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Hypoxic methane oxidation coupled to denitrification in a membrane biofilm

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HIGHLIGHTS

- Nitrate removal increased by 85% in the methane-utilizing MBfR.
- Slow kinetics of MOD microorganisms caused methane leakage to liquid.
- Metagenome identified all genes essential for aerobic methane oxidation.
- *Methylocystaceae* and diverse denitrifiers conducted MOD syntrophically.

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ABSTRACT

Methane oxidation coupled to denitrification (MOD) was tested in a membrane biofilm reactor (MBfR) using methane gas as the sole electron donor. Nitrate reduction to nitrite was rate limiting, and CH_4 was present in the effluent. Slow kinetics of methane oxidation by bacteria were the factors that led to slow kinetics and incomplete removals. *Methylocystaceae* contained the largest fraction (21%) of bacterial SSU rRNA genes, and Archaea were nearly absent. The functional metagenome included all the genes essential for aerobic methane oxidation (*pmo, mdh, mtdB, folD,* and *fdh*) and nitrate reduction to dinitrogen (*nap/nar, nir, nor* and *nos*), but not for reverse methanogenesis (*mcr*). The functional metagenome supports that *Methylocystaceae* conducted MOD in syntrophy with heterotrophic denitrifiers (e.g., *Comamonadaceae* and *Brucellaceae*), suggesting aerobic MOD. DO measurements, serum-bottle tests, and calculation of O_2 permeation bolster hypoxically aerobic MOD would mainly account for denitrification in the MBfR.

1. Introduction

Methane oxidation coupled to denitrification (MOD) has gained attention as a potentially economical and simple means of biological nitrogen removal from water and wastewater. Economy and simplicity are possible because methane is inexpensive compared to typical organic electron donors (e.g., methanol, ethanol, acetate) [1] and can be produced onsite by anaerobic digestion of biosolids.

MOD consists of two mechanisms depending on oxygen presence, anaerobic MOD and aerobic MOD [2–4]. Anaerobic MOD, also called denitrifying anaerobic methane oxidation, can occur via an intracellular oxygenic pathway, found in *Candidatus* Methylomirabilis oxyfera (bacterium of NC 10 phylum), that uses nitrite as the electron acceptor [4]. However, more common is anaerobic MOD that involves anaerobic methanotrophic archaea (ANME) that do reverse methanogenesis coupled to nitrate reduction in a microbial consortium [3,5]. For example, *Candidatus* Methanoperedens nitroreducens (ANME-2d, an archaeon capable of anaerobic methanotroph) used reverse methanogenesis coupled to nitrate reduction to nitrite, which was further reduced to N_2 in syntrophy with anammox bacteria or *Methylomirabilis oxyfera* [1,6].

Aerobic MOD can occur syntrophically between aerobic

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methanotrophs and heterotrophic denitrifiers [7,8]. The denitrifiers can utilize intermediates accumulated from aerobic methane oxidation such as methanol, formaldehyde or formate [2]. A few studies found that methanotrophs such as *Methylomicrobium*, *Methylomonas* and *Methylobacter* could denitrify using methane as the electron donor, but incomplete denitrification accumulating N_2O has been observed [9–11], limiting application of aerobic MOD for nitrogen control in water and wastewater.

Recent works have reported the application of anaerobic MOD to remove nitrogen in water and wastewater using a membrane biofilm reactor (MBfR). A three-way culture of ANME archaea, NC 10 bacteria, and anammox bacteria was successfully enriched in an MBfR using methane gas as the sole electron donor for nitrite reduction [1]; however, the rate of nitrite reduction was relatively slow, 147 mg N/L-d. More recent work improved the nitrite-removal rate to 560 mg N/L-d in an MBfR in which ANME-2d and a member of the *Phycisphaerales* family (potential anammox bacteria) dominated the biofilm, which also had a small population of *M. oxyfera* (3%) [6]. These MBfR results support that reverse methanogenesis (by ANME-2d) coupled to anammox can be important for anaerobic MOD. However, the non-trivial presence of other microorganisms (e.g., *Rhodocyclaceae*) in the bioreactors [6,12] means that the communities may have contained other microorganisms involved in anaerobic MOD, or the MBfRs may have been run in hypoxic conditions, diversifying MOD communities.

A completely different consortium was involved in a CH_4-utilzing MBfR inoculated with an anaerobic MOD culture enriched with ~80% Proteobacteria and exposed to multiple oxyanion electron acceptors (i.e., nitrate, perchlorate, and selenate) [13–15]. Instead of *Methylomirabilis oxyfera*, ANME-2d archaea, and anammox bacteria, the key players for anaerobic MOD were aerobic methanotrophic bacteria in the biofilms of the CH_4-utilizing MBfR. For instance, Lai et al., (2016) reported that the genera *Methylomonas, Methylophilus,* and *Methylocystis* dominated the biofilm in an MBfR using methane as the sole electron donor for simultaneous reductions of selenate and nitrate [14]. They interpreted that microaerophilic conditions, probably caused by O_2 production from reduction of oxyanions, triggered O_2-dependent methane oxidation by aerobic methanotrophs [13–16]. If methanotrophic bacteria can carry out MOD via the intracellular oxygenic pathway, our understanding of anaerobic MOD in natural and engineered systems will be significantly changed, such as global methane and nitrogen cycles. However, they had no direct, quantitative evidence to support this hypothetical interpretation. No literature has directly proved anaerobic MOD in MBfRs, like measuring dissolved oxygen (DO) concentration, although intrusion of small O_2 to MBfRs could shift anaerobic to hypoxically aerobic MOD. In fact, it is almost impossible to keep continuous MBfRs completely anaerobic conditions due to O_2 permeation through tubing or other connections. No studies have assessed O_2 permeation effects on anaerobic MOD in continuous MBfRs, although hypoxic conditions could be created in the MBfRs.

While nitrogen removal is the primary goal of methane-utilizing denitrification in an MBfR, the MBfR effluent should have a low dissolved methane concentration in order to preclude release of a potent greenhouse gas [17]. Hence, minimizing dissolved methane in the effluent is a second criterion for success. To date, experimental evaluation of how the dissolved methane concentration in MBfR effluent coincides with denitrification performance is absent. Can a CH_4-based MBfR simultaneously achieve low nitrate (and nitrite) and dissolved methane concentrations?

This study has four goals. The first goal is to evaluate experimentally the reduction of nitrate and nitrite in a methane-oxidizing MBfR operated over relevant ranges of hydraulic retention time (HRT) and methane pressures. The second is to identify the conditions leading to (undesired) accumulation of aqueous methane in the MBfR effluent. The third is to prove hypoxically aerobic MOD or strictly anaerobic MOD in the MBfR continuously pressurized with methane gas, and the final is to characterize the microbial community (using metagenomics) as a means to identify the pathway used for methane-based denitrification in the MBfR.

2. Materials and methods

2.1. Membrane biofilm reactor (MBfR)

An MBfR was built with Plexiglas tubes (diameter of 1.1 cm, length of 30 cm, and working volume of 86 mL), as shown in Fig. 1-a; this MBfR is called $MBfR_{main}$. Three more MBfRs were used for supplementary tests to evaluate the biofilm community (comparison between biofilm and planktonic cells) and the possible input of dissolved oxygen. For all MBfRs, two membrane modules were prepared with gas-permeable hollow-fiber membranes (MHF 200TL, Mitsubishi Rayon, Japan), and each module consisted of 8 membrane fibers, giving a total specific surface area of 35 m^2/m^3. The hollow-fiber membranes were bundled together using a hydrophobic silicone sealant (Model 908570, Loctite, USA) that cured for 24 h in dry conditions.

$MBfR_{main}$ was inoculated with an MOD culture (50 mL) that had been enriched in serum bottles using methane as the sole electron donor and carbon source. The MOD culture had been inoculated with activated sludge sampled from Waterloo wastewater treatment plant (Waterloo, ON, Canada) and incubated with nitrate medium. To supply methane gas as the sole electron donor, we purged serum bottles with CH_4 gas (99.0%, Praxair, Canada) for 30 min. The bottles were then placed in an incubator (SI-300, Lab Companion, USA) shaken at 150 rpm at a temperature of 25 °C. After withdrawing supernatant from serum bottles in a week, we added the fresh nitrate medium in the bottles, sparged them with the methane gas, and incubated them again in the incubation shaker. We repeated the enrichment of methane-oxidizing microorganisms for over a year. $MBfR_{main}$, filled with nitrate medium, was operated in batch mode, and methane gas (99.0%, Praxair, Canada) was supplied to the membrane modules as the sole electron donor and carbon source. The chemical composition of the nitrate medium was (mg/L) $NaNO_3$ 184, $FeSO_4{\cdot}7H_2O$ 1, $CaCl_2{\cdot}2H_2O$ 1, $MgSO_4{\cdot}7H_2O$ 200, Na_2HPO_4 434,KH_2PO_4 128, and trace mineral (1 mL/L). The trace mineral included (mg/L) $ZnSO_4{\cdot}7H_2O$ 100, $MnCl_2{\cdot}4H_2O$ 30, H_3BO_3 300, $CoCl_2{\cdot}6H_2O$ 200, $CuCl_2{\cdot}2H_2O$ 10, $NiCl_2{\cdot}2H_2O$ 10, $Na_2MoO_4{\cdot}2H_2O$ 30, and Na_2SeO_3 30. The medium was autoclaved for 20 min at 121 °C and cooled to room temperature. Then, we purged the medium with N_2 gas (99.0%, Praxair, Canada) for 45 min, connected it with a N_2 gas bag (1 Liter Tedlar – Sigma-Aldrich, USA), and fed the medium to $MBfR_{main}$ with a peristaltic pump. The anaerobic condition of the feed medium and constant methane pressure applied to the membranes would ensure that $MBfR_{main}$ was anaerobic. The liquid in $MBfR_{main}$ was circulated at a rate of 10 mL/min using a peristaltic pump (Masterflex L/S economy variable speed Drive, RK-07554-80).

After nitrate and nitrite concentrations were less than 1 mg/L in batch operation with a methane pressure of 7 psig (1.48 atm absolute pressure) inside the fibers (i.e., after 151 d in batch operation), we operated $MBfR_{main}$ in continuous mode with nitrate medium (20 ± 1.2 mg N/L) delivered using a peristaltic pump (Masterflex L/S economy variable speed Drive, RK-07554-80) and with a methane pressure of 7 psig (1.48 atm). The initial pH of the nitrate medium was 7.2 ± 0.1. To assess the effects of methane pressure and hydraulic retention time (HRT) on nitrate reduction, the HRT was stepwise decreased from 12 to 4 h, and the methane pressure was stepwise reduced from 7 to 5 psig (1.48 to 1.34 atm) and then to 2 psig (1.13 atm). Table 1 summarizes the operating conditions. $MBfR_{main}$ was run at a constant temperature of 24 ± 1 °C, and the effluent pH ranged from 7.2 to 7.4. $MBfR_{main}$ was operated for ~180 days in continuous mode after 151 days in batch run. For a given condition, the bioreactor was continuously operated at 30-105 HRT cycles to a given HRT.

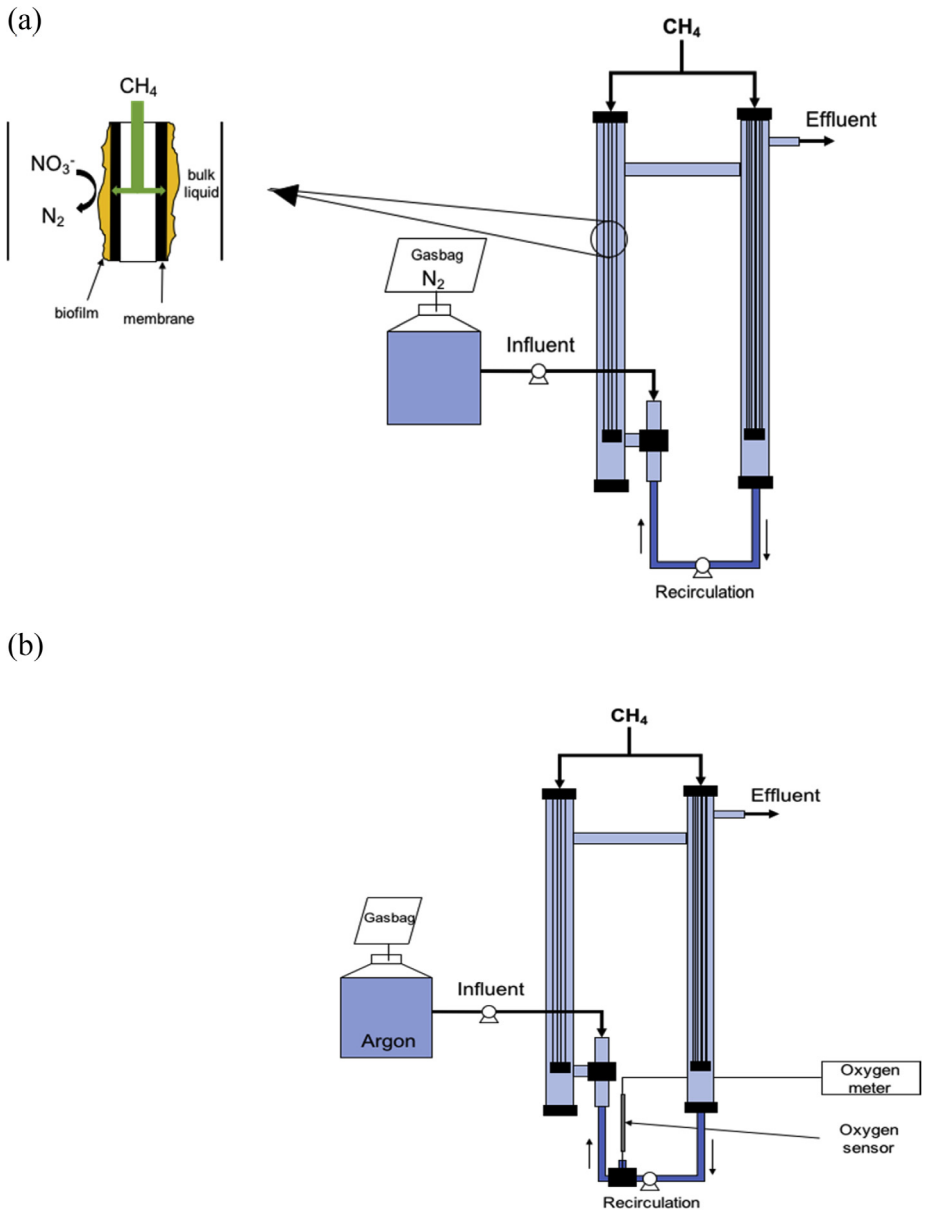


Fig. 1. Schematic of the membrane biofilm reactors (MBfRs). (a) main MBfR ($MBfR_{main}$) used for evaluation of denitrification performance and (b) supplementary MBfR ($MBfR_{sub}$) for assessment of hypoxic or anaerobic conditions in MBfRs. To stop O_2 intrusion into the MBfRs, a gas-sparged medium bottle was closed with a rubber stopper, and a gas-bag was connected with the medium bottle. N_2 or Ar gas was used for the gas-bag.

Table 1
Operating conditions and periods for $MBfR_{main}$.

Methane pressure (psig [atm])	HRT (h)		
	12	8	4
7 [1.48]	Day 178–192	Day 193–207	Day 208–220
5 [1.34]	Day 221–242	Day 243–257	Day 258–270
2 [1.13]	Day 271–299	Day 300–322	Day 323–337

The $MBfR_{main}$ was acclimated for 151 days in batch mode and another 26 days in continuous mode before the start of these experiments. Methane pressure inside the membranes was fixed at 7 psig during acclimation (1 atm = 14.7 psig).

2.2. Calculation of O_2 permeation and monitoring the DO concentration in MBfRs

Although $MBfR_{main}$ was continuously pressurized with methane gas during the experiments, O_2 might have permeated into the MBfR via tubing, creating hypoxic conditions. Then, small O_2 might allow mono-oxygenation of methane to methanol. To compute O_2 permeation and methane oxidation to methanol from the permeated O_2, we calculated an amount of O_2 that can permeate through tubing of $MBfR_{main}$ with Eq (1).

$$\text{Permeation rate of } O_2 = (O_2 \text{ permeability} \times A \times t \times P_{O2})/z \quad (1)$$

where A: surface area of tubing (cm^2), t: time (s), P_{O2} is the atmosphere's partial pressure of O_2 (0.21 atm), z: tubing thickness (mm). The information on tubing (Masterflex, Norprene A60 G) is as follows: the total length of tubing 75 cm, tubing thickness 1.8 mm, tubing diameter 3.1 mm (surface area of tubing: 73.2 cm^2), and O_2 permeability 20×10^{-10} $cm^3 \times mm/cm^2$-s-mmHg (https://www.masterflex.com/tech-article/norprene-a-60-g-tubing).

To investigate DO concentration in the $MBfR_{main}$ without disturbing the biofilm community and denitrification performance, we conducted supplemental experiments with an additional MBfR ($MBfR_{sup}$) and measured the DO concentration with an *in-situ* O_2 microsensor

(PreSens, Regensburg, Germany) installed in recirculating tubes of the $MBfR_{sup}$ (see Fig. 1-b). The $MBfR_{sup}$ were inoculated with biofilms collected from the $MBfR_{main}$ in an anaerobic chamber and run with nitrate medium for about 5 months. Reactor materials and dimensions of the $MBfR_{sup}$, and operating conditions were equivalent to the $MBfR_{main}$, including medium preparation (e.g., gas sparging). The $MBfR_{sup}$ achieved nitrate removal by 39% before installation of *the in-situ* O_2 microsensor. We measured the DO concentration with 2, 5, and 7 psig (1.13, 1.34, and 1.48 atm) methane pressure at a constant HRT of 12 h. The DO detection limit of the microsensor was 3 μg/L.

2.3. Biomass sampling, DNA extraction, and metagenome analysis

Because long O_2 exposure to the membrane biofilm could affect the structure of the biofilm community, we avoided taking biofilm samples. Instead, we collected effluent from $MBfR_{main}$ during steady-state operation on day 297, when the methane pressure was 2 psig (1.13 atm) and the HRT was 12 h. We collected a 10-mL sample using multiple micro-centrifuge tubes. Sampling suspended biomass is representative of the biofilm community if all suspended cells are derived from detachment of the biofilm. We confirmed this assumption by performing Illumina MiSeq sequencing of planktonic and biofilm samples in another MBfR (Supplementary Material provides details): The microbial communities from the biofilm and planktonic biomass were very close to each other: 37% of the rDNA of the total population in the biofilm and 38% for the planktonic cells (Fig. S1).

Genomic DNA (gDNA) of the pellets was extracted by the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Inc., Carlsbad, USA). Sequencing libraries were prepared according to the protocol of the TruSeq DNA PCR-free Sample Preparation Kit (Illumina, Inc., San Diego, CA, USA). DNA (1 μg), fragmented by the adaptive focused acoustic technology (AFA; Covaris), was end-repaired, followed by selection of DNA size and A-tailing to the 3′ ends of the fragments and ligation of adapters [18]. Macrogen, Inc. (Seoul, Republic of Korea) sequenced the DNA using an Illumina Hiseq 1999 (Illumina, San Diego, USA).

A total of 5.38 Gb of paired-end reads was obtained by sequencing forward and reverse strands of DNA fragments. 100-base length of MOD paired-end reads were uploaded to the MG-RAST server (IDs: 4620719.3) [19]. The paired-end reads were proceeded by merging and filtering using default options of MG-RAST pipeline, including removal of artificial replicates-contained affiliated with *Homo sapiens* DNA (NCBI v36). We filtered out reads containing low quality bases more than 5 bp with < 15 Phred score. Microbial taxa were assigned using Best Hit Classification based on the SILVA Small Subunit (SSU) rRNA database [20,21] by filtering with a minimum identity cutoff of 60%, an E-value cutoff of 10^{-5}, and a minimum alignment length of 50 bases. Functions of MOD were annotated by All Annotations tool based on Genbank database at a minimum identity threshold of 60%, an E-value cutoff of 10^{-5}, and a minimum alignment length of 17 amino acids [22]. To characterize taxa for each gene in an MOD pathway, MOD-related nucleotide sequences were selected from the All Annotations results.

2.4. Serum-bottle tests

After confirming that methanotrophic bacteria had been enriched in the membrane biofilm of $MBfR_{main}$, we conducted serum-bottle experiments with control to ensure that the enriched methanotrophs could perform anaerobic MOD. The control indicates a microcosm lacking methane (microorganisms + nitrate): endogenous decay. We collected 100 mL of $MBfR_{main}$ effluent and centrifuged it at 5000 rpm at a temperature of 24 ± 0.1 °C for 15 min. Cell pellets were added to serum bottles filled with nitrate medium (50 mL of working volume and 110 mL of headspace in the bottles). Then, the serum bottles were sparged with CH_4 gas (99.999%) for 30 min and then incubated in a shaker (MaxQ™ 4450, Thermo Scientific™, USA) at a temperature of 25 °C and with 170 rpm. The initial pH in the serum bottles was 7.0 ± 0.2. To measure CH_4 gas composition and nitrate concentration in serum bottles, we sampled the headspace with a gas-tight syringe (1000 Series Gastight™, Hamilton TM, USA) and the liquid with plastic syringes (3-ml Norm-Ject™, Air-Tite™, USA) in an anaerobic chamber (Vinyl TypeB, Coy, USA) at days 0, 10, and 20. We carefully monitored anaerobic conditions in the anaerobic chamber with a high sensitivity DO sensor (detection limit of 0.007% gaseous O_2) and resazurin solution (0.1%). Anaerobic conditions were confirmed by no color change in resazurin solution and negative DO concentrations read in the microsensor (i.e., below the detection limit). Serum bottle tests including control were conducted in duplicate and reported average data with standard deviations (four measurements at each point).

2.5. Chemical analysis

Nitrate and nitrite were analyzed with an ion chromatograph (IC-1100, Dionex, USA) equipped with an AS9-SC analytical column (IonPac, 4 × 250 mm, Dionex, USA) using 9 mM sodium carbonate as an eluent at a flow rate of 1.00 mL/min. All samples were filtered with syringe filters (pore size: 0.45 μm, VWR International Inc., Canada) before analysis. We regularly sampled influent and effluent three times per week, measured nitrate and nitrite concentration in duplicate, and reported average data of 12–24 measurements with standard deviations at given conditions. N_2O gas was quantified using a gas chromatography (GC-2014, Shimadzu, Japan) equipped with an electron capture detector (ECD) and a molecular sieve column (6 ft × 1/5 in., 85/100 mesh, all tech, USA). The temperatures of the column and the detector were constant at 250 °C and 80 °C, respectively, and helium gas was used as the carrier gas (99%, PraxAir, Canada) at a flow rate of 10 mL/L and a pressure of 21 psig. We regularly sampled gases from headspace of the MBfR using a gas-tight syringe (Hamilton Gastight Syringe, Hamilton, USA) for N_2O analysis.

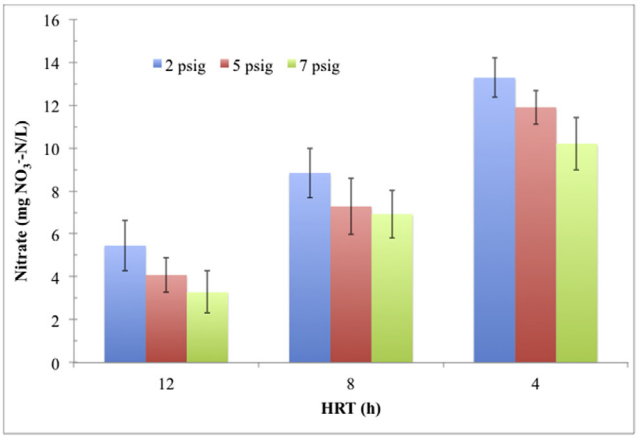
We quantified the concentration of dissolved methane in the MBfR effluent according to the literature [20,21]. Briefly, liquid samples taken from the MBfR using a syringe were immediately transferred to a vial (20 mL) that had been sparged with N_2 gas (99%, Praxair, Canada). The vial was vigorously mixed with a vortex mixer (Fisher STD, USA) for 6 min to reach equilibrium between gas and liquid phases. We then sampled gas from the headspace of the vial and quantified gas composition with a GC equipped with a thermal conductivity detector (TCD) (SRI 310C, SRI instruments, USA). We computed dissolved methane concentration in liquid samples using Henry's law constant of methane (25.6 mg/L-atm at 25 °C) and the volumes of gas and liquid [23]. We measured dissolved methane concentration twice at every week, measured it in duplicate, and reported average data of 8–16 measurements with standard deviation at given conditions.

3. Results and discussion

3.1. Nitrate reduction and nitrite accumulation in the MBfR

Fig. 2A shows how nitrate reduction was related to HRT and methane pressure. At methane pressure 2 psig, nitrate concentration in MBfR effluent was 5.5 ± 1.18, 8.8 ± 1.15, and 13.3 ± 0.91 mg N/L, respectively, for HRT 12, 8, and 4 h. Similar trends were found at higher methane pressure. Effluent nitrate was 4.1 ± 0.81, 7.3 ± 1.31, and 11.9 ± 0.79 mg N/L, respectively, for HRT 12, 8, and 4 h, at 5 psig methane pressure, and at 7 psig the nitrate was 3.3 ± 0.98, 6.9 ± 1.11, and 10.2 ± 1.23 mg N/L in the same order. Higher methane pressure slightly improved nitrate reduction for a fixed HRT, but decreasing the HRT dramatically increased the effluent nitrate concentration. This result means that delivery of methane to methane-oxidizing microorganisms in the biofilm was not the main limiting factor for nitrate reduction for these conditions; instead, the limiting

(a)



(b)

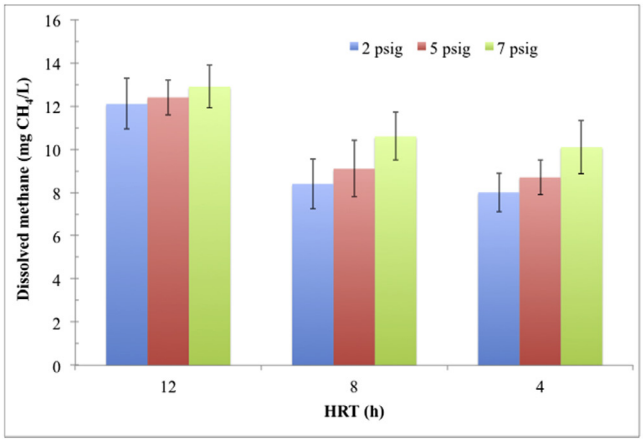


Fig. 2. Effluent concentrations for the operating conditions (HRT and methane pressure) in $MBfR_{main}$: (a) nitrate and (b) dissolved methane. Not shown is the effluent nitrite concentration, which was ≤0.2 mgN/L throughout. The influent nitrate concentration was constant at 20 ± 1.2 mg N/L, and the $MBfR_{main}$ was run at a temperature of 24 ± 1 °C.

factor was related to the kinetics of methane oxidation and nitrate reduction by the microorganisms. The nitrite concentration, summarized in Table 2, was less than 0.2 mg/L throughout all experiments, which means that nitrate reduction, not nitrite reduction, limited the overall denitrification rate. We attempted to establish the nitrogen balance in $MBfR_{main}$, but could not build the balance because of continuous methane supply to the bioreactor. Intermediate compounds, such as nitrite or nitrous oxide, might be accumulated during denitrification in $MBfR_{main}$. However, we did not detect N_2O gas in the headspace of $MBfR_{main}$, implying full denitrification to dinitrogen. Metagenome analysis also identified *nos* gene accounting for N_2O reduction to N_2. (see Section 3.4).

Table 2
Nitrite concentrations (mgN/L) in the $MBfR_{main}$ effluent.

Methane pressure (psig [atm])	HRT (h)		
	12	8	4
2 [1.13]	0.021 ± 0.005	0.07 ± 0.06	0.12 ± 0.005
5 [1.34]	0.017 ± 0.008	0.055 ± 0.07	0.091 ± 0.006
7 [1.48]	0.01 ± 0.009	0.051 ± 0.005	0.085 ± 0.004

1 atm = 14.7 psig.

3.2. Dissolved methane in the MBfR effluent

Fig. 2B shows that the MBfR effluent had a relatively high dissolved-methane concentration (8 ± 0.21 – 12.9 ± 0.19 mg CH4/L) for all experimental conditions. Table S1 provides average data and standard deviations of dissolved methane. The concentration of dissolved methane became slightly higher with a longer HRT and a higher methane pressure. These results document that methane leaked from the biofilm to the bulk liquid, which further supports that methane oxidation, not delivery, was rate-limiting, since the methane concentration inside the biofilm was higher than in the bulk liquid. The high concentration of effluent methane contrasts to the results with the H_2-based MBfR, in which dissolved H_2 in the effluent normally was negligible [24,25]. Eq. (2) is the steady-state mass balance for methane for a location in the biofilm, given that methanotrophic bacteria mainly use O_2 for methane oxidation. We confirmed this assumption with metagenome (see Section 3.4. Metagenome Analysis).

$$0 = D_{f1}\frac{dS_{f1}^2}{dz^2} - q_{max} f_1 X_f \frac{S_{f1}}{K_1 + S_{f1}} \frac{S_{f2}}{K_2 + S_{f2}} \quad (2)$$

where S_{f1} = methane concentration in the biofilm (g CH_4/m^3), S_{f2} = DO concentration in the biofilm (g O_2/m^3), D_{f1} = methane diffusion coefficient within the biofilm (m^2/d), z = biofilm thickness (m), q_{max} = maximum specific methane utilization rate in methanotrophic bacteria (g CH_4/g cells-d), f_1 = fraction of methanotrophic bacteria, X_f = biomass density (g cells/m^3), K_1 = half-maximum-rate concentration for methane (g CH_4/m^3), and K_2 = half-maximum-rate concentration for O_2 (g O_2/m^3). The first term is the rate of methane transport by diffusion inside the biofilm according to Fick's second law, and the second term is the rate of methane consumption based on multiplicative Monod kinetics [26]. Accumulation of dissolved methane means slower consumption rate of methane than its delivery. Methane pressure, methane percentage, or both can be adjusted for minimizing dissolved methane concentration in MBfR effluent. In addition, the methane consumption kinetic in Eq. (2) that includes the kinetic parameters (q_{max}, K_1, and K_2), biofilm thickness, and the density of methanotrophic bacteria (X_f) should be optimized for achieving low dissolved methane and nitrate in the effluent together. Given that the chemical formula of methanotrophic bacteria is $C_5H_7O_2N$ (113 g cells/mol) [27], the maximum specific methane utilization rate (q_{max}) for methanotrophic bacteria is computed at 2.9 g COD_{CH4}/g $COD_{biomass}$-d [28], which is smaller than 5 g COD_{donor}/g$COD_{biomass}$-d for H_2-utilizing autotrophic denitrifiers [29]. Thus, a small q_{max} is part of the reason for slow methane consumption in the biofilm. The half-maximum-rate concentration for methane (K_1) is very small at 0.45 mg CH_4/L in methanotrophic bacteria [28], which means that the Monod term ($S_{f1}/(K_1 + S_{f1})$) should have been close to unity for the methane concentrations inside the biofilm (> 8–12.9 mg CH_4/L). The half-maximum-rate concentration for O_2 (K_2) for methanotrophic bacteria is also small at 61 μg O_2/L [28]. However, DO concentration in the biofilm would be much less than K_2 because DO concentration was steady at 0.2–0.3 μg/L in bulk liquid of an $MBfR_{sup}$. This means that DO concentration mainly governs methane oxidation and denitrification rate, and consequently dissolved methane concentration. Hence, DO control will be key for achieving high denitrification simultaneously with low dissolved methane in methane-utilizing denitrification of MBfRs.

3.3. Calculation of denitrification from O_2 permeation and DO concentration in an MBfR

The maximum O_2 permeation rate to $MBfR_{main}$ was calculated at 1.12 mL O_2/d (= 20×10^{-10} $cm^3 \times mm/cm^2$-s-mmHg) × 73.2 cm^2 × 760 mmHg × 0.21 × (3600 × 24)s/1d/1.8 mm), according to Eq. (1). Using the Ideal gas law with temperature of 298.15 K, the volume is converted to mass: 0.046 mmol O_2/d. From the O_2 permeation, we computed

the maximum production rate of methanol using the mono-oxygenation stoichiometry of $CH_4 + O_2 + 2H = CH_3OH + H_2O$, which requires one mole of O_2 and 2 intracellular electron equivalents (2H) per one mole of methane oxidized to methanol. The maximum methanol production rate via O_2 permeation is calculated at 0.046 mmol CH_3OH/d. We then computed the maximum nitrate reduction rate coupled to oxidation of the methanol given that the methanol is completely utilized via denitrification in $MBfR_{main}$. The maximum nitrate-reduction rate coupled to the methanol generated from the O_2 can be calculated with denitrification stoichiometry from methanol:

$$1/6CH_3OH + 1/5NO_3^- + 1/5H^+ = 1/10N_2 + 1/6CO_2 + 4/15H_2O$$

The maximum nitrate reduction rate to N_2 using the methanol is calculated at 0.055 mmol NO_3^-/d, which equals 0.77 mg NO_3^-N/d. The observed nitrate removals in $MBfR_{main}$ ranged from 2 to 4 mg NO_3^--N/d (see Table 3), which indicates that the methanol produced

Table 3
Daily nitrate reduction to N_2 and (maximum percentage) of nitrate removal using the methanol produced from the maximum permeated O_2 out of observed nitrogen removal in $MBfR_{main.}$

Methane pressure (psig)	HRT (h)		
	12	8	4
2 [1.13 atm]	2.0 mg N/d (39%)	2.3 mg N/d (33%)	2.5 mg N/d (31%)
5 [1.34 atm]	2.2 mg N/d (35%)	2.7 mg N/d (29%)	3.4 mg N/d (23%)
7 [1.48 atm]	2.4 mg N/d (32%)	2.8 mg N/d (28%)	4.0 mg N/d (19%)

( ): percentage of nitrate reduction to N_2 using the methanol produced from permeated O_2 out of the observed nitrate removal, (0.71 mg N/d/observed N removal × 100), 1 atm = 14.7 psig.

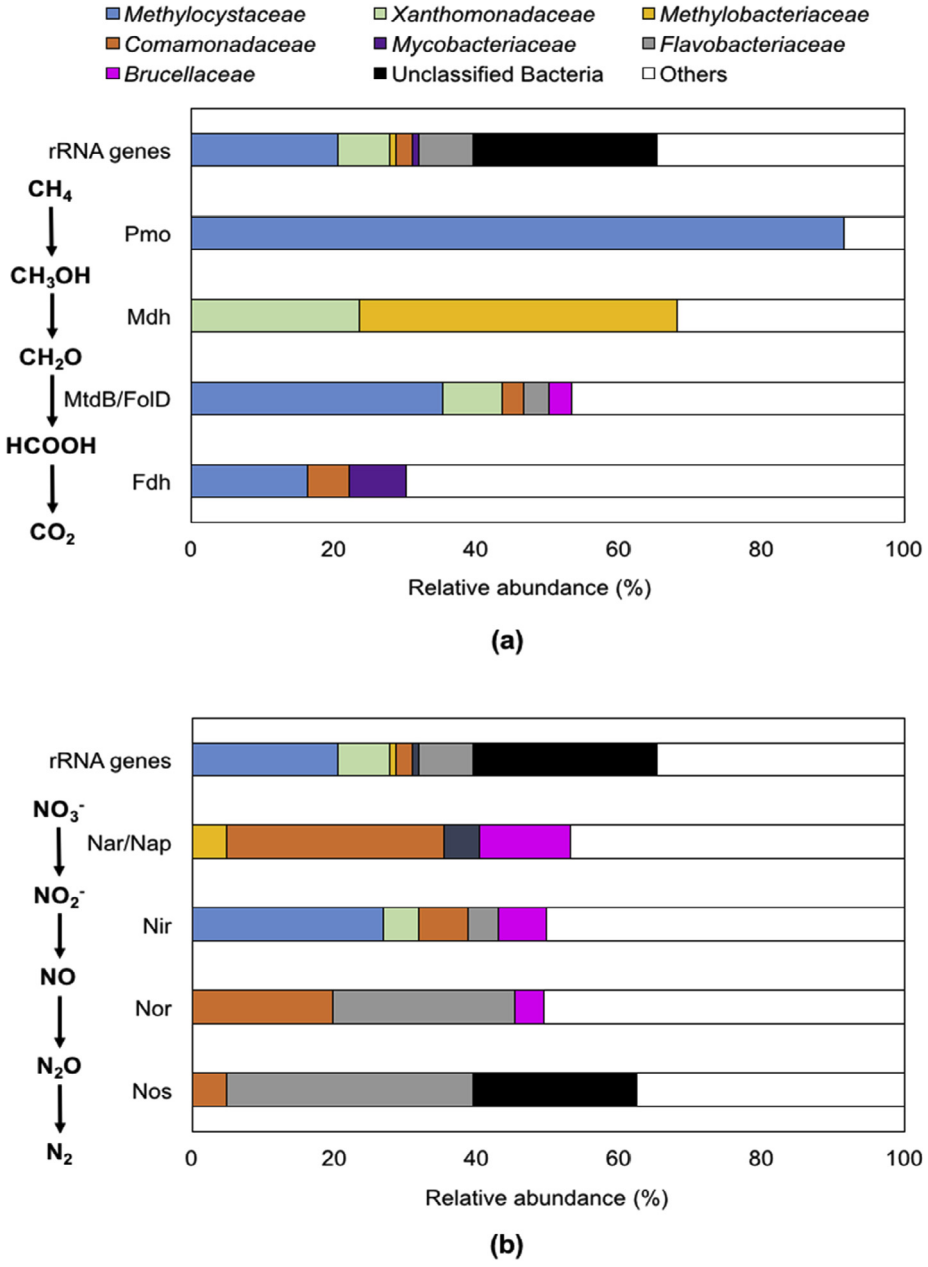


Fig. 3. (a) Relative abundances of SSU rRNA genes and major functional genes for methane oxidation-related enzymes at the family level. (b) Relative abundance of SSU rRNA genes and major functional genes for denitrification-related enzymes at the family level. "Others" indicates genera with relative abundance below 1% for the rRNA genes and below 4% for the functional gene cases.

from the maximum O_2 permeation could have accounted for 19–39% of observed nitrogen removal in $MBfR_{main}$. This calculation suggests that methane-utilized denitrification could occur partially aerobically (aerobic MOD) in the membrane biofilm.

The *in-situ* O_2 microsensor equipped with an $MBfR_{sup}$ showed very small DO concentration from 0.2 to 0.3 μg/L, as mentioned above, which seems like hypoxic conditions. This result might indicate negligible O_2 permeation to the MBfR, but not necessarily because aerobic methanotrophs can quickly consume the permeated O_2, keeping such small DO in the MBfR. Hence, the *in-situ* O_2 monitoring did not enable us to determine between hypoxic and anaerobic conditions, but only confirm the presence of trivial DO maintained in the MBfR.

3.4. Metagenome analysis

Most SSU rRNA reads (> 84%) were from the bacterial domain. Archaea, responsible for reverse methanogenesis (e.g., ANME clades) [3,30,31], were negligible (0.003% of the total rRNA reads), indicating no involvement of ANME-2d (reverse methanogenesis) in MOD reaction for the MBfR (see Fig. S2). Approximately 16% of the reads could not be classified at the domain level.

Fig. 3 shows the distribution of bacterial SSU rRNA genes (34,195 reads). *Methylocystaceae* (21%) was the highest, followed by *Flavobacteriaceae* (8%) and *Xanthomonadaceae* (7%). All three members have been found in denitrification environments [32–34]. The dominance of *Methylocystaceae* (a type II aerobic methanotroph) [35–38] supports that O_2 molecules could permeate $MBfR_{main}$, leading to hypoxia conditions.

We searched functional genes responsible for the steps of aerobic methane oxidation, nitrate reduction to N_2, and reverse methanogenesis. Fig. 4 identifies the genes for each step of those pathways. The functional annotation of the metagenome showed no evidence of the *mcr* gene, which is essential for an initial step of reverse methanogenesis (see Fig. 4a) [39,40], a finding consistent with absence of methanogens based on the gene for the SSU rRNA. Instead, the functional metagenome shows all the genes essential for aerobic methane oxidation: *pmo, mdh, mtdB, folD,* and *fdh*, which encode for the particulate methane monooxygenase, methanol dehydrogenase, methylene-H_4F dehydrogenase/methenyl-H_4F cyclohydrolase, methylene-H_4MPT dehydrogenase, and formate dehydrogenase, respectively (see Fig. 4a). This result is consistent with the dominance of *Methylocystaceae* for the SSU-16rRNA genes. The *pmo* genes were mostly present in *Methylocystaceae* (92%). In comparison, more diverse bacteria, such as *Methylobacteriaceae*, *Xanthomonadaceae*, *Comamonadaceae Flavobacteriaceae*, and *Brucellaceae,* harbored the functional genes *mdh, mtdB, folD,* and *fdh*, (Fig. 3a). Despite phylogenetic and functional diversity, *Methylocystaceae* seems to have been the key player for the oxidation of formaldehyde and formate.

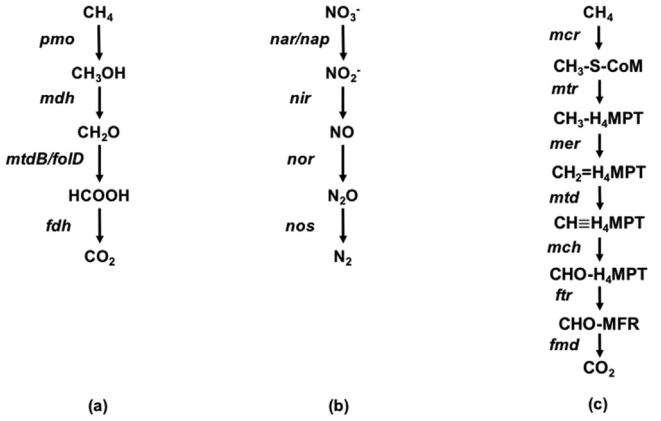


Fig. 4. Microbial pathways on (a) aerobic methane oxidation, (b) nitrate reduction to N_2, and (c) reverse methanogenesis. Genes encoding specific enzymes for aerobic methane oxidation, *pmo*: particulate methane monooxygenase, *mdh*: methanol dehydrogenase, *mtdB*: methylene-H_4F dehydrogenase/methenyl-H_4F cyclohydrolase, *folD:* methylene-H_4MPT dehydrogenase, and *fdh*: formate dehydrogenase. Genes encoding specific enzymes for denitrification, *nar/nap*: nitrate reductase, *nir*: nitrite reductase, *nor*: nitric oxide reductase, and *nos*: nitrous oxide reductase. Genes encoding specific enzymes for reverse methanogenesis, *mcr*: methyl-coenzyme M reductase, *mtr*: terahydromethanoprotein S-methyltransferase, *mer*: coenzyme F_{420}-dependent N5N10-methylene tetrahydromethanopterin reductase, *mtd*: methylene tetrahydromethanopterin dehydrogenase, *mch:*, methenyl tetrahydromethanopterin cyclohydrolase, *ftr*: formylmethanofuran tetrahydromethanopterin N-formyltransferase, *fmd*: formylmethanofuran dehydrogenase.

Functional genes for denitrification, shown in Fig. 3b and Fig. 4b, indicate that diverse bacteria were involved in nitrate reduction to dinitrogen. The *nap/nar* genes for nitrate reduction to nitrite were identified in *Comamonadaceae* (30%), *Brucellaceae* (13%), *Mycobacteriaceae* (5%), and *Methylobacteriaceae* (5%), all of which are heterotrophic denitrifiers [41–45]. No *nap/nar* genes were found for *Methylocystaceae*, but this family was the most dominant for *nir* genes (27%), followed by *Brucellaceae* (7%), *Comamonadaceae* (7%), *Xanthomonadaceae* (5%), and *Flavobacteriaceae* (4%). The *nor* and *nos* genes, encoding nitric oxide and nitrous oxide reductases, respectively, were mainly identified in *Flavobacteriaceae, Comamonandaceae,* and *Brucellaceae*.

The metagenome results support that hypoxia methane oxidation coupled to denitrification occurred via a syntrophic interaction between a methanotroph (*Methylocystaceae*) and diverse denitrifying bacteria in the membrane's biofilm (Fig. 5). Denitrifying bacteria (e.g., *Comamonadaceae* and *Brucellaceae*) were the main reducers of nitrate to nitrite probably using metabolic intermediates from methane oxidation (i.e., methanol, formaldehyde or formate based on functional genes in Fig. 3). The functional genes suggest that *Methylocystaceae* oxidized methane coupled to nitrite reduction to nitric oxide, while denitrifiers (e.g., *Comamonadaceae* and *Flavobacteriaceae*) further reduced nitric oxide to dinitrogen.

3.5. Microcosm experiments

We conducted microcosm tests using biofilm samples enriched with *Methylocystaceae* as inocula. Nitrate concentration slowly decreased from 28 to 18 mg N/L with time for over 60 d, as shown in Fig. 6, but change of methane composition in headspace was trivial over nitrate reduction. Almost same amount of nitrate was reduced in control lacking methane, supporting that anaerobic MOD using methane as the electron donor would not mainly account for denitrification in $MBfR_{main}$. DO was also not detected during the microcosm experiments, supporting no involvement of intracellular oxygenic pathway to MOD in *Methylocystaceae* enrichment culture. This result evidences that methanotrophic bacteria enriched in $MBfR_{main}$ do not carry out anaerobic

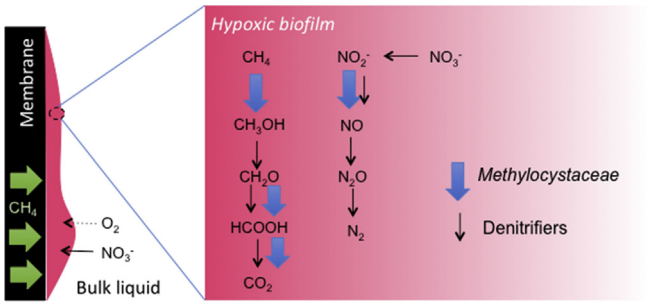


Fig. 5. Conceptual diagram describing syntrophic interactions between methanotrophic bacteria and heterotrophic denitrifiers in the hypoxic membrane biofilm.

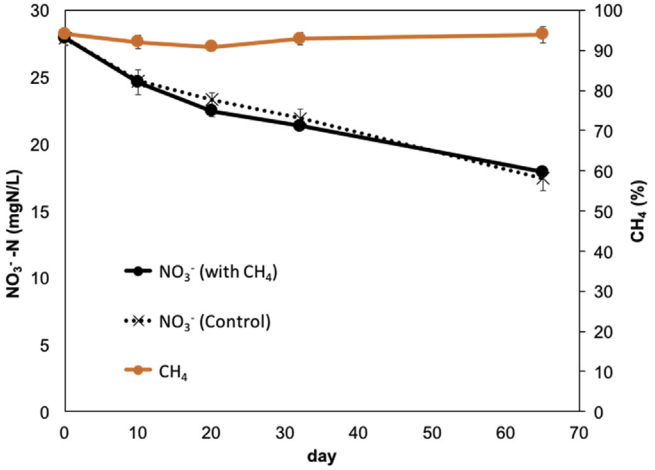


Fig. 6. Trends of CH_4 gas composition and NO_3^- concentration in serum-bottle tests. Duplicate tests were conducted for over 60 d incubation at 25 °C. Control means endogenous-decay denitrification without methane.

MOD via the intracellular oxygenic pathway. This well accords to DO concentration and metagenome outcomes. In addition, control tests imply that part of nitrate reduction could occur via endogenous decay in $MBfR_{main}$, although we could not quantify endogenous denitrification in the bioreactor. Simple organics (i.e., methanol, formate, acetate, propionate, butyrate, valerate, and caproate) were not detected in the supernatant and MBfR effluent, implying that complex, polymeric organics would be excreted during endogenous decay [46] and they might be used as the electron donor to denitrification [47].

3.6. Implication of methane-utilizing MBfRs for denitrification

Several literatures have suggested the possibility that methanotrophic bacteria might anaerobically conduct MOD via the intracellular oxygenic pathway in MBfRs pressurized with methane gas, given that O_2 intrusion to the MBfRs would be negligible [14,16,48]. If some methanotrophic bacteria can anaerobically oxidize methane coupled to denitrification, our understanding of global methane and nitrogen cycles will be significantly changed, such as contribution of ANME to global methane consumption. This study proves that methanotrophic bacteria mainly affiliated with *Methylocystaceae* family oxidize methane using O_2 evidenced by identification of *pMMO* genes and DO detection in $MBfR_{main}$. Calculation of O_2 permeation through tubing, identification of *nor* and *nos* genes, and microcosm experiments further support that anaerobic MOD does not occur via the intracellular oxygenic pathway in $MBfR_{main}$. Instead, heterotrophic denitrification bacteria in syntrophy with methanotrophic bacteria mainly reduced nitrate to dinitrogen in the membrane biofilm.

Biological nitrogen removal processes have been applied for mitigating eutrophication of surface water. Among several processes, pre-denitrification (e.g., Ludzack-Ettinger processes) using organics in wastewater or exogenous electron donor has been widely used for nitrogen control in WWTPs. However, pre-denitrification cannot address the demand of current society for energy-efficient, economical wastewater treatment and strict nitrogen limits in treated wastewater. Pre-denitrification is energy-intensive, requiring high pumping costs for sludge recirculation, and dose of exogenous electron donor (e.g., methanol) can be needed due to insufficient organics in wastewater. For instance, methanol has been used for denitrification for over 200 WWTPs in USA [49]. Methanol dose adds operating costs to energy-intensive pre-denitrification processes.

Methane-utilizing denitrification of MBfRs can save costs substantially as compared to heterotrophic denitrification using methanol, since methane ($120/ton) is much cheaper than methanol ($500/ton) [50,51]: Methane cost is about 10 times lower than methanol per mole of electrons ($0.24/kmol electrons of methane vs. $2.7/kmol electrons of methanol). In addition, the MBfRs can use biogas generated from anaerobic digesters, typically operated for sludge stabilization in most WWTPs, and in this coupling process methane cost will be negligible. Hence, methane-based denitrifying MBfRs can be a competitive biological nitrogen removal process to existing WWTPs mainly designed for organic removal and nitrification (called, activated sludge); in North America, activated sludge processes are still used for most WWTPs. Moreover, the MBfRs can be used as a tertiary nitrogen removal process to existing biological nitrogen removal WWTPs for further decreasing nitrogen concentration in wastewater effluent.

4. Conclusions

We tested denitrification in the MBfR using methane gas as the sole electron donor. *Methylocystaceae* family dominated the membrane biofilm in which nitrate reduction to nitrite was rate limiting. Dissolved methane was 8–13 mg CH_4/L in the effluent, mainly due to slow kinetics of microbial reactions, not methane mass transport. The functional metagenome showed all the genes essential for aerobic methane oxidation and nitrate reduction to dinitrogen, but the genes for reverse methanogenesis was not found in the metagenome. This study proposes that O_2 could permeate the MBfR and it could allow *pMMO*-dependent methane oxidation coupled to denitrification in hypoxic conditions.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.cej.2018.04.202.

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