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ORIGINAL PAPER

Methane oxidation coupled to vanadate reduction in a membrane biofilm batch reactor under hypoxic condition

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Abstract This study shows vanadate (V(V)) reduction in a methane (CH_4) based membrane biofilm batch reactor when the concentration of dissolved oxygen (O_2) was extremely low. V(IV) was the dominant products formed from V(V) bio-reduction, and majority of produced V(IV) transformed into precipitates with green color. Quantitative polymerase chain reaction and Illumina sequencing analysis showed that archaea *methanosarcina* were significantly enriched. Metagenomic predictive analysis further showed the enrichment of genes associated with reverse methanogenesis pathway, the key CH_4-activating mechanism for anaerobic methane oxidation (AnMO), as well as the enrichment of genes related to acetate synthesis, in archaea. The enrichment of aerobic methanotrophs *Methylococcus* and *Methylomonas* implied their role in CH_4 activation using trace level of O_2, or their participation in V(V) reduction.

Keywords Methane oxidation · Vanadate reduction · Membrane biofilm batch reactor · Hypoxic condition

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Introduction

Vanadium (V), a transition heavy metal, is derived from a variety of natural and human activities, including V-rich rocks and V associated industries (Bosch et al. 1989; Nriagu 1998; Wright and Belitz 2010; Yoo 1998). Although trace amount of V is beneficial to the organisms, V in water is always a concern due to its mutagenicity and cytotoxicity to human bodies (Altamirano-Lozano 1998). The maximum permitted V concentration in drinking water established by China was 0.05 mg/L (Standardization Administration of People's Republic of China, 2006). It has been reported that the V concentration in the surface water in Panzhihua region, China, reached up

to 0.3 mg/L, far exceeding the permitted limit (Zhang et al. 2019a). V in natural water usually exist as pentavalent (V(V)) and tetravalent (V(IV)) (Crans et al. 2004). V(V) is highly toxic and soluble, while V(IV) has much less toxicity and easily form into precipitates (Patel et al. 1989). Therefore, transforming V(V) to V(IV) is considered as an effective method to remove V from wastewaters (Ortiz-Bernad et al. 2004).

Several physico-chemical technologies have been applied to remove V in wastewaters, such as adsorption by metal (hydr) oxide and nanoparticles, ion exchange and co-precipitation (Naeem et al. 2007; de Godoi et al. 2013; Keränen et al. 2015; Blackmore et al. 1996). Due to the low expense and simplicity, bio-reduction of V(V) for V detoxification draws more attention in recent years (Carpentier et al. 2003; Xu et al. 2015; Zhang et al. 2014, 2018, 2019b). Electron donors, e.g., lactate, acetate, and glucose were needed for the bio-reduction process (Ortiz-Bernad et al. 2004; Carpentier et al. 2003; Zhang et al. 2014). In recent years, CH_4 has been deemed to be a more promising electron donor compared with the traditional electron donors, as CH_4 is nontoxic to environment, leaves less electron residues (due to its low water solubility), and has lower cost per electron equivalent (Lai et al. 2016a, b, 2018a, b, c; Lv et al., 2019). A variety of electron acceptors, including nitrate (NO_3^-), nitrite (NO_2^-), selenate (SeO_4^{2-}), chromate (CrO_4^{2-}), and bromate (BrO_3^-) have been documented to be reduced in CH_4 supplying systems (Lai et al. 2016a, b, 2018a, b, c; Haroon et al. 2013; Ettwig et al. 2010; Lu et al. 2016).

Based on stoichiometric and energetic calculation, the occurrence of methane oxidation coupled to V(V) reduction is feasible (Without considering biomass synthesis, Rittmann and McCarty 2012):

$$8VO_2^+ + CH_4 + 8H^+ = 8VO^{2+} + CO_2 + 6H_2O \quad (1)$$
$$\Delta G = -951.2\,\mathrm{kJ/mol\,CH_4}$$

In our previous work, we demonstrated bio-reduction of V(V) and other electron acceptors in CH_4 based membrane biofilm reactors (MBfR) (Lai et al. 2016a, b, 2018a, b; Luo et al. 2015). In that work, aerobic methane oxidation (AMO) played the major role, and released intermediate electrons that were utilized by oxyanions-reducing bacteria, thus transferring oxidized oxyanions to reduced form. Due to the involvement of O_2, AMO process is energy intensive and has low electron utilizing efficiency. In contrast, anaerobic methane oxidation (AnMO) process does not need O_2, saving more energy and expense. However, AnMO coupled to V(V) reduction (AnMO-VR) has never been reported.

AnMO could be carried out by bacteria affiliated to NC10 and anaerobic methanotrophic archaea (ANME) (Haroon et al. 2013; Ettwig et al. 2010). The bacteria affiliated to NC10 activated CH_4 by "Intra-Aerobic" pathway. In this process, electron acceptor NO_2^- was reduced to NO, and further disproportionated to O_2 intracellularly, which was used to oxidize CH_4. ANME activated CH_4 by "Reverse Methanogesis" pathway. Raghoebarsing et al. (2006) hypothesized ANME activate CH_4 by reverse methanogenesis to generate electrons, which were then shuttled to denitrifying bacteria for denitrification. Moreover, Haroon et al. (2013) reported ANME-2d alone was able to carry out NO_3^- dependent AnMO through "Reverse Methanogesis" pathway. Key enzymes, e.g., methylcoenzyme M reductase (*Mcr*), tetrahydromethanopterin S-methyltransferase (*Mtr*), and methylenetetrahydromethanopterin cyclohydrolase (*Mch*) were involved in the "Reverse Methanogesis" pathway (Haroon et al. 2013). Although AnMO coupled to bio-reduction of NO_3^-, NO_2^-, SO_4^{2-}, and Fe^{3+} have been reported (Haroon et al. 2013; Ettwig et al. 2010; Beal et al. 2009; Ettwig et al. 2016; Milucka et al. 2012), whether AnMO is able to reduce more oxyanions remained unknown.

Compared with AnMO, AMO is usually carried out by two bacterial groups: methanotrophs and oxyanions-reducing bacteria such as denitrifying bacteria. In this process, methanotrophs derive electrons from CH_4 by incorporating O_2, and methane mono-oxygenase plays a crucial role in activating CH_4 (Modin et al. 2007). Electrons were released as intermediates such as acetate, methanol, formic acid and then used by co-existing denitrifying bacteria for bi-reduction of NO_3^- and NO_2^- (Modin et al. 2007).

However, a series of researches demonstrated methanotrophs played an important role in CH_4 supplied culture when the concentration of surrounding O_2 was extremely low or even zero. Bar-Or et al. (2017) reported increasing gene copies of methane monooxygenase *pmoA*, a key enzyme for AMO, in an iron-dependent AnMO culture. Martinez-Cruz et al. (2017) carried out anaerobic incubation for sub-Arctic lake sediments, finding aerobic methanotrophs, e.g.,

Methylobacter, assimilated most of carbon from CH_4. Moreover, Siniscalchi et al. (2017) found the high abundance of aerobic methanotrophs dominating in a culture which performed AnMO coupled to denitrification process. So the absolute distinction between AMO and AnMO under low oxygen concentration was vague.

Thus, the first aim of this study is to test bioreduction of V(V) in a CH_4 based membrane biofilm batch reactor (MBBR) when the concentration of surrounding O_2 was extremely low, and the second objective is to reveal the possible microbial mechanisms in the process. We assessed the microbial community in the biofilm by quantitative polymerase chain reaction (qPCR) and Illumina sequencing targeting methanogens' and bacterial 16S rRNA genes, and predicted the metabolic genes associated with CH_4 oxidation and V(V) reduction through PICRUSt pipline.

Materials and methods

Experimental setup

The MBBR system was comprised of a 1 L glass container, 42 hollow fibers, plastic caps and norprene tubings (Lai et al. 2018c). The fibers were inserted into a glass tube, glued together with AB gel (EC2216, Scotch-Weld, USA) and sealed within Norprene tubing. The MBBR system was similar to that of Lai et al. (2018c) except that a gas collection bag (Delin, Dalian) was connected to the system all the time without clamp by pinchcocks, which may cause O_2 permeation at a very low concentration. All fibers were supplied with CH_4 gas from both ends at the pressure of 10 psi, while a magnetic stirrer was applied to mix the liquids. O_2 in the gas collection bag was determined every 5 days.

Startup and continuous operation

A culture from our previous V(V) reducing MBfR was inoculated into the MBBR (Lai et al. 2018a). The medium contained following ingredients (per L of demineralized water): $Na_2HPO_4{\cdot}12H_2O$ 0.8 g, $MgSO_4{\cdot}7H_2O$ 5 mg, NH_4Cl 20 mg, $CaCl_2$ 1 mg, KaH_2PO_4 0.4 g, trace element solution 1 mL (Luo et al. 2015). The medium was degassed with argon (Ar) for 15 min before use. The MBBR operation was in sequencing batch mode and was divided into three stages, in which the introduced V(V) concentration was 10, 10 and 20 mg V/L in Stage 1, 2 and 3, respectively. V(V) was added again after V(V) was completely consumed for each stage. The MBBR contains 200 mL of headspace and 800 mL of liquid, and the working temperature was kept at 35 ± 1 °C. O_2 in the MBBR was determined by GC (model 7890A, Agilent Technologies Inc., U.S.A), and the concentration in headspace was bellow detection limit (200 ppm, equivalent to 8 μg/L dissolved O_2 in liquids by Henry's law).

Chemical analysis

We sampled 0.5 mL liquids from the MBBR and filtered them using a 0.22 μm membrane filter for removing microbial biomass. The liquid samples were centrifuged ($15{,}000 \times g$, 10 min) to remove V(IV) precipitates. The concentrations of V(V) and V(IV) were assayed by spectrophotometric method as previously reported by Ensafi et al. (1999) and Safavi et al. (2000), respectively. pH in the liquids of MBBR system were measured by a pH meter and it was in the range of 7.0–7.5.

Biofilm sampling, DNA extraction and imagining

We conducted biofilm samplings in an anaerobic glove box (AW200SG, Electrotek, England). At the end of each stage, we cut off a section from a biofilm attached fiber ($\sim$ 5 cm in length) for DNA extraction, and the remained fiber was sealed. We extracted DNA from the biofilms using the DNeasy Blood and Tissue Kit as described previously (Lai et al. 2016a). At the end of Stage 3, ten fibers were cut off for SEM observation (Lai et al. 2014). After more than 250 days of running time (the V(V) removal data after day 156 was not shown in Fig. 1), precipitates were collected for characterization.

qPCR analysis of mcrA, and archaeal and bacterial 16S rRNA genes

We amplified the MBBR biofilms with primes MLf/MLr targeting *mcrA* (unit of methyl-coenzyme reductase) (Ceccarelli et al. 2014), ARC787F/ARC1059R targeting archaeal 16S rRNA gene (Yu et al. 2005), and 16SF/16SR targeting bacterial 16S rRNA gene

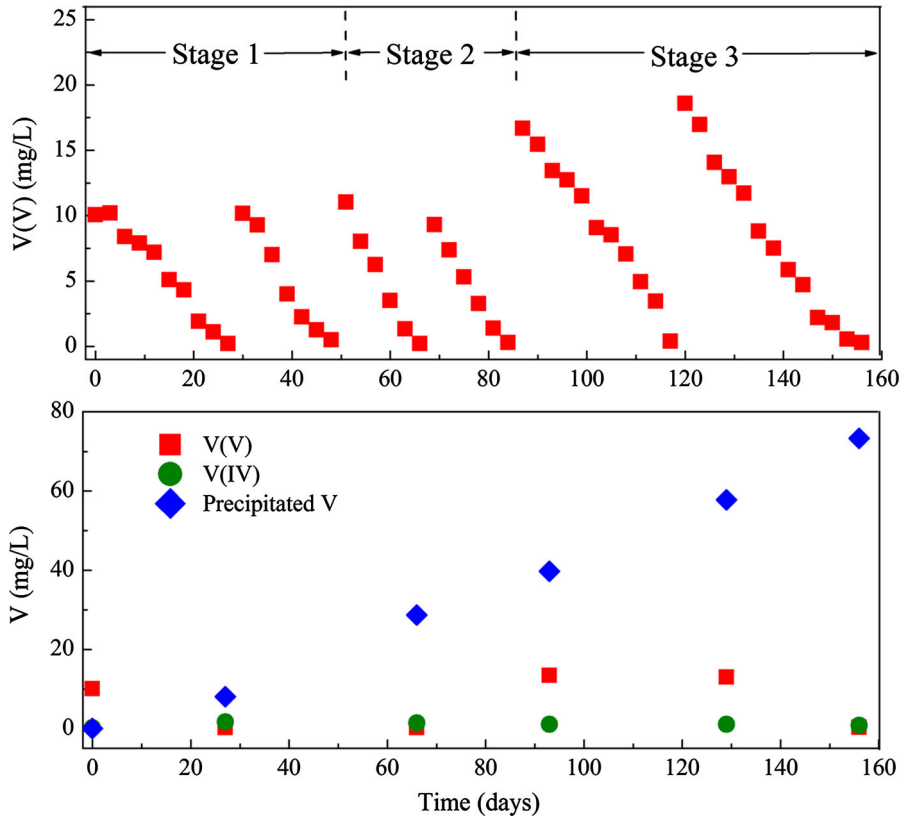

Fig. 1 Performance profiles during the three stages. Top: measured concentration of V(V) in the CH_4-based MBBR. Bottom: measured concentrations of V(V), V(IV) and calculated concentration of precipitated V

(Maeda et al. 2003). We used plasmids that contain target fragments to build standard curves and serve as positive control for qPCR analysis. We utilized the SYBR Premix to perform qPCR for DNA samples and standard plasmids according to the instruction. Sterile water served as negative template, and three repetitions in qPCR reaction were performed for all samples.

Illumina sequencing

We amplified V9 and V4–V5 regions of 16S rRNA genes for methanogens and bacteria, respectively using Premix Taq 2.0 plus dye (Takara, Japan). The primer sets were 1106F/1378R (targeting methanogens) and 515F/907R (targeting bacteria) (Lai et al. 2016b; Feng et al. 2012). We purified the amplicons and sent them to Novogene Technology company to perform Illumina sequencing. The raw data were trimmed and processed using QIIME (version 1.9.1) (Lai et al. 2014). The metagenomic information associated with archaeal and bacterial 16S rRNA gene data were predicted using PICRUSt piplines (Langille et al. 2013; Xie et al. 2011).

V(IV) precipitates characterization

We recovered the V(IV) precipitates by sequential washing using deionized water, and 100% acetone for three times, and centrifugation (10,000 × *g*, 2 min) was applied to separate the precipitates and supernatant. We applied SEM (Hitachi TM 1000) and EDS analyzer (Hitachi Se3400N VP-SEM–EDS) to determine the constituent of V(IV) precipitates, and conducted XPS (ESCALAB 250, Thermo Electron, USA) to measure the valent state.

Results and discussion

V(V) reduction in the CH4 based MBBR

Figure 1a, b illustrated the measured V(V) concentrations throughout the whole experiments. CH_4 supply was not limited, since the theoretical CH_4 demand was much lower than the supplied CH_4 flux shown in Table S1. The V(V) removal rates in Stage 2 (0.66 mg/L day) were higher than that in Stage 1 (0.43 mg/

L day) (Table S1), as the microorganisms in the biofilm need adaption in Stage 1. However, the V(V) removal rates decreased slightly in Stage 3 (0.52 mg/L day) compared with Stage 2, might due to the negative impact of higher loading of V(V). Table S2 showed that the V(V) reduction mainly followed zero-order kinetics elucidated by linear regression model.

The color of the bulk liquids in the MBBR turned into green during the running of MBBR, implying the occurrence of V(IV) resulting from V(V) reduction. However, after centrifugation of the liquid samples, precipitates with green color occurred and the liquid phase turned into colourless. Figure 1b shows that less than 1.7 mg/L of V(IV) was detected by spectrophotometry. The concentration of precipitated V was up to 73.3 mg/L that was calculated by subtracting measured V(IV) from total added V(V) after 156 days' running. These results suggest most of V(IV) formed as precipitates during V(V) reduction, which is further confirmed by SEM–EDS, and XPS characterization (Fig. S1).

We previously achieved bio-reduction of V(V) in a CH_4 based MBfR, and the O_2 concentration in the influent and effluent was between 0.1 and 0.2 mg/L. However, in this study, we found V(V) could be reduced in the CH_4 based MBBR when O_2 concentration was extremely low, as O_2 in the MBBR system was undetectable by using GC (the lowest detection limit was 8 μg/L O_2). Besides, V(IV) precipitates as the major production formed form this bio-process might also bring additional commercial benefits. V has been used in multiple industries, including pharmaceutical and atomic energy industry, petroleum refining, phthalic anhydrides and sulfuric acid production (Bosch et al. 1989; Nriagu 1998; Wright and Belitz 2010; Yoo 1998), and is also an important alloying element which can improve the hardness and durability of many kinds of materials (Gupta and Krishnamurthy 1992). The expanding demand and wide application of V makes it one of the most important metals for modern technology (Nriagu 1998; Ortiz-Bernad et al. 2004).

Microbial community

Figure 2a illustrated the qPCR results normalized to copies/cm^2 of biofilm for archaeal 16S rRNA and *mcrA*, and bacterial 16S rRNA genes. The abundance of these genes increased continuously through whole experiments, implying both of archaea and bacteria were enriched. Figure 2b showed two archaeal methanogens, *Methanobacterium* (Kim et al. 1995) and *Methanosarcina* (Rotaru et al. 2014), dominated in the biofilm by Illumina sequencing targeting methanogenic 16S rRNA gene. The proportion of *Methanosarcina* increased from 13.5 to 34.5%, accompanied with decreased abundance of *Methanobacterium* from 38.6% to 4.6%, through Stage 1 to Stage 3.

Three clades of archaea, ANME-1, ANME-2, and ANME-3, has been reported to couple AnMO to bio-reduction of electron acceptors such as SO_4^{2-}, NO_3^-, and Fe^{3+} (Haroon et al. 2013; Ettwig et al. 2016; Meyerdierks et al. 2010). Among these three clades, ANME-2 and ANME-3 belong to the *Methanosarcinales*, while *Methanosarcina* also belongs to this family (Meyerdierks et al. 2010; Sheehan et al. 2015). Therefore, the genus *Methanosarcina* has intimate

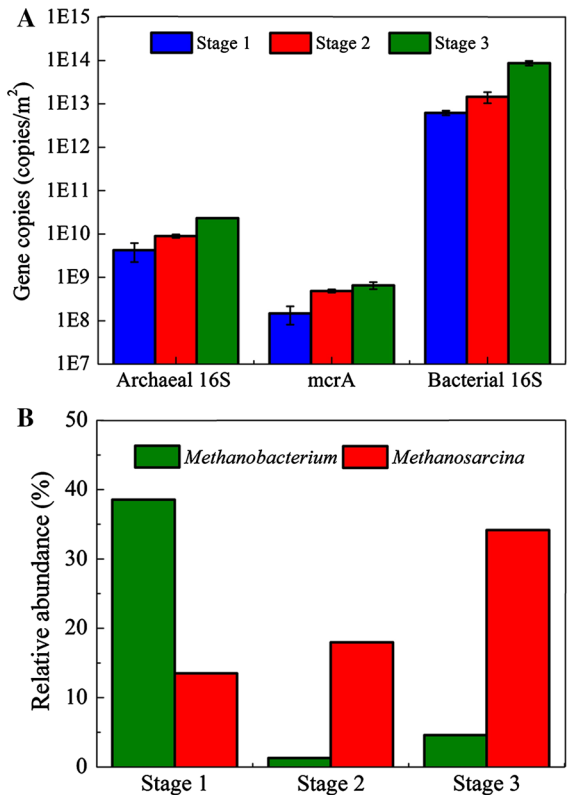

Fig. 2 **a** Copy numbers of archaeal mcrA and 16S rRNA gene, and bacterial 16S rRNA gene in the V(V) reducing CH_4 fed biofilms. Results are the mean values calculated from three replicates. **b** Relative abundance of two dominant methanogens in the biofilms during Stage 1 to Stage 3

phylogenetically relationship with ANME, implying *Methanosarcina* might have the similar metabolic capability with ANME. Considering some *Methanosarcina* species harbor the ability to reduce ferric iron (Fe(III)), this genus probably also played as vanadate reducer (Liu et al. 2011).

Figure 3 shows that methanotrophs, e.g., *Methylococcus* and *Methylomonas*, were the predominant bacteria in the biofilm. Although the relative abundance of these two genera decreased from inoculum to Stage 1, it increased again in the latter two stages. Notably, the relative abundance of *Methylococcus* increased from 5.4 to 23.7% throughout the whole experiments.

Here we propose three possible reasons explaining the persistence of aerobic methanotrophs *Methylococcus* and *Methylomonas*. Firstly, the inoculum contained high abundance of these aerobic methanotrophs and they were robust and able to bear hypoxia. *Methylomonas denitrificans* has been found to carry out CH_4 oxidation even the surrounding O_2 concentration was lower than 50 nM (Kits et al. 2015), while *Methylococcaceae* was able to live where O_2 was scarce (Danilova et al. 2016). These two genus might oxidize CH_4 using trace level of O_2 in the system. Secondly, these aerobic methanotrophs might also play a role in AnOM, although their real contribution hasn't been identified. Bar-Or et al.(2017) incubated anoxic slurry which had iron-dependent AnMO activity and found ^{13}C-labeled CH_4 was mainly assimilated by aerobic methanotrophs They proposed AnMO might be carried out by synergy between methanogens and aerobic methanotrophs. Similarly, Martinez-Cruz et al.(2017) applied stable isotope probing technologies targeting sub-Arctic lake sediments and found type I methanotroph *Methylobacter* assimilated carbon from CH_4 in anaerobic condition. Thirdly, these aerobic methanotrophs might act as vanadate-reducers, as aerobic methanotrophs have versatile functions and they contain multiple reductases. *Methylococcus capsulatus* (Bath) has been peported to detoxify mercury ions (Hg(II))

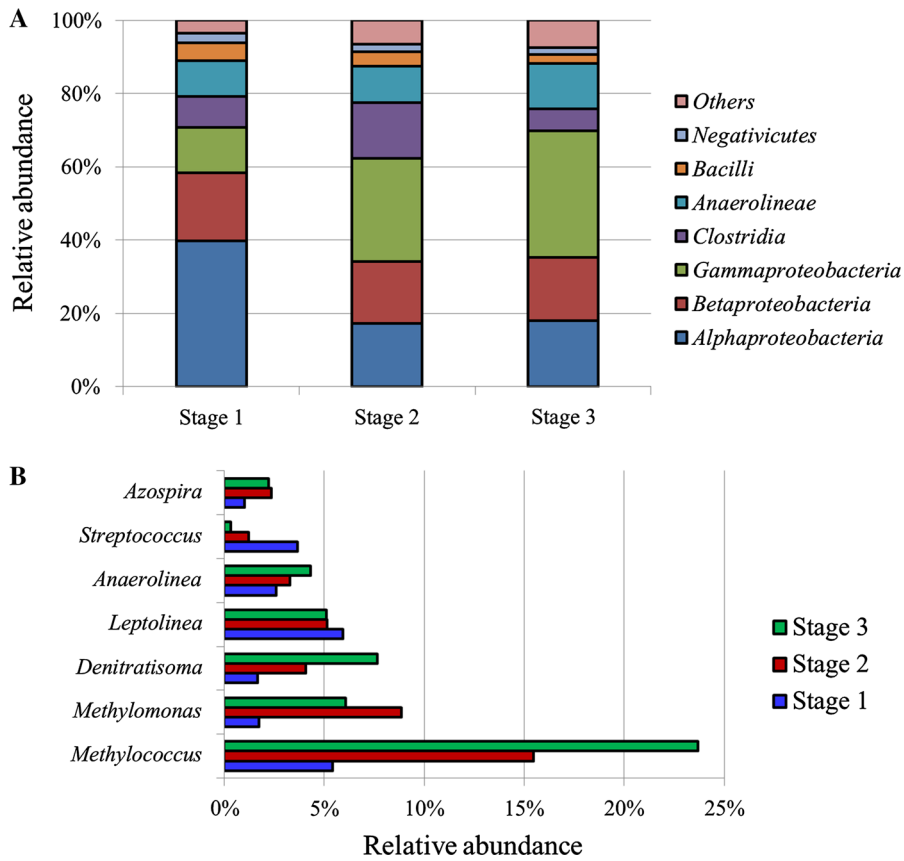

Fig. 3 Phylogenetic profiling of the dominant bacteria in the biofilms at the levels of class (**a**) and genus (**b**)

through dissimilatory Hg(II) reduction (Boden and Murrell 2011), while *Methylomonas denitrificans*, sp. nov. type strain FJG1 had metabolic modules for NO_3^- reduction (Kits et al. 2015).

Proposed microbial mechanisms

We applied PICRUSt pipeline to in-depth excavate predicted metagenomic information to reveal the possible microbial mechanisms in the CH_4 fed biofilm. As to methanogens, predicted genes encoding key enzymes related to (reverse) methanogenesis pathway, e, g., methyl-coenzyme M reductase (*Mcr*), tetrahydromethanopterin S-methyltransferase (*Mtr*), and methylenetetrahydromethanopterin cyclohydrolase (*Mch*), and formylmethanofuran dehydrogenase (Fmd), were detected (Fig. 4). Besides, majority of these genes were enriched during Stage 1 to Stage 3, suggesting the possible occurrence of AnMO by *Methanosarcina*-like methanogen through reverse-methanogenesis pathway. The detection of predicted gene encoding acetate kinase (*Ack*) in methanogens' community suggested *Methanosarcina*-like archaea might be able to produce acetate, a versatile intermediate electron donor that can be used by other group for V(V) reduction (Ortiz-Bernad et al. 2004). The obvious increased abundance of predicted genes encoding ATP synthase (*Atps*) and sodium/proton antiporter (*Mrp*) suggested the AnMO coupled to V(V) reduction process might produce large amount of energy (ATP) for *Methanosarcina*'s biomass synthesis and metabolic activities (Stock et al. 1999; Beck and Rosen 1979).

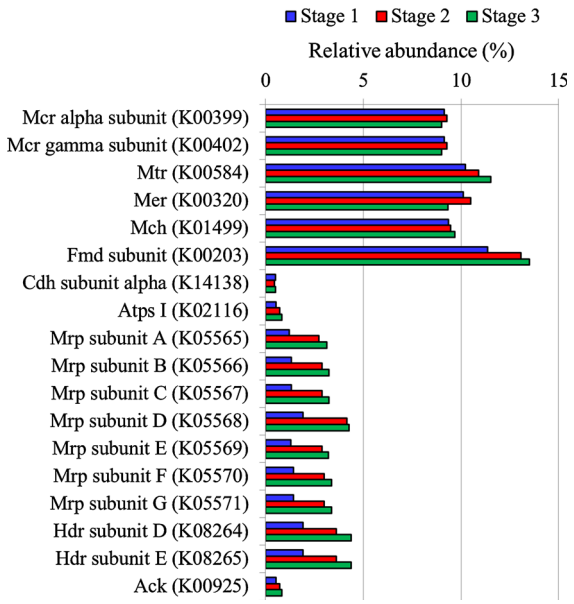

Fig. 4 Relative abundances of predictive genes coding functional enzymes associated with reverse-methanogenesis pathway for methanogens by PICRUSt analysis

For bacterial community, the predicted genes encoding nitrate reductase (*Nar*) and cytochrome C (*Cytc*) were enriched during Stage 1–3 (Fig. 5). Nitrate reductase has multiple functions and has been reported to reduce many types of oxyanions including SeO_4^{2-}, ClO_4^- and BrO_3^- (Lai et al. 2016b, 2018b; Zhao et al. 2011), while Antipov and Khijiniak (2016) found nitrate reductase in *Haloalkaliphilic Halomonas* was involved in V(V) reduction. Myers et al. (2004) found *Shewanella oneidensis* MR-1 required Cytochrome C for carrying out V(V) reduction. These evidences supported that nitrate reductase and cytochrome C might also contribute to V(V) reduction in the CH_4 fed biofilm. The genes associated with these enzymes might be assigned to aerobic methanotrophs such as *Methylococcus* and *Methylomonas* (Kits et al. 2015), or assigned to other bacterial groups performing V(V) reduction.

These results indicate the possible microbial mechanisms in the V(V) reducing CH_4 fed biofilm when the concentration of O_2 was extremely low: simultaneous AnMO that was carried out by *Methanosarcina* through reverse-methanogenesis pathway, and AMO that was carried out by *Methylococcus* and *Methylomonas* using trace level of O_2 occurred, releasing intermediates such as acetate. *Methanosarcina*, aerobic methanotrophs or other bacterial group then utilized these intermediates as electron donors for V(V) bio-reduction.

Conclusion

In this research, continuous V(V) reduction was achieved in a CH_4 fed biofilm with extremely low concentration of O_2. Archaea *Methanosarcina* was enriched in the biofilm, accompanied with the increased of abundance of predictive genes associated with AnOM, implying its possible role in CH_4 activation. Aerobic methanotrophs *Methylococcus* and *Methylomonas* might also activated CH_4 by trace level of O_2. More in-depth researches by using

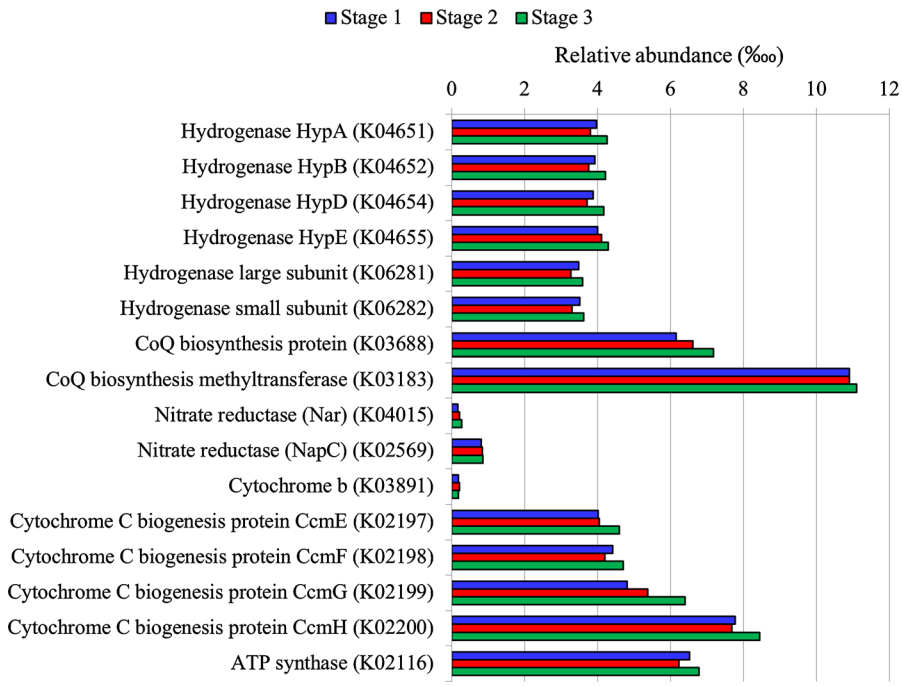

Fig. 5 Relative abundances of predictive genes involved in potential V(V) reductase (e.g., nitrate reductase and cytochrome C) and intracellular electron transfer for methanotrophs or other V(V) reducing bacteria by PICRUSt analysis

advanced molecular technologies including metatranscriptomics and proteomics are needed to confirm the microbial mechanisms.

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