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RESEARCH ARTICLE

Evolution of the microbial community of the biofilm in a methane-based membrane biofilm reactor reducing multiple electron acceptors

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Abstract Previous work documented complete perchlorate reduction in a membrane biofilm reactor (MBfR) using methane as the sole electron donor and carbon source. This work explores how the biofilm's microbial community evolved as the biofilm stage-wise reduced different combinations of perchlorate, nitrate, and nitrite. The initial inoculum, carrying out anaerobic methane oxidation coupled to denitrification (ANMO-D), was dominated by uncultured *Anaerolineaceae* and *Ferruginibacter* sp. The microbial community significantly changed after it was inoculated into the CH_4-based MBfR and fed with a medium containing perchlorate and nitrite. Archaea were lost within the first 40 days, and the uncultured *Anaerolineaceae* and *Ferruginibacter* sp. also had significant losses. Replacing them were anoxic methanotrophs, especially *Methylocystis*, which accounted for more than 25 % of total bacteria. Once the methanotrophs became important, methanol-oxidizing denitrifying bacteria, namely, *Methloversatilis* and *Methylophilus*, became important in the biofilm, probably by utilizing organic matter generated by the metabolism of methanotrophs. When methane consumption was equal to the maximum-possible electron-donor supply, *Methylomonas*, also an anoxic methanotroph, accounted for >10 % of total bacteria and remained a major part of the community until the end of the experiments. We propose that aerobic methane oxidation coupled to denitrification and perchlorate reduction (AMO-D and AMO-PR) directly oxidized methane and reduced NO_3^- to NO_2^- or N_2O under anoxic condition, producing organic matter for methanol-assimilating denitrification and perchlorate reduction (MA-D and MA-PR) to reduce NO_3^-. Simultaneously, bacteria capable of anaerobic methane oxidation coupled to denitrification and perchlorate reduction (ANMO-D and ANMO-PR) used methane as the electron donor to respire NO_3^- or ClO_4^- directly.

Keywords Methane oxidation · Perchlorate reduction · Membrane-biofilm reactor · Microbial community

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Introduction

Perchlorate (ClO_4^-) causes serious human health problems by interfering with the production of thyroid hormones needed for growth and development (Coates and Achenbach 2004), and California is planning to lower its drinking water

maximum contaminant level (MCL) to 1 μg/L (Water Resource Foundation 2015). The main environmental sources of perchlorate are rocket fuel, explosives, and certain fertilizers (Coates and Achenbach 2004). ClO_4^- contamination often is accompanied by pollution from nitrate (NO_3^-; USEPA 2001), which has an MCL of 10 mg N/L because it causes methemoglobinemia in infants (USEPA 2015).

Extensive studies have proven that ClO_4^- can be biologically reduced using organic substrates (e.g., *Dechloromonas*, *Azospira*, and *Dechlorspirillum* bacteria use acetate and lactate) (Coates and Achenbach 2004) and inorganic substrates as electron donors (e.g., *Dechloromonas* bacteria use hydrogen gas (H_2)) (Logan et al. 2001; Nerenberg et al. 2002; Coates et al. 1999; Rikken et al. 1996). We recently reported complete ClO_4^- reduction using anaerobic methane oxidation coupled to denitrification (ANMO-D), in which methane (CH_4) was the sole electron donor and carbon source (Luo et al. 2015). We described the process as *anaerobic methane oxidation coupled to perchlorate reduction* (ANMO-PR). The ANMO-D biofilm reduced up to 5 mg/L of ClO_4^- to a non-detectable concentration when CH_4 delivery was sufficient; NO_3^- was completely reduced as well when its surface loading (SL) was ≤0.302 g N/m^2 day. When CH_4 delivery was insufficient, NO_3^- inhibited ClO_4^- reduction by competing for the scarce electron donor. Nitrite (NO_2^-) inhibited ClO_4^- reduction at a lower SL (0.10 g N/m^2 day), probably due to cellular toxicity. Similarly, although some bacteria, e.g., *Azospira suillum* and *Dechloromonas*, were reported to use both nitrate and perchlorate as electron acceptors (Chaudhuri et al. 2002; Nerenberg et al. 2008), Ziv-El and Rittmann reported that, when hydrogen was supplied as electron donor and in a limited condition, the nitrate had a clear H_2 utilization priority than perchlorate.

Aerobic methane oxidation coupled to denitrification (AMO-D) is performed by the combined actions of methane oxidizers (types I, II, and X methanotrophs) and denitrifiers (Eisentraeger et al. 2001; Knowles 2005; Yao et al. 2013; Modin et al. 2007; Osaka et al. 2006). When methanotrophs oxidize methane using oxygen (O_2) for the initial monooxygenation step, they release soluble organic compounds such as methanol, acetate, and citrate. These organics can be oxidized by denitrifiers to reduce NO_3^- or NO_2^- (Eisentraeger et al. 2001; Modin et al. 2010; Hu et al. 2009).

ANMO-D involves two processes conducted by two distinct microbial groups. Archaea phylogenetically positioned between *Methanosaeta* and ANME-II (candidate *Methanoperedens nitroreducens*) carry out reverse methanogenesis to generate H_2 for themselves to reduce NO_3^- to NO_2^- (Raghoebarsing et al. 2006; Haroon et al. 2013). NO_2^- is then reduced by denitrifying bacteria such as NC10 (*Candidate* division) using methane as the electron donor through an "intra-aerobic" pathway (Ettwig et al. 2010; Wu et al. 2011). During the intra-aerobic pathway, *Methylomirabilis oxyfera* stepwise reduces NO_2^- to NO using nitrate reductase (narGHJI) and nitrite reductase (nirSJFD/GH/L); then, it intracellularly dismutates NO to O_2 and N_2. The O_2 is used by a membrane-bound particulate methane monooxygenase (pMMO) for methane oxidation.

AMO-D and ANMO-D have three distinct differences. First, ANMO-D microorganisms are much slower growers than are the AMO-D bacteria (He et al. 2015). Second, AMO-D bacteria grow with a dissolved oxygen (DO) concentration ≤ 0.5 mg/L (Sun et al. 2013), while the ANMO-D microorganisms work with much lower dissolved oxygen concentration, since the oxygen is disproportionated intracellularly from NO (Ettwig et al. 2010). Third, the ratios of methane consumption to nitrate reduction (CH_4/NO_3^-) are significantly different between the two types of methane consumers, 5/4 mol CH_4/mol NO_3^- for AMO-D (according to Eq. 3) versus 5/8 mol CH_4/mol NO_3^- for ANMO-D (according to Eq. 5) (Modin et al. 2007, 2010; Osaka et al. 2006; Hu et al. 2009; Raghoebarsing et al. 2006). For aerobic methane oxidation, a half of the electrons from CH_4 eventually flow to O_2 (shown in Eqs. 1, 2, and 3) and the other half flow to NO_3^- (shown in Eq. 3). For intra-aerobic-type anaerobic methane oxidation, the oxygen which is used to activate methane comes from the dismutation of electron acceptors; thus, all the electrons from CH_4 eventually flow to NO_3 (shown in Eq. 5). Consequently, for AMO-D, 1-mol NO_3^- reduction needs 5/4 mol CH_4 and 5/4 mol O_2, while for ANMO-D, only 5/8 mol CH_4 is needed. The electron flow pattern for ClO_4^- is the same as that for NO_3^-. Based on Eqs. 1–6 and electron acceptors' concentrations in the membrane biofilm reactor (MBfR) influent and effluent, we calculated the DO demand for two pathways.

$$CH_4 + O_2 + 2H^+ + 2e^- = CH_3OH + H_2O \quad (1)$$

$$CH_3OH + H_2O = CO_2 + 6H^+ + 6e^- \quad (2)$$

$$CH_4 + O_2 + 4/5NO_3^- + 4/5H^+ = 2/5N_2 + 12/5H_2O + CO_2 \quad (3)$$

$$CH_4 + O_2 + 1/2ClO_4^- = 1/2Cl^- + 2H_2O + CO_2 \quad (4)$$

$$CH_4 + 8/5NO_3^- + 8/5H^+ = 4/5N_2 + 14/5H_2O + CO_2 \quad (5)$$

$$CH_4 + ClO_4^- = Cl^- + 2H_2O + CO_2 \quad (6)$$

Perchlorate reduction process, especially chlorite dismutation, is similar to ANMO-D, since they both involve a step to produce oxygen. During ClO_4^- reduction, perchlorate-reducing bacteria (PRB) reduce ClO_4^- to ClO_2^- using perchlorate reductase (*pcrA*). Disproportion of ClO_2^- to Cl^- and O_2 is carried out by a chlorite dismutase enzyme (*cld*) (Bender et al. 2005).

Here, we evaluate which microorganisms were responsible for reducing NO_3^- and ClO_4^- and their likely metabolic pathways. Specifically, we use high-throughput sequencing and principal component analysis (PCoA) to identify the key microorganisms responsible for ANMO-D and ANMO-PR and

important relationships between the community structure and the fluxes of the different electron acceptors. To understand the likely metabolic pathways, we first review pathways of methane oxidation coupled to denitrification or perchlorate reduction.

Materials and methods

Inoculum

The inoculum was from an ANMO-D culture donated by Dr. Wei Xiang Wu at Zhejiang University (China) and maintained anoxic (dissolved oxygen concentration ≤0.2 mg/L) in a sequencing batch reactor in which the inoculum was able to reduce NO_3^- at 7 mg N/L day. Using this inoculum, Luo et al. (2015) achieved complete perchlorate reduction in a CH_4-based MBfR.

MBfR configuration and performance

Detailed information about the ANMO-PR MBfR is given by Luo et al (2015). In brief, the MBfR was composed of two glass tubes that held bundles of composite hollow fibers (composite polyethylene fibers, 280 μm o.d., and a 180 μm i.d., pore size 0.1–0.15 μm) manufactured by Mitsubishi Rayon (Model MHF-200TL, Mitsubishi, Ltd., Japan). One tube held the main bundle of 32 fibers, and the second tube held 10 fibers used for biofilm sampling. The liquid contents (65 mL) were completely mixed by recirculation with a peristaltic pump (Longer Pump, model 1515X, Longer Precision Pump Co., Ltd., China) at 100 mL/min. The influent feeding rate was 0.5 mL/min, giving a hydraulic residence time (HRT) of 130 min. Influent and effluent samples were assayed for NO_3^-, NO_2^-, ClO_4^-, and dissolved O_2 by the methods reported in Luo et al. (2015).

To accumulate biomass, we fed the MBfR with 2 mg N/L of NO_2^- continuously for 40 days, when complete NO_2^- reduction was achieved. The MBfR was then operated for eight experimental stages: stages 1 and 7 were fed with ClO_4^- and NO_2^-; stages 3, 4, 5, and 8 were fed with ClO_4^- and NO_3^-; and stages 2 and 6 were fed with ClO_4^- only. The actual influent and effluent concentrations of all stages were in Luo et al (2015); we list the surface loading and fluxes of all stages in Table 1. We moved to a new stage once acceptor-removal rates reached steady state (effluent concentrations having <10 % variation for a minimum of three HRTs). Actually, it took less than 2 days for all the stages to reach a steady state, except stage 4, which took almost 2 weeks to reach a steady state. The feed medium was de-gassed with N_2 for at least 15 min to maintain an anaerobic condition (DO ≤ 0.2 mg/L, actually anoxic), after which the pH was adjusted to 7.0 ± 0.2 with hydrochloric acid. The CH_4 pressure was 10 psig (1.7-atm absolute pressure) for stages 1–4 and 15 psig (2 atm absolute) for stages 5–8. The DO concentration was ~0.2 mg/L for the influent and ≤0.1 mg/L for the effluent. The experiment was conducted at a temperature of 29 ± 1 °C for all stages.

Biofilm microbial ecology analyses

We collected biofilm samples when the reactor reached steady state for all stages except stage 2. We cut off one ~10-cm-long section from one of the coupon fiber, then sealed the remaining fiber by tying the end into a knot. N_2 gas was sparged at the sampling point to minimize any effects of O_2 exposure during sampling. We then extracted DNA using the DNeasy Blood and Tissue Kit (Qiagen, USA) as previously described by Zhao et al. (2011).

DNA samples were sent to Shanghai Majorbio Technology (Shanghai, China) to perform amplicon pyrosequencing with standard Illumina MiSeq sequencing protocols. We used primers 338F (5′-ACTCCTACGGGAGGCAGC-3′) and 806R (5′-CCGTCAATTCMTTTRAGTTT-3′) to target the conserved V3–V5 regions of the bacteria 16S ribosomal ribonucleic acid (rRNA) gene. We processed the data analysis using QIIME (version 1.17) pipeline as described previously (Lai et al. 2014). Operational units (OTUs) with 97 % similarity cutoff were clustered using UPARSE (version 7.1) after chimeric sequences were removed by HCHIME (Edgar 2010). We analyzed the phylogenetic affiliation of representative sequences by RDP Classifier against the silva (SSU115)16S rRNA database using confidence threshold of 70 % (DeSantis et al. 2006; Caporaso et al. 2010). All sequences shorter than 200 bps, having homopolymers of 6 bps and primer mismatches, and a quality score lower than 25 were removed. Since all of the DNA samples were treated in same condition at the same time, we used the sequence numbers of each OTUs, with a total of 260,099 high-quality sequences for the 16S rRNA gene for all stages, to quantify the relative abundance of microbial communities in all the stages. But, the 16S rRNA gene sequence numbers for different species are various; thus, the quantification only represents estimation.

We evaluated the overall community composition using the unweighted UniFrac distance matrix and the relationships among samples with Cytoscape and PCoA (Lozupone et al. 2006; Shannon et al. 2003). The PCoA is a scaling or ordination method that starts with a matrix of similarities (close) or dissimilarities (distance) between a set of individuals and produce a low-dimensional graphical, in which the distances between points in the plot are close to original dissimilarities. We used MEGA 6 to align functional gene nucleotide sequences and the neighbor-joining algorithm of ARB and MEGA 6 program packages to generate the phylogenetic trees based on distance analysis for 16S rRNA gene afterward (Tamura et al. 2013). The robustness of inferred topologies was tested

Table 1 Summary of the acceptor surface loadings and methane flux for all stages

Stages	ClO_4^-		NO_2^--N		NO_3^--N		CH_4	
	SL[a] (g/m^2 day)	Removal percentage (%)	SL (g N/ m^2 day)	Removal percentage (%)	SL (g N/ m^2 day)	Removal percentage (%)	Computed flux[b] (mmol CH_4/m^2 day)	CMR[c] (%)
1	0.080 ± 0.005	23.5	0.102 ± 0.0004	98.2	NA	0	6.26 ± 0.06	10.8
2	0.061 ± 0.001	100	NA	0	NA	0	0.82 ± 0.09	1.42
3	0.064 ± 0.001	100	NA	0	0.076 ± 0.006	100	7.35 ± 0.60	12.7
4	0.067 ± 0.003	1.92	NA	0	0.745 ± 0.026	68.9	47.5 ± 7.20	82.0
5	0.068 ± 0.0002	100	NA	0	0.302 ± 0.003	100	27.0 ± 0.26	31.2
6	0.393 ± 0.001	100	NA	0	NA	0	5.91 ± 0.09	6.83
7	0.380 ± 0.007	46.6	0.392 ± 0.010	100	NA	0	26.0 ± 0.59	30.0
8	0.101 ± 0.006	5.51	NA	0	2.212 ± 0.033	45.4	87.7 ± 6.14	100

[a] SL means surface loading, which is based on the measured influent and effluent concentrations in Luo et al. (2015)

[b] The methane fluxes are computed based on stoichiometric relationships in Luo et al. (2015)

[c] CMR means consumed methane ratio, which stands for the ratio of computed methane fluxes versus maximum methane delivery flux computed from Luo et al (2015) and Tang et al. (2012)

by bootstrap re-sampling using the same distance model (1000 replicates).

All sequencing data were deposited into NCBI with accession numbers SAMO03468106 for stage 0, SAMO03468114 for stage 1, SAMO03470077 for stage 3, SAMO03470079-80 for stages 4 and 5, SAMO03470084 for stage 6, and SAMO03470086-87 for stages 7 and 8.

Results and discussion

Summary of removal performance of electron acceptors

Table 1 summarizes the performance of the CH_4-based MBfR. In brief, the CH_4-delivery capacity exceeds the CH_4 demand in stages 1, 2, 3, 5, 6, and 7. In stages 2 and 6, perchlorate was the only electron acceptor, and the biofilm was able to reduce up to ~5 mg/L of ClO_4^- to a non-detectable level (Luo et al. 2015). Stages 1and 7 demonstrated that the presence of ≥0.10 g N/m^2 day of NO_2^- inhibited ClO_4^- reduction, although NO_2^- itself was completely reduced. They also demonstrated that NO_3^- had no effect on ClO_4^- reduction when its loading was <0.302 g N/m^2 day. When the CH_4-delivery capacity was not much larger than the CH_4 demand (stages 4 and 8), the presence of NO_3^- inhibited ClO_4^- reduction due to electron-donor competition (Luo et al. 2015).

Microbial community change

We used pyrosequencing targeting the V3–V5 regions of the 16S rRNA gene to analyze the diversity and structure of the MBfR bacterial communities in the MBfR biofilm samples. Pyrosequencing of the 16S rRNA gene yielded a total of 658,054 sequences with a median length of 468 bp for all biofilm samples of all stages.

Figure 1 shows the unweighted UniFrac analysis of the biofilm samples for all stages except stage 2. Stages having similar influent loadings are marked with the same color stage label. Once ClO_4^- was added in the first stage, the biofilm community structure significantly changed, since stage 0 was very distinct from stage 1 and all other stages. Clearly, the ANMO-D microbial community was greatly changed in the biofilm after the addition of ClO_4^-.

Figure 2 shows the unweighted PCoA based on the absence or presence of phylotypes. The most important trends is that samples for stages 1 to 8 grouped together, having much lower PC2 values compared to stage 0. The stages 5 and 6 were grouped comparatively distant because of much higher perchlorate loading in stage 6 than stage 5. Thus, the PCoA analysis reinforces that introducing ClO_4^- had the highest impact on shaping the microbial community structure. Lai et al. reported that an autotrophic nitrate-reducing community was significantly changed after the introduction of selenite (Lai et al. 2014). Ontiveros-Valencia et al. found that high SO_4^{2-} flux reshaped the original NO_3^--reducing microbial community in a H_2-based MBfR (Ontiveros-Valencia et al. 2014).

The biofilm samples from stages 4 to 8 were close to each other along the PC1 vector, while the biofilm samples from stages 1 and 3 were distant. Compared to the latter stages, stages 1 and 3 had much lower average total acceptor loadings (<8 mmol CH_4/m^2 day) so that competition for the electron donor was not strong (Table 1). Therefore, the availability of the electron donor significantly affected the microbial community. Probably, only the bacteria that were competitive in an

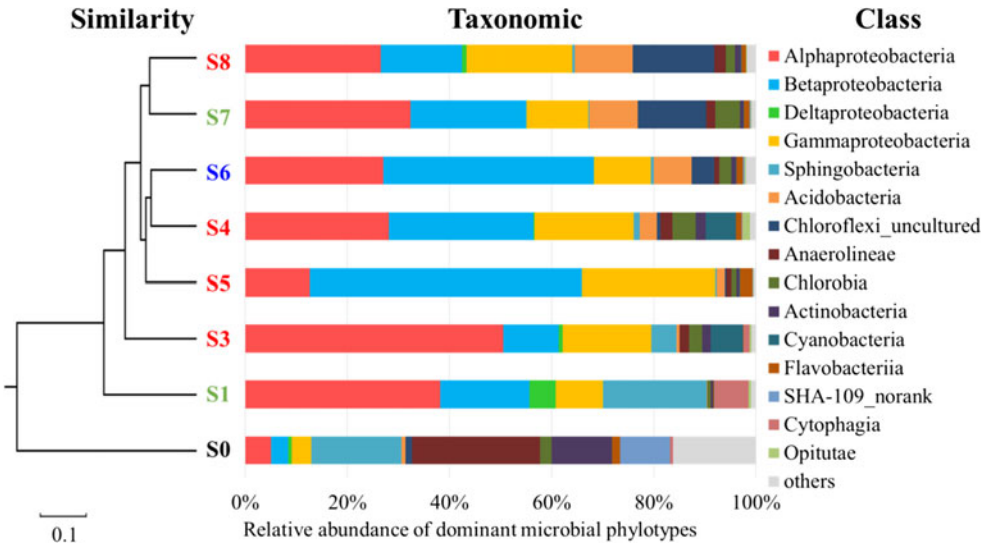

Fig. 1 Clustering based on the unweighted UniFrac analysis of the microbial community structure at the class level (relative abundances of dominant microbial phylotypes). Stage 0 was the inoculum. Stages 1 and 7 were fed with ClO_4^- and NO_2^-; stages 3, 4, 5, and 8 were fed with ClO_4^- and NO_3^-, and stage 6 was fed with ClO_4^- only

environment that had limited electron donor survived in stages 4 to 8 (>25 mmol CH_4/m^2 day, except for stage 6).

Another significant difference between the inoculum (stage 0) and the stages with perchlorate addition is with Archaea. Archaea contain the functional methylcoenzyme M reductase (coded by *mcrA* gene) that conducts the reverse methanogenesis supporting ANMO-D. Figure S1 shows that Archaea were present in stage 0 at low abundance compared to bacteria but Archaea were absent in latter stages. The result is consistent with our finding that the *mcrA* gene (very low abundance) had no correlation with genes associated with respiration of NO_3^- or ClO_4^- (Luo et al. 2015).

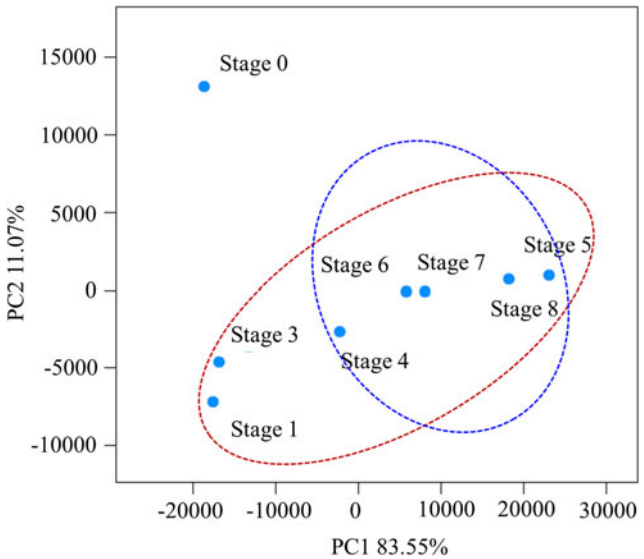

Fig. 2 Principle coordinate analysis (PcoA) based on the unweighted UniFrac analysis showing the microbial community groupings. The *red oval* is for all MBfR biofilm samples, and the *blue oval* is for biofilm samples with the highest total electron acceptors loadings (stages 4–8 refer to the total surface loadings in Table 1). The inoculum community (stage 0) was very different from all other stages

The phylogenetic tree analysis

Figure 3 shows the phylogenetic tree constructed from the bacterial communities in the MBfR biofilm and selected known perchlorate-reducing bacteria (PRBs), methanol-assimilating denitrifiers (MADs), methanotrophs containing denitrifying genes (AMO-D), and nitrite-dependent anaerobic methane oxidizing bacteria (n-DAMO).

Detected genuses *Methylomonas* and *Methylocystis* are phylogenetically close to the known AMO-D *Methylomonas methanica*, *Methylomonas koyamae*, and *Methylocystis trichosporium*. *Methylomonas* was almost absent in stages 0 to 3 but became important from stage 4 (~10 %), when electron-acceptor fluxes greatly increased, but were >15 % of bacterial abundance in stages 5 and 8, which had higher CH_4 pressure and total acceptor flux. *Methylocystis* was remarkably enriched in stage 1, started to decrease in stage 4, but remained important through stage 8 (relative abundance range from 4 to 10 %). The enrichment of AMO-D *Methylomonas* and *Methylocystis* in the MBfR probably was caused by the significant delivery of CH_4 through the MBfR fibers; they initiated methane oxidation using the limited DO in the influent and respired NO_3^- for energy generation (Luo et al. 2015; Tang et al. 2012). Dam et al. reported that *Methylocystis* sp. strain SC2 was able to reduce NO_3^- to N_2 with DO-limiting (0.4 mg/L) condition (Dam et al. 2013). Very recently, Dimitri et al. reported that *Methylomonas denitrificans* sp. nov., strain FJG1T coupled methane oxidation to reduction of NO_3^- to N_2O with very low DO (1.6 μg/L) concentration (Dimitri et al. 2015).

Methylophilus are phylogenetically close to known methanol-assimilation denitrifiers (MA-D, green color in Fig. 3), such as *Methylophilus quaylei*, *Methylotenera mobilis*, *Methyloversatilis universalis*, and to selected known PRBs (blue color in Fig. 3), such as *Dechloromonas agitate*,

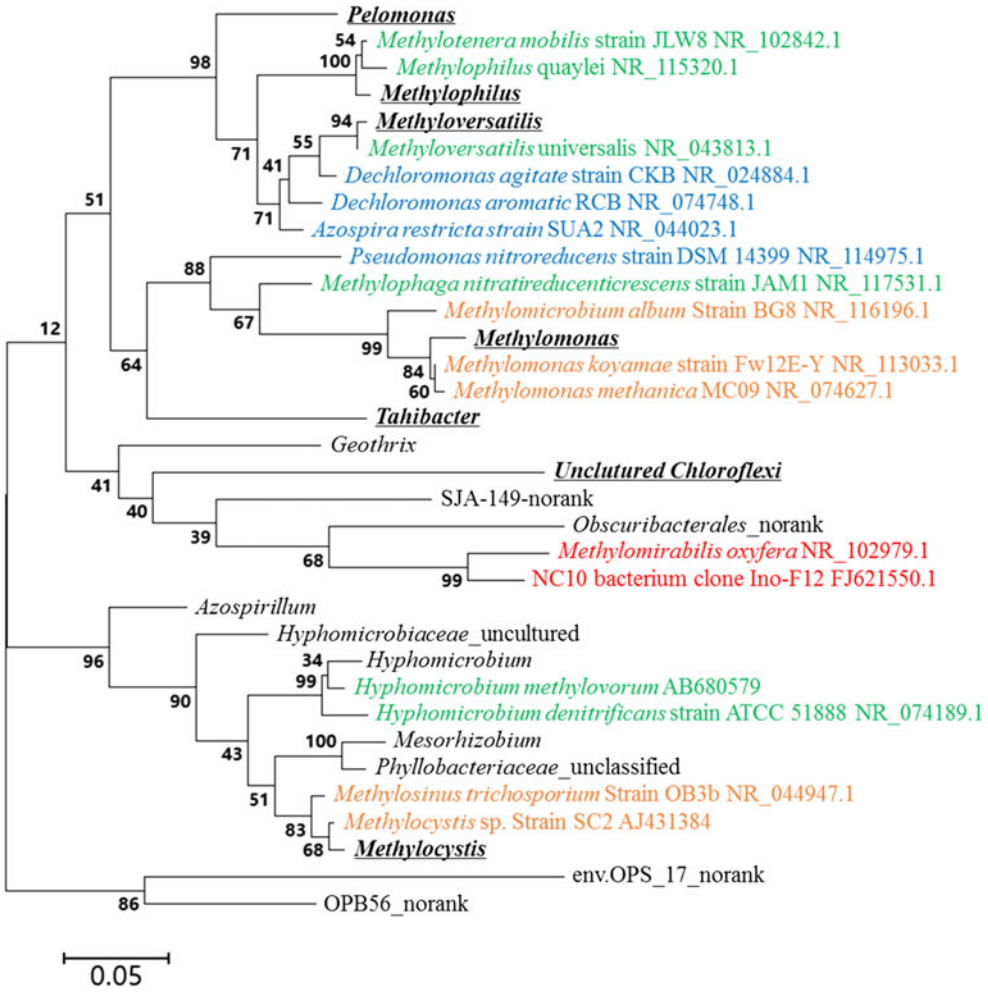

Fig. 3 Phylogenetic analysis of the predominant bacterial genera in the MBfR biofilm (stages 1 to 8) (*black*), known PRBs (*blue*, most of them reduce NO_3^- as well), bacteria from phylum NC10 (*red*), known methanol-assimilating denitrifers (*green*), and methanotrophs known to have denitrifying gene content (*orange*). An MBfR genus with *bold and underline* was among the relatively most abundant (≥10 %) in one of the stages; detailed relative abundance was shown in the supporting information Fig. S2

Dechloromonas aromatic, and *Azospira restricta*. While *Methylophilus* was almost absent in stage 0, it became important in stage 1 after perchlorate was introduced, reached ~30 % of bacterial abundance in stage 5, and then decreased sharply. The increase of the *Methylophilus* abundance in the first five stages may have been due to the accumulation of methanotrophs that provided organic electron donors, such as methanol, acetate, and amino acids. Ginige et al. and Doronina et al. reported that *Methylophilus* can use simple organics, such as methanol and methylamine, to reduce NO_3^- in anoxic conditions, but they cannot grow on methane (Ginige et al. 2004; Doronina et al. 2005).

Pelomonas started to grow in stage 3, and became dominant from stages 4 to 8, consistently following the development of *Methylomonas*. The genera of *Pelomonas* is phylogenetically close to known PRBs, e.g., *Dechloromonas agitate* CKB and *Dechloromonas aromatic* RCB. The accumulation of *Pelomonas* may have been due the increasing flux of perchlorate and organic donors produced by methanotrophs. *Dechloromonas* is the main genera known to reduce NO_3^- and ClO_4^- in the MBfR (Nerenberg et al. 2008), where its abundance increased with increasing ClO_4^- loading relative to NO_3^-.

Uncultured *Chloroflexi* existed in all the stages and were significantly enriched after stage 6. The phylotype of uncultured *Chloroflexi* is phylogenetically close to bacteria in the NC10 phylum, e.g., *M. oxyfera*. Therefore, the uncultured *Chloroflexi* may work similarly like NC10 phylum bacteria in the ANMO-D processes. In fact, Ettwig et al. (2009) reported that *Chloroflexi* existed in *M. oxyfera* enrichment culture that performed n-DAMO. Beal et al. and Hu et al. found that *Chloroflexi* present during anaerobic methane oxidation were coupled to manganese and iron-reducing culture and a reactor containing ammonium oxidation (anammox) and ANMO-D processes (Beal et al. 2009; Hu et al. 2015).

Metabolic pathways based on the DO and methane/N ratio analysis

Although the AMO-D (AMO-PR) and MA-D (MA-PR) bacteria were important in the biofilm samples, they may not be the only methane oxidation manners in MBfR. Figure 4 shows the DO demand for both computed aerobic-type and intra-aerobic-type anaerobic methane oxidation coupled to denitrification and perchlorate reduction. Clearly, the actual DO

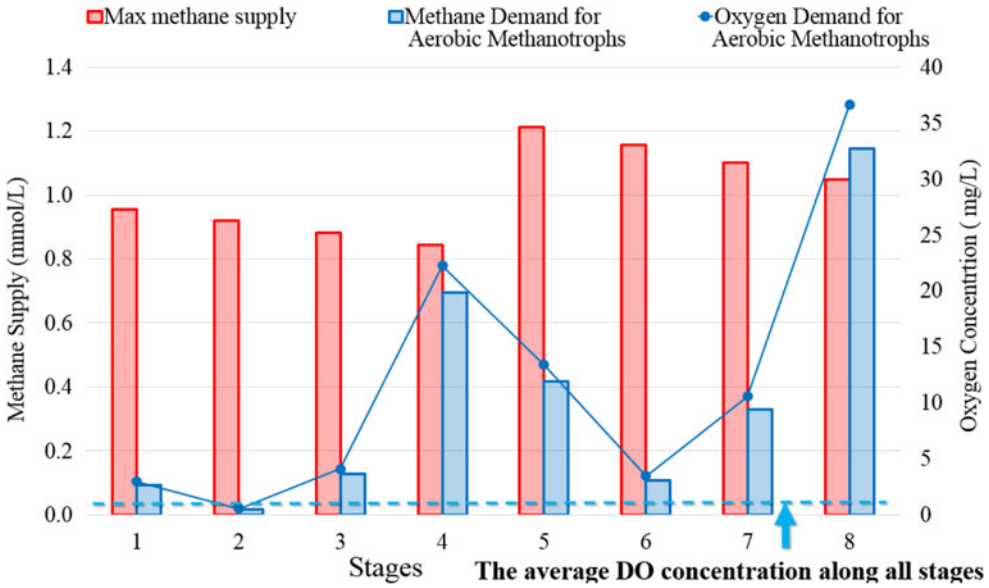

Fig. 4 The calculated oxygen required for different metabolic pathways. Max methane supply is the theoretical maximum methane supply through the MBfR (decrease because considering the membrane surface area loss due sampling); it gradually declines by stage due to volume loss through sample. Methane demand for aerobic methanotroph is the methane required for aerobic methane oxidation coupled to denitrification and perchlorate reduction. Oxygen demand for aerobic methanotrophs is the oxygen required for aerobic methane oxidation coupled to denitrification and perchlorate reduction. The *dashed line* represents the actual average DO concentration along all stages

concentrations in the MBfR system were much lower than the theoretical demand for aerobic methane oxidation. Ettwig et al. reported that during the intra-aerobic-type ANMO-D pathway, the oxygen produced by *M. oxyfera* intracellularly for methane mono-oxygenation, was not detectable (Ettwig et al. 2010). Considering the conflict between actual DO concentrations and theoretical demand, we propose that beside AMO-D (AMO-PR) and MA-D (MA-PR), the ANMO-D (ANMO-PR) were also important in all the stages except stage 2 in the MBfR system (Fig. 5). To quantify the comprehensive processes of different electron acceptor/donor utilizations, further studies on the effect of oxygen and microbial community are required.

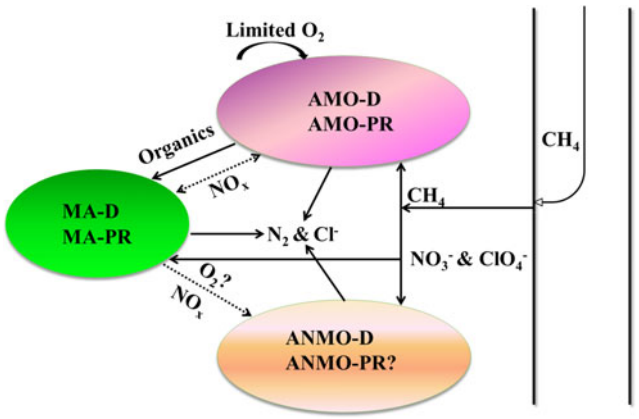

Fig. 5 Proposed microbial community functions in the CH_4-based MBfR. *MA-D* methanol-assimilating denitrification reduction, *MA-PR* methanol-assimilating perchorate reduction, *AMO-D* aerobic methane oxidation coupled to denitrification perchlorate reduction, *AMP-PR* aerobic methane oxidation coupled to perchlorate reduction, *ANMO-D* anaerobic methane oxidation coupled to denitrification, and *ANMO-PR* anaerobic methane oxidation coupled to perchlorate reduction

Besides the DO concentration, we analyzed the CH_4/NO_3^- mole ratios through all stages. When CH_4 delivery was limited at stages 4 and 8, the calculated C/N ratios values were of 1.52 and 1.15, respectively. Both of the two observed C/N ratios were much lower than the minimum ration for AMO-D. Thus, the ANMO-D (ANMO-PR) might also play important roles in the MBfR.

Overall, we propose that in the MBfR system, the AMO-D (AMO-PR) directly oxidize methane and reduce NO_3^- to NO_2^- or N_2O under anoxic condition, producing organic matters for MA-D (MA-PR) to reduce NO_3^-. Simultaneously, the ANMO-D (ANMO-PR) bacteria use methane as electron donor to respire NO_3^- or ClO_4^- directly. The metabolic intermediates of the AMO-D (AMO-PR) may improve the metabolism of ANMO-D (ANMO-PR). For example, Wu et al. 2015 reported that the genome of "*M. oxyfera*" lacks known pyrroloquinoline quinone (PQQ) biosynthesis machinery, which is necessary during the methane metabolism. However, the PQQ biosynthesis exists in aerobic methanotrophs, so AMO-D (AMO-PR) could possibly supply PQQ for ANMO-D (ANMO-PR).

In summary, we found that the microbial community of an ANMO-D culture significantly changed after it was inoculated to the CH_4-based MBfR reducing perchlorate, nitrate, and nitrite. Original, the ANMO-D culture was dominated by uncultured *Anaerolineaceae* and *Ferruginibacter*. After being introduced to the anoxic MBfR fed with nitrite and perchlorate

initially, all Archaea were removed out, the abundances of both uncultured *Anaerolineaceae* and *Ferruginibacter* significantly decreased, while the anoxic methanotrophs greatly increased, especially *Methylocystis*. With the development of methanotrophs, the methanol-assimilating denitrifying bacteria, e.g., *Methloversatilis* and *Methylophilus*, became important in the biofilm. When nitrate loading increased to 0.75 g N/m^2 day in stage 4, the genus *Pelomonas* accounted ~15 % of total bacteria and remained >10 % to the end, while another methanotrophs *Methylomonas* accounted >10 % of total bacteria. The genera *Azospirillum* significantly increased in stage 6 when perhchlorate loading was up to 0.39 mg/m^2 day. Based on the DO and C/N ratio analyses, we propose that beside anoxic AMO-D, AMO-PR processes, MA-D, and MA-PR processes, the anaerobic ANMO-D and ANMO-PR processes played important roles in the methane oxidation coupled to multiple electron acceptors reduction in the MBfR.

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