Complete perchlorate reduction using methane as the sole electron donor and carbon source

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Abstract

Using a CH_4-based membrane biofilm reactor (MBfR), we studied perchlorate (ClO_4^-) reduction by a biofilm performing anaerobic methane oxidation coupled to denitrification (ANMO-D). We focused on the effects of nitrate (NO_3^-) and nitrite (NO_2^-) surface loadings on ClO_4^- reduction and on the biofilm community's mechanism for ClO_4^- reduction. The ANMO-D biofilm reduced up to 5 mg/L of ClO_4^- to a non-detectable level using CH_4 as the only electron donor and carbon source when CH_4 delivery was not limiting; NO_3^- was completely reduced as well when its surface loading was $\leq$ 0.32 g N/m^2-d. When CH_4 delivery was limiting, NO_3^- inhibited ClO_4^- reduction by competing for the scarce electron donor. NO_2^- inhibited ClO_4^- reduction when its surface loading was $\geq$ 0.10 g N/m^2-d, probably due to cellular toxicity. Although Archaea were present through all stages, Bacteria dominated the ClO_4^--reducing ANMO-D biofilm, and gene copies of the particulate methane mono-oxygenase (*pMMO*) correlated to the increase of respiratory gene copies. These pieces of evidence support that ClO_4^- reduction by the MBfR biofilm involved chlorite (ClO_2^-) dismutation to generate the O_2 needed as a co-substrate for the mono-oxygenation of CH_4.

Key Words: methane, perchlorate, oxidation, reduction, membrane-biofilm reactor

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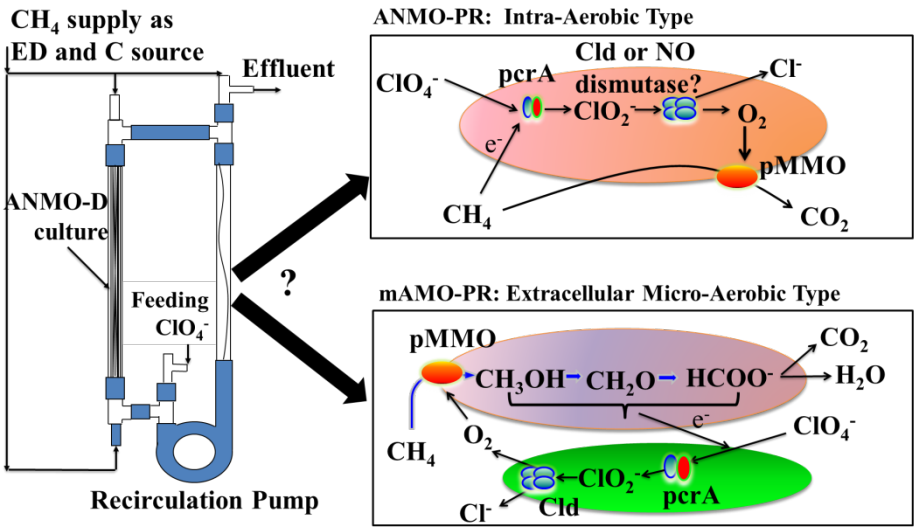
TOC Art



ANMO-D: Anaerobic methane oxidation coupled to nitrate reduction
ANMO-PR: Anaerobic methane oxidation coupled to perchlorate reduction
mAMO-PR: micro-Aerobic methane oxidation coupled to perchlorate reduction

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Introduction

Perchlorate (ClO_4^-) is a strong oxidizing agent that has been widely used in rocket fuel, munitions, and explosives (EPA, 2005).[1] It causes serious health problems by interfering with the production of thyroid hormones needed for growth and development (Coates & Achenbach, 2004).[2] The typical perchlorate concentration in groundwater is lower than 100 μg/L, but in some cases it can reach concentrations of 20 mg/L or more.[3, 4] Although a nationwide maximum contaminant level (MCL) for ClO_4^- has not yet established by the US EPA, some states have established cleanup levels ranging from 2 to 18 μg/L for ClO_4^- in drinking water (Gu & Coates, 2006).[5] Nitrate (NO_3^-) is an oxyanion commonly co-occurring with ClO_4^- in groundwater, for example, at military bases that house rockets (USEPA, 2001).[6] Because NO_3^- causes methemoglobinemia in infants, the MCL for NO_3^- in drinking water is regulated at 10 mg N/L (USEPA, 2009).[7] NO_3^- inhibits ClO_4^- reduction due to competition for the common electron donor when the electron donor is insufficient.[8, 9]

Different electron donors have been applied to achieve complete ClO_4^- and NO_3^- removal by microbiological reduction.[10-12] An interesting example is nitrogen reduction using methane as the sole electron donor and carbon source,[13, 14] since methane is inexpensive and widely available.[15-17]

Methane oxidation coupled to denitrification (MO-D) has been extensively studied during the past decade.[14, 15, 18-21] Two microbial processes are capable of carrying out MO-D. One is aerobic methane oxidation coupled to denitrification (AMO-D),[22, 23] which is performed by the combined actions of two distinct bacterial groups: methane oxidizers (methanotrophs) and denitrifiers.[22, 24, 25] The second is anaerobic

59 methane oxidation coupled to denitrification (ANMO-D).[21, 26] ANMO-D
60 microorganisms include a bacterial group affiliated with the candidate division NC10
61 and an archaeal group distantly related to anaerobic methanotrophic archaea.[19, 27-29]

62 AMO-D occurs in the presence of O_2, because methanotrophs require O_2 for the initial
63 mono-oxygenation step. The methanotrophs can release organic intermediates from
64 their catabolism and anabolism, for example, methanol, acetate, and citrate, and the
65 intermediates can be further utilized by denitrifiers as electron donors.[15, 22, 30] These
66 steps are illustrated schematically in panel A of Figure S1 in Supplemental
67 Information. Although a high concentration of O_2 inhibits denitrification, a certain
68 amount of O_2 is necessary to promote AMO-D.[30, 31]

69 ANMO-D can follow two pathways, illustrated schematically in panels B and C of
70 Figure S1. Raghoebarsing et al. hypothesized the "Reverse Methanogesis" pathway,
71 which involves the combined action of Archaea and denitrifying bacteria.[19] The
72 Archaea carry out reverse methanogenesis to generate H_2 that is shuttled to the
73 denitrifying bacteria that respire NO_2^- to N_2. Haroon et al. reported that Archaea
74 population ANME-2d (*Methanoperedens nitroreducens*) catalyzed CH_4 oxidation by
75 methylcoenzyme M reductase (*mcrABCDG*) through a reverse-methanogenesis
76 pathway using NO_3^- as their terminal electron acceptor and generating NO_2^-; the
77 NC10 bacteria then reduced NO_2^- to N_2.[14]

78 The second ANMO-D pathway is called the "Intra-Aerobic" pathway,[21, 32] and only
79 one denitrifying microorganism (*Candidatus Methylomirabilis oxyfera*) was involved.

Denitrification was carried out by stepwise reduction of NO_2^- to NO using nitrate reductase (*narGHJI*) and nitrite reductase (*nirSJFD/GH/L*); then, NO was disproportionated to produce O_2 intracellularly and N_2 using an unknown dismutase enzyme. The O_2 was then used by the same microorganism as a co-substrate for methane mono-oxygenation by a membrane-bound particulate methane mono-oxygenase (*pMMO*).

While the true mechanism of ANMO-D is unresolved, it is thermodynamically feasible, as shown by redox equations 1 and 2 for NO_3^- or NO_2^- as the terminal electron acceptor:[19]

$$5CH_4 + 8NO_3^- + 8H^+ = 5CO_2 + 4N_2 + 14H_2O \qquad \Delta G^{0'} = -765\ KJmol^{-1}CH_4 \qquad (1)$$

$$3CH_4 + 8NO_2^- + 8H+ = 3CO_2 + 4N_2 + 10H_2O \qquad \Delta G^{0'} = -928\ KJmol^{-1}CH_4 \qquad (2)$$

When perchlorate (ClO_4^-) is the electron acceptor, a similar reaction between ClO_4^- and CH_4 also is thermodynamically feasible: [33]

$$CH_4 + ClO_4^- = HCO_3^- + Cl^- + H_2O \qquad \Delta G^{0'} = -792\ KJmol^{-1}CH_4 \qquad (3)$$

The pathway for dissimilatory perchlorate reduction begins with reduction of ClO_4^- to chlorite (ClO_2^-, catalyzed by perchlorate reductase, *pcrA*) and ends with dismutation of ClO_2^- to yield chloride (Cl^-, catalyzed by chlorite dismutase, *cld*) and molecular oxygen (O_2), which is essential for the methane oxidation.[2] Miller et al. confirmed complete CH_4 removal coupled with ClO_2^- dismutation by a mixture of methanotrophs and the perchlorate-reducing bacterium *Dechloromonas agitate* CKB.[34] The methanotrophs used extracellular O_2 derived from disproportion of ClO_2^- by *D. agitate* CKB to oxidize CH_4 aerobically; thus, it was an extracellular-aerobic pathway

that required that the substrate for dismutation, ClO_2^-, be supplied. The mixed culture did not oxidize CH_4 when supplied with ClO_4^- or ClO_3^-; thus, they concluded that O_2 produced via ClO_4^- reduction was unavailable for the aerobic methanotrophs.

So far, no study has successfully reduced ClO_4^- using CH_4 as the sole electron donor and carbon source. However, thermodynamics and the reality that most denitrifiers are able to reduce ClO_4^- (using either nitrate or perchlorate reductase) means that a ANMO-D or AMO-D culture has the possibility to reduce ClO_4^- using CH_4 as electron donor and carbon source.

The H_2-based membrane biofilm reactor (MBfR) has been applied successfully for microbial removal of oxidized contaminants, including NO_3^- and ClO_4^-.[11, 35-37] The non-porous walls of hollow-fiber membranes transfer H_2 directly to a biofilm of H_2-oxidizing bacteria that reduce one or more electron acceptors.[35] The use of "bubbleless" membranes and the rapid oxidation of H_2 in the biofilm allow nearly 100% utilization of H_2, preventing H_2 losses to the atmosphere or effluent liquid.[36, 38]

The MBfR also could provide a means for the safe and efficient supply of CH_4 to drive ANMO-D, AMO-D, and ClO_4^- reduction. Supporting the concept, Sun et al reported that an aerobic methane-based MBfR removed up to 97% of NO_3^- applied at a concentration of 30 mg N/L,[39] and Shi et al achieved 86 mg N/m^2-d NO_3^- removal in an anaerobic MBfR provided with CH_4 as carbon source and electron donor.[40]

The objective of this study was to evaluate ClO_4^- reduction in a CH_4-based MBfR.

Specifically, we studied the reduction patterns of NO_2^-, NO_3^-, and ClO_4^- when we exposed the biofilm to different relative loadings. We quantified the CH_4-permeation coefficient through the membrane wall to determine if the delivery rate of CH_4 was limiting, and we used quantitative real-time PCR (qPCR) to monitor how the abundances of functional genes key to respiration reactions were affected by the acceptor loadings. Based on several types of evidence, we are able to provide mechanistic interpretation about what controls ClO_4^- reduction by the biofilm and the likely pathways by which NO_3^- and ClO_4^- reductions occur when CH_4 is the sole electron donor.

Materials and Methods

CH_4 permeation

Steady-state CH_4-permeation experiments were carried out in the same system (shown schematically in Supporting Information (SI) Figure S2) Tang et al. used for quantifying H_2 permeation.[41] Deionized water was pumped through the serum bottle (total volume of 1.6×10^{-4} m^3, liquid volume of 0.6×10^{-4} m^3) at a flow rate of 7.2×10^{-4} m^3/d. The hollow fibers in the serum bottle were pressurized with CH_4 (99.99% purity, Shanghai Gas Company, China) at a pressure of 1.0 bar (14.5 psi). CH_4 diffused through the hollow-fiber wall, dissolved in the water, and partitioned into the headspace. A magnetic stirring bar ensured complete mixing of the liquid and that CH_4 was rapidly partitioned to the gas phase. We took the headspace gas samples to measure its CH_4 partial pressure. Steady state was achieved when the CH_4 partial pressure was stable for at least 40 hydraulic retention times (HRTs).[41] We then calculated the CH_4 permeability of the membrane fiber based on the method in Tang et al.[41] The equations and experimental parameters for the CH_4-permeation test are summarized in the Supplementary Information (Table S1 & S2).

MBfR Setup

We used a two-column MBfR system similar to Zhao et al.[8] The MBfR had composite hollow fibers (hydrophobic microporous polyethylene fiber, 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). The fibers were glued into a CH_4-supply manifold at the bottom of the MBfR column, and the top of each fiber was sealed. The total volume of the MBfR was 65 mL, and the total membrane surface area was 7.0 cm^2. The MBfR was completely mixed by recirculation with a peristaltic pump (Longer Pump, model 1515X, Longer Precision Pump Co, Ltd, China) at 100 mL/min.

Start up and continuous operation of the MBfR

We inoculated the MBfR with 10 mL of ANMO-D culture (original maintained anaerobic) donated by Dr. Wei Xiang Wu at Zhejiang University (China) and enriched the community by recirculating a mineral salt medium (described below) containing 2 mg N/L NO_2^- for 2 days. To accumulate enough biomass, we fed the MBfR with 2 mg N/L of NO_2^- continuously for 40 days, when complete NO_2^- reduction was achieved. To investigate ClO_4^- reduction in the presence of NO_2^-, we fed the MBfR with ClO_4^- and NO_2^- at influent concentrations of 1 mg/L and 2 mg N/L, respectively, in Stage 1. Since NO_3^- may inhibit ClO_4^- reduction at high surface loadings (> 0.6 g N/m^2-d) or improve ClO_4^- removal at medium (0.1-0.6 g N/m^2-d) or small (<0.1g N/m^2-d) loadings in a H_2-based MBfR,[9] we then systematically changed the influent concentrations of NO_3^- and ClO_4^- in Stages 2 through 6. We allowed each stage to reach steady state, which was defined as effluent concentrations stable (<10% variation) for a minimum of three HRTs. The influent concentrations were: Stage 2: 1 mg/L ClO_4^-; Stage 3: 1 mg/L ClO_4^- and 1.1 mg N/L of NO_3^-; Stage 4: 1

mg/L ClO_4^- and 11.3 mg N/L of NO_3^-; Stage 5: 1 mg/L ClO_4^- and 4.5 mg N/L of NO_3^-; and Stage 6: 5 mg/L ClO_4^-. To investigate ClO_4^- reduction in the presence of NO_2^- after high ClO_4^--removal efficiency had been achieved, we fed the MBfR 5 mg/L of ClO_4^- and 5 mg N/L of NO_2^- in Stage 7. Actual influent concentrations varied slightly from the targets and are presented in Table S3 and Figure 1.

The influent feeding rate was 0.5 mL/min (HRT of 130 min), the CH_4 pressure was 10 psi (0.69 bar) for Stage 1-3 and 15 psi (1.03 bar) for the latter stages, and the temperature was 29±1^oC for all experiments. The medium pH was adjusted to 7.0±0.2 with hydrochloric acid and contained the following mineral salts (analytical grade or purer) per L of demineralized water: $CaCl_2$ 1 mg, $NaHCO_3$ 0.3 g, $MgSO_4•7H_2O$ 5 mg, KaH_2PO_4 0.2 g, $Na_2HPO_4•12H_2O$ 0.4 g, 1 mL acid trace element solution (HCl 100 mM, 2.085 g of $FeSO_4•7H_2O$, 68 mg of $ZnSO_4•7H_2O$, 14 mg of H_3BO_3, 120 mg of $CoCl_2•6H_2O$, 500 mg of $MnCl_2•4H_2O$, 320 mg of $CuSO_4$, 95 mg of $NiCl_2•6H_2O$ per liter), and 1 mL alkaline trace element solution (NaOH 10 mM, 67 mg of SeO_2, 50 mg of $Na_2WO_4•2H_2O$, 242 mg of $Na_2MoO_4•2H_2O$ per liter). The medium was de-gassed with N_2 to maintain an anaerobic condition.

During Stage 4, the CH_4 supply was accidently lost for 48 hours, and we immediately substituted N_2 gas to keep the fibers pressurized. To re-evaluate the response found in Stage 4, we intentionally stopped the CH_4 supply for 30 hours in Stage 5. The CH_4 supply was reinstated when the removal percentages were zero for NO_3^- and ClO_4^-.

Analyses

We measured the CH_4 partial pressure (P_{CH4}) of gas samples using a gas chromatograph (Agilent Technologies GC system, model 7890A, Agilent Technologies Inc., U.S.A) equipped with a flame ionization detector and a packed column (30 m long, 0.32 mm i.d., 0.5 μm thickness, cross-linked polydimethysiloxane film, J&W scientific, U.S.A.). N_2 was the carrier gas fed at a constant pressure of 0.96 bar and a constant flow rate of 0.065 m^3/d, and the temperature conditions for injection and detector were 200 and 260^oC, respectively. Analytical grade CH_4 was used for standard calibration curves and for the experiments.

We took liquid samples from the MBfR with 5-mL syringes and filtered them immediately through a 0.2-μm membrane filter (LC+PVDF membrane, Shanghai Xinya, China). We assayed for NO_3^- and NO_2^- using ion chromatography (Metrohm 833 Basic IC plus, Switzerland) with an A-Supp-5 column, an eluent containing 3.2 mM $NaHCO_3$, 1.0 mM Na_2CO_3, and 5% Acetone in a flow rate of 1 mL/min. ClO_4^- was measured using ion chromatography (Metrohm 833 Basic IC plus, Switzerland) with an AS 16 column and AG 16 pre-column, eluent concentration of 35 mM KOH, and a 1.5 mL/min flow rate. Dissolved O_2 was measured with a dissolved oxygen probe (Starter, model 300D, Ohaus Instruments Company, Germany), and the concentrations for ~0.2 mg/L for the influent and ≤0.1 mg/L for the effluent. The pH values of the influent and effluent were measured by a pH meter (Seven Easy, Mettler Toledo, Switzerland) and were between 7.4 and 7.7 for all stages.

214 *Flux Calculations*

215 We calculated the NO_3^- and ClO_4^- removal fluxes (g/m^2-d) according to:

216 $$J = (S^o\text{-}S)Q/A \quad (4)$$

217 in which S° and S are the influent and effluent NO_3^- or ClO_4^- concentration (g/L), Q is
218 the influent flow rate to the MBfR system (L/d), and A is the membrane surface area
219 (m^2). The CH_4 flux was calculated from the removal fluxes and reaction
220 stoichiometries shown in equations 5 through 7.[42]

$$NO_2^- + 0.828CH_4 + H^+ = 0.04CO_2 + 0.42N_2 + 0.158C_5H_7O_2N + 1.6H_2O \quad (5)$$

$$NO_3^- + 1.2CH_4 + H^+ = 0.2CO_2 + 0.4N_2 + 0.2C_5H_7O_2N + 2.2H_2O \quad (6)$$

$$ClO_4^- + 1.613CH_4 + 0.175NO_3^- + 0.175H^+ = Cl^- + 0.737CO_2 + 2.7H_2O + 0.175C_5H_7O_2N \quad (7)$$

221 We compared the actual CH_4 flux to the maximum CH_4 flux that can be delivered
222 through the composite hollow fiber at the applied CH_4 pressure to indicate if CH_4
223 delivery was limiting.[41]

224 *Biofilm Sampling and DNA extraction*

225 We collected biofilm samples when the reactor reached a steady state for all stages
226 except Stage 2. Sparging with N_2 gas at the sampling point to preclude O_2 exposure,
227 we cut off one ~10-cm-long section from the coupon fiber and then sealed the
228 remaining by tying the end into a knot. We then extracted DNA using the DNeasy
229 Blood & Tissue Kit (Qiagen, USA) as previously described by Zhao et al (2011).[38]

230

Quantification of 16S rRNA genes for Bacterial, Archaea and other functional genes

We used plasmids containing target fragments as positive controls and to produce calibration curves.[8] The primers and qPCR conditions were the same as previously described for *pcrA* – reductase for ClO_4^-,[43] *narG* – reductase for NO_3^-,[44] *nirS* – reductase for NO_2^-,[45] *mcrA* – formation of methane from most of methanogens,[46] *pMMO* – CH_4 mono-oxygenase,[47] the*16S rRNA* gene for bacteria,[48] and the *16S rRNA* for archaea.[49] We used the SYBR Premix Ex Taq Kit (Takara Bio Inc, Japan) and performed qPCR as previously described by Zhao et al. (2011).[8] The copy numbers of each gene were calculated by comparison to standard curves. Negative controls included water instead of template DNA in the PCR reaction mix. We performed triplicate PCR reactions for all samples and negative controls. The slopes of the plasmid standard curves and efficiency values for quantification by qPCR are shown in Table S4.

Results and Discussion

Methane permeability

Figure 1 shows the headspace pressures during the CH_4-permeation experiment. Steady state was achieved at ~15 hours for the composite fiber. The CH_4 permeability for the composite fiber was 1.03×10^{-7} m^3 CH_4 at standard temperature and pressure - m membrane thickness/m^2 hollow fiber surface area - d - bar. This permeability is about 10-fold smaller than the H_2 permeability for the same composite fiber and temperature.[41] Although the Henry's law constant of CH_4 is only slightly smaller than for H_2, its mass-to-mole ratio (16 g/mol) is about 8 times greater than for H_2 (2 g/mol), making the CH_4 molecule bulkier and more slowly diffusing through the membrane wall.

Perchlorate reduction in the presence of nitrate and nitrite

Figure 2-A shows the influent and effluent concentrations of NO_2^-, NO_3^-, and ClO_4^- for the entire set of experiments, and Figure 2-B shows the corresponding removal percentages. ClO_4^- reduction could be achieved when CH_4 was the sole electron donor and carbon source. 100% reductions occurred in Stages 2, 3, 5, and 6, and major partial reduction was achieved in Stages 1 and 7. Though Miller et al established a link between ClO_2^- and CH_4 consumption in soils and mixed cultures by *D. agitate* CKB and methanotrophs (*Methylococcus capsulatus* Bath or *Methylomicrobium album* BG8) using acetate as the electron donor and carbon source,

they did not find any upstream connection between ClO_4^- or ClO_3^- reduction and methane oxidation.[34] They concluded that oxygen generation during perchlorate reduction was negligible or unavailable for aerobic methanotrophs. In contrast, our results clearly show that the MBfR biofilm was able to reduce ClO_4^- using CH_4 as the sole electron donor. This success of coupling perchlorate reduction with anaerobic methane oxidation suggests that reduction of other anions might also be coupled to anaerobic methane oxidation. Hence, it would be interesting to explore whether bromate, selenate, chromate and other anion contaminants could be reduced in similar CH_4-based systems.

Comparison among Stages 1, 2, 6, and 7 shows that NO_2^- inhibited ClO_4^- reduction: ClO_4^- reduction was complete when NO_2^- was absent in the influent in Stages 2 and 6, but it decreased to < 50% when NO_2^- was present in the influent at a surface loadings of 0.1-0.4 g N/m^2-d (1.69±0.006 mg N/L for Stages 1, 5.22±0.13 mg N/L for Stage 7 in the influent, respectively).

Comparison among Stages 2, 3, 4, 5, and 6 shows that NO_3^- also inhibited ClO_4^- reduction, but only at high NO_3^- surface loadings. When the NO_3^- surface loadings were <0.32±0.003 g N/m^2-d (Stages 2, 3, 5, and 6), ClO_4^- and NO_3^- reductions were complete. However, when the NO_3^- surface loading increased to 0.78±0.09 g N/m^2-d in Stage 4, ClO_4^- reduction dropped to ≤5%, with NO_3^- reduction declining to ≤85%. This trend is consistent with Tang et al,[9] who used biofilm modeling to quantify the impact of NO_3^- loading on perchlorate reduction when H_2 was the electron donor.

High NO_3^- loading slowed ClO_4^- reduction by competing for the common donor (H_2 for Tang et al.[9] and CH_4 here).

The MBfR accidently lost its CH_4 supply for 48 hours (days 75-77), and we provided N_2 gas to keep the fibers pressurized (Figure S3-A). NO_3^- removal dropped sharply to 2% before the CH_4 supply was recovered, but it returned to 70% within 12 hours. However, ClO_4^- removal remained low (2%) after recovery of the CH_4 supply, although it recovered to 100% in Stage 5, when the nitrate loading was smaller. To reinforce that methane was the electron donor responsible for perchlorate and nitrate reduction, we repeated the CH_4-loss experience during Stage 5 by intentionally replacing the CH_4 supply with N_2 gas for 30 hours beginning on day 94 (Figure S3-B). NO_3^- and ClO_4^- removals dropped to 0 within 12 hours for ClO_4^- and 24 hours for NO_3^-, but both returned to 100% after the CH_4 supply was recovered.

We calculated the consumption fluxes of NO_2^-, NO_3^-, and ClO_4^-, along with the stoichiometric fluxes of CH_4 (from equations 5 – 7). The fluxes are summarized in Table 1 for each steady state. One important comparison is between the actual CH_4 consumption and the maximum possible CH_4 flux. The maximum fluxes were 57.9 mmol CH_4/m^2-d for Stages 1 – 4 and 86.8 mmol CH_4/m^2-d for Stages 5-7, both calculated from the K_m of CH_4 of the composite fiber for the experimental conditions. The maximum CH_4 delivery flux for Stages 1, 2, 3, 5, 6, and 7 was substantially greater than the observed CH_4 fluxes, and CH_4 delivery should not have been limiting.

Stage 4 may have been limited by CH_4 delivery, because the actual CH_4 flux

(47.5±7.20 mmol/m^2-d) was close to the maximum CH_4 flux (57.9 mmol CH_4/m^2-d). In Stage 4, the effluent concentration of nitrate was stable at 1.39±0.21 mg N/L for 2 days before the methane supply was lost (Figure S3). Based on stoichiometry, the maximum methane-delivery rate could remove NO_3^- at a maximum flux of 0.67 g N/m^2-d, which corresponds to 100% removal of an influent concentration at 10.2 mg N/L. The actual influent concentration was 11.3±0.40 mg N/L in Stage 4, which explains the partial NO_3^- removal and that competition of CH_4 is why ClO_4^- reduction remained very low throughout Stage 4.

Because donor limitation was not an issue for Stages 1 and 7, the negative impact of NO_2^- on ClO_4^- reduction probably was due to toxicity of NO_2^-, not to competition for CH_4. Kluber & Conrad reported that methanogenesis activity could be significantly inhibited by adding NO_2^-.[50] King & Schnell reported that NO_2^- could inhibit the methane oxidation by methanotrophs, and the inhibition was inversely proportional to headspace methane concentrations.[51]

Functional Community Structure through Functional Gene Analysis

Figure 3 shows the 16S rRNA gene copies for Bacteria and Archaea, functional-gene copy numbers, and fluxes of the tested electron acceptors through all stages. The copy number of the *pcrA* gene gradually increased from Stage 1 to Stage 5, and this was parallel to overall increasing flux of ClO_4^- + NO_3^- and accumulation of more bacteria, illustrated by the increasing gene copies for the 16S rRNA gene. The copy number of the *pcrA* gene decreased when the flux of all electron acceptors

significantly decreased in Stage 6 (due to the absence of NO_3^- and NO_2^-), but increased again when NO_2^- was re-introduced at a flux of 0.39±0.01 g N/m^2-d in Stage 7. Since most denitrifying bacteria (DB) are able to reduce ClO_4^- and may harbor the *pcrA* gene,[52, 53] it is not surprising that the abundance of the *pcrA* gene was significantly related with the NO_3^- flux in our study (Table S5), as well as in previous MBfR studies with H_2.[8, 54, 55]

Similar to the *pcrA* gene, *nirS* and *narG* genes gradually increased from Stage 1 to Stage 5, though the fluxes of NO_3^-+NO_2^- decreased from Stage 4 to 5; again, the increases likely were due to accumulating bacteria overall. When NO_3^- and NO_2^- were absent in Stage 6, the *nirS* and *narG* abundances dropped by 0.5 to 1 order of magnitude. Also similar with the *pcrA* trend, the *nirS* and *narG* genes increased in Stage 7 when NO_2^- was re-introduced into the MBfR system. However, the *narG* abundance was similar to *nirS* in Stage 1, when NO_2^- was fed at a low loading, and became much lower than *nirS* in Stage 7 when NO_2^- was fed at a higher loading. Because the *NarG* gene is not selective for all DB,[56] *nirS* is mostly used to quantify the DB.[57]

Overall, Bacteria (16S rRNA gene) were ~2 orders of magnitudes higher than Archaea (Archaeal 16S rRNA gene) through all stages, suggesting that Bacteria dominated Archaea. While the abundances of *mcrA* and *pMMO* genes were about the same in Stages 1 and 3, the *pMMO* gene increased much more by Stage 5 and in parallel to the large increase in the flux of CH_4. The *mcrA* and *pMMO* genes decreased in Stage 6,

when NO_3^- and NO_2^- were absent in the system, resulting in a much lower CH_4 flux. The *pMMO* gene abundance returned to its Stage 5 level with the increase of CH_4 flux Stage 7, but the *mcrA* gene remained low in Stage 7.

Archaea are necessary for the "Reverse Methanogesis" ANMO-D pathway, as they produce electrons for denitrification. The low abundance of Archaea (Fig. 3) supports the "Reverse Methanogenesis" was not important in the ClO_4^--reducing, CH_4-oxidizing biofilm. Further support is given in Figure 4, which shows that the gene copies of *mcrA* and respiration genes had no correlation.

Intracellularly generated O_2 is essential for the "Intra-Aerobic Type" ANMO-D pathway, in which *Candidatus M. oxyfera* (or a similar methanotroph) oxidizes CH_4 via an initial mono-oxygenation reaction. Figure 4 shows that the gene copies of *pMMO* correlated to the gene copies for *narG+nirS+pcrA*, which supports an essential role of O_2 generation associated with ClO_4^- reduction. This association is logical if the key bacteria reducing ClO_4^- used a chlorite dismutase in a manner similar to NO disproportionation in denitrification.[21] If O_2 were produced and consumed intracellularly, ClO_4^- reduction occurred via an "Intra-Aerobic Type" ANMO-PR pathway, which is illustrated in Figure 5-A.

Rikken et al found that O_2 was released extracellularly during complete ClO_4^- reduction.[58] Thus, another possibility is that CH_4 oxidation was coupled to ClO_4^- reduction by a mixture of methanotrophs and perchlorate-reducing bacteria using *pMMO* and *pcrA* separately. Miller et al. reported that a variety of

methane-oxidizing bacteria, e.g., *M. capsulatus* Bath, *M. album* BG8, and *M. trichlsporium* OB3b, were able to utilize O_2 released from the disproportion of ClO_2^- by dissmilatory perchlorate-reducing bacteria.[34] Sun et al. reported that,[39] in an AMO-D process, co-existing methanotrophs consumed O_2 preferentially, creating a micro-aerobic environment conducive for denitrification. In addition, the methanotrophs released organic intermediates that served as electron donors for denitrification.[15, 22, 30] We name this potential mechanism "micro-Aerobic Methane Oxidation coupled to Perchlorate Reduction," or "mAMO-PR". It is illustrated in Figure 5-B.

In summary, we found that the biofilm in an MBfR was able to reduce up to 5 mg/L of ClO_4^- to non-detectable levels using CH_4 as the only electron donor and carbon source in the presence of NO_3^- at a surface loading of $\leq$ 0.32 g N/m^2-d. While NO_3^- at high surface loadings (e.g., 0.78 g N/m^2-d) inhibited ClO_4^- reduction due to electron-donor competition, NO_2^- inhibited ClO_4^- reduction at low surface loadings (e.g., 0.1 g N/m^2-d), probably due to toxicity of NO_2^- to the ClO_4^--reducing cells. Bacteria were much more abundant than Archaea in the biofilm, and *pMMO* gene copies correlated to the increase of respiratory gene copies, while *mcrA* did not; thus, the CH_4-oxidizing biofilm likely respired ClO_4^- by a pathway that involved generating O_2 using ClO_2^- dismutation, with the O_2 utilized as a co-substrate for the mono-oxygenation of CH_4. Two options are possible: (1) ANMO-PR via a single strain producing and utilizing intracellular O_2, and (2) mAMO-PR, in which ClO_4^--reducing bacteria produce extracellular O_2 by ClO_2^- dismutation, while methanotrophs uses O_2 as a co-substrate

to initiate oxidation of CH_4. This study shows that it is feasible to use methane as an electron donor to biologically remove perchlorate, which is a new option for perchlorate reduction and a new application for the MBfR. Should further study demonstrate that methane is a versatile electron donor, like hydrogen, for reducing oxidized contaminants in water and wastewater treatment, then methane could be used as an inexpensive electron donor.

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Supporting Information Available

Table S1-5 and Figure S1-3. This material is available free of charge via the Internet at http://pubs.asc.org.

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