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Anaerobic consortia mediate Mn(IV)-dependent anaerobic oxidation of methane

Wenbo Liu^{1,2}, Sai Xu³, Hongpu Ma¹, Yuanyuan Li¹, Jacek Mąkinia⁴, Jun Zhai^{1,2*}

Affiliations

¹College of Environment and Ecology, Chongqing University, Chongqing, 400045, China

² Institute for Smart City of Chongqing University in Liyang, Chongqing University, Jiangsu, 213300, China

³ School of Environmental and Biological Engineering, Nanjing University of Science and Technology, Nanjing, 210094, China

⁴ Faculty of Civil and Environmental Engineering, Gdansk University of Technology, 80-233, Gdańsk, Poland

*Corresponding author. E-mail: zhaijun@cqu.edu.cn (J.Z.)

1 Abstract

2 Manganese-dependent anaerobic oxidation of methane (Mn-AOM) is a major methane sink and vital to mitigating global warming. However, it is difficult for 3 microorganisms to mediate electron transfer between the hardly dissolved CH₄ and 4 5 insoluble Mn(IV) minerals, leading to poor understanding of species mediating Mn-AOM. This study successfully enriched an anaerobic consortium mediating AOM 6 driven by Mn-dependent respiratory growth, and for the first time, revealing a 7 syntrophic pathway for Mn-AOM. The Mn-AOM occurrence was confirmed by long-8 9 term bioreactor performance and ¹³C-labelling batch experiment. Metagenomic and metatranscriptomic analyses demonstrated that the Candidatus Methanoperedens sp. 10 BLZ1 was responsible for CH₄ oxidation. The Luteitalea pratensis mediated 11 extracellular electron transfer crossing S-layer of Ca. M. BLZ1 by conductive pili, and 12 mediated microbial Mn(IV) reduction via multi-heme c-type cytochromes. This study 13 offers an alternative syntrophic pathway for Mn-AOM by a microbial consortium 14 instead of previously reported pathway by ANME alone. These outcomes provided new 15 insight into migrating global climate change and manganese cycles. 16

17 **Keywords:** Anaerobic oxidation of methane; manganese; multi-heme *c*-type

18 cytochromes; extracellular electron transfer

19 1. Introduction

20 Methane (CH₄) is the second most important greenhouse gas after carbon dioxide (CO₂) [1], with the global warming potential 28 times higher than CO₂ [1]. Anaerobic 21 oxidation of methane (AOM), coupled with reduction of nitrate and nitrite [2, 3], sulfate 22 23 [4, 5], metal oxides [6, 7], humic acids [8], and biochar [9], is important to regulate global CH₄ emission [10]. The Mn(IV)- and Fe(III)-bearing minerals are widely 24 distributed in the biosphere. They are also thermodynamically favourable and 25 biochemically feasible electron acceptors for AOM. The Mn(IV)- and Fe(III)-26 27 dependent AOM (Mn-AOM and Fe-AOM) has been observed in in marine [7] and freshwater sediments [11]. They both are major global CH₄ sinks and considered as 28 29 vital energy sources for the early Earth [6]. However, the growth of species mediating Mn-AOM is slow and poorly explored. This is attributed to the difficulty of electron 30 transfer between the hardly dissolved CH₄ and insoluble Mn(IV). Up to now, only two 31 ANMEs (anaerobic methanotrophic archaea) were enriched from AOM mediated by 32 Mn-dependent respiratory growth, namely Candidatus Methanoperedens manganicus 33 and Candidatus Methanoperedens manganireducens [12]. These species can perform 34 Mn-AOM independently via extracellular electron transfer and microbial Mn(IV) 35 reduction. In the extracellular electron transfer, both two ANMEs can use the multi-36 heme *c*-type cytochromes (MHCs) to bridge the nonconductive S-layer by forming 37 MHC/S-layer fusion protein [12]. Then, these ANMEs make contact with the minerals 38 for Mn(IV) reduction, with a certain amino acid motif at the C-terminus of the MHC 39 protein ([ST]-[AVILMFYW]-[ST]-P-[ST]) [13, 14]. 40

Some ANMEs can use Mn(IV) as alternative electron acceptor mediating AOM. 41 The Candidatus Methanoperedens sp. BLZ1 (formerly known as Methanoperedens 42 nitroreducens MPEBLZ), cultured under anaerobic, nitrate-reducing conditions, is able 43 to mediate Mn(IV)-AOM[15]. However, the Ca. M. BLZ1 lacks the proteins for 44 crossing the archaeal S-layer. It is still mysterious for extracellular electron transfer of 45 Ca. M. BLZ1[16]. Besides, this species also lacks the specific mineral-binding 46 47 motifsfor electron transfer between microorganism and insoluble Mn(IV) minerals [16]. Further studies are needed to reveal the mechanisms for Mn-AOM mediating by Ca. 48 49 *M*. BLZ1.

This study aimed to enrich and characterize the Mn-dependent respiratory microorganisms that can perform AOM. The microbial process was determined through mass and electron balance and isotopic tests. Microorganisms involved in Mn-AOM were identified and characterized through metagenomic and metatranscriptomic analyses. These results increase the understanding of Mn-AOM and its role in global methane and Mn cycling. Moreover, the results can also provide new insight into the mitigation of global warming and climate change.

57 2. Methods and Materials

58 **2.1 Bioreactor setup and operation**

A 2.5 L sequencing batch reactor (SBR) was operated for over 820 days to enrich the microorganisms mediating the Mn-AOM process (Fig.S1). The culture was collected from a lab-scale constructed wetland filled with natural Mn ores (Text S1) [17]. In total, a 1.7 L mixture of culture and synthetic medium (Text S2) was filled, leaving 0.8 L headspace. Every 30 days, about 500 mL supernatant of the bioreactor 64 was replaced by the newly synthesized medium.

65 Meanwhile, synthetic δ -MnO₂ slurry (stock solution at 4 gMn/L), prepared according to the modified Murry's method (Text S3) [17], was added as the sole electron 66 acceptor. Subsequently, the reactor was bubbled with pure N2 for maintain anaerobic 67 68 conditions. During the entire experiment, the O_2 in the headspace was consistently below the detection limit (< 0.002% v/v). Next, the CH₄ (99.999%) was supplied as the 69 sole electron donor by flushing the liquid and headspace for 10 min. The reactor was 70 operated at 30 ± 2 °C in a thermostatic water cabinet in the dark, and was mixed gently 71 72 with a magnetic stirrer at 150 rpm. The pH was maintained at ~7.0 by dosing 1 M HCl.

Generally, liquid samples were withdrawn regularly (Text S4). After extraction and filtration, the samples were used to measure the final products of Mn-AOM, including generated Mn(II), soluble Mn(III), dissolved CO₂, and precipitated carbonate. The gas samples were also withdrawn for chemical analysis of CH₄ and CO₂, performed as described previously [17] (see in Text S4).

78 **2.2 Isotopic labeling and stoichiometry test**

On day 820, the reactor was refreshed by the new medium, including δ -MnO₂ and CH₄. After that, about 150 mL ¹³CH₄ (Sigma-Aldrich, 99 atom % ¹³C, USA) was manually injected into the reactor. Every three days, gas samples were withdrawn from the headspace to measure CH₄ and CO₂, and ¹³CO₂. The mixed liquid and solid samples were withdrawn simultaneously to detect the δ^{13} C, Mn(II) and Mn(IV) (see in Text S4). The δ^{13} C stable isotopic ratio of CO₂ was measured with a stable isotope ratio mass spectrometer (isoprime precisION, Elementar, Germany).

The stoichiometry test was performed by using a subsample of bioreactor biomass in duplicate. In total, 300 mL subsamples were transferred anaerobically to a 500 mL of a glass vessel. Every 3 days, gas samples were withdrawn from the headspace for measurement of CH_4 and CO_2 and the Mn(II) concentration in the liquid. The consumption rate of CH_4 (rCH₄) and the production rate of Mn(II) (rMn(II), representing the consumption of Mn(IV)) were calculated via linear regression. The quantitative ratio between rCH₄ and rMn(II) was calculated for the stoichiometry test.

93 2.3 Metagenomic and metatranscriptomic analysis

(1) Metagenomic analysis. Sample collected from the bioreactor on day 820 was
used for metagenomic analysis. A paired-end library was prepared using the TruSeq PE
Cluster Kit v3-cBot-HS and TruSeq SBS Kit v3-HS (Illumina, USA). The library was
sequenced on the HiSeq 2000 (Illumina) platform.

98Paired-end reads for the initial metagenome were trimmed using Seqprep99(https://github.com/jstjohn/SeqPrep)andSicklev1.33100(https://github.com/najoshi/sickle) using the default settings. The reads were assembled101using IDBA-UD [19](http://i.cs.hku.hk/~alse/hkubrg/projects/idba_ud/), a de novo102assembler. The resulting assembly consisted of 676561 contigs \geq 300 bp with an N50103of 1470 bp, and a total of ~ 691 Mbp of sequence data.

Population genomes were recovered from the assembled contigs and unmapped reads using MetaBat (https://bitbucket.org/berkeleylab/metabat, v2.12.1) [20]. 106 Therecovered draft genomes were further treated by refineM
107 (https://github.com/dparks1134/RefineM) and dRep (https://github.com/MrOlm/drep)
108 [21]. Completeness and contamination of the population bins were assessed using
109 CheckM [22].

110 The annotation of assembled contigs for taxonomy was used AMPHORA2 111 (https://github.com/martinwu/AMPHORA2), while the functional annotation was used 112 BLASTP (BLAST Version 2.2.31+, http://blast.ncbi.nlm.nih.gov/Blast.cgi) against 113 protein family databases (Pfam, COG, and KEGG).

(2) Metatranscriptomic analysis. The metatranscriptomic paired-end reads were 114 quality trimmed using bmtagger with the default settings and the contig was obtained 115 by using Trinity. Subsequently, the assembled contigs used TransGeneScan for gene 116 protein annotation and MMseqs2 for annotation. The soap.coverage 117 (https://github.com/aguaskyline/SOAPcoverage) was used to determine the counts for 118 the contigs, and the RNA-TPM was calculated the same as described in other studies 119 120 [12].

121 2.4 Visualization of Mn-AOM microbial aggregates by transmission electron 122 microscopy (TEM)

The fluorescence in situ hybridization (FISH) analysis was performed as described by Ettwig et al. [23] to locate the ANME-2d (Cy-3(red) labeled AAA-641 probe [24]), other archaea (FITC (green) dye-labeled Arch-0915 probe) and bacterial species (DAPI, blue) potentially involving Mn-AOM (Text S5). Then the sample were used to examine by the TEM.

128 The TEM analysis was performed based on a modified protocol described in the previous studies [25]. The samples examined by FISH analysis were embedded into LR 129 white by a graded ethanol series (15 minutes each of 25%, 50%, 75%, 100% \times 3 times). 130 Subsequently, the samples were embedded by 50% LR White Resin and 50% ethanol 131 for 30 min on a shaker at room temperature and then moved to 100% LR White Resin 132 for 1 hour. The polymerization was conducted for 48 h at 56°C. The target areas marked 133 by FISH were taken from the slides and transferred into a tube containing 5% 134 135 phosphotungstic acid hydrate in 100mM HEPES (pH 7.8) and then incubated 90 min on ice. The samples were embedded again as described above (see in Fig.S2). The block 136 was then sectioned at 200nm and examined using a FEI Talos F200S transmission 137 138 electron microscope (ThermoFisher Scientific Co., USA).

139 **3. Results and Discussion**

140 3.1 Observation of Mn(IV)-dependent AOM in the laboratory-scale sequencing 141 batch reactor (SBR)

A laboratory-scale sequencing batch reactor (SBR) was performed for about 820 days to enrich microorganisms mediating Mn-AOM. The inoculum, from a constructed wetland filled with natural Mn ores [17], was fed with CH_4 and δ -MnO₂ as the sole electron donor and electron acceptor, respectively. After 200-day pre-cultivation, stable CH_4 oxidation was observed (Fig. 1a). The observed stoichiometric ratio of $r[Mn(IV)]/r[CH_4]$ (ratio between Mn(IV) reduction rate and CH_4 consumption rate) was 4.01:1, while that of $r[Mn(II)]/r[CH_4]$ (ratio between Mn(II) generation rate and CH_4

149 consumption rate) was 3.83:1 (Fig.1b). Both values are close to the theoretical stoichiometric ratio of 4:1 for Mn-AOM (Table S1). Other soluble electron acceptors 150 were found at low concentrations in the SBR (NO3⁻, <20µg/L; NO2⁻, <10µg/L; SO4²⁻, 151 4.36 mg/L; Fe(III), 0.05mg/L, Table S2). Therefore, the AOM occurred in the 152 bioreactor was mainly coupled with Mn(IV) reduction, namely Mn-dependent AOM. 153 The final product of CH₄ oxidation was CO₂, remaining as carbonate in the solid and 154 liquid phases as reported [12] (Fig.1c). As a result, the CH₄ consumption rate is higher 155 than the CO₂ production rate in the bioreactor's headspace. The observed microbial 156 CH₄ oxidation rate (0.6 pmol/d/cell) was comparable with the reported Mn-AOM [12] 157 (Table 1). 158



Fig. 1. Performance of the laboratory-scale sequencing batch reactor and batch experiments. (a) Total CH_4 reduction and total CO_2 production during the long-term cultivation. The blue arrows indicate δ -MnO₂ additions (the continuing cultivation in Fig.S1); (b) The profiles of Mn(II) and CH_4 during the stoichiometry (batch) test on day 820; (c) Conversion of ¹³CH₄ during the isotopic (batch) test on day 820. The error bars stand for standard deviation of replicated bottles (n=3).

165

166Table 1. Observed CH4 consumption rates in anaerobic oxidation of167methane (AOM) coupled with reduction of Mn.

Key microorganisms for AOM	Observed microbial CH ₄ consumption rate (pmol/d/cell)	Reference
Ca. M. manganicus Ca. M. manganireducens	0.4 - 0.5	[12]
ANME-2d lineage	0.6	[17]
Ca. M. BLZ1	0.3×10 ⁻³	[15]
Ca. M. BLZ1	0.2 (initial period, day 150-460)	This study
	0.6 (stable period, day 460-820)	

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169

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171 **3.2 Genome recovery and microbial community analysis**

172 The metatranscriptomic analysis showed high expression of mcrA gene (methylcoenzyme M reductase alpha subunit gene, TPM=2211.39), which was the key gene in 173 "reverse methanogenesis" pathway [26] and thus proved this pathway was the primary 174 175 CH₄ oxidation process in this study (Fig.2). The particulate methane monooxygenase (pmo) genes, both for Methylomirabilis oxyfera (NC10 phylum) mediating nitrite-AOM 176 via intracellular O_2 production [3, 27] and for aerobic methane oxidation [28], were 177 hardly expressed (TPM=70.20). Besides, the O₂ content in the bioreactor's headspace 178 179 was extremely low (< 0.002%, v/v). Therefore, the possibility of aerobic methane 180 oxidation pathway and nitrite-dependent AOM pathway by *M. oxyfera* were excluded.

The metagenomic analysis revealed that an archaeon containing genes encoded complete "reverse methanogenesis" (Fig.2) was enriched (from 1.4% to 96.3% in the archaeal community), and finally accounting for 6.6% of the whole microbial community (Fig.S2). This archaeon was proven to be *Ca. M.* BLZ1 by comparative analysis (Table S4, S5), with the average amino acid identity (AAI) of 97.7% and average nucleotide identity (ANI) of 98.1% (Text S6, Table S4, S5). Besides, the CH₄

- 187 oxidation rate had a linear relationship with the number of *Ca. M.* BLZ1 (R²=0.94). In
- addition to "reverse methanogenesis", the Ca. M. BLZ1 also contained genes encoding
- 189 enzymes catalyzed for multiple energy conversion and intracellular electron transfer,
- 190 including heterodisulfide reductase (*hdr*), Na⁺ translocating methyltransferases (*Mtr*),
- 191 coenzyme B, ferredoxin, and Cofactor F_{420} (Fig. 2b, Fig.4).





Fig. 2. Metagenomic analysis of the microbial consortia in the bioreactor. (a) Reads number of the key genes encoded CH_4 metabolism pathway; (b) Multi-heme c-type cytochrome with predicted S-layer domains from the reconstructed genomes. Protein schematics are simply positioned to show compositional similarity, not a sequence alignment. Each vertical tick-mark denotes 250 amino acids. The gene identifier
numbers are shown on the right; (c) Heatmap of genes (reads number) encoded enzymes
possibly involving electron transfer and Mn(IV) reduction.

199

200 However, Ca. M. BLZ1 could not mediate Mn-AOM independently because it lacked proteins for extracellular electron transfer crossing the archaeal S-layer, which 201 202 was non-conductive and non-penetrative [29], and also lacked the specific binding motifs for electron transfer to insoluble Mn(IV) minerals. Therefore, archaea or bacteria 203 capable of transferring electrons to insoluble Mn(IV) and mediating microbial Mn(IV) 204 205 reduction, were expected to be the consortia microbial partner. In the archaeal community, the proportion of other archaea was less than 0.1%. It was unlikely that the 206 207 archaea with small proportions could mediate extracellular electron transfer for Mn(IV) 208 reduction.

The *L. pratensis* (1.71% of total microbial community), rather than widely reported metal-reducing bacteria (*Shewanella*, 0.007% of the community; *Geobacter*, not found in our bioreactor), was the bacterial partner in Mn-AOM in the SBR. The *L. pratensis* contained genes encoding MHCs for extracellular electron transfer and metal reduction [30, 31], and the MHCs contained the specific amino acid sequence for Mn(IV)-mineral binding.

215 Instead of enriching Ca. M. manganicus and Ca. M. manganireducens, Ca. M. 216 BLZ1 was successfully enriched in our bioreactor with continuous long-term incubation. This microorganism was earlier enriched through nitrate/nitrite-AOM [16], 217 but the low concentrations of NO₃⁻ (< $20\mu g/L$) and NO₂⁻ (< $10\mu g/L$) excluded their 218 contribution to AOM in this study. In the Mn-AOM, microbial Mn(IV) reduction was 219 performed via two one-electron transfer steps [32], and Mn(III) was the intermediate. 220 The generated Mn(III) will be reduced immediately to Mn(II) by the metal reductase 221 on the surface of bacteria, or rapidly converted into Mn(II) and Mn(IV) via 222 disproportionation [33], leading no Mn³⁺ detected in our bioreactor via 223 spectrophotometric method (absorbance at 258 nm of Mn(III) complex with 224 pyrophosphate) [33]. It is a coincidence that the Ca. M. BLZ1 was enriched both from 225 226 nitrate-AOM and Mn-AOM cultures, indicating there were some specific ecological niches that respiratory growth of Ca. M. BLZ1 in both cultures, but requiring further 227 228 investigation.

229 **3.3 Extracellular electron transfer between** *Ca. M.* BLZ1 and *L. pratensis*

The Ca. M. BLZ1 cannot perform extracellular electron transfer across the S-layer, 230 because its genes encoded MHC/S-layer fusion protein incapable of forming complete 231 "bridge" crossing the S-layer (Fig.2c), and it has no genes encoded conductive 232 233 archaellum(archaeal conductive nanowire). On the contrary, the Ca. M. manganicus contained MHC/S-layer proteins possessing 12 and 22 hemes, while Ca. 234 M.manganireducens contained MHC/S-layer proteins processing 113, 52 and 19 hemes 235 [12]. Besides, the Ca. M. manganicus can also use conductive archaellum for electron 236 237 transfer in Mn-AOM [12].

238

The Ca. M. BLZ1 contains no genes for formate formation, H₂ production, or

239 membrane-bound H₂-uptake NiFe hydrogenases. Besides, no formate in liquid or H₂ in the headspace was detected in our bioreactor (Table S2). The genes encoding the 240 enzymes for the complete acetoclastic methanogenesis pathway were found in Ca. M. 241 BLZ1 [16], but the key genes of acetyl-CoA decarbonylase/synthase (cdh) were not 242 expressed, and expression of acetyl-CoA synthetase (ACS) was low (TPM=41.1). 243 Besides, the acetate was not found in the water stream. No microorganisms in our 244 bioreactor contained genes encoding the complete dissimilatory sulfate reduction, 245 resulting in that the novel Mn(IV)-supported sulfate-AOM [34], using Mn(IV) to 246 oxidize sulfur species from sulfate-AOM, was excluded in our bioreactor. 247

The conductive pili were shown by the TEM image (Fig.3, in red circle). The conductive pili connected the *Ca. M.* BLZ1 (spherical shape) and *L. pratensis* (rodshaped), mediating extracellular electron transfer (Fig.3a,c). Besides, the direct contact between *L. pratensis* and Mn(IV)-minerals was observed (Fig.3), which was also suitable for extracellular electron transfer in Mn(IV) reduction [35].



Fig. 3. Spatial location of microbial consortia mediating Mn-AOM in our bioreactor. A: *Candidatus Methanoperedens* sp. BLZ1 (spherical shape); B: *Luteitalea pratensis* (rod-shaped [31]); M: δ -MnO₂. The conductive nanowire structure was marked in the red circle. The scale was listed in the picture. (a), (d), (g) and (h) were the TEM image; (b) and (e) the image of element Mn by TEM; (c) and (f) the image of element W by TEM (representing for microorganisms).

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In our bioreactor, *L. pratensis* encoded conductive pili (Fig. 2c), assist the electron crossing the S-layer of the *Ca. M.* BLZ1. Then, these electrons were transported through the MHCs containing in the *L. pratensis* and finally to terminal electron acceptor (Mn(IV)-minerals). (Fig.4). The MHCs, including OmcS, MtrC and OmcA, was highly expressed (TMP= 1240.63). Both MtrC and OmcA were metal reductase [36, 37] while the OmcS could form the specific bond with the mineral surface [38]. These MHCs participated in microbial Mn(IV) reduction by direct transfer of the electrons to

269 insoluble MnO₂.

270



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272 Fig. 4. Putative syntrophic pathway for Mn-AOM by *Candidatus Methanoperedens*

sp. BLZ1 and Luteitalea pratensis. Abbreviations for enzymes and co-factors: mcr, 273 274 methyl-coenzyme M reductase; *mtr*, Na⁺-translocating methyl-H₄MPT:coenzyme M methyltransferase; mer, F₄₂₀-dependent methylene-H₄MPT reductase; mtd, F₄₂₀-275 dependent methylene H₄MPT dehydrogenase; *mch*, methenyl-H₄MPT cyclohydrolase; 276 formylmethanofuran-H₄MPT formyltransferase; *fmd*, formyl-methanofuran 277 ftr, dehydrogenase (also known as *fwd*); *frh*, F₄₂₀-reducing hydrogenase; *hdr*, 278 heterodisulfide reductase; *nrf*, cytochrome c nitrite reductase; *cyt*b, b-type cytochrome; 279 cvtc, c-type cytochrome; FeS, ferredoxin iron sulfur protein; MtrABC, metal-reducing 280 outer membrane decahaem cytochrome c; CoB-SH, coenzyme B; CoM-SH, coenzyme 281 CoB-S-S-CoM. Coenzvme heterodisulfide: 282 M: M-HTP H₄MPT. tetrahydromethanopterin; Fd, ferredoxin; MQ, menaquinone; MQH₂, menaquinol. 283

284

285 **4. Conclusion**

In conclusion, unlike previously reported Mn-AOM mediated by ANME alone, this study revealed a syntrophic pathway by an anaerobic consortium. In this consortium, *Ca. M.* BLZ1 encoded and expressed genes for the "reverse methanogenesis", converting CH_4 to CO_2 . The *L. pratensis*, as a bacterial partner, assisted the extracellular electron transfer from archaea to terminal Mn(IV) minerals and microbial Mn(IV) reduction. The electrons generated from CH_4 oxidation acrossed

the archaeal S-layer via the conductive pili of *L. pratensis*. Then, those electrons were used for Mn(IV) reduction through MHCs, including metal reductases like MtrA and OmcA, and OmcS. This study expands the knowledge and provides new insight into the metabolic characteristics of *Methanoperedenaceae* family and Mn(IV)-AOM. The syntrophic pathway for Mn-AOM is vital for migtagating the global climate change and manganese cycles.

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423	Declaration of interests
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425	\boxtimes The authors declare that they have no known competing financial interests or personal
426	relationships that could have appeared to influence the work reported in this paper.
427	
428	\Box The authors declare the following financial interests/personal relationships which may be
429	considered as potential competing interests:
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437	Highlights
438	Anaerobic consortia mediate Mn-dependent AOM via syntrophic pathway
439	• Ca. Methanoperedens sp. BLZ1 mediates CH_4 oxidation via reverse
440	methanogenesis
441	• Interspecies electron transfer is by conductive pili of <i>Luteitalea pratensis</i>
442	• Luteitalea pratensis perform Mn(IV) reduction via multi-heme c-type cytochrome
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