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ENVIRONMENTAL BIOTECHNOLOGY

Vertical distribution of *Candidatus* Methylomirabilis and Methanoperedens in agricultural soils

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Abstract

Candidatus Methylomirabilis-related bacteria conduct anaerobic oxidation of methane (AOM) coupling with NO_2^- reduction, and *Candidatus* Methanoperedens-related archaea perform AOM coupling with reduction of diverse electron acceptors, including NO_3^-, Fe (III), Mn (IV) and SO_4^{2-}. Application of nitrogen fertilization favors the growth of these methanotrophs in agricultural fields. Here, we explored the vertical variations in community structure and abundance of the two groups of methanotrophs in a nitrogen-rich vegetable field via using illumina MiSeq sequencing and quantitative PCR. The retrieved *Methylomirabilis*-related sequences had 91.12%-97.32% identity to the genomes of known *Methylomirabilis* species, and *Methanoperedens*-related sequences showed 85.49%-97.48% identity to the genomes of known *Methanoperedens* species which are capable of conducting AOM coupling with reduction of NO_3^- or Fe (III). The *Methanoperedens*-related archaeal diversity was significantly higher than *Methylomirabilis*-related bacteria, with totally 74 and 16 operational taxonomic units, respectively. In contrast, no significant difference in abundance between the bacteria (9.19×10^3–3.83×10^5 copies g^{-1} dry soil) and the archaea (1.55×10^4–3.24×10^5 copies g^{-1} dry soil) was observed. Furthermore, the abundance of both groups of methanotrophs exhibited a strong vertical variation, which peaked at 30–40 and 20–30 cm layers, respectively. Soil water content and pH were the key factors influencing *Methylomirabilis*-related bacterial diversity and abundance, respectively. For the *Methanoperedens*-related archaea, both soil pH and ammonium content contributed significantly to the changes of these archaeal diversity and abundance. Overall, we provide the first insights into the vertical distribution and regulation of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea in vegetable soils.

Key points

- *The archaeal diversity was significantly higher than bacterial.*
- *There was no significant difference in the abundance between bacteria and archaea.*
- *The abundance of bacteria and archaea peaked at 30–40 and 20–30 cm, respectively.*

Keywords Anaerobic methanotrophs · Vertical variation · Community structure · Abundance · Agricultural ecosystems

Introduction

The process of nitrite-driven anaerobic oxidation of methane (AOM) is mediated by the NC10 phylum bacteria that are closely associated with *Candidatus* Methylomirabilis oxyfera (Ettwig et al. 2010), *Candidatus* Methylomirabilis sinica (He et al. 2016) or *Candidatus* Methylomirabilis lanthanidiphila (Versantvoort et al. 2018), which can oxidize methane (CH_4) through the intra-aerobic pathway using NO_2^- as the electron acceptor (Ettwig et al. 2010). The nitrate-driven AOM is performed by the anaerobic methanotrophic archaea (ANME)—ANME-2d that are closely

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associated with *Candidatus* Methanoperedens nitroreducens, oxidizing CH_4 via the pathway of reverse methanogenesis with NO_3^- as the electron acceptor (Haroon et al. 2013). Furthermore, *Methanoperedens*-related archaea were also reported to have the capability to conduct AOM coupling with Fe (III) (Ettwig et al. 2016; Cai et al. 2018; Li et al. 2021; Chen et al. 2022; Zhang et al. 2023), Mn (IV) (Leu et al. 2020) and SO_4^{2-} reduction (Nie et al. 2021). The discovery of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea greatly extends the understanding of the microbial CH_4 cycle.

Currently, a lot of studies demonstrated that *Methylomirabilis*-related bacteria were present in freshwater wetlands sediments (Zhu et al. 2015; Shen et al. 2017a; Xie et al. 2020b), water columns or sediments of lakes (Graf et al. 2018; Yang et al. 2018; Mayr et al. 2020), reservoirs sediments (Wang et al. 2016; Long et al. 2017a; Shen et al. 2020b), rivers (Shen et al. 2014b, 2019; Long et al. 2017b), paddy fields (Wang et al. 2012; Vaksmaa et al. 2016; Yang et al. 2022) and coastal sediments (Zhang et al. 2018; Wang et al. 2019; Zheng et al. 2020). Furthermore, there is increasing evidence that *Methanoperedens*-related archaea occur in different habitats, including freshwater wetlands (Shen et al. 2017a; Huang et al. 2020; Xie et al. 2020b), freshwater aquatic systems (Weber et al. 2017; Shen et al. 2019, 2020b), paddy fields (Vaksmaa et al. 2016; Shen et al. 2021b) and coastal wetlands (Zheng et al. 2020; Chen et al. 2021; Niu et al. 2022). The widespread distribution of the two groups of methanotrophs in diverse environments suggests their potential importance in regulating global CH_4 emissions.

Agriculture releases a significant amount of CH_4, which accounts for 52% of global emissions from anthropogenic sources (Smith et al. 2008). China's vegetable production accounts for 49% of global production (Jia et al. 2012), and vegetable soils occupy about 11.4% of China's total planting area and are a major sink of CH_4 for the croplands. Until now, some researchers have examined the CH_4 flux from vegetable fields (Jia et al. 2012; Qi et al. 2020; Fan et al. 2021b). *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea are novel players in controlling CH_4 emissions from paddy (Vaksmaa et al. 2016; Yang et al. 2023) and upland fields (Zhu et al. 2018). However, there is no study has reported the distribution of the two groups of anaerobic methanotrophs in vegetable soils, and therefore whether these methanotrophs are present and their distribution characteristics in vegetable soils are poorly known. For promoting crop yields, vegetable fields receive intensive nitrogen (N) fertilization, and the total rate of fertilization was reported to be 300–700 kg N ha^{-1} during individual vegetable growing seasons in China (Zheng et al. 2004; He et al. 2009). Theoretically, the high-input of N fertilizers into vegetable soils can make them as suitable habitats for the two groups of methanotrophs. In addition, the physicochemical properties vary with soil depths in vegetable fields (Chen et al. 2014; Zhao et al. 2019; Qin et al. 2020), which has been shown to have a great impact on the communities and function of soil microorganisms (Shen et al. 2017c; Zhao et al. 2019; Qin et al. 2020). Here, we hypothesized that the vertical variation in soil properties can also affect the abundance and community structures of the two groups of methanotrophs.

To explore the distribution characteristics of *Methylomirabilis*-related bacterial and *Methanoperedens*-related archaeal communities in vegetable fields, the vertical variations in their community composition, diversity and abundance were examined along the soil profile (0–100 cm). In addition, the vertical variation in soil properties was also examined to explore its impact on these anaerobic methanotrophs.

Materials and methods

Soil sampling

Soils were retrieved from a vegetable field which experienced periodic drying and wetting as previously reported (Shen et al. 2017c), and the sample site (32°12′N, 118°43′E) was located in Nanjing City, Jiangsu Province, China. Five soil cores (0–100 cm) were taken from the field, and then sliced into 10 layers with an interval of 10 cm.

DNA isolation and PCR amplification

The soil genomic DNA was exacted via the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, US). The *pmo*A and *mcr*A are two important functional genes of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea, respectively (Ettwig et al. 2010; Vaksmaa et al. 2017b). In this study, a nested PCR approach with the primers of A189_b-cmo682 (for first PCR round) and cmo182-cmo568 (for the second PCR round) was employed to amplify the *pmo*A genes of *Methylomirabilis*-related bacteria. However, no positive PCR products can be generated from any examined soil samples. Instead, the 16S rRNA genes of these bacteria were successfully amplified through a nested PCR approach using the primers of 202F-1545R (for the first PCR round) and qp1f-qp2r (for the second round) (Table S1), and the PCR thermal cycle programs were conducted by referring to the protocol reported previously (Shen et al. 2017a). The archaeal *mcr*A genes were also amplified through a nest PCR approach using the primers of McrA169F-McrA1360R (for the first round) and McrA997F-McrA1360R (for the second round) (Table S1), and the thermal cycle programs were conducted according to Shen et al. (2017a). Through the nested PCR protocols, the average amplification lengths of the bacterial 16S rRNA

genes and the archaeal *mcr*A genes were 418 bp and 318 bp, respectively. Purified amplicons with correct size were used to construct the *Methylomirabilis*-related bacterial 16S rRNA gene and *Methanoperedens*-related archaeal *mcr*A gene libraries on the Illumina MiSeq (PE300) sequencing platform.

Sequencing data processing

Methylomirabilis-related bacterial 16S rRNA gene sequences and *Methanoperedens*-related archaeal *mcr*A gene sequences were denoised, and the operational taxonomic units (OTUs) were generated. *Methylomirabilis*-related and *Methanoperedens*-related OTUs were clustered with 97% (Tian et al. 2021) and 95% (Shen et al. 2021b) sequence similarity cutoff, respectively, using UPARSE. The taxonomic assignments of the recovered OTUs were performed by RDP Classifier. Furthermore, each OTU's representative sequences were aligned in GenBank to exclude any non-*Methylomirabilis*-related or non-*Methanoperedens*-related sequences (Zhang et al. 2022). In accordance with the previous studies (Tian et al. 2021; Xu et al. 2018), the 16S rRNA gene sequences showing > 90% identity to known *Methylomirabilis* species (Ettwig et al. 2010; He et al. 2016), and the *mcr*A gene sequences showing > 80% identity to known *Methanoperedens* species (Haroon et al. 2013; Berger et al. 2017; Cai et al. 2018; Leu et al. 2020) were remained. In order to ensure the evenness and consistency of data, sequences from each sample were normalized at the same sequencing depth. Subsequently, Chao1 estimators and Shannon index were calculated for assessing alpha diversity. The vertical distribution of communities of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea was examined at the OTU level.

Phylogenetic analyses

The top 10 *Methylomirabilis*-related OTUs (occupying > 99% of the total sequences), and the top 20 *Methanoperedens*-related OTUs (occupying > 88% of the total sequences) were used to construct the phylogenetic trees using the neighbour-joining method by software MEGA 6. Additionally, some sequences deposited in the GenBank database that were closely related to our obtained sequences were also included in the phylogenetic analyses.

Quantitative PCR

The copy numbers of *Methylomirabilis*-related 16S rRNA genes and *Methanoperedens*-related *mcr*A genes were analyzed through quantitative PCR (qPCR) method. The primers of qp1f-qp1r and McrA159F-McrA345R (Table S1) were employed to quantify the 16S rRNA and *mcr*A gene abundance, respectively (Ettwig et al. 2009; Shen et al. 2021b). The standard curves for quantifying the gene abundance of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea in individual samples were established according to a series of dilutions of pMD18-T simple plasmid vector containing the target gene.

Statistical analyses

Principal coordinates analysis (PCoA) and canonical correspondence analysis (CCA) were employed to examine the vertical variations in *Methylomirabilis*-related bacterial or *Methanoperedens*-related archaeal communities and their potential relationships with soil properties, respectively. One-way analysis of variance (ANOVA) was employed to examine the vertical variation in abundance of *Methylomirabilis*-related bacteria or *Methanoperedens*-related archaea. Pearson correlation analysis was applied to study the relationships among the diversity, abundance of these anaerobic methanotrophs and different soil factors. The above analyses were performed using the SPSS 25 software or *R* software.

Results

Detection of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea

Positive PCR products of *Methylomirabilis*-related bacterial 16S rRNA genes were obtained at 0–80 cm soil layers, while no positive products can be retrieved at 80–100 cm layers. Miseq sequencing generated a total of 148,737 sequences from 0–80 cm layers. After discarding the non-*Methylomirabilis*-related sequences and normalizing at the same depth, each sample had 13,038 effective sequences (Table 1). In contrast, positive PCR products of *Methanoperedens*-related archaeal *mcr*A genes were retrieved throughout 0–100 cm layers, and a total of 224,008 sequences were recovered from these layers. Each sample contained 15,063 effective sequences after discarding non-*Methanoperedens*-related sequences and normalization (Table 1).

Diversity of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea

The coverage values of *Methylomirabilis*-related and *Methanoperedens*-related sequences were both greater than 99% (Table 1), suggesting our sequencing results can well represent the diversity of these methanotrophs. Sixteen OTUs of *Methylomirabilis*-related 16S rRNA genes were generated from the soil samples according to 97% sequence similarity cut-off, and each sample had 8–13 OTUs (Table 1). The ranges of Chao1 estimators and Shannon index were

Table 1 Diversity of the recovered 16S rRNA gene sequences of *Methylomirabilis*-like bacteria and *mcr*A gene sequences of *Methanoperedens*-like archaea in the examined soil samples

Samples	Effective sequences		Coverage		Number of OTUs		Shannon index		Chao1 estimator	
	Methylomirabilis	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*
S10	13,038	15,063	100%	99.99%	13	41	1.47	2.22	13	41
S20	13,038	15,063	100%	99.99%	9	61	1.06	3.01	9	61
S30	13,038	15,063	100%	99.97%	8	47	1.33	3.17	8	50
S40	13,038	15,063	99.99%	99.98%	9	45	1.57	2.90	9	46.5
S50	13,038	15,063	99.98%	99.99%	12	42	1.38	2.81	13	42
S60	13,038	15,063	100%	99.99%	10	40	1.15	2.91	10	40
S70	13,038	15,063	99.99%	99.99%	9	32	0.90	2.49	9	32
S80	13,038	15,063	100%	100%	11	40	1.08	2.75	11	40
S90	N.A	15,063	N.A	99.99%	N.A	43	N.A	2.95	N.A	43.3
S100	N.A	15,063	N.A	99.99%	N.A	29	N.A	2.85	N.A	30

The designations S10, S20, S30, S40, S50, S60, S70, S80, S90, and S100 correspond to soil depths of 0–10, 10–20, 20–30, 30–40, 40–50, 50–60, 60–70, 70–80, 80–90, and 90–100 cm, respectively. N.A. denotes not available

8–13 and 0.90–1.57, respectively, in individual samples (Table 1). For the *Methanoperedens*-related archaea, totally 74 OTUs of *mcr*A genes were recovered according to 95% sequence similarity cut-off. The ranges of OTU numbers, Chao1 estimators and Shannon index of these archaeal *mcr*A genes varied from 29 to 61, 30 to 61 and 2.22 to 3.17, respectively, in individual samples (Table 1), which were all significantly greater than those of *Methylomirabilis*-related bacterial 16S rRNA genes (Fig. 1A). Moreover, no obvious vertical variation in diversity of the bacteria or the archaea was observed.

Community composition of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea

It was found that the OTU7 was the most dominant *Methylomirabilis*-related lineage at the soil layers of 10–20 and 40–80 cm. For the layers of 20–40 cm, the OTU7 and OTU34 co-dominated the bacterial communities. The OTU7 and OTU4 were the co-dominant lineages at the layer of 0–10 cm (Fig. 2A). The top 10 *Methylomirabilis*-related OTUs (occupying 99.8% of the total sequences) were classified into two separate clusters (Fig. 3A). Sequences from Cluster A showed 92.24%-97.32% and 91.12%-95.89% identities to the reported genome sequences of *Methylomirabilis oxyfera* and *Methylomirabilis sinica*, respectively (Table S2). These sequences were most closely associated with the *Methylomirabilis*-related sequences detected in wetland soils (Hu et al. 2014; Shen et al. 2015; Zhu et al. 2015) and river sediments (Shen et al. 2014b), with 95.32%-100% identity (Table S2). Sequences from Cluster B (occupying over 85% of the total sequences) exhibited 92.46%-94.16% and 92.01%-93.05% identities to the genome sequences of *Methylomirabilis oxyfera* and *Methylomirabilis sinica*, respectively (Table S2). This cluster was most closely associated with the *Methylomirabilis*-related sequences reported in wetland soils (Zhu et al. 2015), peatlands (Zhong et al. 2020) and estuarine sediments (Yan et al. 2015), with 98.08%-99.76% identity (Table S2).

For the *Methanoperedens*-related archaeal communities, the OTU10 was the most dominant lineage at the soil layer of 0–10 cm, and OTU12 was the most dominant lineage at the layer of 10–20 cm. The OTU49 dominated the archaeal communities at the layer of 30–40 cm, and OTU49 and OTU9 co-dominated the communities at the layer of 20–30 cm. For the layers of 40–60 cm, the communities were co-dominated by OTU49, OTU64 and OTU12, and the OTU49 and OTU64 co-dominated the communities at the layers of 60–100 cm (Fig. 2B). The top 20 OTUs (occupying 88.1% of the total sequences) were classified into three different clusters (Fig. 3B). The OTU6 and OTU9

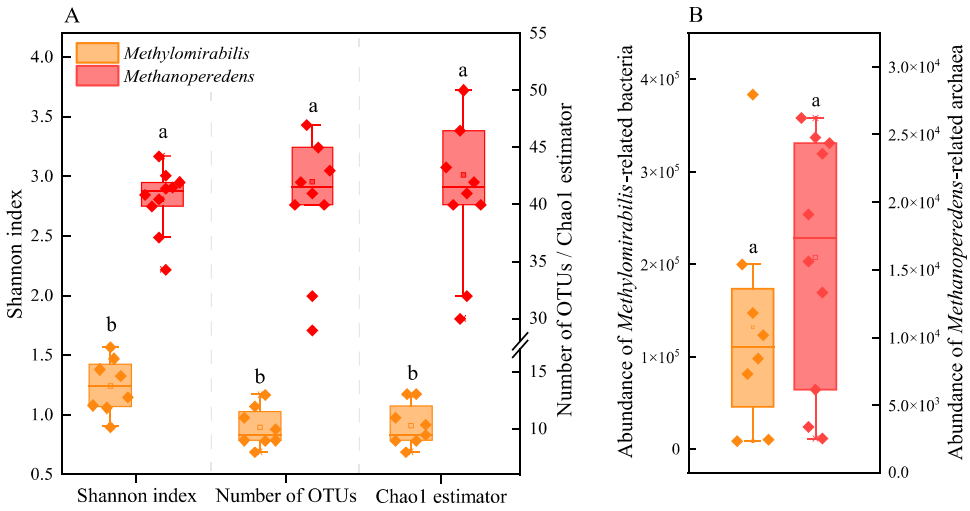

Fig. 1 Comparison of overall diversity (**A**) and abundance (copies g^{-1} dry soil) (**B**) between *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea across different soil layers. Different lowercase letters demonstrate a significant difference in diversity or abundance between the bacteria and archaea

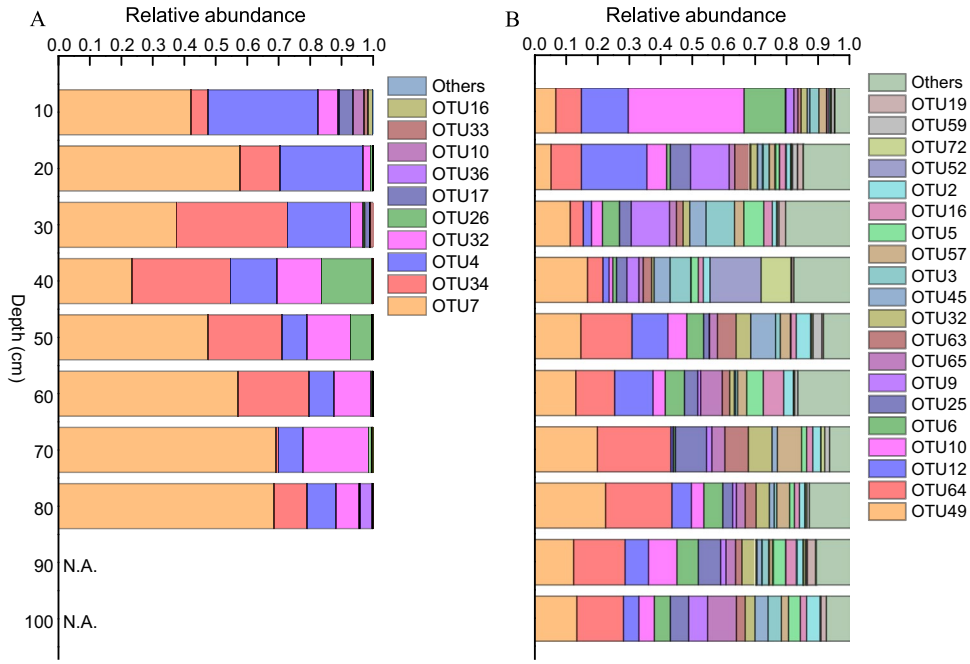

Fig. 2 Community composition of *Methylomirabilis*-related bacteria (**A**) and *Methanoperedens*-related archaea (**B**) at different soil layers. N.A. denotes not available

in Cluster A were associated with the genome sequence of *Methanoperedens* sp. ANME2D, with 85.49%-91.17% identity (Table S3), while the remaining OTUs (OTU64, OTU32, OTU45, OTU57, OTU52, OTU72 and OTU59) in this cluster were associated with the genome sequence of *Methanoperedens* sp. Mnv1, with 93.69%-97.48% identity (Table S2). Sequences from Cluster B were all associated with the genome sequence of *Methanoperedens ferrireducens*, with 93.06%-95.58% identity (Table S3). Sequences from Cluster C were also associated with the genome sequence of *Methanoperedens ferrireducens*, with 87.74%-90.85% identity (Table S3).

Abundance of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea

The *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea were present at every soil layer examined (Fig. 4). The gene abundance of *Methylomirabilis*-related bacterial 16S rRNA varied between 9.19×10^3 and 3.83×10^5 copies g^{-1} dry soil, peaking at the 30–40 cm layer and then decreased sharply with depth (Fig. 4A). The *Methanoperedens*-related archaeal *mcr*A gene abundance ranged between 1.55×10^4 and 3.24×10^5 copies g^{-1} dry soil. The *mcr*A gene abundance peaked at the 20–30 cm

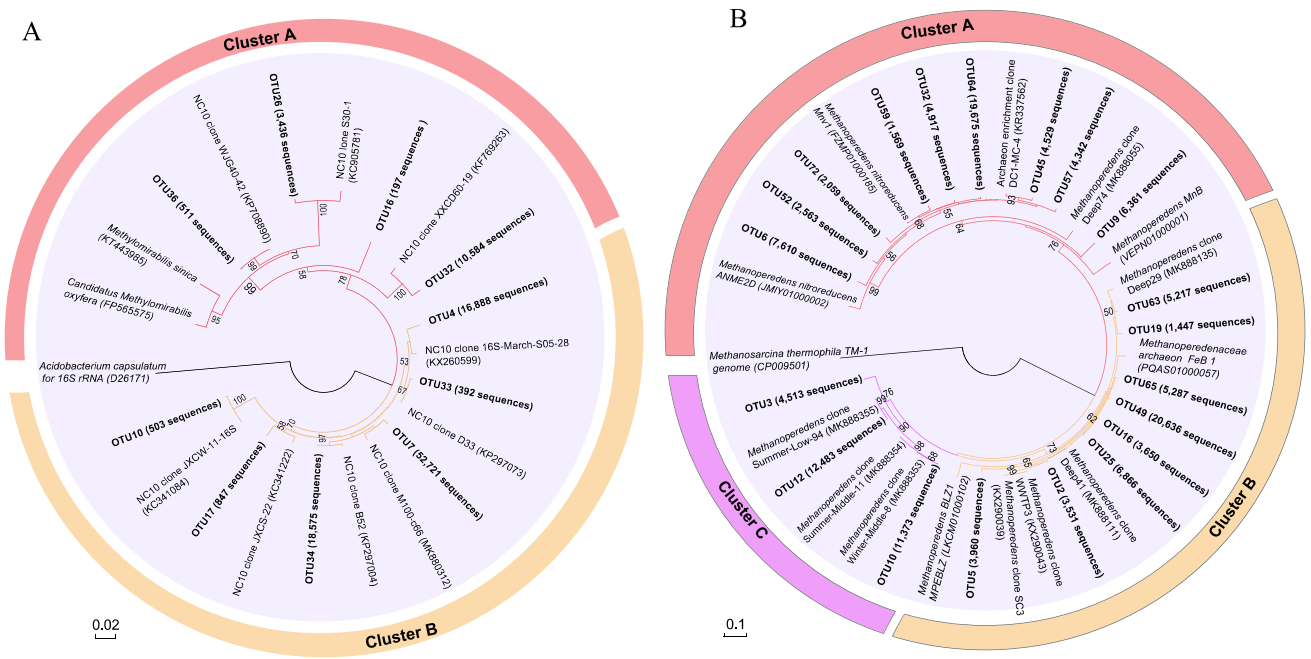

Fig. 3 Neighbor-joining tree of *Methylomirabilis*-related sequences from the top 10 OTUs (**A**) and *Methanoperedens*-related sequences from the top 20 OTUs (**B**). The numbers in bracket denote the number of sequences for each OTU. The scale bar represents 2% sequence divergence in panel (**A**) and 10% divergence in panel (**B**)

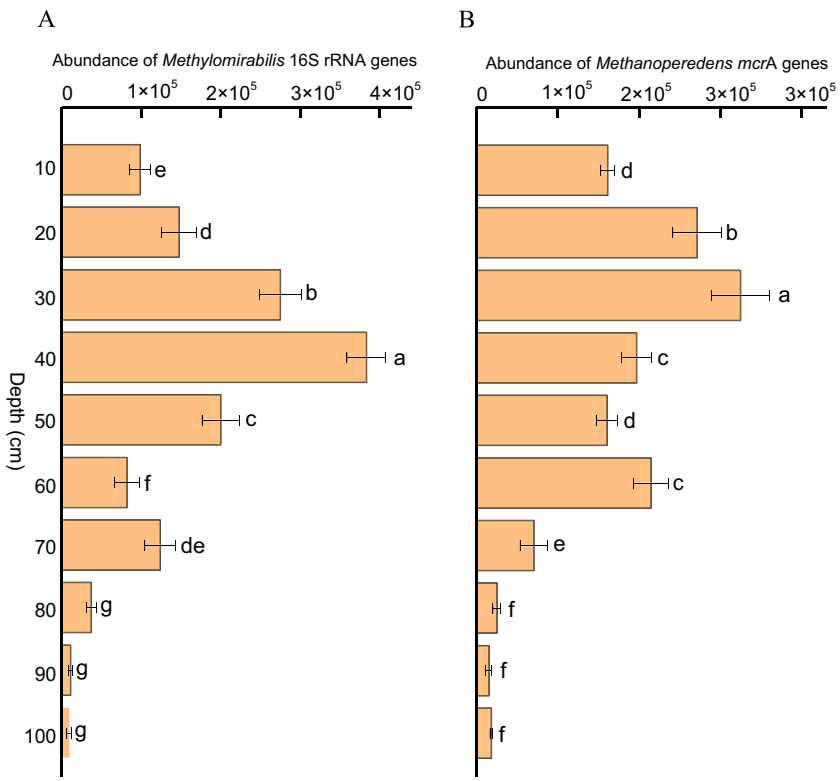

Fig. 4 Abundance (copies g^{-1} dry soil) of *Methylomirabilis*-related bacteria (**A**) and *Methanoperedens*-related archaea (**B**) at different soil depths. Different lowercase letters demonstrate a significant difference in abundance among depths

layer (Fig. 4B). The ratio of the abundance of *Methylomirabilis*-related bacteria to *Methanoperedens*-related archaea was 0.38 (at 50–60 cm layer)-5.95 (at 20–30 cm layer), but there was no significant variation in overall abundance between the two groups of anaerobic methanotrophs (Fig. 1B).

Factors affecting the communities and abundance of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea

The soil physicochemical characteristics have been reported previously (Shen et al. 2017c). The soil water content varied between 23.4% and 34.1%, and pH varied from 6.2 to 6.8. Contents of both soil NH_4^+ and NO_3^- decreased with depth, and their contents were in the ranges of 0.8–16.7 and 0.2–25.4 mg N kg^{-1}, respectively. Contents of soil total nitrogen (TN) and organic carbon (OrgC) were 0.8–1.5 and 8.6–16.5 g kg^{-1}, respectively. The RDA indicated that the soil factors of water content, NH_4^+, NO_3^-, TN and OrgC contents had significant impacts on the vertical distribution

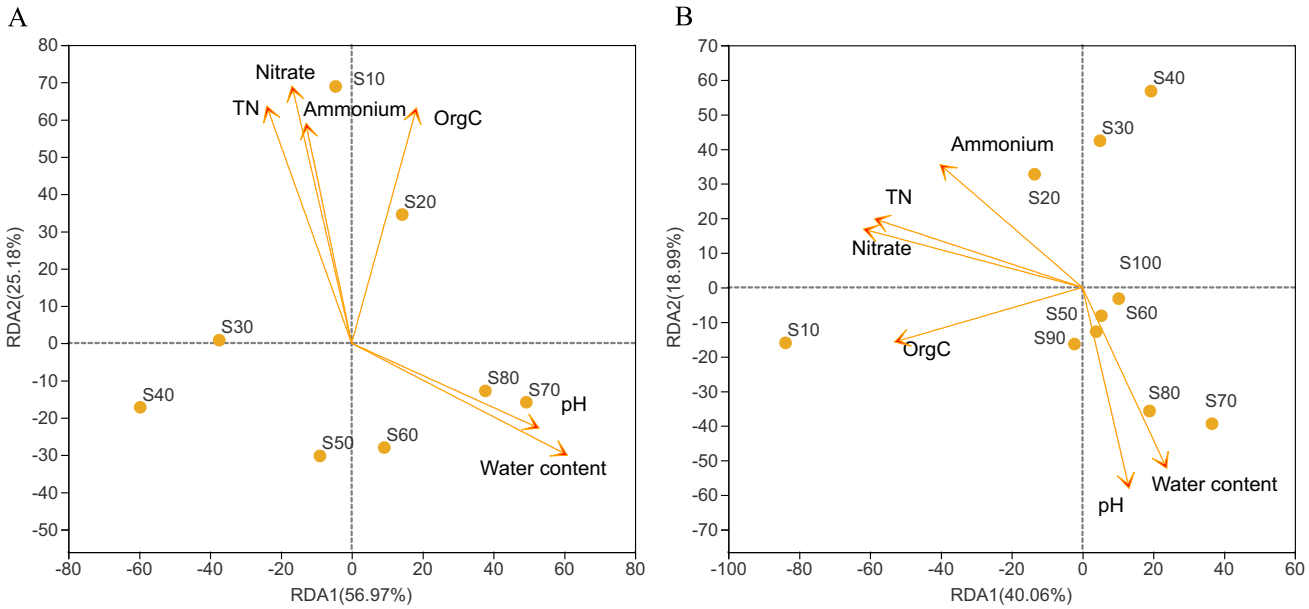

Fig. 5 RDA ordination plots showing the relationships between the community composition of *Methylomirabilis*-related bacteria (**A**), *Methanoperedens*-related archaea (**B**) and the different soil factors. TN—total nitrogen; OrgC—organic carbon. The same below

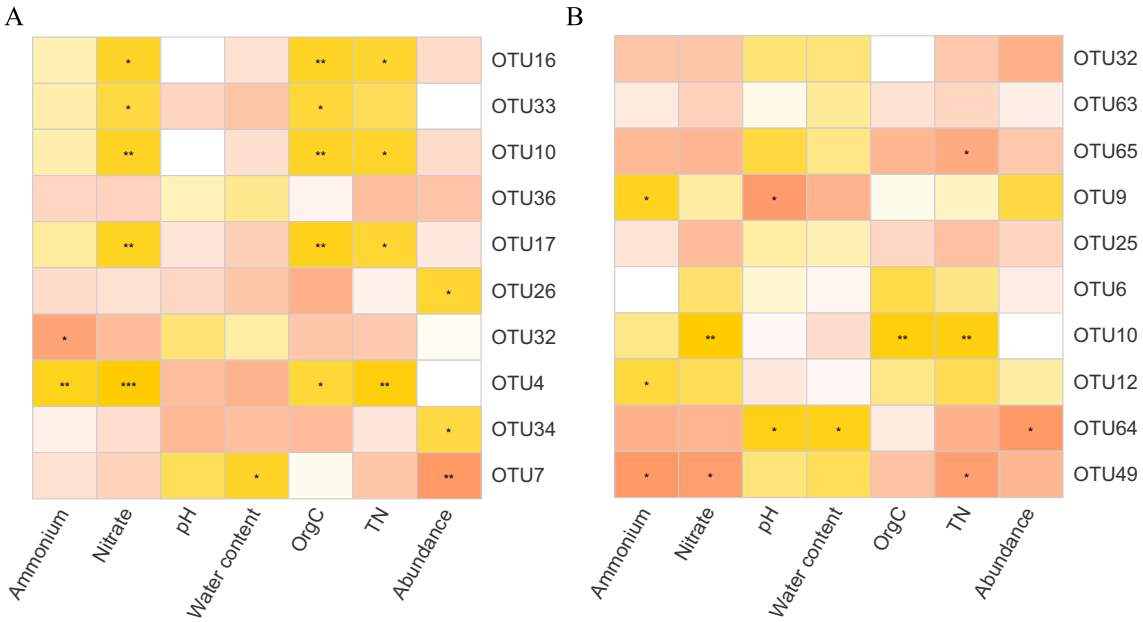

Fig. 6 The heatmaps showing relationships among soil factors, proportions of the 10 dominant OTUs and abundance of *Methylomirabilis*-related bacteria (**A**) or *Methanoperedens*-related archaea (**B**). "*" and "**" demonstrate a significant correlation at $p = 0.05$ and 0.01, respectively

Table 2 Potential correlations between diversity, abundance of *Methylomirabilis*-like bacteria or *Methanoperedens*-like archaea and different soil factors

Soil factors	Pearson correlation coefficients (r)								
	Number of OTUs		Shannon index		Chao1 estimator		Abundance		Abundance ratio
	Methylomirabilis	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis*	*Methanoperedens*	*Methylomirabilis/Methanoperedens*
pH	0.361	-0.718*	-0.489	-0.407	0.319	-0.776**	0.744*	**-0.768****	0.695*
Water content	0.152	-0.411	-0.712*	-0.268	0.122	-0.473	-0.528	-0.393	0.263
NH_4^+-N	-0.110	0.782**	0.098	0.022	-0.155	0.781**	0.269	0.668*	-0.465
NO_3^--N	0.377	0.443	0.401	-0.438	0.308	0.434	0.186	0.472	-0.174
TN	0.355	0.514	0.479	-0.402	0.300	0.498	0.298	0.531	0.072
OrgC	0.433	0.266	-0.017	-0.551	0.343	0.240	-0.138	0.212	0.272

* and ** denote $p < 0.05$ and 0.01, respectively

of *Methylomirabilis*-related bacterial community composition (Fig. 5A). The soil pH, water content, NO_3^-, TN and OrgC contents had significant impacts on the vertical distribution of *Methanoperedens*-related archaeal community composition (Fig. 5B). Furthermore, the above factors also had significant impacts on the proportions of dominant OTUs of the bacteria (Fig. 6A) or the archaea (Fig. 6B).

The soil water content was negatively associated ($p < 0.05$) with the Shannon index of *Methylomirabilis*-related bacteria (Table 2). The soil pH was negatively associated ($p < 0.05$) with both Chao1 estimators and of OTU numbers of *Methanoperedens*-related archaea, while the NH_4^+ content was positively associated ($p < 0.05$) with the two diversity indexes. In addition, the abundance of both *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea was negatively associated ($p < 0.05$) with soil pH, and the later was also positively associated ($p < 0.05$) with NH_4^+ content. The bacterial abundance correlated well ($p < 0.05$) with the archaeal abundance (Figure S1), with their abundance ratio being positively associated ($p < 0.05$) with pH.

Discussion

The anaerobic methanotrophs of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea were reported to be novel players in mitigating CH_4 emissions from paddy ecosystems (Shen et al. 2014a; Vaksmaa et al. 2016; Fan et al. 2021a). Thus far, the distribution and community composition of the two groups of anaerobic methanotrophs in vegetable fields is poorly known. Here, we provide the first evidence for the co-occurrence of the two groups of methanotrophs in different depths of vegetable soils. The number of OTUs of *Methylomirabilis*-related bacteria ranged from 8 to 13 in individual soil samples according to 3% sequence divergence, which was lower than that (7–33 OTUs in individual samples) reported in paddy soils (Shen et al. 2021a; Tian et al. 2021) using the same sequencing approach. The soil water content was negatively related to the Shannon index of *Methylomirabilis*-related bacteria (Table 2), suggesting that a lower level of water content allows the distribution of more diverse lineages of these bacteria. This is not consistent with the results reported in mangrove sediment, where sediment water content showed a positive effect on *Methylomirabilis*-related bacterial diversity (Zhang et al. 2018).

Compared to *Methylomirabilis*-related bacteria, a significantly higher *Methanoperedens*-related archaeal diversity (29–61 OTUs in individual samples) was detected in the studied soils (Fig. 1A). Currently, the NO_2^- was reported to be the only electron acceptor that can be used via *Methylomirabilis*-related bacteria (Ettwig et al. 2010). However,

diverse electron acceptors, including NO_3^-, Fe (III), Mn (IV) and SO_4^{2-}, can be used via *Methanoperedens*-related archaea (Haroon et al. 2013; Ettwig et al. 2016; Cai et al. 2018; Leu et al. 2020; Li et al. 2021; Nie et al. 2021; Chen et al. 2022). The potential of *Methanoperedens*-related archaea to use multiple electron acceptors could contribute to their relatively higher diversity compared to *Methylomirabilis*-related bacteria. The higher *mcr*A gene diversity also indicates that different AOM pathways catalyzing by *Methanoperedens*-related archaea may co-occur in the examined soils. The *mcr*A gene diversity detected in our study was higher than that in paddy soils (3–20 OTUs for each sample, with 95% similarity cutoff) (Shen et al. 2021b), wetland soils (1–26 OTUs for each sample, with 95% similarity cutoff) (Shen et al. 2022) and coastal wetlands (7–12 OTUs for each sample, with 97% similarity cutoff) (Chen et al. 2020). The soil pH and NH_4^+ content were two key factors impacting the *Methanoperedens*-related archaeal diversity in the studied soils (Table 2). The soil pH has been found to have a great effect on the diversity of these archaea in freshwater wetland soils (Shen et al. 2022). The soil NH_4^+ may affect *Methanoperedens*-related archaeal diversity through influencing the nitrification potential, which can produce NO_3^- for these archaea.

Phylogenetic analysis showed two distinct clusters of *Methylomirabilis*-related bacteria (Fig. 3A). The majority of the dominant OTUs exhibited 91.12%-94.16% identity (Table S2) to the 16S rRNA genes of known *Methylomirabilis* species, while only OTU36 (occupying 0.5% of the total sequences) exhibited a relatively higher identity (95.89%-97.32%) to the known species. Our results is consistent with the results reported in paddy fields, where only a minor part of the retrieved *Methylomirabilis*-related sequences showed greater than 95% identity to the known *Methylomirabilis* species (Shen et al. 2021a; Tian et al. 2021). Among the dominant OTUs, the proportions of OTU34 and OTU26 were significantly positively associated with *Methylomirabilis*-related bacterial 16S rRNA gene abundance (Fig. 6A). Nevertheless, it should be mentioned that no positive PCR products of *pmo*A genes of these bacteria can be recovered from any examined soil samples. Hence, the actual role of nitrite-driven AOM in methane oxidation and the main species responsible for this process in vegetable soils need to be further explored. RDA results suggested that the soil water content, NO_3^- and OrgC contents exhibited significant effects on the *Methylomirabilis*-related bacterial community (Fig. 5A). These soil factors may greatly alter the availability of NO_2^- or CH_4 for *Methylomirabilis*-related bacteria via affecting denitrification and methanogenesis (Shen et al. 2021a), and NO_2^- and CH_4 were also reported to impact the vertical distribution of *Methylomirabilis*-related bacterial community in paddy soils (Hui et al. 2017; Vaksmaa et al. 2017c; Tian et al. 2021).

A total of three clusters that were closely related to the genomes of *Methanoperedens* sp. Mnv1 and *Methanoperedens* sp. ANME2D (Fig. 3B) which were reported to conduct AOM coupling with NO_3^- reduction or *Methanoperedens ferrireducens* (Fig. 3B) which was reported to conduct AOM coupling with Fe (III) reduction were detected. The presence of these distinct clusters further suggests the co-occurrence of different methane-oxidizing pathways driven by *Methanoperedens*-related archaea. Shen et al. (Shen et al. 2019) have reported that different active pathways of AOM coupling with NO_2^-, NO_3^-, Fe^{3+} and SO_4^{2-} reduction co-occurred in anoxic riverbed sediments. In agroecosystems, Vaksmaa et al (2017a) reported nitrate-driven AOM activity and the occurrence of *Methanoperedens*-related archaea in an Italian paddy soil. Fan et al (2020) identified multiple AOM pathways driven by various electron acceptors (NO_3^-, Fe_3^+, SO_4^{2-}, and humic acids) in a Chinese paddy field, and also showed the presence of *Methanoperedens*-related archaea (Fan et al. 2021a). Here, all the examined soil factors had important impacts on the community composition of *Methanoperedens*-related archaea (Fig. 5B). A previous study indicated that the sharp decrease of NH_4^+ content and OrgC content with the soil depth had a great effect on the vertical distribution of community composition of *Methanoperedens*-related archaea in paddy soils (Shen et al. 2021b). Recently, Shen et al. (Shen et al. 2022) reported that the soil water content significantly affected these archaeal community composition in freshwater wetland soils.

Quantitative PCR suggested the co-occurrence of *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea throughout 0–100 cm layers, although very low abundance of these methanotrophs was observed at deep layers (80–100 cm) (Fig. 4). This reduced abundance of these methanotrophs in the 80–100 cm layers can be attributed to the comparatively lower nitrate content (0.2–0.5 mg N kg^{-1}) in these layers in contrast to the levels found in the 0–80 cm layers (0.9–25.4 mg N kg^{-1}). The diminished nitrate content in the deep layers may act as a limiting factor for the growth of *Methanoperedens*-related archaea and the symbiotic *Methylomirabilis*-related bacteria. The gene abundance of *Methylomirabilis*-related bacterial 16S rRNA ranged between 9.19×10^3 and 3.83×10^5 copies g^{-1} dry soil, which was lower than the range (10^3–10^7 copies g^{-1} dry soil) reported in rice paddies (Wang et al. 2012; Shen et al. 2014a; Hui et al. 2017; Vaksmaa et al. 2017c; Tian et al. 2021). Although both vegetable and paddy fields receive intensive N fertilizers, the frequent water-logging condition in paddies can provide more anoxic environments for *Methylomirabilis*-related bacteria. Furthermore, the abundance of *Methylomirabilis*-related bacteria exhibited a strong vertical variation, with a higher abundance being recorded in 20–30 cm soils (Fig. 4A). Some previous studies reported an obvious vertical variation in *Methylomirabilis*-related bacterial abundance

in paddy fields (Wang et al. 2012; Shen et al. 2014a; Hui et al. 2017). Wang et al. (2012) found a higher abundance of these bacteria in upper 40 cm paddy soils, while Hui et al. (2017) showed a higher abundance in 40–60 cm soils. The detected abundance of *Methylomirabilis*-related bacteria was negatively related to the soil pH (Table 2). The pH ranged from 6.2 to 6.8 in the studied soils, indicating that this bacterial growth favored under weak acidic conditions. These bacterial abundance was also shown to be negatively related to sediment pH in estuarine wetlands (Chen et al. 2021).

A similar range of *Methanoperedens*-related *mcr*A gene abundance was detected, varying from 1.55×10^4 to 3.24×10^5 copies g^{-1} dry soil, which was lower than that (10^3–10^7 copies g^{-1} dry soil) reported in rice paddies (Shen et al. 2021b; Vaksmaa et al. 2017c). An obvious vertical change of *Methanoperedens*-related archaeal abundance was found, with the abundance peaking at 30–40 cm layer (Fig. 4B). In upland fields, like vegetable soils, though with a high NO_3^- concentration, intermittent flooding indicates that the surface soil layer experiences periodic aerobic-anoxic conditions (Nie et al. 2018), which can negatively affect the growth of *Methanoperedens*-related archaea. These archaeal abundance was also reported to be increased with soil depth in a paddy field, with higher value in 20–40 cm soils (Shen et al. 2021b). Here, the soil pH and NH_4^+ content were negatively and positively correlated with *Methanoperedens*-related archaeal abundance, respectively (Table 2). A recent study demonstrated that these archaeal abundance was also negatively related to soil pH in freshwater wetland soils (Shen et al. 2022). A relatively higher NH_4^+ content can stimulate the nitrification potential, which produce sufficient NO_3^- for *Methanoperedens*-related archaea and then promote these archaeal growth. However, the abundance of *Methanoperedens*-related archaea exhibited a weak positive correlation with the content of soil NO_3^- (Table 2). This can be caused by the competition of NO_3^- between *Methanoperedens*-related archaea and denitrifiers.

In addition, there was no significant variation in overall abundance between the two groups of anaerobic methanotrophs (Fig. 1B), and their abundance correlated well in the examined soils (Figure S1), suggesting the cooperation between the two groups of methanotrophs. Although they may compete for the common substrate of CH_4, *Methanoperedens*-related archaea can produce NO_2^- for *Methylomirabilis*-related bacteria through NO_3^- reduction. Some previous studies also found that the abundance of the two groups of methanotrophs was significantly positively correlated in riverbed (Shen et al. 2019) and coastal wetland sediments (Niu et al. 2022).

Overall, this study provided molecular biological evidence supporting the existence of the two groups of anaerobic methanotrophs across varying depths of vegetable soils. Although previous studies have demonstrated higher AOM activity driven by nitrite or nitrate was correlated with higher gene abundance of *Methylomirabilis*-related bacteria or *Methanoperedens*-related archaea, across diverse ecosystems such as marsh wetland sediments (Shen et al. 2017b), freshwater reservoirs (Shen et al. 2020a), and Tibetan alpine wetlands (Xie et al. 2020a). The extent of nitrite- or nitrate-driven AOM activity carried out by the two groups of anaerobic methanotrophs, along with their proportional role in reducing CH_4 emissions from vegetable soils remain unknown and require additional investigation through $^{13}CH_4$ isotope tracer methodologies. Furthermore, a combination of DNA/RNA-based stable isotope probing techniques is expected to be used to elucidate the functional microbial species catalyzing each AOM pathway (Luo et al. 2021).

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Author contribution LS and YB conceived and designed research. YH and QH conducted experiments. YY and BR contributed new reagents or analytical tools. WY and GG analyzed data. LS and YB wrote the manuscript. All authors read and approved the manuscript.

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Data availability The sequencing data obtained in this study has been submitted to the NCBI BioSample database, the accession numbers for *Methylomirabilis*-related bacteria and *Methanoperedens*-related archaea are SAMN36189722-SAMN36189729 and SAMN36189712-SAMN36189721, respectively.

Declarations

Ethics approval This article does not contain any studies with human participants or animals performed by any of the authors.

Conflict of interest The authors declare no competing interests.

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