and a winch was placed with a Niskin water collector (8 L, Keruiou, Tianjin) mounted for water sample collection. All water samples were collected on the buoyant platform and immediately transferred to the research vessel for subsequent processing. Water samples were taken at a total of 13 depths, including 0, 10, 40, 70, 80, 85, 90, 100, 120, 140, 170, 190 and 230 m within YBH. About 800 of ml seawater was filtered through 3 μm and 0.22 μm polycarbonate membranes (Millipore, USA) to obtain PA and FL fractions. The membranes were immediately frozen in liquid nitrogen and then stored on board at -20°C before being transferred to -80°C in the laboratory. Environmental factors, such as temperature, salinity, concentrations of dissolved oxygen (DO), NO_2^-, NO_3^-, NH_4^+ and H_2S, were determined on site as described previously (Yao et al., 2020). The environmental factors involved in this paper are presented

in Table S1. Unfiltered seawater was collected for bacterial culture experiments.

Based on previous studies (Yao et al., 2020) on the anoxic marine zones (AMZs) (Canfield et al., 2010; Garcia-Robledo et al., 2017; Zakem et al., 2020) and the oxygen minimum zones (OMZs) (Trouwborst et al., 2006; Moore et al., 2009), the water column within YBH in this study was divided according to the DO concentration, with 0-80 m being the oxic zone (DO≥3 mg/L), 80-100 m being the suboxic zone (0<DO<3 mg/L). Under 100 m was the anoxic zone, which can be further subdivided into two zones, that were 100-140 m being the anoxic zone I (DO below the lower detection limit of the sensor or assay but with markers of metabolic processes that can demonstrate the presence of oxygen, such as nitrate and small amounts of hydrogen sulfide) and below 140 m being the anoxic zone II (nitrate and nitrite were not available, but a significant amount of hydrogen sulfide can be detected, indicating that the DO was zero).

DNA extraction and quantitative PCR (qPCR)

The total genomic DNA was extracted from membranes following the methods described previously (Liu et al., 2015). The bacterial 16S rRNA gene, archaeal 16S rRNA gene, and genes encoding for dissimilatory sulfite reductase β subunit (dsrB) and SoxB, were amplified with Eub338F/Eub518R (Lane, 1991), 967F/1060R (Cadillo-Quiroz et al., 2006), DSRp2060F/DSR4R (Cabrera et al., 2006) and soxB1164F/soxB1446B (Krishnani et al., 2010), respectively. PCR reactions were carried out as follows: initial denaturation at 95℃ for 3 min followed by 35 cycles of 95℃ for 30 s, primer specific annealing temperature for 30 s and 72℃ for 30 s. The melting curve was then performed as follows: denaturing at 95℃ for 1 min, starting at annealing temperature in 0.5℃ increments and collecting the signal. A standard curve was generated using a plasmid containing the target gene. In addition to template DNA, each reaction included serial dilutions of the plasmid and a negative control to ensure that the qPCR assay was free from contamination and stable. All samples were run on the StepOne^{TM} real-time PCR system (Applied Biosystems, USA) and the data obtained were analyzed by the StepOne software (version 2.2).

High-throughput sequencing and analyses

Primers 338F (5'-ACTCCTACGGGAGGCAGCA-3') and 806R (5'-GGACTACHVGGGTWTCTAAT-3') were used to amplify the V3-V4 region of the bacterial 16S rRNA gene. Amplification was performed in triplicate for each sample in a reaction system containing 2 μl of 10X polymerase chain reaction (PCR) buffer, 2 μl of 2.5 mM dNTPs, 0.5 μl of each primer (5 μM), 0.2 μl of rTaq polymerase, 0.2 μl of bovine serum albumin and 10 ng of template DNA. The PCR reaction was 95℃ for 3 min, 25 cycles of 95℃ for 30 s, 55℃ for 30 s, 72℃ for 30 s and a final extension at 72℃ for 5 min. After the amplification, the triplicate PCR products were mixed for the downstream analysis. The PCR amplicons were purified using the AxyPrepDNA Gel Extraction Kit (Axygen, China) and quantified using the Quant-iT PicoGreen Double Stranded DNA Detector (Invitrogen, USA). The purified amplicons were pooled together at equimolar concentrations and sequenced on the Illumina PE 250 platform at Majorbio Bio-pharm Tech. Co., Ltd. (Shanghai, China). The raw data were merged and filtered for subsequent analyses. All screened reads were clustered into operational taxonomic units (OTUs) with a similarity cutoff of 97%. The taxonomy of each 16S rRNA gene sequence was analyzed by the RDP classifier against the SILVA v128 16S rRNA database with a 70% confidence level. Taxa assigned to sulfur-oxidizing bacteria (SOB) and sulfate-reducing bacteria (SRB) were based on the 16S rRNA gene sequencing data according to the taxonomy (Ghosh and Dam, 2009; Rosenberg et al., 2014). The raw data were deposited in the National Center for Biotechnology Information Short Read Archive database under the accession number SRP178367.

Enrichment cultures, isolation and identification of bacteria

Seawater from different depths were diluted in a serial dilution (10^{-1} to 10^{-5}), and aliquots (100 μl) of each dilution were spread on marine agar 2216E (MA) (Patrick, 1978) or marine R₂A (Gibbs and Hayes, 1988) plates, respectively. The inoculated plates were incubated at room temperature on board and at 20℃ in the laboratory. The numbers of colonies (colony forming units, CFU) were counted after 7 days' cultivation when most morphologically distinct colonies appeared on MA plates. Individual colonies were randomly selected and purified by streaking three times on MA or R₂A plates at 20℃. The purified isolates were stored at -80℃ in sterile 0.85% (w/v) NaCl supplemented with 15% (v/v) glycerol. The isolates were identified using 16S rRNA gene sequencing and the EzBioCloud server (Fu et al., 2018). For the enrichment of sulfur-oxidizing bacteria, seawater samples from 10, 110, 140 and 270 m were inoculated at 2% inoculum in serum bottles containing 50 ml liquid SPG medium (the components of media are available in Table S2) on board, respectively. Once the color of the culture changed from red to yellow or purplish-red, the subculture was performed using 2% inoculum for three times. After the enrichment, cultures were spread onto SPG plates for isolation and purification. For anaerobic bacteria, 1 ml water sample from 90, 130, 160 and 190 m was injected by syringe into an anaerobic flask containing 5 ml anaerobic marine broth 2216E (MB) on board, respectively. After incubation at 16℃ for one month, or the enrichment becoming turbid, subsequent purification and identification were carried out (Fu et al., 2018).

Statistical analysis

Alpha diversity indices, including Chao 1 and Shannon, were performed by Mothur (Schloss, 2020) to estimate the community richness and diversity. For beta diversity analysis, non-metric multidimensional scaling (NMDS) was performed based on Bray-Curtis dissimilarity. Differences in community structure between the two lifestyles were tested for significance by analysis of similarities (ANOSIM). Both NMDS and ANOSIM analyses were conducted

using the "vegan" package of R software (version 4.1.1) (Oksanen et al., 2013). Relationships between bacterial community structure and environmental factors were assessed by distance-based redundancy analysis (db-RDA), and were performed using Canoco software (version 5.0) (Šmilauer and Lepš, 2014) with a 9,999 Monte Carlo permutation test. Correlations between environmental factors and the abundance of functional genes were tested using Pearson correlation tests. The relative contributions of nutrition and environmental factors were estimated by Variance Partitioning Analysis (VPA) using the "vegan" package of R software (version 4.1.1) (Oksanen et al., 2013), with adjusted R^2 coefficients based on RDA. The relationship between the two lifestyles and environmental factors was tested using the "ade4" package of R software (version 4.1.1) (Dray and Dufour, 2007).

Neutral community model (NCM) (Sloan et al., 2006; Zhou and Ning, 2017) was used to assess the potential contribution of stochastic processes to the assembly of bacterial communities, which can be expressed simply as the following equation.

$$Nm = N*m$$

N described the metacommunity size, and it was the total abundance of all OTUs in each sample here. m quantified the migration rate at the community level. Nm was the product of N and m, quantifying the estimate of dispersal between communities. The calculations were performed using the "Hmisc", "minpack.lm" and "stats4" packages in R (version 4.1.1) (Chen et al., 2019). Statistical analyses were performed by SPSS version 24.0 (SPSS Inc., USA).

Results

Abundance, diversity and richness of bacteria

The abundances of bacterial and archaeal 16S rRNA genes (Figure 1A) in YBH were analyzed by qPCR. The trends in the abundance of 16S rRNA genes in FL (1.74×10^7-4.87×10^8 copies/L) and PA fractions (1.49×10^6-3.85×10^7 copies/L) were approximately the same. The abundance of bacteria remained essentially constant in the oxic zone, increased slightly in the suboxic zone, and reached the highest values in the anoxic zone I and decreased gradually in the anoxic zone II. The results for archaea were similar, with the difference that FL archaea had a substantial increase in the oxic zone, and the 16S rRNA gene abundance of FL and PA archaea were 8.36×10^3-2.09×10^6 copies/L and 9.65×10^2-1.76×10^4 copies/L, respectively.

For the 26 samples from 13 water layers, a total of 947,543 sequences were obtained after high-throughput sequencing. After rarefaction, the number of sequences in each sample was 27,593, and the coverage of all samples was above 99%. Divided by a threshold of 97% similarity, a total of 1,988 OTUs were generated. The indices for alpha diversity analyses are shown in Figures 1B, C and Figure S1, including estimators for community richness (OTU numbers, Chao 1, Ace), community diversity (Shannon, Simpson) and coverage. Generally, these indices revealed that the diversity and richness of the prokaryotic community in the PA lifestyle were moderately higher than that in the FL lifestyle but with no significant difference ($P>0.05$). In the vertical direction, the community richness and diversity remained
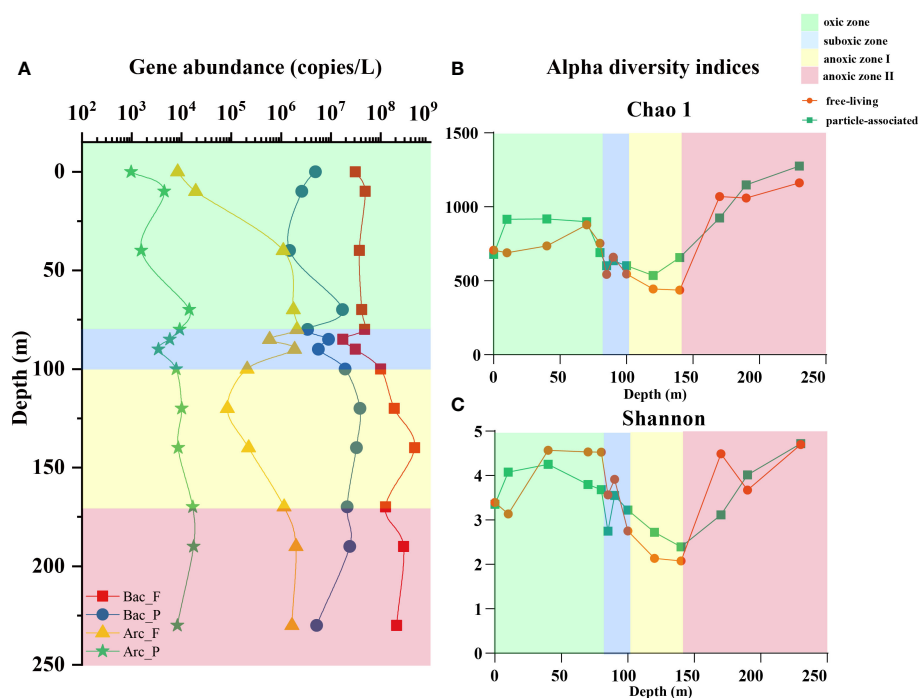
FIGURE 1
(A) The abundance of bacterial and archaeal 16S rRNA genes for free-living and particle-associated lifestyles. F, free-living lifestyle. P, particle-associated lifestyle. Alpha indices including **(B)** Chao 1 index and **(C)** Shannon index for bacteria in the two lifestyles. The orange dots represent free-living bacteria and the green squares represent particle-associated bacteria. Green background represents the oxic zone, the blue background represents the suboxic zone, the yellow background represents anoxic zone I, and the red background represents the anoxic zone II.

relatively stable in the oxic zone, then consistently decreased in the suboxic zone and anoxic zone I, and increased in the anoxic zone II.

In the NMDS analysis at the OTU level, the bacterial communities in YBH could be clearly distinguished according to the two lifestyles, FL and PA (ANOSIM, R^2 = 0.48, $P<0.05$; Figure 2A). As can be seen, along the first axis, the samples were well differentiated by DO concentration and could be clustered into oxic, suboxic and anoxic groups. At the same time, the two lifestyles within the same zone were separated. Furthermore, bacterial community structure varied significantly between lifestyles within each zone defined (ANOSIM, R^2 = 0.51 for the oxic zone; R^2 = 0.42 for the suboxic zone, R^2 = 0.60 for anoxic zone I; R^2 = 0.46 for anoxic zone II, respectively; $P<0.05$; Figure 2B).

Bacterial community structure

Although the depth of YBH was only 300 m, the community structure of bacteria showed substantial variations among depths. At the phylum/class level (for bacteria within the phylum *Pseudomonadota*, the individual classes were used for analysis), the most dominant taxon was *Gammaproteobacteria* for both PA (41.17%-86.62%) and FL fractions (16.88%-78.31%) across all depths, with a significantly higher proportion ($P<0.05$) in the PA lifestyle (Figures 3A, B). The oxic zone was dominated by *Gammaproteobacteria* and *Alphaproteobacteria*, with FL fraction having more *Alphaproteobacteria* and less *Gammaproteobacteria* compared to PA fraction ($P<0.01$). Additionally, more *Bacteroidetes*, *Chloroflexi*, SAR406 clade and *Actinobacteria* were observed among the FL fraction ($P<0.01$). There were a number of *Cyanobacteria* in the 10 m and 40 m samples for both lifestyles in this zone. As for the suboxic zone, the main distinction from the oxic zone was the emergence of *Campylobacterota*. In the anoxic zone I, the abundance of *Campylobacterota* increased in both lifestyles, while *Alphaproteobacteria* almost disappeared, and *Gammaproteobacteria* changed little compared to the oxic and suboxic zones. In the anoxic zone II, the diversity of microbial communities was much higher, and

Desulfobacterota, SAR406 clade and *Parcubacteria* were more abundant in the FL fraction. Meanwhile, there were more *Alphaproteobacteria* in the PA than in the FL lifestyle (all $P<0.01$).

The detailed results of the community structure at the genus level are presented in Figure 3C. In the oxic zone, the main taxa were *Alteromonas*, *Pseudoalteromonas* and *Alcanivorax*. Notably, *Rhodobacteraceae* (not assigned to the genus, 35.08%) and *Synechococcus* (14.27%) showed a high abundance in the FL fraction of 10 m layer and *Vibrio* (11.36%) in the PA fraction of 0 m layer, respectively. In the suboxic zone, it was mainly composed of *Alteromonas*, *Pseudoalteromonas*, *Alcanivorax* and *Oleiphilus*, while the abundance of the two genera *Thiomicrorhabdus* and *Sulfurimonas* belonging to SOB also began to increase. It was remarkable that the two SOB genera were significantly more abundant in PA fraction in this zone ($P<0.01$). However, in the anoxic zone I, there was a massive difference from the oxic and suboxic zones, which was dominant by two SOBs, *Thiomicrorhabdus* (56.90% for FL fraction and 46.30% for PA fraction) and *Sulfurimonas* (15.38% for FL fraction and 6.84% for PA fraction), and both taxa were more abundant in FL lifestyles ($P<0.01$). In the anoxic zone II, the abundance of these two SOB genera, *Thiomicrorhabdus* and *Sulfurimonas* decreased, while *Prosthecochloris* (SOB, ranked 33rd in relative abundance) and *Desulfatiglans* (SRB) showed an increasing trend. In addition, *Thiomicrorhabdus* was more abundant in PA lifestyle ($P<0.01$) and *Prosthecochloris* was more abundant in FL fraction ($P<0.05$).

The above analyses found that several taxa were associated with the sulfur cycle in YBH, and the abundance and composition of bacteria involved in sulfur metabolism were further analyzed. Among them, bacteria identified as SOB could be clustered into the four main categories, which were green sulfur bacteria (GSB), purple sulfur bacteria (PSB), purple non-sulfur bacteria (PNSB) and colorless sulfur bacteria (CSB). The results are shown in Figure 3D. The relative abundance of SOB varied dramatically among all 26 samples (3.83% in the 80 m PA sample to 89.99% in the 140 m FL sample), with the taxon distribution shifting considerably with depth. The SOB in YBH were
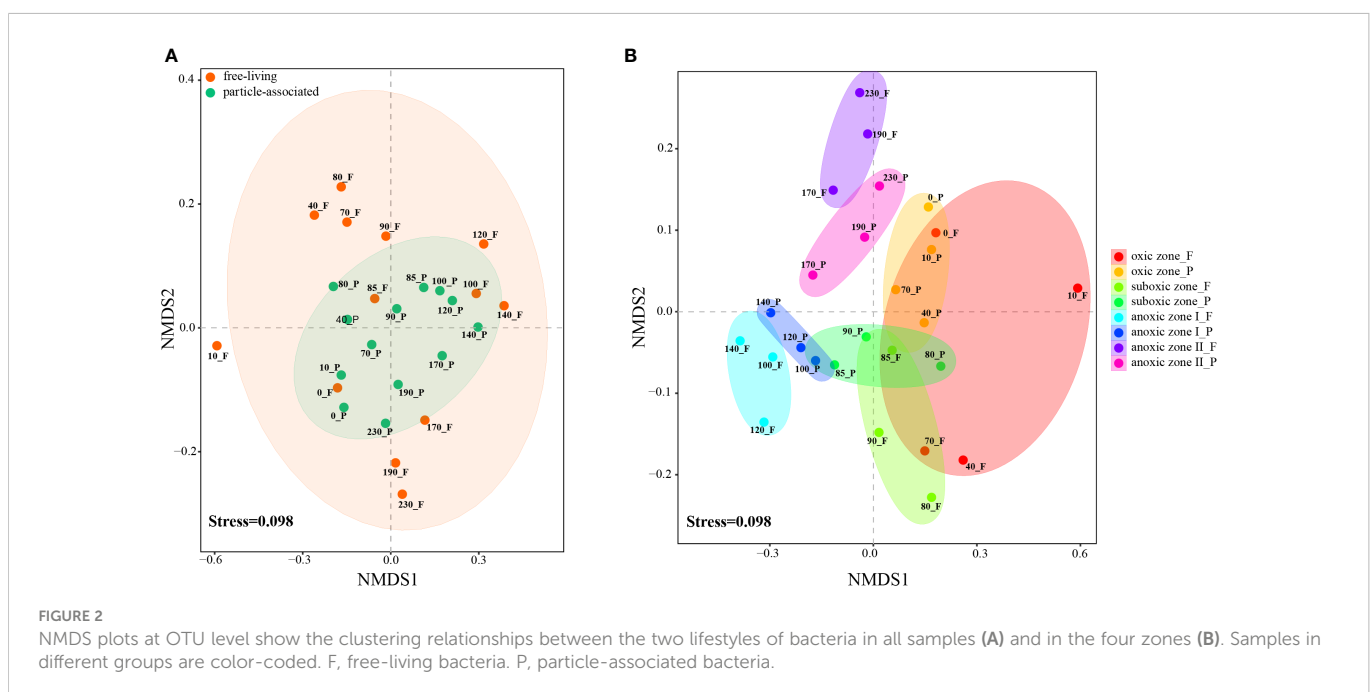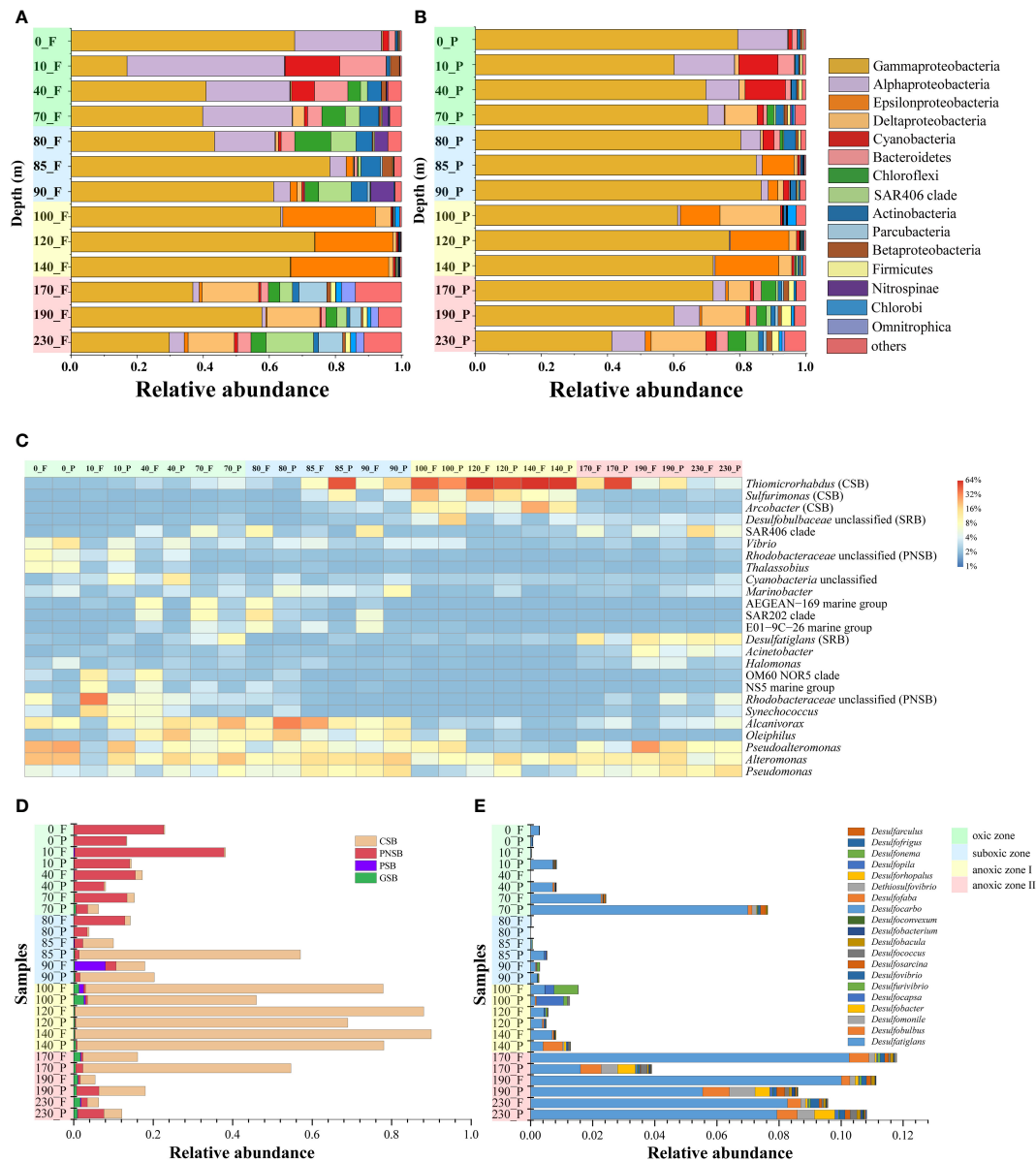

FIGURE 2
NMDS plots at OTU level show the clustering relationships between the two lifestyles of bacteria in all samples **(A)** and in the four zones **(B)**. Samples in different groups are color-coded. F, free-living bacteria. P, particle-associated bacteria.

FIGURE 3

Bacterial communities at phylum level (for bacteria within the phylum *Pseudomonadota*, the individual classes under it were used for analysis) for free-living **(A)** and particle-associated bacteria **(B)**. **(C)** Heatmap for TOP 25 genus within the bacterial communities. F, free-living bacteria. P, particle-associated bacteria. Green background represents the oxic zone, the blue background represents the suboxic zone, the yellow background represents anoxic zone I, and the red background represents the anoxic zone II. CSB, colorless sulfur bacteria. PNSB, purple non-sulfur bacteria. SRB, sulfate-reducing bacteria. The composition of sulfur-oxidizing bacteria **(D)** and sulfate-reducing bacteria **(E)**. F, free-living bacteria. P, particle-associated bacteria. CSB, colorless sulfur bacteria. PNSB, purple non-sulfur bacteria. PSB, purple sulfur bacteria. GSB, green sulfur bacteria. Green background represents the oxic zone, the blue background represents the suboxic zone, the yellow background represents anoxic zone I, and the red background represents the anoxic zone II.

dominated by CSB and PNSB. For PNSB, it prevailed in the oxic zone (mainly in the FL lifestyle) and anoxic zone II (mainly in the PA lifestyle). Whereas, CSB was abundant in the suboxic zone and anoxic zone I. The relative abundances of both GSB and PSB were low, and GSB was concentrated at 100 m and in the anoxic zone II, while PSB was presented at 90 and 100 m. For SRB, they were primarily occurred at 70 m and in the anoxic zone II, with *Desulfatiglans* as the predominant genus (Figure 3E). For the two genes involved in sulfur metabolism, the range of variation was $9.53×10^2$-$9.35×10^5$ copies/L for *dsrB* and $3.19×10^2$-$1.52×10^5$ copies/L for *soxB*, respectively, with a dramatic and very rapid increase in the suboxic zone (Figure S2).

Correlation between bacterial composition and environmental factors

Environmental factors that determined the bacterial structure at each depth were analyzed by db-RDA (Figure 4A). The RDA1 and RDA2 axes explained 28.63% and 15.61% of the variation in bacterial composition, respectively. All the samples were clustered along the first axis into oxic, suboxic and anoxic groups. The 15 environmental factors included in the analysis had significant roles in shaping the community structure of bacteria within YBH, and the top 4 environmental factors in order of influence were silicate (F=6.5,

P=0.002), phosphate (F=6.3, P=0.003), pH (F=6.2, P=0.012) and temperature (F=6.2, P=0.022). The VPA analysis showed that nutrition and physicochemical properties could explain more for the PA fraction than for the FL fraction (Figure S3, 0.4951 Vs. 0.3119). At the same time, both lifestyles were mainly influenced by a combination of nutrition and physicochemical properties with an explanation degree of 0.3691 and 0.3690 for FL and PA fraction, respectively. The mental test revealed that the components of the two lifestyles were mainly controlled by temperature, salinity, pH, DO and silicate. In addition, the effects of dissolved inorganic nitrogen (DIN), N/P and Si/N varied between the two lifestyles (Figure S4).

The abundance of bacteria in the PA fraction was mainly negatively regulated by temperature, DO and pH, and positively regulated by silicate as well as salinity, while the abundance of bacteria in the FL fraction was significantly positively regulated by ammonium, phosphate, DIN, hydrogen sulfide, depth, silicate and salinity. As for archaea, in the PA lifestyle were negatively regulated by temperature and pH, and positively regulated by ammonium, silicate, DIN, hydrogen sulfide, phosphate and depth. No environmental factors were significantly correlated with the abundance of FL archaea. Meanwhile, of the 15 environmental factors included in the study, the abundance of the $dsrB$ gene in the FL fraction was significantly correlated with 10 factors, while the $dsrB$ gene in the PA fraction was only positively correlated with Si/N. For $soxB$ in both lifestyles of bacteria, it was primarily influenced by temperature, pH, ammonium, phosphate, silicate, DIN, hydrogen sulfide and depth. Furthermore, the abundance of $soxB$ in the PA fraction was also correlated with salinity and DO. The detailed values of the correlation results are shown in Figure 4B.

The neutral community model at the OTU level explained more of the microbial community variation for the PA fraction (R^2 = 0.595) than that for the FL fraction (R^2 = 0.285) in YBH. The Nm-value (metacommunity size times immigration) was higher for the PA fraction (Nm=3,903) than the FL fraction (Nm=2,188). Since the number of sequences in each sample was 27,593, the m (migration rate) value was estimated to be 0.141 in PA and 0.079 in FL fraction, respectively (Figures 5A, B).

Diversity of culturable bacteria

The numbers of culturable bacteria on MA plates were found highest at the depths of 40 m (7,488 CFU/ml), 95 m (3,874 CFU/ml) and 0 m (2,232 CFU/ml). A total of 125 bacterial strains were isolated from MA and R2A plates. At the phylum/class level, 99 strains of *Gammaproteobacteria*, 19 strains of *Alphaproteobacteria*, 5 strains of *Actinobacteria* and 2 strains of *Firmicutes* were isolated. At the genus level (Figure 6A), the dominant taxa were *Alcanivorax* (34), *Alteromonas* (28), *Vibrio* (11), *Pseudomonas* (10), *Erythrobacter* (7), *Marinobacter* (7), and *Pseudovibrio* (6).

For the enrichment of SOB, a total of 88 bacterial strains were obtained by enrichment and isolation of seawater samples from the four water layers (Figure 6B). All these strains belong to *Pseudomonadota*, including 77 strains of *Gammaproteobacteria*, 10 strains of *Alphaproteobacteria* and 1 strain of *Betaproteobacteria*. For samples from different depths, *Vibrio* was abundant at 10 m, 140 m and 270 m. Besides, more *Halomonas* were isolated at 10 m, whereas *Alcanivorax* at 110 m, *Pseudomonas*, *Alcanivorax* and *Roseovarius* at 140 m, and *Pseudomonas* at 270 m, respectively.

For the cultivation of anaerobic bacteria, 81 strains of anaerobic bacteria were isolated and identified from samples of 90, 130, 160 and 190 m. At the phylum level, *Bacteroidetes* had the highest abundance (21%, 17/81). At the genus level, the dominant genera were *Saccharicrinis* and *Halodesulfovibrio*, both in equal proportions (14%, 11/81 of each). Eighteen bacterial strains in 10 genera showed the 16S rRNA gene sequence similarity lower than 98.65% (90.24%-97.90%) with their closely related bacteria, which could be considered novel bacteria and need further analyses.

Discussion

As a specific habitat, studies on the abundance and community composition of bacteria in blue holes mainly focused on the Bahamas Blue Hole and YBH. However, current research on microorganisms in YBH was mainly based on the whole microbial communities, and
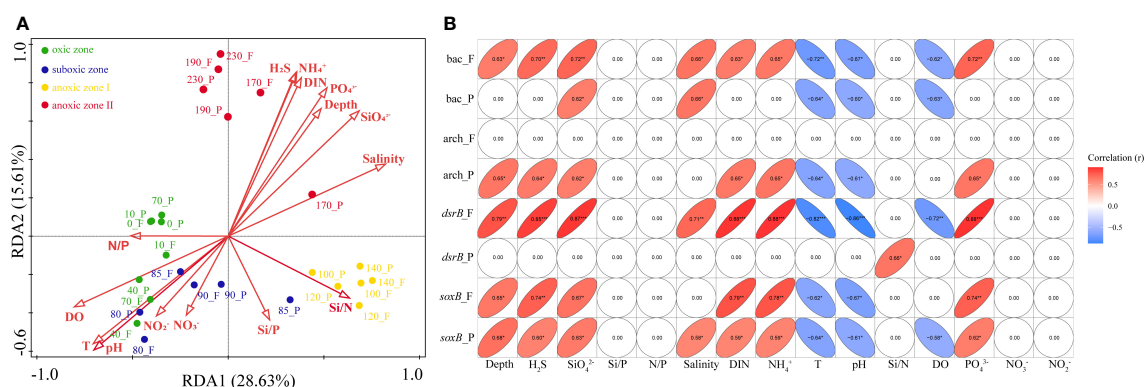
FIGURE 4
(A) Redundancy analysis (RDA) diagram illustrating the relationship between samples and environmental factors. Environmental factors are indicated by red arrows, and dots in different color represent samples from different zones. **(B)** Correlations between gene abundance and environmental factors. *0.01<P< 0.05; **=0.001<P ≤ 0.01; ***=P ≤ 0.001. bac, bacterial 16S rRNA gene. arch, archaeal 16S rRNA gene. F, free-living bacteria. P, particle-associated bacteria.

FIGURE 5

Fitting of a neutral community model (NCM) to the community assemblage at OTU level. The frequencies of occurrence of free-living **(A)** and particle-associated bacteria **(B)** are predicted respectively.

there was a lack of understanding of the culturability of microorganisms in blue holes. Our results primarily showed differences in community structure, gene abundance and response to the environmental factors between bacteria with FL and PA lifestyles within YBH. We also used culture-dependent methods as a complementary to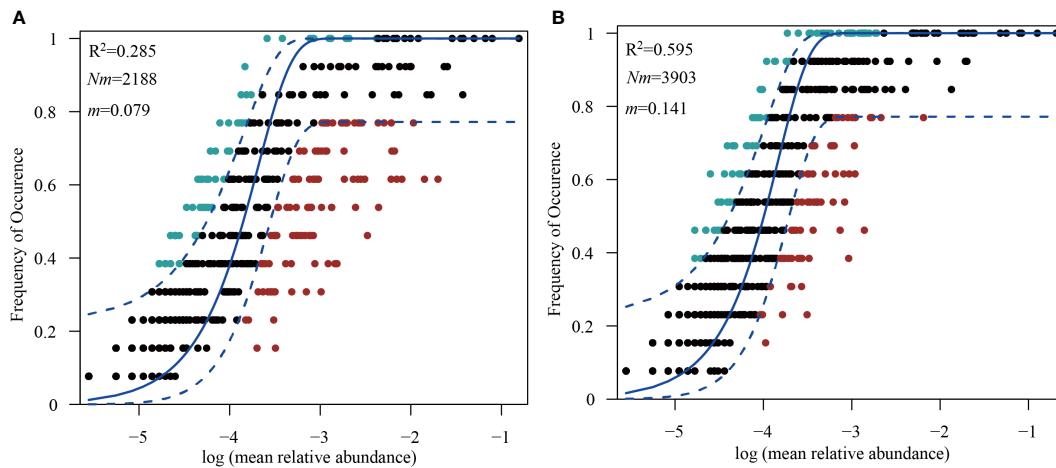 expand the knowledge of the diversity of culturable bacteria living in this specific habitat. The results indicated the presence of a large number of novel species in YBH.

Different lifestyles lead to separate bacterial composition

The two modes of bacterial lifestyles, FL and PA, have been reported in many studies, and there was a clear difference between the bacteria in these two lifestyles, both in terms of community structure and biogeochemical processes (DeLong et al., 1993; Tarn et al., 2016; Mestre et al., 2017; Liu et al., 2020b). Our previous studies on *Vibrio* in YBH also demonstrated that *Vibrio* preferred to FL lifestyle and had a different dominant composition in the two lifestyles (Li et al.,

2020a). In this study, the bacteria from the two lifestyles showed significant differences in several aspects, such as gene abundance, diversity and richness. Both bacteria and archaea favored a FL lifestyle, which was also consistent with the results of previous studies (Li et al., 2015; Zhang et al., 2020; Zheng et al., 2020). The differences in abundance and diversity between the two lifestyles might be due to the suspended matters as well as the lower water fluidity in YBH. Specifically, the particulate matters in YBH consisted mainly of biogenous debris, terrestrial debris, authigenic particles, organic membrane-marine snow and debris from modern artificial materials (Sun et al., 2018). The suspended particles could provide bacteria with nutrients and were enriched in particular bacterial groups, which may exhibit less abundance and diversity than the FL fraction. Further, the abundance of PA bacteria might be less influenced by environmental factors compared with that of FL bacteria because of the particulate matters (Figure 5B). In addition, the bacterial community structure of the two lifestyles was distinctive. For example, the relative abundance of SOB in FL fraction was higher than in PA fraction in anoxic zone I, whereas the opposite was true in anoxic zone II. Meanwhile, in the oxic zone and suboxic zone, PNSB

FIGURE 6

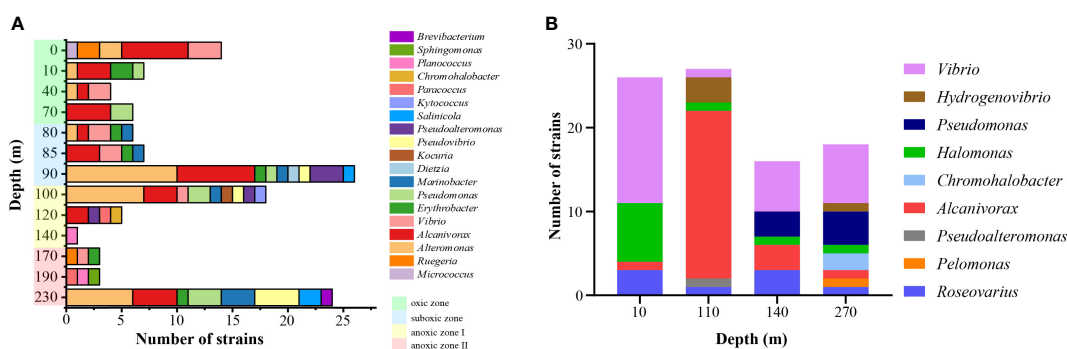The number of culturable bacteria at the genus level in YBH. **(A)** Numbers of strains isolated on MA and R2A plates. **(B)** Numbers of sulfur-oxidizing bacteria enriched and isolated from SPG liquid medium. Green background represents the oxic zone, the blue background represents the suboxic zone, the yellow background represents anoxic zone I, and the red background represents the anoxic zone II.

was more abundant in FL fraction. The reason was likely to be the different substrates oxidized by the different types of SOB and their different sulfur-oxidation pathways (Ghosh and Dam, 2009), combined with the interaction with the specific physicochemical properties in YBH. Certainly, these inferences needed to be supported by more detailed physicochemical parameters, as well as more in-depth analyses. Furthermore, the results for the effects of both nutrition and physicochemical properties, as well as the corresponding differences for each specific environmental factor, reflected the different distribution patterns of microorganisms in YBH for the two lifestyles.

Moreover, the differences brought about by the two lifestyles in YBH can be better revealed from the perspective of assembly process, which reflected the impact of deterministic or stochastic processes on the two lifestyles. The NCM model was a tool commonly used to explore this issue (Sloan et al., 2006; Zhou and Ning, 2017). Our results showed that the NCM model (Figures 5A, B) successfully estimated most of the relationship between the frequency of occurrence of OTUs and their relative abundance variation in YBH. The higher degree of explanation implied that the assembly of the PA fraction was more affected by stochastic processes, while that of the FL fraction was more affected by deterministic processes. This was because FL bacteria may have greater motility capability in YBH and were influenced more by environmental factors including temperature and salinity, which were deterministic processes. The stochastic processes played a greater influence on PA bacteria, and this was in accordance with previous findings in a subtropical river-bay system (Wang et al., 2020), but was quite different from previous studies in the marginal sea (Yuan et al., 2021). It was suggested that the bacterial assembly processes in different environments showed different results. The structure of bacterial composition with different lifestyles in YBH may be affected by various environmental factors because of the distinctive physicochemical factors in YBH from other marine environments.

Extremely specific and dynamic sulfur cycle

Current studies on the microbial constituents of the blue hole all found that bacteria involved in the sulfur cycle, particularly SOB, were dominated in the suboxic and anoxic zones, where rapid oxygen depletion and hydrogen sulfide production occurred (Gascoyne et al., 1979; Bottrell et al., 1991; Gonzalez et al., 2011; Haas et al., 2018; He et al., 2019; Gordon, 2020; He et al., 2020; Zhang et al., 2021). Bacteria belonging to *Thiomicrospira* was reported to be the most abundant SOB in YBH. Bacteria in the family *Piscirickettsiaceae* were reclassified in 2017, and a portion of species in the former genus *Thiomicrospira* was reclassified as the genera *Hydrogenovibrio*, *Thioalkalimicrobium*, and the newly named *Thiomicrorhabdus* (Boden et al., 2017; Kojima and Fukui, 2019; Liu et al., 2020a; Liu et al., 2021; Updegraff et al., 2022). In our study, based on the SILVA v128 database, six OTUs were annotated as *Thiomicrospira* (Figure S5), and the phylogenetic tree confirmed that five OTUs were affiliated with *Thiomicrorhabdus* and one was affiliated with *Hydrogenovibrio* (Figure S6). Our results confirmed that the relative abundance of SOB, represented by *Thiomicrorhabdus*, *Sulfurimonas*, *Sulfurovum*, *Arcobacter* and *Rhodobacteraceae*, reached almost 90% at

120 m water depth in YBH, agreed with previous studies (He et al., 2019; He et al., 2020; Zhang et al., 2021).

Meanwhile, the above-mentioned taxa all belonged to CSB. They were found to obtain energy from the oxidation of reduced sulfur compounds and fix dissolved inorganic carbon (DIC) through the Calvin-Benson-Bassham (CBB) or reverse tricarboxylic acid cycles (Li et al., 2018b; Scott et al., 2018). $S_2O_3^{2-}$ could be oxidized under acidic and low O_2 conditions through extracellular deposition of S^0 by *Thiomicrospira*, and transient accumulation of sulfite and even polythionates could be detected as products of thiosulfate and S^0 oxidation (Javor et al., 1990; Scott et al., 2006). It was suggested that the *Thiomicrospira* was able to oxidize reductive sulfur-containing compounds, especially at low pH and hypoxic conditions. Both oxygen (reduction from 5 mg/L to 0) and pH (reduction from 8.2 to 7.6) decreased rapidly in the anoxic zone I of YBH (Yao et al., 2018), providing an adequate material basis for the proliferation of the *Thiomicrospira*. In addition, *Thiomicrorhabdus*, *Sulfurovum*, *Arcobacter* and *Thiothrix* were also found to be important participants in the process of sulfur cycling. For example, *Thiomicrorhabdus* can form extracellular S^0 spheres at low oxygen/pH and transient accumulation of SO_3^{2-} or polysulfate during S^0 spheres or $S_2O_3^{2-}$ oxidation (Liu et al., 2020a; Liu et al., 2021).

SOB were the most abundant bacteria in blue holes, but the dominant taxa of SOB varied from different habitats. *Chlorobium* (Gonzalez et al., 2011; Gordon, 2020) and *Arcobacter* (Iwanowicz et al., 2021) were the most abundant SOB in the Bahamian Blue Hole, while *Thiomicrorhabdus* was the dominant taxa in YBH. *Chlorobium* was a member of GSB, which was also a group of strictly anaerobic bacteria that can undergo photoautotrophy in very low light conditions (Imhoff, 2014; Nielsen et al., 2016). In YBH, the two photoautotrophic taxa, PSB and GSB, were much less abundant than CSB and PNSB ($P<0.05$). Furthermore, although *Arcobacter* was also found in YBH, its relative abundance (7.24% of SOB) was much lower than that in the Bahamas Blue Hole (over 50% of SOB). It was demonstrated that the composition of SOB and possible sulfur oxidation processes in YBH were very different from those in the Bahamas Blue Hole.

In addition, chemoautotrophic bacteria derived from the genera *Thiomicrorhabdus*, *Sulfurovum*, *Arcobacter*, *Thiomicrospira*, *Hydrogenovibrio* were also dominant SOBs in other sulfide-rich habitats, including OMZs, hydrothermal vents, cold spring, soda and salt lakes and marine sediments (Cao et al., 2014; Li et al., 2018b; Scott et al., 2019; Magnuson et al., 2021). For example, SUP05 was dominant in the OMZ and hydrothermal vent (Crowe et al., 2018; van Vliet et al., 2021), and it could account for up to 50% of the entire community in some samples, which would be a high level to be reported (Crowe et al., 2018; van Vliet et al., 2021). In the present study, the relative abundance of *Thiomicrohabdus* in YBH exceeded 50% in five samples (64.25% in 120 m FL, 59.78% in 140 m FL, 50.61% in 120 m PA, 57.87% in 140 m PA and 51.48% in 170 m PA), that was extremely rare in marine environments.

The Sox system was reported to be a prevalent mechanism of SOB (Friedrich et al., 2001; Friedrich et al., 2005; Ghosh and Dam, 2009), and the *soxB* gene was used as a biomarker of SOB in hydrothermal vents (Li et al., 2018b). The results of *soxB* gene abundance in our study were similar to previous studies in the same region (Luo et al., 2018). However, there were differences with the results from

hydrothermal vents (Li et al., 2018b), where they found that SOB was positively correlated with *soxB* abundance. At the same time, we observed considerable amounts of *Alcanivorax* and *Oleiphilus* within YBH, suggesting that the sulfur oxidation process in YBH maybe also related to the carbon cycle. It was found that the sulfur oxidation processes in YBH were distinctive from other blue holes and the hydrothermal vents where sulfur oxidation is active. Further data, including but not limited to the transcriptome as well as the metagenome, will certainly be essential to fully reveal the extremely unique sulfur cycle within YBH.

A rich source of unknown culturable bacteria

The bacterial structure in blue holes was well studied using high-throughput sequencing, but for such a unique environment, there was a high probability that novel microorganisms could be obtained using different culture methods. While next-generation sequencing methods can provide a comprehensive and complete picture of the microbial community, in many studies, such as the physiological and biochemical characteristics of strains, the utilization of different substrates could only be carried out if the strain resources were available. At the genus level, *Alcanivorax* and *Vibrio* were found to be isolated at almost all depths (Figure 6A), indicating that both genera were well adapted to the environment in YBH, especially the absence of oxygen. This was consistent with previous studies of the Marianas Trench (Zhao et al., 2020), where both genera were also found in the water column from the surface to nearly 10,000 m. In addition, the numbers of bacteria isolated at 90, 100 and 230 m were much higher than other depths, most likely due to the rapid change in environmental factors represented by DO from the suboxic zone to the anoxic zone I and the accumulation of large amounts of dissolved inorganic carbon in the anoxic zone II (Yao et al., 2020).

Our anaerobic bacterial isolation and identification in YBH yielded a large number of potentially new species. Of the 81 anaerobic bacterial strains obtained, 18 strains had 16S rRNA similarities below the 98.65% criterion for new species determination. The relative abundance of novel bacteria in YBH was 22.2% (18/81), which was significantly higher than that in Marianas Trench waters (7.2%) (Zhao et al., 2020), Red Sea corals (10.0%) (ElAhwany et al., 2015), Antarctic soils (1.5%) (Ouyang et al., 2021) and South Korean Yellow Sea sediments (3.8%) (Velmurugan et al., 2011). In contrast, for the isolation of aerobic strains, only 2.4% of the strains were potentially new species. From the results of culturable anaerobic bacteria, it could be assumed that a considerable proportion (approximately 25%) of the anaerobes in YBH were unknown. At the same time, these anaerobic bacteria should play an important role in the unique biogeochemical cycle in YBH, as well as possess a special adaptation mechanism to the anaerobic environment in YBH. This suggested that the novelty and specificity of the culturable anaerobic bacteria in YBH provided a great opportunity to study their metabolic characteristics and further application.

As expected, there were differences in the microbial composition identified in the samples using culture-dependent methods compared to culture-independent methods (Demko et al., 2021). For example, *Thiomicrorhabdus*, which had the highest relative abundance in the high-throughput sequencing results, was not obtained under any of the various culture conditions used. However, four strains of *Hydrogenovibrio*, which belonged to the same family as *Thiomicrorhabdus* and had a similar phylogenetic relationship, was isolated in the SOB-enriched SPG medium (Figure 6B). Three of the strains were isolated from 110 m and one from 270 m. This was in agreement with the high-throughput sequencing result that *Thiomicrorhabdus* was predominantly distributed in the anoxic zone I. This demonstrated that, for specific habitats such as YBH, a culture-dependent approach that restores as much of the real-life condition as possible will help to obtain a larger, more representative number of strains. In addition to the differences, a number of strains that were abundant and important in high-throughput sequencing were also obtained, such as *Alcanivorax*, *Vibrio*, *Alternomonas*, *Pseudomonas* and *Pseudoalteromonas*. It was suggested that the results for culturable bacteria reflect to some extent the structure of the microbial community in YBH. Further, it also provided the strain resources for in-depth studies of the adaptation mechanism of bacteria to the unique environment of YBH.

Conclusion

In this article, the bacterial composition in YBH was studied by both culture-dependent and -independent methods. In YBH, there were many differences between bacteria with FL and PA lifestyles. FL bacteria show stronger variation internally compared to PA bacteria. Also, FL bacteria are relatively more influenced by deterministic processes. A large number of sulfur-oxidizing bacteria, represented by *Thiomicrorhabdus*, drive a very complex sulfur cycling process in YBH. In addition, a large number of new taxa have been identified among culturable anaerobic bacteria. Our study expanded current knowledge on microbial community structure from a lifestyle-based perspective in deep ocean blue holes, and provided a vast resource of bacterial strains for further studies on their adaptation to extreme marine environments.

Data availability statement

The datasets presented in this study can be found in online repositories. The names of the repository/repositories and accession number(s) can be found in the article/Supplementary Material.

Author contributions

X-HZ and MY designed the experiments and analyzed the data. SZ conducted most of the experiments, analyzed the data, prepared graphs and tables, and wrote the manuscript. ZY, PY, LF, and LC attended the cruise, collected the samples and analyzed the data. YZ and JCL carried out the culture, isolation and analysis of aerobic bacteria. RD carried out the culture, isolation and analysis of sulfur-

oxidizing bacteria. CJ and XS carried out the culture, isolation and analysis of anaerobic bacteria. XW performed the quantitative PCR analysis. MY, SZ, X-HZ, and JWL revised the manuscript. All authors contributed to the article and approved the submitted version.

Conflict of interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fmars.2023.1086117/full#supplementary-material

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