Stable-isotopic and metagenomic analyses reveal metabolic and microbial link of aerobic methane oxidation coupled to denitrification at different O_2 levels

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Abstract: Aerobic methane (CH_4) oxidation coupled to denitrification (AME-D) can not only mitigate CH_4 emission into the atmosphere, but also potentially alleviate nitrogen pollution in surface waters and engineered ecosystems, and it has attracted substantial research interest. O_2 concentration plays a key role in AME-D, yet little is understood about how it impacts microbial interactions. Here, we applied isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ and metagenomic analyses to investigate the metabolic and microbial link of AME-D at different O_2 levels. Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% and a CH_4 concentration of 8% (i.e., the O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the AME-D system incubated at the O_2 concentration of 10%. Methanol and acetate may serve as the trophic linkage between aerobic methanotrophs and denitrifers in the AME-D systems. Methylotrophs including *Methylophilus*, *Methylovorus*, *Methyloversatilis* and *Methylotenera* were abundant under the O_2-sufficient condition with the O_2 concentration of 21%, while denitrifiers such as *Azoarcus*, *Thauera* and *Thiobacillus* dominated in the O_2-limited environment with the O_2 concentration of 10%. The competition of denitrifiers and methylotrophs in the AME-D system for CH_4-derived carbon, such as methanol and acetate, might be influenced by chemotactic responses. More methane-derived carbon flowed into methylotrophs under the O_2-sufficient condition, while more methane-derived carbon was used for denitrification in the O_2-limited environment. These findings can aid in evaluating the distribution and contribution of AME-D and in developing strategies for mitigating CH_4 emission and nitrogen pollution in natural and engineered ecosystems.

Keywords: Aerobic CH_4 oxidation coupled to denitrification; aerobic methanotrophs; denitrifers; O_2 concentration; metagenomic analyses

1. Introduction

Methane (CH_4) is a globally important greenhouse gas due to its global warming potential being approximately 28 times of CO_2 (IPCC, 2014). In the past 300 years, the average CH_4 concentration in the atmosphere increase at an average rate of 0.6%-0.8% per year (Chistoserdova et al., 2005). Microbial CH_4 oxidation is critical to reduce CH_4 emissions and can be achieved through a variety of physiological processes, including aerobic CH_4 oxidation coupled to denitrification (AME-D) (Modin et al., 2007; Sun et al., 2013; Kits et al., 2015). Since AME-D can not only mitigate CH_4 emission into the atmosphere, but also potentially alleviate nitrogen pollution in surface waters and engineered ecosystems such as wastewater treatment plants and landfills, it has attracted substantial research interest (Modin et al., 2010; Zhu et al., 2016).

The AME-D process is mainly considered that the release of organic compounds from aerobic CH_4 oxidation can provide electron donors for coexisting denitrifiers and indirectly participate in denitrification (Modin et al, 2007). AME-D process is mainly performed by two microbial guilds working in concert: aerobic methanotrophs and denitrifiers. Aerobic methanotrophs are generally members of the *Proteobacteria* and can be divided into two groups: type I (belonging to the family *Methylococcaceae* in γ-*Proteobacteria*) and type II methanotrophs (including the genera *Methylocella*, *Methylosinus*, *Methylocystis*, *Methylocapsa* and *Methyloferula*, which belong to α-*Proteobacteria*), based on cell morphology, phylogeny, ultra-structure, and metabolic pathways (Vorobev et al., 2011; Bodelier et al., 2013; Deutzmann et al., 2014). Denitrifiers are widely distributed phylogenetically and most are facultative anaerobes using ionic and gaseous nitrogen oxides as electron accepters in absence of O_2 (Zumft, 1997). Aerobic denitrifiers, such as *Hyphomicrobium* and *Methylotenera* have been reported to utilize both O_2 and NO_3^-/NO_2^- as their terminal electron acceptors

(Meschner and Hamer, 1985; Kalyuhznaya et al., 2009). Aerobic denitrifiers usually preferentially utilize O_2 over NO_3^-/NO_2^- as electron acceptors under aerobic condition (Ji et al., 2015). However, some aerobic denitrifiers such as *Pseudomonas stutzeri* TR2 can reduce NO_3^-/NO_2^- to produce N_2 and a very low level of N_2O at a DO concentration of 1.25 mg l^{-1} (Miyahara et al., 2010). Some aerobic methanotrophs also have been reported to encode partial denitrification pathways and can simultaneously reduce NO_3^- /NO_2^- to N_2O via NO_3^-, NO_2^-, and NO reductases during CH_4 oxidation (Nyerges et al., 2010; Campbell et al., 2011; Stein and Klotz, 2011). Additionally, methanotrophs can also contribute to denitrification by nitrogen assimilation (Amaral et al., 1995; Bishoff et al., 2021).

Many factors can affect the activity of aerobic methanotrophs and denitrifiers in the AME-D process such as temperature, CH_4 and O_2 concentrations, O_2/CH_4 ratio and nutrients (Semrau et al., 2010; Zhu et al., 2016). Among them, the availability of O_2 as a substrate for aerobic CH_4 oxidation is a key factor influencing the AME-D process. An O_2 concentration of 5% has been observed to be enough to sustain the activity of methanotrophs and increasing O_2 concentrations do not influence CH_4 oxidation of landfill cover soil (Wang et al., 2011). As the O_2 concentration is less than the saturated value, an increased O_2 level can lead to increased CH_4 oxidation, and thus may result in more organic compounds released for denitrifiers and improvement of NO_3^- removal (Sun et al., 2013). When CH_4 is supplied in stoichiometric excess and methanotrophs are sufficiently abundant, they can consume nearly all of the supplied O_2. High aerobic CH_4 oxidation mainly occurs in the hypoxic zone in the presence of a high CH_4 concentration in natural systems such as lakes and wetlands, due to the opposite direction of CH_4 and O_2 diffusion gradients (Lopes et al., 2011; He et al., 2012). O_2 is also a known inhibitor for most denitrifiers. Additionally, O_2 has been reported to be an

inhibitor for the chemotaxis of some denitrifiers such as *Rhodobacter sphaeroides* 2.4.1, R*hodopseudomonas palustri*, *and Pseudomonas fluorescens* F113 towards NO_3^--N/NO_2^--N (Lee et al., 2002; Muriel et al., 2015). However, O_2 is a requirement for the AME-D process (Sun et al., 2013; Modin et al., 2018). If O_2 is supplied in stoichiometric excess, the dissolved oxygen (DO) concentration increases and denitrification is inhibited (Thalasso et al., 1997; Modin et al., 2007). Although NO_3^- removal has been reported to increase at higher O_2 partial pressures due to the rapid growth of microorganisms such as methanotrophs for assimilating NO_3^--N into biomass (Bishoff et al., 2021), a high NO_3^- removal attributed to denitrification is usually observed at lower O_2 partial pressures (Thalasso et al., 1997). Thus, an optimal O_2 concentration is required to improve the performance of AME-D process.

O_2 concentrations can also influence the secretion of metabolites critical to AME-D by aerobic methanotrophs during CH_4 oxidation. Generally, CH_4-derived carbon is converted into CO_2 and biomass under O_2-sufficient conditions, while more CH_4-derived carbon can be secreted into the environment in O_2-limited conditions (Wei et al., 2015; He et al., 2020). Aerobic methanotrophs can assimilate CH_4 with a highly efficient pyrophosphate-mediated glycolytic pathway and produce organic compounds, such as formate, acetate and lactate under O_2-limited conditions (5%) (Kalyuzhnaya et al., 2013). In the AME-D process, the organic compounds secreted by aerobic methanotrophs can work as carbon sources not only for denitrifiers, but also for other heterotrophic microorganisms such as methylotrophs in the community (Stock et al., 2013; Oshkin et al., 2015). Competition for organic compounds might occur between denitrifiers and other heterotrophic bacteria, which may influence the NO_3^-/NO_2^- removal in the AME-D process. However, little information is available about microbial interactions among methanotrophs, denitrifiers, and other heterotrophic

bacteria in the AME-D process.

In this study, we applied isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ and metagenomics analyses to investigate the metabolic linkage between aerobic CH_4 oxidation and denitrification at different O_2 concentrations. We analyzed metabolites generated and also experimentally added exogenous carbon sources including methanol, citrate, acetate, formaldehyde and formate to identify putative CH_4-derived carbon sources for denitrifiers in the AME-D systems. With isotopic and metagenomic analyses, we identified the main microorganisms and functional genes predominantly involved in CH_4 oxidation, denitrification and chemotaxis in the AME-D systems at different O_2 levels.

2. Materials and methods

2.1. Experimental set-up and operation

The inoculum was prepared by mixing landfill cover soil, waste biocover soil, stabilized waste from a laboratory landfill reactor, and activated sludge (Chu et al., 2020) and transferred into 1000-ml bottles and incubated at a shaker without the addition of carbon and nitrogen sources for 7 days as described previously (Chu et al., 2020). After that, simulated NO_3^- wastewater prepared with the nitrate mineral salts (NMS) medium with the NO_3^--N concentration of about 30 mgN l^{-1} was added into the bottle, which was then sealed and flushed with high purity N_2 (99.999%) (Chu et al., 2020). The NMS medium composition was as follows (g l^{-1}): KNO_3, 0.216; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 0.2; FeEDTA, 0.0038; $FeSO_4 \cdot 7H_2O$, 0.0005; Na_2MoO_4, 0.00026; $CuSO_4 \cdot 5H_2O$, 0.0002; $ZnSO_4 \cdot 7H_2O$, 0.0004; EDTA disodium salt, 0.00025; H_3BO_3, 0.000015; $CoCl_2 \cdot 6H_2O$, 0.00005; $MnCl_2 \cdot 4H_2O$, 0.00002; $NiCl_2 \cdot 6H_2O$, 0.00001;

141 $Na_2HPO_4 \cdot 12H_2O$, 0.716; KH_2PO_4, 0.26. Four O_2 levels (i.e., 21%, 10%, 5%, 2.5%(v/v))
142 that represented the aerobic to anoxic environment were selected for operational
143 conditions. According to the inoculum quantity and activity, a CH_4 concentration of 8%
144 (v/v) was used. After a certain amount of gas was taken from the bottle, CH_4 and O_2
145 were injected into the bottles to establish a CH_4 concentration of 8% (v/v) and O_2
146 concentrations of 21%, 10%, 5%, 2.5%(v/v) with the O_2/CH_4 ratios of 2.62, 1.26, 0.63
147 and 0.31 in triplicate for each treatment, and identified hereafter as O_{21}, O_{10}, O_5 and
148 $O_{2.5}$, respectively. All bottles were incubated in a shaker at 130 rpm and 30 °C and
149 operated as a sequencing batch reactor with a hydraulic retention time of 64 h (Chu et
150 al., 2020). CH_4 and O_2 were replenished into the bottle to the initial concentrations at
151 each 24 h-cycle. The experiment lasted for 178 d. Granular sludge was formed at the
152 end of the experiment. At the end of the cycle, gas samples in the headspace of the
153 bottles and the effluent samples were collected periodically to analyze the
154 concentrations of CH_4 and NO_3^--N as described previously (He et al., 2017).
155
156 *2.2. Quantification of ^{13}C and ^{15}N in the AME-D systems*
157 In order to investigate the fate of CH_4-C and NO_3^--N in the AME-D systems,
158 isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ were applied and the amount of ^{13}C and ^{15}N in
159 the gas, solid (i.e., sludge) and liquid phases was quantified. Approximately 0.1 g (dry
160 weight) of granular sludge from the O_{21} and O_{10} systems was used for the test. The
161 sludge was transferred into 80-ml serum bottles with 24 replicates created per treatment
162 (O_{21} and O_{10}). In order to avoid the interference of other impurities in the liquid phase,
163 the sludge was washed with the NMS medium (without NO_3^--N) twice. Then, 20 ml

NMS medium with the $^{13}NO_3^-$-N concentration of 30 mgN l^{-1} (KNO_3, 99% of ^{15}N) was added to the serum bottles. After mixing, 1-ml water sample was withdrawn from each bottle to measure the initial NO_3^--N concentration. Then the serum bottles were flushed with high purity N_2 (99.999%) at a flow rate of 200 ml min^{-1} for 5 min, and then sealed with a butyl rubber stopper. After extracting a certain amount of gas from the headspace of the serum bottles, O_2 was injected to achieve the O_2 concentrations of 21% and 10% (v/v), respectively. $^{13}CH_4$ (99% of ^{13}C, Cambridge Isotope Laboratories, Inc., United States) was injected to a concentration of 8% (v/v). The serum bottles were incubated on a shaker at 30 °C and 130 rpm.

Three serum bottles were randomly selected to measure the concentrations of $^{13}CH_4$, $^{13}CO_2$, $^{15}N_2$ and $^{15}N_2O$ at periodic intervals within a 24-h cycle using a gas chromatograph-mass spectrometer (Agilent 7890B inert 5977A MSD, Agilent, United States) as described previously (Ettwig et al., 2009). The O_2 concentration was detected as described by Wang et al. (2011). The total amount of $^{15}N_2O$ and $^{13}CO_2$ (including in the headspace of the serum bottles and liquid) produced in the AME-D systems was calculated as described by He et al. (2016) and standard curves of N_2O and CO_2 concentrations in the headspace of bottles with NMS medium and the same test conditions.

After detecting the concentrations of ^{13}C and ^{15}N compounds in the headspace, the three serum bottles were destructively harvested to collect the water and sludge samples. After centrifugation at 10,000 rpm for 5 min, an aliquot of the supernatant was used to detect the NO_3^--N concentrations. Another portion of supernatant was dried using a rotary evaporator (Eyela N-1200AS-W, Tokyo Kikakikai Co., Ltd., Japan) at 60 °C, and dissolved with acetone and then freeze-dried to detect the concentrations of ^{13}C and ^{15}N in the water samples. The remaining precipitate and sludge sample was mixed and

freeze-dried. After grinding and sieving through 0.15 mm-mesh, approximately 1 g of the frozen dried sample was used to detect the contents of ^{13}C and ^{15}N with an Elementar Vario MICRO cube elemental analyzer coupled to the GV Isoprime 100 isotope ratio mass spectrometer (GV Instruments, UK) as described previously (Zong et al., 2018).

2.3. Analysis of intermediate metabolites of AME-D systems

Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems was added into the 80-ml serum bottles and incubated on a shaker as described above in section 2.2. Three serum bottles were randomly sampled for water analyses. After passing through a 0.45 μm filter, the water sample was added with barium chloride to precipitate sulfate, and then was centrifuged at 8000 rpm for 2 min. The supernatant was sequentially filtered through a 0.22 μm filter and an IC-Ag column, and then was analyzed for organic metabolites generated from aerobic CH_4 oxidation using a thermoelectric AQUION ion chromatograph equipped with a AS11-HC protection column (4 μm × 5 cm) and a AS11-HC analysis column (4 μm × 25 cm). KOH was used as the eluent at the flow rate of 1ml min^{-1}. The gradient run of the eluent was as follows: 0-5 min, 5 mM; 5-20 min, 5-30 mM; 20-23 min, 30 mM; 23-31 min, 5 mM. Detection was performed using a suppressed conductivity detector. The column temperature was 30°C.

2.4. Batch test of CH_4 oxidation and denitrification kinetics

(1) CH_4 oxidation

Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems was added into 80-ml serum bottles containing 20 ml NMS medium with the NO_3^--N concentration of 30 mgN l^{-1} (the determined NO_3^--N concentration of 29.1-30.5 mgN l^-

214 ^{1} in the bottles), and then sealed with butyl rubber stoppers (prepared as described above
215 in section 2.2). After extracting a certain amount of gas from the headspace of the serum
216 bottles, O_2 and CH_4 were injected to obtain O_2 concentrations of 21% and 10% (v/v),
217 and CH_4 concentrations of 1%, 2%, 4%, 8%, 10%, 15%, 20% and 30% (v/v).

218 (2) Denitrification

219 Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems
220 was added into 80-ml serum bottles as described above and washed with NMS medium
221 with the NO_3^--N concentrations of 3, 8, 10, 15, 30, 40, 50 and 80 mgN l^{-1}, respectively,
222 twice to avoid the interference of other impurities in the liquid phase. Then, 20 ml NMS
223 with the NO_3^--N concentrations of 3, 8, 10, 15, 30, 40, 50 or 80 mgN l^{-1} was added the
224 serum bottles containing granular sludge samples previously washed with the medium
225 having corresponding NO_3^--N concentrations. The serum bottles were sealed and
226 injected with O_2 and CH_4 to obtain the O_2 concentrations of 21% or 10% and the CH_4
227 concentration of 8%.

228 Sterilized sludge was used as control for each treatment. All treatments were
229 performed in triplicate. The serum bottles were incubated at 30 °C and 130 rpm for 0.5-
230 3 h. Gas and liquid samples were withdrawn to detect the concentrations of CH_4 and
231 NO_3^--N at the beginning and the end of incubation. The CH_4 oxidation activity and
232 NO_3^--N removal rate was calculated by a zero-order decrease (Wang et al., 2011) and
233 expressed as milligram per gram volatile solid per hour (mg gVS^{-1} h^{-1}). The differences
234 in the CH_4 oxidation activity and NO_3^--N removal rate of the sludge samples from the
235 O_{21} and O_{10} systems were analyzed by ANOVA (one-way analysis of variance) using
236 SPSS 19.0 software.

237

238 *2.5. Exogenous carbon sources test*

239 Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems
240 was added into 80-ml serum bottles containing 20 ml NMS medium with the NO_3^--N
241 concentration of 30 mgN l^{-1} and a series of concentrations between 10 and 500 mg l^{-1}
242 of sodium acetate, methanol, sodium citrate, sodium formate and formaldehyde in
243 triplicate and incubation as described above in section 2.2. These metabolite
244 concentrations were selected based on the hypotheses that the conversion rate of
245 methane-to-methanol was 64% (Duan et al., 2011). Liquid samples were withdrawn to
246 detect the NO_3^--N concentration at the beginning and the end of incubation, and then
247 the NO_3^--N removal rate was calculated.
248
249 *2.6. Metagenomic analyses*
250 Sludge samples were taken from the O_{21} and O_{10} systems at the end of the
251 experiment. Genomic DNA was extracted from the samples using E.Z.N.A.TM Soil
252 DNA Kit (Omega Bio-Tek, Inc., Norcross, USA), verified using gel electrophoresis,
253 and quantified using a Qubit Fluorometer (Thermo, USA). Shotgun metagenomic
254 analysis was conducted by BGI (Shenzhen, China). Specifically, after fragmentation,
255 paired-end fragment library with the insert size of 350 bp was constructed. Adaptor-
256 appended fragments were sequenced on Illumina HiSeq 4000 platform. Reads were
257 excluded from further analysis if they had 10% or more ambiguous bases, contained 15
258 bp or more overlapping regions with adapter sequences, had 40% or more low quality
259 (Q<20) bases.
260 Qualified sequencing data that produced by Illumina platform were preprocessed
261 and then assembled *de novo* with SOAP denovo2 (Luo et al., 2012) and Rabbit (You et
262 al., 2013). MetaGeneMark (version 2.10, default parameters) was used to predict open
263 reading frames based on assembly results (Zhu et al., 2010; Guo et al., 2016). Genes

from different samples were combined together and clustered using CD-HIT. CD-HIT (version 4.6.1) was used to remove redundant sequences (sequence identity threshold 95% and alignment coverage threshold 90%) and to determine gene abundance and statistics among the samples (Guo et al., 2016).

The gene catalogs were blasted against public databases including nr, Swiss-Prot, COG, KEGG, GO and eggNOG with an e-value cutoff of 10^{-5} (Guo et al., 2016). Reads mapping to multiple genes were then reassigned to a gene using Pathoscope v1.0 (Francis et al., 2013), which used a Bayesian framework to examine each read's sequence and mapping quality within the context of a global reassignment. The gene abundance of each sample was calculated. Taxonomic classification of nr BLAST output files was assigned to NCBI taxonomies with MEGAN (version 5.3) by using the lowest common ancestor algorithm. After that, we summed the relative abundance of each taxonomy level from the same taxonomy. Significance analysis of gene abundance was performed as described by Audic and Claverie (1997).

The taxonomic affiliation of the genes was estimated the correlation of their abundances between the O_{21} and O_{10} systems with ccrepe (version 1.7.0). The species network of the top 300 correlation coefficient was visualized by Cytoscape 3.4.0 (Shannon et al., 2003). The phylogenies and relative abundance of the top 500 taxonomically assigned species were constructed using GraphlAn (Asnicar et al., 2015). The difference in the gene abundance of nitrogen metabolism and CH_4 oxidation between the two groups was analyzed by ANOVA (one-way analysis of variance) using SPSS 19.0 software.

The metagenomic data sets were deposited in NCBI Short Read Archive under the accession number of SAMN08813215.

3. Results

3.1. Nitrogen removal in the AME-D systems

Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% with the CH_4 concentration of 8% (i.e., O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the O_{10} system, reaching about 100% after day 169, followed by the O_{21} system, while the O_5 and $O_{2.5}$ systems had the lowest NO_3^--N removal (Fig. 1a). The CH_4 removal efficiency increased with the increasing O_2 concentration and the highest was observed in the O_{21} system (Fig. 1b). From day 76, the CH_4 removal efficiency in the O_{21} system reached 100%, while it was kept stable at 12%-70% in the other systems. At the end of experiment, the sludge formed granules, likely due to the secretion of extracellular polymeric substances, microbial self-immobilization, or the growth of filamentous microorganisms. Compared with the other sludge samples, the sludge in the O_{10} system was slightly black at the end of the experiment (Fig.1c).

3.2. Fate of CH_4-C and NO_3^--N

Based on the NO_3^--N removal in the AME-D systems, we selected the granular sludge samples in the O_{21} and O_{10} systems to further characterize the AME-D process. The fate of CH_4-C and NO_3^--N in the AME-D systems was investigated by applying isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$. After CH_4 was completely consumed at 13 h, the NO_3^--N concentration decreased slowly over time in the O_{21} system (Fig. 2). $^{15}NO_3^-$-N was mainly converted into $^{15}N_2O$-N and sludge-^{15}N (mainly biomass-N) in the O_{21} system, accounting for 50.3% and 35.8% of the $^{15}NO_3^-$-N loss, respectively. In the O_{10} system, after O_2 was used up at 13 h, NO_3^--N was still removed quickly between 13 and 18 h. $^{15}NO_3^-$-N was mainly converted into $^{15}N_2$-N, $^{15}N_2O$-N and sludge-^{15}N, accounting for 57.1%, 25.3% and 5.2% of the $^{15}NO_3^-$-N loss, respectively, in the O_{10} system. After

314 a 24-h cycle, the NO_3^--N removal efficiency was 45.2% in the O_{21} system, while it
315 reached 88.3% in the O_{10} system.

316 As CH_4 was consumed, the amount of CO_2-C and sludge-C (mainly biomass-C)
317 increased in the AME-D systems. Compared with the O_{10} system, more CO_2-C and
318 sludge-C were produced in the O_{21} system. Moreover, the conversion of $^{13}CH_4$-derived
319 carbon to $^{13}CO_2$-C and sludge-^{13}C was also higher in the O_{21} system, accounting for
320 70.8% and 22.6%, respectively, while they were 48.7% and 9.3%, respectively, in the
321 O_{10} system.

322

323 *3.3. Kinetics of CH_4 oxidation and denitrification*

324 When the CH_4 concentration was below 8%, the CH_4 oxidation rate increased with
325 the increasing CH_4 concentration and no significant difference was observed in the CH_4
326 oxidation rate at the two experimental O_2 concentrations (P=0.136-0.783) (Fig. 3a). The
327 CH_4 oxidation rate reached 60.1 mg gVS^{-1} h^{-1} at the CH_4 concentration of 15% and then
328 kept stable at higher concentrations in the O_{21} system. However, when the CH_4
329 concentration was above 8%, the CH_4 oxidation rate did not change significantly and
330 fluctuated around 36.1 mg gVS^{-1} h^{-1} in the O_{10} system. These data suggested that the
331 CH_4 concentration was a limiting factor for the CH_4 oxidation rate at concentrations
332 below 15% CH_4 for the O_{21} system and below about 8% for the O_{10} system. Above
333 these concentrations, the CH_4 oxidation rate did not increase, indicating saturation.
334 When the CH_4 concentration ranged from 1% to 30%, the kinetics of CH_4 oxidation of
335 the AME-D systems fit the Michaelis–Menten model well (R^2=0.932-0.974, P<0.001).

336 When the NO_3^--N concentration was less than 40 mgN l^{-1}, the NO_3^--N removal rate
337 increased with the influent NO_3^--N concentration (Fig. 3b). Compared with the O_{21}
338 system, the NO_3^--N removal rate was higher in the O_{10} system. When the NO_3^--N

concentration was between 40 and 80 mgN l^{-1}, the NO_3^--N removal rate in both systems showed no obvious variation. When the NO_3^--N concentration was 3-80 mgN l^{-1}, the Michaelis–Menten model fit the NO_3^--N removal rate in the AME-D systems well (R^2=0.892-0.975, P<0.001). Compared with the O_{21} system, the maximum NO_3^--N removal rate was higher in the O_{10} system, which was about 3.7 times of the former. And, the affinity of denitrifiers for NO_3^--N was higher with the K_m value of 6.9 mgN l^{-1} in the O_{10} system relative to the O_{21} system.

3.4. Trophic linkage in AME-D

In this study, we investigated organic compounds that may serve as trophic linkages between methanotrophs and denitrifiers in the AME-D systems. Ion chromatography analysis showed that acetate and oxalate were the only organic acids detected in the AME-D systems (Fig. 4a and Fig.4b). The highest oxalate concentration was determined at 7-13 h with 311.2 and 611.0 ng l^{-1} in the O_{10} and O_{21} systems, respectively. Compared with oxalate, the concentration of acetate was lower, with the highest concentration of 3.5 ng l^{-1} in the O_{10} system at 13 h and 5.6 ng l^{-1} in the O_{21} system at 7 h, respectively. At 3, 18 and 23 h, acetate was undetectable in the two systems with the detection limit of ~0.4 ng l^{-1}.

In order to examine the possible carbon sources for denitrification in the AME-D systems, exogenous carbon sources including methanol, sodium acetate, sodium citrate, sodium formate, sodium oxalate and formaldehyde were added to the AME-D systems. Little or no NO_3^--N removal was observed with the addition of sodium citrate, sodium oxalate and sodium formate as carbon sources (Fig. S1). When the methanol concentration was below 25 mg l^{-1}, almost no NO_3^--N was removed in the O_{21} system (Fig. 4d). An obvious increase in the NO_3^--N removal rate occurred in the O_{21} system

364 at the methanol concentration of 30 mg l^{-1}. The NO_3^--N removal rate rapidly increased
365 to 2.71 mgN gVS^{-1} h^{-1} when the methanol concentration was 200 mg l^{-1}, and then kept
366 steady at the methanol concentration between 300 and 500 mg l^{-1}. However, in the O_{10}
367 system, the NO_3^--N removal rate increased with the increasing methanol concentration,
368 and reached 12.13 mgN gVS^{-1} h^{-1} at the methanol concentration of 500 mg l^{-1}, which
369 was about four times of that at the acetate concentration of 500 mg l^{-1} (Fig. 4e and Fig.
370 4f).

371 In the O_{21} system, when the concentration of sodium acetate increased to 20 mg l^{-1}
372, the NO_3^--N removal rate increased rapidly to 1.46 mgN gVS^{-1} h^{-1} and then remained
373 steady (Fig. 4c). In the O_{10} system, when the concentration of sodium acetate increased
374 to 150 mg l^{-1}, the NO_3^--N removal rate increased rapidly to 3.21 mgN gVS^{-1} h^{-1} and
375 then kept steady (Fig. 4e). Compared with methanol, the addition of acetate had lower
376 NO_3^--N removal in the O_{10} system. There was no significant difference in the NO_3^--N
377 removal between acetate and methanol treatments in the O_{21} system at the O_2
378 concentration of ～0. However, the NO_3^- removal rate increased quickly to the
379 maximum with the acetate addition of 20 mg l^{-1}, while there was a lag phase (little or
380 no NO_3^--N removal) with the methanol addition in the O_{21} system. When the O_2
381 concentration was decreased to 10% and ～0, no lag phase was observed in the O_{21}
382 system. Of the two exogenous carbon sources, the K_m value of Michaelis-Menten model
383 was lower for acetate treatments at the O_2 concentrations of 21% and 10%, while it was
384 lower for methanol treatments at the O_2 concentration of ～0 (Table S1).
385

386 *3.5. Taxonomic profiling of the metagenomes*

Average contig lengths were 1674 and 1973 bp for the metagenomic assemblies from the O_{21} and O_{10} systems, respectively (Table S2). Taxonomic assignment for the gene catalogue using nr annotation and Megan showed that there was no significant difference in the total gene number and the annotated gene number in the two experimental treatments (P=0.667-0.852).

Proteobacteria, Chloroflexi, Bacteroidetes, Planctomycetes, Firmicutes, Verrucomicrobia and Cyanobacteria predominated in the two AME-D systems, accounting for 56.4% and 58.5% of the metagenomic reads in the O_{21} and O_{10} systems, respectively (Fig. 5a). Compared with the O_{10} system, higher microbial diversity was observed in the O_{21} system (Figure S2). In the top 30 genera of the two AME-D systems, methanotrophs, methylotrophs and denitrifiers accounted for about 50% (Fig. S3). Among the top 30 genera, 18 genera had significant difference in the relative abundance between the O_{21} and O_{10} systems (Fig. 5b). Methanotrophs including *Methylocaldum*, *Methylomicrobium*, *Methylomonas*, *Methylobacter*, *Methylococcus* had similar relative abundance in the two treatments, but *Methylosarcina* and *Methylocystis* were more abundant in the O_{21} system than in the O_{10} system. Methylotrophs including *Methylophilus*, *Methylovorus, Methyloversatilis* and *Methylotenera* were abundant in the O_{21} system with the relative abundance of 1.7%, 0.6%, 0.3% and 0.5%, respectively. The relative abundance of *Methylophilus*, *Methylovorus* and *Methylotenera* was significantly lower in the O_{10} system than in the O_{21} system, while the relative abundance of *Methyloversatilis* was significantly higher in the former. Denitrifiers including *Azoarcus*, *Pseudomonas*, *Thauera* and *Thiobacillus* dominated in the two treatments. Among them, *Azoarcus*, *Thauera* and *Thiobacillus* were more abundant in the O_{10} system than in the O_{21} system.

The top 300 species associations in the two AME-D systems showed that many

412 microorganisms had significantly higher relative abundance in the O_{21} system,
413 including methanotrophs *Methylosarcina fibrate*, *Methylocystis* sp. SB2, *Methylocystis*
414 *rosea*, *Methylocystis* sp. SC2, *Methylocystis parvus*, *Methylosinus trichosporium* and
415 methylotrophs *Methylophilus methylotrophus*, *Hyphomicrobium* sp. MC1,
416 *Hyphomicrobium* sp. 802, *Bacillus mycoides*, *Methylibium petroleiphilum*, *Thiobacillus*
417 *prosperus* (Fig. 6). Moreover, complicated associations occurred between aerobic
418 methanotrophs and non-methanotrophs in the O_{21} system. In the O_{10} system, fewer
419 microorganisms had obviously higher relative abundance than in the O_{21} system and the
420 associated species mainly belonged to methylotrophs and denitrifiers.
421
422 *3.6. Differences in gene abundances of CH_4 oxidation, denitrification and chemotaxis*
423 CH_4 is first converted to methanol by methane monooxygenase (MMO) during
424 CH_4 oxidation, which has two forms, soluble MMO (sMMO) and particulate MMO
425 (pMMO). The genes of *pmo*A and *mmo*X encode key subunits of pMMO and sMMO,
426 respectively. Compared with *mmo*X, *pmo*A was more abundant in the AME-D system
427 accounting for 0.0131%-0.0135% of the total genes. The relative abundance of *mmo*X
428 in the O_{21} system was 0.00084%, which was two orders of magnitude higher than in
429 the O_{10} system, while the relative abundance of *pmo*A was similar in the two AME-D
430 systems (Fig. 7a and Fig. 7c). The gene *mxa*F encoding key subunit of methanol
431 dehydrogenase was more abundant in the O_{21} system. In the Kyoto Encyclopedia of
432 Genes and Genomes (KEGG) pathway of CH_4 oxidation, there are three pathways
433 involved in formaldehyde oxidation with the first stage catalyzed by enzymes, i.e.,
434 glutathione-independent formaldehyde dehydrogenase (*fdhA*), S-hydroxymethyl
435 glutathione synthase (*gfa*) and 5,6,7,8-tetrahydromethanopterin hydro-lyase (*fae*). *fae*
436 was the most abundant gene, accounting for 81.8% and 88.3% of the total abundance

437 of the three genes. *fae* was mainly associated with methanotrophs, accounting for 50.3%
438 in the O_{21} system, while it was less abundant in the O_{10} system (Table S3). In addition,
439 the gene encoding formate dehydrogenase (FDH) was also more abundant in the O_{21}
440 system.

441 NO_3^- can be removed by assimilatory NO_3^- reduction, dissimilatory NO_3^- reduction
442 and denitrification. The metagenomic data revealed that *nar*B and *nas*A associated with
443 assimilatory NO_3^- reduction and *nor*B and *nor*C denitrification genes were significantly
444 more abundant in the O_{21} system than in the O_{10} system (Fig. 7b and Fig. 7d). Genes
445 involved in dissimilatory NO_3^- reduction including *nar*G, *nar*J, *nap*A and *nap*B, and
446 *nir*S were significantly more abundant in the O_{10} system than in the O_{21} system. Some
447 methanotrophs have genes involved in nitrogen metabolism such as *haoAB*, *nirS*, *nirK*
448 and *norCB* (Stein and Klotz, 2011). In the two AME-D systems, some *nas*A, *nar*G,
449 *nar*H, *nar*I, *nar*J, *nir*B, *nir*D, *nir*K, *nor*B and *nor*C were affiliated with methanotrophs
450 (Table S4). Among them, *nar*G, *nar*H, *nar*I, *nar*J, *nir*B, *nir*K, *nor*B and *nor*C associated
451 with methanotrophs were all significantly more abundant in the O_{21} system.

452 Since the relative abundance of genes involved in bacterial chemotaxis appeared
453 in the top 10 list of genes with significantly different abundances in the two AME-D
454 systems (Fig. S4), the chemotactic genes and associated with microorganisms were
455 analyzed. Genes involved in chemotaxis including MCP, Aer, CheA, CheB, CheR and
456 CheV, CheX and FliG were significantly more abundant in the O_{10} system (Fig. 8a).
457 Methylotrophs and denitrifiers were the main two type microorganisms associated with
458 the chemotactic genes with the average relative abundance of 28.9-35.9% and some
459 even reaching the relative abundance of 81.9% in the two AME-D systems. Except for
460 CheV, the genes involved in bacterial chemotaxis that were associated with
461 methylotrophs were significantly more abundant in the O_{21} system than in the O_{10}

system (Fig. 8b). However, the genes involved in bacterial chemotaxis associated with denitrifiers were significantly more abundant in the O_{10} system (Fig. 8c).

Based on these data, we hypothesized proposed schemes for the AME-D process at different O_2 levels (Fig. 8d). In this study, methanol and acetate might serve as the trophic linkage between aerobic methanotrophs and denitrifiers. Under the O_2-sufficient condition (the O_2 concentration of 21% and O_2/CH_4 ratio of 2.62 with the O_2 concentration kept at above 8% within the cycle), methylotrophs could outcompete denitrifiers for CH_4-derived carbon, such as methanol and acetate, thus resulting in the low NO_3^- removal in the AME-D process. The competition of denitrifiers and methylotrophs for CH_4-derived carbon could be influenced by chemotaxis. Under the O_2-sufficient condition, methylotrophs had a competitive advantage in obtaining CH_4-derived carbon, while they might not outcompete denitrifiers in the O_2-limited environment (the O_2 concentration of 10% and O_2/CH_4 ratio of 1.25 with the O_2 concentration lower than the detection limit for half a cycle). Additionally, methanotrophs might participate in incomplete denitrification and produce N_2O.

4. Discussion

The data presented in this study suggested that O_2 level played an important role in regulating the AME-D process. Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% at a CH_4 concentration of 8% (i.e., O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the O_{10} system, followed by the O_{21} system, while the O_5 and $O_{2.5}$ systems had the lowest NO_3^--N removal. This indicated that the nitrate removal in the AME-D system highly depended on the carbon sources generated by CH_4 oxidation that correlated well with O_2 concentrations and O_2/CH_4

ratios. Similarly, Sun et al. (2013) reported that an apparently improved NO_3^- removal rate when the O_2/CH_4 ratio ranged from 1.0 to 1.5, due to the suitable O_2 levels for the biomass mixture, by which sufficient organic substrate could be generated from CH_4 oxidation for denitrification in the AME-D system.

The relative abundance of the genes involved in bacterial chemotaxis (Bonnie et al., 2006; Bi and Sourjik, 2018) differed significantly between the experimental AME-D systems with the O_2 concentration of 21% and the O_2/CH_4 ratio of 2.62 (where the O_2 concentrations were kept at above 8% within the cycle, referred to as "the O_2-sufficient condition") and the O_2 concentration of 10% and the O_2/CH_4 ratio of 1.25 (where the O_2 concentrations were lower the detection limit for half a cycle, referred to as "the O_2-limited condition") (Fig. S4). The genes associated with methylotrophs were significantly more abundant under the O_2-sufficient condition, while the genes associated with denitrifiers were significantly more abundant in the O_2-limited environment. This might be attributed to the difference in aerotaxis between methylotrophs (most are strongly positively aerotactic) and denitrifiers (usually are attracted by low O_2 concentration (Grishanin and Bibikov, 1997), which resulted in the flow of methane-derived carbon into different heterotrophic species. In the experimental AME-D systems, more methane-derived carbon flowed into methylotrophs under the O_2-sufficient condition, while more methane-derived carbon was used for denitrification in the O_2-limited environment (Fig. S3).

Organic metabolites generated from aerobic CH_4 oxidation including methanol, citrate, acetate, formaldehyde and formate have been reported to be the possible carbon sources for coexisting denitrifiers in the AME-D process (Zhu et al., 2016). In this study, acetate and oxalate were detectable in the AME-D systems. Between 13 and 23 h into a cycle, the oxalate concentration was high in the experimental AME-D systems (Fig.

4b), yet little NO_3^- was removed (Fig. 2a). Additionally, NO_3^- removal was negligible in the AME-D systems with the addition of exogenous oxalate. This demonstrated that oxalate was unlikely the direct trophic linkage between aerobic methanotrophs and denitrifiers in the AME-D systems. The highest concentration of acetate was observed at 7 h under the O_2-sufficient condition when CH_4 was rapidly consumed, while it occurred at 13 h in the O_2-limited environment when O_2 was consumed (Fig. 4a). This might be attributed to the highly efficient pyrophosphate-mediated glycolytic pathway of aerobic methanotrophs under the O_2-limited condition, which converted less CO_2 but produced more organic compounds, such as formate, acetate and lactate in the environment (Kalyuzhnaya et al., 2013). After O_2 was depleted, acetate was quickly consumed and not detected in the O_2-limited environment at 18 h. Meanwhile, a high NO_3^--N removal occurred in the O_2-limited environments. It suggested that acetate was an important trophic linkage between aerobic methanotrophs and denitrifiers in the AME-D system under the O_2-limited condition.

Although methanol was not detectable in the AME-D systems (the detection limit was about 200 ng l^{-1} in this study), the addition of exogenous methanol enhanced the denitrification capacity in the AME-D systems. This suggested that methanol might work as the trophic linkage between aerobic methanotrophs and denitrifiers in the two AME-D systems. Among the possible carbon sources, methanol is considered as a critical intermediate for the cooperative AME-D process performed by the microbial consortium (Zhu et al., 2016). In this study, the wastewater was simulated with NMS medium including phosphate and ethylenediaminetetraacetic acid ferric sodium salt. Aerobic methanotrophs could excrete methanol to adjust their metabolic pathways for survival under specific environments such as phosphate, NaCl and sodium formate (Mehta et al., 1987; Duan et al., 2011; Hur et al., 2017). There was a lag phase in the

536 NO_3^- removal efficiency in the O_{21} system at the O_2 concentration of 21% with the
537 addition of methanol, while it was absent when the O_2 concentration decreased to 10%
538 and ～0. This might be attributed to two causes: 1) some bacteria such as
539 methylotrophs competed with denitrifiers for methanol under the O_2-sufficient
540 condition (Veillette et al., 2011); 2) some denitrifiers could use both NO_3^- and O_2 as
541 electron acceptors and preferentially utilize O_2 over NO_3^- at high O_2 concentrations (Ji
542 et al., 2015).

543 Methylotrophs were observed to dominate in various methanotrophic
544 environments such as lake sediments and landfill cover soils (Hernandez et al., 2015;
545 He et al., 2020). Among the two AME-D systems, a higher relative abundance of
546 methylotrophs was observed in the O_{21} system. Compared with the O_{10} system,
547 *Methylophilus* were more abundant in the O_{21} system, which was in agreement with
548 previous studies (Oshkin et al., 2015; Hernandez et al., 2015). Oshkin et al. (2015)
549 observed that *Methylophilus* was more competitive at higher O_2 concentration of 150
550 mM and resembled with the organism was not capable of respiratory denitrification and
551 possessed MxaFI type methanol dehydrogenase, while *Methylotenera* dominated at low
552 O_2 concentration of 15 mM, which could potentially denitrify and utilized a more
553 efficient variant of the ribulose monophosphate pathway, likely due to a lack of an
554 MxaFI methanol dehydrogenase (Beck et al., 2014). The occurrence of specifc
555 Methylophilaceae types was considered as oxygen-dependent (Hernandez et al., 2015).
556 Methylotrophs such as Methylophilaceae also have been found to dominate in a
557 methanol-denitrifying bioreactor (Osaka et al., 2006). Compared with the O_{21} system,
558 *Methyloversatilis* was significantly more abundant in the O_{10} system. It was plausible
559 that *Methyloversatilis* can utilize several C1 and multicarbon compounds (Kalyuzhnaya
560 et al., 2006). A similar result was observed by Baytshtok et al. (2008) who showed that

561 *Methyloversatilis* dominated in methanol and ethanol-fed denitrifying bioreactors. In
562 the top 30 genera, *Azoarcus*, *Thauera*, *Acidovorax* and *Dechloromonas* were
563 significantly more abundant in the O_{10} system and have also been reported to dominate
564 in methanol or acetate-fed denitrifying bioreactors (Hallin et al., 2006; Baytshtok et al.,
565 2008; Osaka et al., 2008). These findings further confirmed that methanol and acetate
566 might be the trophic linkage between aerobic CH_4 oxidation and denitrification in the
567 AME-D systems.

568 In addition to denitrifiers, some genes such as *nas*A, *nar*G, *nar*H, *nar*I, *nar*J, *nir*B,
569 *nir*D, *nir*K, *nor*B and *nor*C were associated with methanotrophs in the AME-D systems.
570 Many methanotrophs have been reported to encode partial denitrification pathways and
571 have the ability to reduce NO_3^- and/or NO_2^- to N_2O (Nyerges et al., 2010; Campbell et
572 al., 2011). The methanotrophs having *nar*G, *nar*H, *nar*I, *nar*J, *nir*B, *nir*K, *nor*B and
573 *nor*C were all significantly more abundant in the O_{21} system than in the O_{10} system.
574 This might be attributed to methanotrophs with denitrifying capacity outcompeting
575 other methanotrophs under aerobic conditions. Nyerges et al. (2010) also found that
576 methanotrophs with denitrifying capacity might outcompete other methanotrophs in
577 ecosystems with high nitrogen loading as they have the capacity to withstand NO_3^-
578 and/or NO_2^- stress and may even derive a growth benefit in the presence of both oxygen
579 and nitrite. However, it cannot be ruled out that the high O_2 concentration might result
580 in high numbers of methanotrophs with denitrifying capacity in the O_{21} system. Further
581 investigation is needed to understand the effects of oxygen and NO_3^- on the denitrifying
582 activity of methanotrophs.

583 NO_3^--N was mainly converted into N_2O-N and sludge-N (mainly biomass-N) in the
584 O_{21} system, while the majority was converted into N_2-N in the O_{10} system. Compared
585 to the O_2-limited condition, microorganisms grew more quickly under the O_2-sufficient

586 condition and more NO_3^--N was converted into biomass-N. Denitrification can proceed
587 under both aerobic and anaerobic conditions. In anaerobic respiratory NO_3^- reduction,
588 O_2 is toxic to anaerobic denitrifiers. However, most aerobic denitrifiers, such as
589 *Hyphomicrobium*, *Methylotenera* and *Pseudomonas stutzeri* TR2 can utilize both O_2
590 and NO_3^- or NO_2^- as their terminal electron acceptors (Meschner and Hamer, 1985;
591 Kalyuhznaya et al., 2009; Ji et al., 2015). Under aerobic condition, O_2 can inhibit N_2O
592 reductase and result in the production of N_2O, an important greenhouse gas with a
593 global warming potential of 298 times of CO_2, rather than N_2 (Thomson et al., 2012).
594 Under the O_2-limited condition, aerobic denitrifiers might utilize NO_3^- or NO_2^- as their
595 terminal electron acceptors and convert the majority of NO_3^- into N_2. Additionally,
596 aerobic methanotrophs have the capacity for denitrification, but they only reduce NO_3^-
597 and/or NO_2^- to N_2O (Nyerges et al., 2010; Campbell et al., 2011). Thus, an optimal O_2
598 concentrations is needed to control the AME-D process to mitigate nitrogen pollution
599 and the emission of greenhouse gases such as CH_4 and N_2O.
600
601 **5. Conclusion**
602 O_2 level was an important factor influencing the performance of AME-D process
603 and the microbial communities and metabolic pathways involved. Acetate and
604 methanol could serve as the key compounds trophically linking aerobic methanotrophs
605 to methylotrophs and denitrifiers in the AME-D process. The chemotactic abilities of
606 the bacterial community might influence the trophic link of aerobic methanotrophs to
607 methylotrophs and denitrifiers in the AME-D systems at different O_2 levels. Under the
608 O_2-sufficient condition, methylotrophs dominated the assimilation of CH_4-derived
609 carbon, while more methane-derived carbon was used for denitrification in O_2-limited
610 environments. These findings may help in future efforts to evaluate the distribution and

contribution of AME-D and develop strategies for mitigating CH_4 emission and nitrogen pollution in natural and engineered ecosystems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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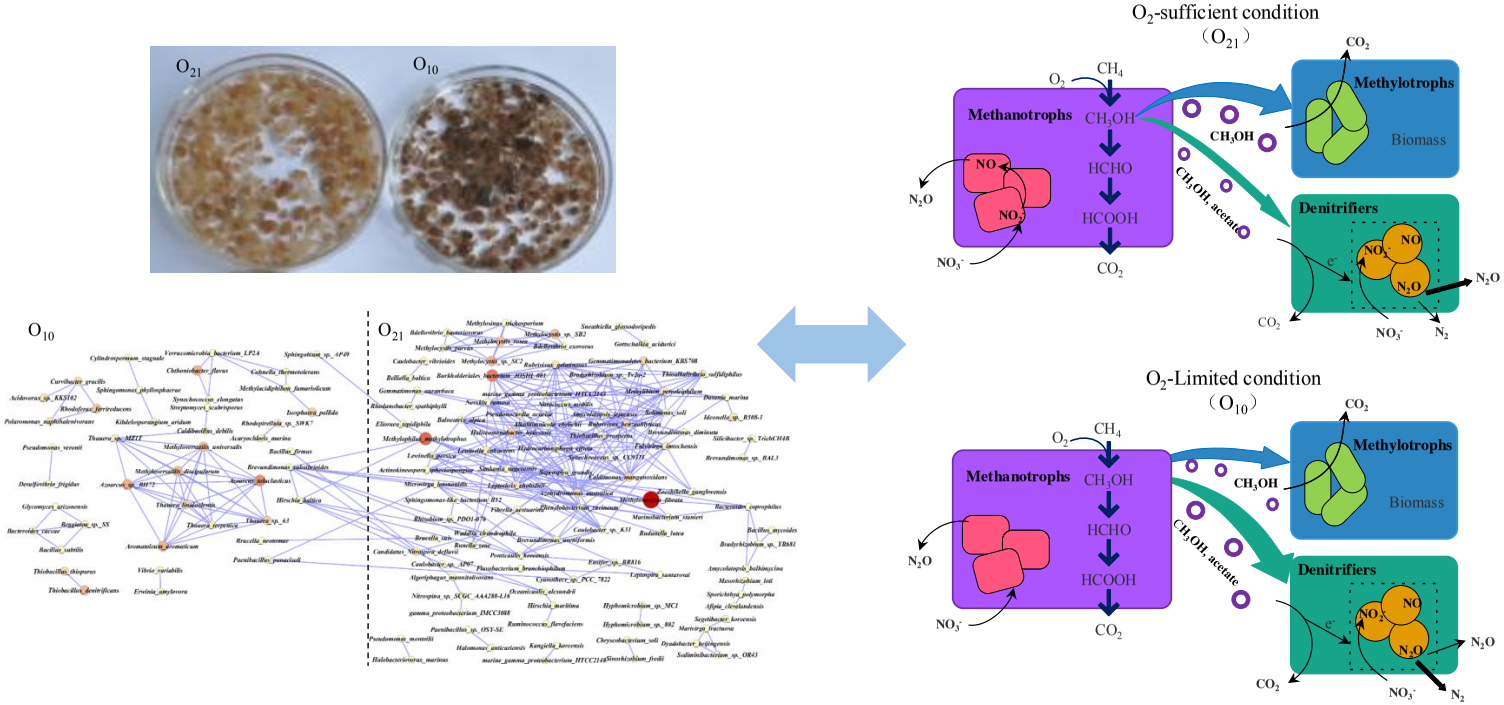
*Graphical Abstract

Graphical abstract



*Highlights (for review : 3 to 5 bullet points (maximum 85 characters including spaces per bullet point)

Highlights

- Methanol and acetate serve as trophic linkage between denitrifiers and methanotrophs in AME-D
- O_2 concentrations influence the microbial community and contribution of AME-D
- Methylotrophs and denitrifiers dominate in O_2-sufficient and O_2-limited environment, respectively
- Bacterial chemotaxis affects the competition of denitrifiers and methylotrophs in AME-D

Stable-isotopic and metagenomic analyses reveal metabolic and microbial link of aerobic methane oxidation coupled to denitrification at different O_2 levels

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Abstract: Aerobic methane (CH_4) oxidation coupled to denitrification (AME-D) can not only mitigate CH_4 emission into the atmosphere, but also potentially alleviate nitrogen pollution in surface waters and engineered ecosystems, and it has attracted substantial research interest. O_2 concentration plays a key role in AME-D, yet little is understood about how it impacts microbial interactions. Here, we applied isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ and metagenomic analyses to investigate the metabolic and microbial link of AME-D at different O_2 levels. Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% and a CH_4 concentration of 8% (i.e., the O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the AME-D system incubated at the O_2 concentration of 10%. Methanol and acetate may serve as the trophic linkage between aerobic methanotrophs and denitrifers in the AME-D systems. Methylotrophs including *Methylophilus*, *Methylovorus*, *Methyloversatilis* and *Methylotenera* were abundant under the O_2-sufficient condition with the O_2 concentration of 21%, while denitrifiers such as *Azoarcus*, *Thauera* and *Thiobacillus* dominated in the O_2-limited environment with the O_2 concentration of 10%. The competition of denitrifiers and methylotrophs in the AME-D system for CH_4-derived carbon, such as methanol and acetate, might be influenced by chemotactic responses. More methane-derived carbon flowed into methylotrophs under the O_2-sufficient condition, while more methane-derived carbon was used for denitrification in the O_2-limited environment. These findings can aid in evaluating the distribution and contribution of AME-D and in developing strategies for mitigating CH_4 emission and nitrogen pollution in natural and engineered ecosystems.

Keywords: Aerobic CH_4 oxidation coupled to denitrification; aerobic methanotrophs; denitrifers; O_2 concentration; metagenomic analyses

1. Introduction

Methane (CH_4) is a globally important greenhouse gas due to its global warming potential being approximately 28 times of CO_2 (IPCC, 2014). In the past 300 years, the average CH_4 concentration in the atmosphere increase at an average rate of 0.6%-0.8% per year (Chistoserdova et al., 2005). Microbial CH_4 oxidation is critical to reduce CH_4 emissions and can be achieved through a variety of physiological processes, including aerobic CH_4 oxidation coupled to denitrification (AME-D) (Modin et al., 2007; Sun et al., 2013; Kits et al., 2015). Since AME-D can not only mitigate CH_4 emission into the atmosphere, but also potentially alleviate nitrogen pollution in surface waters and engineered ecosystems such as wastewater treatment plants and landfills, it has attracted substantial research interest (Modin et al., 2010; Zhu et al., 2016).

The AME-D process is mainly considered that the release of organic compounds from aerobic CH_4 oxidation can provide electron donors for coexisting denitrifiers and indirectly participate in denitrification (Modin et al, 2007). AME-D process is mainly performed by two microbial guilds working in concert: aerobic methanotrophs and denitrifiers. Aerobic methanotrophs are generally members of the *Proteobacteria* and can be divided into two groups: type I (belonging to the family *Methylococcaceae* in γ-*Proteobacteria*) and type II methanotrophs (including the genera *Methylocella*, *Methylosinus*, *Methylocystis*, *Methylocapsa* and *Methyloferula*, which belong to α-*Proteobacteria*), based on cell morphology, phylogeny, ultra-structure, and metabolic pathways (Vorobev et al., 2011; Bodelier et al., 2013; Deutzmann et al., 2014). Denitrifiers are widely distributed phylogenetically and most are facultative anaerobes using ionic and gaseous nitrogen oxides as electron accepters in absence of O_2 (Zumft, 1997). Aerobic denitrifiers, such as *Hyphomicrobium* and *Methylotenera* have been reported to utilize both O_2 and NO_3^-/NO_2^- as their terminal electron acceptors

(Meschner and Hamer, 1985; Kalyuhznaya et al., 2009). Aerobic denitrifiers usually preferentially utilize O_2 over NO_3^-/NO_2^- as electron acceptors under aerobic condition (Ji et al., 2015). However, some aerobic denitrifiers such as *Pseudomonas stutzeri* TR2 can reduce NO_3^-/NO_2^- to produce N_2 and a very low level of N_2O at a DO concentration of 1.25 mg l^{-1} (Miyahara et al., 2010). Some aerobic methanotrophs also have been reported to encode partial denitrification pathways and can simultaneously reduce NO_3^- /NO_2^- to N_2O via NO_3^-, NO_2^-, and NO reductases during CH_4 oxidation (Nyerges et al., 2010; Campbell et al., 2011; Stein and Klotz, 2011). Additionally, methanotrophs can also contribute to denitrification by nitrogen assimilation (Amaral et al., 1995; Bishoff et al., 2021).

Many factors can affect the activity of aerobic methanotrophs and denitrifiers in the AME-D process such as temperature, CH_4 and O_2 concentrations, O_2/CH_4 ratio and nutrients (Semrau et al., 2010; Zhu et al., 2016). Among them, the availability of O_2 as a substrate for aerobic CH_4 oxidation is a key factor influencing the AME-D process. An O_2 concentration of 5% has been observed to be enough to sustain the activity of methanotrophs and increasing O_2 concentrations do not influence CH_4 oxidation of landfill cover soil (Wang et al., 2011). As the O_2 concentration is less than the saturated value, an increased O_2 level can lead to increased CH_4 oxidation, and thus may result in more organic compounds released for denitrifiers and improvement of NO_3^- removal (Sun et al., 2013). When CH_4 is supplied in stoichiometric excess and methanotrophs are sufficiently abundant, they can consume nearly all of the supplied O_2. High aerobic CH_4 oxidation mainly occurs in the hypoxic zone in the presence of a high CH_4 concentration in natural systems such as lakes and wetlands, due to the opposite direction of CH_4 and O_2 diffusion gradients (Lopes et al., 2011; He et al., 2012). O_2 is also a known inhibitor for most denitrifiers. Additionally, O_2 has been reported to be an

inhibitor for the chemotaxis of some denitrifiers such as *Rhodobacter sphaeroides* 2.4.1, R*hodopseudomonas palustri*, *and Pseudomonas fluorescens* F113 towards NO_3^--N/NO_2^--N (Lee et al., 2002; Muriel et al., 2015). However, O_2 is a requirement for the AME-D process (Sun et al., 2013; Modin et al., 2018). If O_2 is supplied in stoichiometric excess, the dissolved oxygen (DO) concentration increases and denitrification is inhibited (Thalasso et al., 1997; Modin et al., 2007). Although NO_3^- removal has been reported to increase at higher O_2 partial pressures due to the rapid growth of microorganisms such as methanotrophs for assimilating NO_3^--N into biomass (Bishoff et al., 2021), a high NO_3^- removal attributed to denitrification is usually observed at lower O_2 partial pressures (Thalasso et al., 1997). Thus, an optimal O_2 concentration is required to improve the performance of AME-D process.

O_2 concentrations can also influence the secretion of metabolites critical to AME-D by aerobic methanotrophs during CH_4 oxidation. Generally, CH_4-derived carbon is converted into CO_2 and biomass under O_2-sufficient conditions, while more CH_4-derived carbon can be secreted into the environment in O_2-limited conditions (Wei et al., 2015; He et al., 2020). Aerobic methanotrophs can assimilate CH_4 with a highly efficient pyrophosphate-mediated glycolytic pathway and produce organic compounds, such as formate, acetate and lactate under O_2-limited conditions (5%) (Kalyuzhnaya et al., 2013). In the AME-D process, the organic compounds secreted by aerobic methanotrophs can work as carbon sources not only for denitrifiers, but also for other heterotrophic microorganisms such as methylotrophs in the community (Stock et al., 2013; Oshkin et al., 2015). Competition for organic compounds might occur between denitrifiers and other heterotrophic bacteria, which may influence the NO_3^-/NO_2^- removal in the AME-D process. However, little information is available about microbial interactions among methanotrophs, denitrifiers, and other heterotrophic

bacteria in the AME-D process.

In this study, we applied isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ and metagenomics analyses to investigate the metabolic linkage between aerobic CH_4 oxidation and denitrification at different O_2 concentrations. We analyzed metabolites generated and also experimentally added exogenous carbon sources including methanol, citrate, acetate, formaldehyde and formate to identify putative CH_4-derived carbon sources for denitrifiers in the AME-D systems. With isotopic and metagenomic analyses, we identified the main microorganisms and functional genes predominantly involved in CH_4 oxidation, denitrification and chemotaxis in the AME-D systems at different O_2 levels.

2. Materials and methods

2.1. Experimental set-up and operation

The inoculum was prepared by mixing landfill cover soil, waste biocover soil, stabilized waste from a laboratory landfill reactor, and activated sludge (Chu et al., 2020) and transferred into 1000-ml bottles and incubated at a shaker without the addition of carbon and nitrogen sources for 7 days as described previously (Chu et al., 2020). After that, simulated NO_3^- wastewater prepared with the nitrate mineral salts (NMS) medium with the NO_3^--N concentration of about 30 mgN l^{-1} was added into the bottle, which was then sealed and flushed with high purity N_2 (99.999%) (Chu et al., 2020). The NMS medium composition was as follows (g l^{-1}): KNO_3, 0.216; $MgSO_4 \cdot 7H_2O$, 1; $CaCl_2 \cdot 2H_2O$, 0.2; FeEDTA, 0.0038; $FeSO_4 \cdot 7H_2O$, 0.0005; Na_2MoO_4, 0.00026; $CuSO_4 \cdot 5H_2O$, 0.0002; $ZnSO_4 \cdot 7H_2O$, 0.0004; EDTA disodium salt, 0.00025; H_3BO_3, 0.000015; $CoCl_2 \cdot 6H_2O$, 0.00005; $MnCl_2 \cdot 4H_2O$, 0.00002; $NiCl_2 \cdot 6H_2O$, 0.00001;

141 $Na_2HPO_4 \cdot 12H_2O$, 0.716; KH_2PO_4, 0.26. Four O_2 levels (i.e., 21%, 10%, 5%, 2.5%(v/v))
142 that represented the aerobic to anoxic environment were selected for operational
143 conditions. According to the inoculum quantity and activity, a CH_4 concentration of 8%
144 (v/v) was used. After a certain amount of gas was taken from the bottle, CH_4 and O_2
145 were injected into the bottles to establish a CH_4 concentration of 8% (v/v) and O_2
146 concentrations of 21%, 10%, 5%, 2.5%(v/v) with the O_2/CH_4 ratios of 2.62, 1.26, 0.63
147 and 0.31 in triplicate for each treatment, and identified hereafter as O_{21}, O_{10}, O_5 and
148 $O_{2.5}$, respectively. All bottles were incubated in a shaker at 130 rpm and 30 °C and
149 operated as a sequencing batch reactor with a hydraulic retention time of 64 h (Chu et
150 al., 2020). CH_4 and O_2 were replenished into the bottle to the initial concentrations at
151 each 24 h-cycle. The experiment lasted for 178 d. Granular sludge was formed at the
152 end of the experiment. At the end of the cycle, gas samples in the headspace of the
153 bottles and the effluent samples were collected periodically to analyze the
154 concentrations of CH_4 and NO_3^--N as described previously (He et al., 2017).
155
156 *2.2. Quantification of ^{13}C and ^{15}N in the AME-D systems*
157 In order to investigate the fate of CH_4-C and NO_3^--N in the AME-D systems,
158 isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$ were applied and the amount of ^{13}C and ^{15}N in
159 the gas, solid (i.e., sludge) and liquid phases was quantified. Approximately 0.1 g (dry
160 weight) of granular sludge from the O_{21} and O_{10} systems was used for the test. The
161 sludge was transferred into 80-ml serum bottles with 24 replicates created per treatment
162 (O_{21} and O_{10}). In order to avoid the interference of other impurities in the liquid phase,
163 the sludge was washed with the NMS medium (without NO_3^--N) twice. Then, 20 ml

NMS medium with the $^{13}NO_3^-$-N concentration of 30 mgN l^{-1} (KNO_3, 99% of ^{15}N) was added to the serum bottles. After mixing, 1-ml water sample was withdrawn from each bottle to measure the initial NO_3^--N concentration. Then the serum bottles were flushed with high purity N_2 (99.999%) at a flow rate of 200 ml min^{-1} for 5 min, and then sealed with a butyl rubber stopper. After extracting a certain amount of gas from the headspace of the serum bottles, O_2 was injected to achieve the O_2 concentrations of 21% and 10% (v/v), respectively. $^{13}CH_4$ (99% of ^{13}C, Cambridge Isotope Laboratories, Inc., United States) was injected to a concentration of 8% (v/v). The serum bottles were incubated on a shaker at 30 °C and 130 rpm.

Three serum bottles were randomly selected to measure the concentrations of $^{13}CH_4$, $^{13}CO_2$, $^{15}N_2$ and $^{15}N_2O$ at periodic intervals within a 24-h cycle using a gas chromatograph-mass spectrometer (Agilent 7890B inert 5977A MSD, Agilent, United States) as described previously (Ettwig et al., 2009). The O_2 concentration was detected as described by Wang et al. (2011). The total amount of $^{15}N_2O$ and $^{13}CO_2$ (including in the headspace of the serum bottles and liquid) produced in the AME-D systems was calculated as described by He et al. (2016) and standard curves of N_2O and CO_2 concentrations in the headspace of bottles with NMS medium and the same test conditions.

After detecting the concentrations of ^{13}C and ^{15}N compounds in the headspace, the three serum bottles were destructively harvested to collect the water and sludge samples. After centrifugation at 10,000 rpm for 5 min, an aliquot of the supernatant was used to detect the NO_3^--N concentrations. Another portion of supernatant was dried using a rotary evaporator (Eyela N-1200AS-W, Tokyo Kikakikai Co., Ltd., Japan) at 60 °C, and dissolved with acetone and then freeze-dried to detect the concentrations of ^{13}C and ^{15}N in the water samples. The remaining precipitate and sludge sample was mixed and

freeze-dried. After grinding and sieving through 0.15 mm-mesh, approximately 1 g of the frozen dried sample was used to detect the contents of ^{13}C and ^{15}N with an Elementar Vario MICRO cube elemental analyzer coupled to the GV Isoprime 100 isotope ratio mass spectrometer (GV Instruments, UK) as described previously (Zong et al., 2018).

2.3. Analysis of intermediate metabolites of AME-D systems

Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems was added into the 80-ml serum bottles and incubated on a shaker as described above in section 2.2. Three serum bottles were randomly sampled for water analyses. After passing through a 0.45 μm filter, the water sample was added with barium chloride to precipitate sulfate, and then was centrifuged at 8000 rpm for 2 min. The supernatant was sequentially filtered through a 0.22 μm filter and an IC-Ag column, and then was analyzed for organic metabolites generated from aerobic CH_4 oxidation using a thermoelectric AQUION ion chromatograph equipped with a AS11-HC protection column (4 μm × 5 cm) and a AS11-HC analysis column (4 μm × 25 cm). KOH was used as the eluent at the flow rate of 1ml min^{-1}. The gradient run of the eluent was as follows: 0-5 min, 5 mM; 5-20 min, 5-30 mM; 20-23 min, 30 mM; 23-31 min, 5 mM. Detection was performed using a suppressed conductivity detector. The column temperature was 30°C.

2.4. Batch test of CH_4 oxidation and denitrification kinetics

(1) CH_4 oxidation

Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems was added into 80-ml serum bottles containing 20 ml NMS medium with the NO_3^--N concentration of 30 mgN l^{-1} (the determined NO_3^--N concentration of 29.1-30.5 mgN l^-

214 ^{1} in the bottles), and then sealed with butyl rubber stoppers (prepared as described above
215 in section 2.2). After extracting a certain amount of gas from the headspace of the serum
216 bottles, O_2 and CH_4 were injected to obtain O_2 concentrations of 21% and 10% (v/v),
217 and CH_4 concentrations of 1%, 2%, 4%, 8%, 10%, 15%, 20% and 30% (v/v).

218 (2) Denitrification

219 Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems
220 was added into 80-ml serum bottles as described above and washed with NMS medium
221 with the NO_3^--N concentrations of 3, 8, 10, 15, 30, 40, 50 and 80 mgN l^{-1}, respectively,
222 twice to avoid the interference of other impurities in the liquid phase. Then, 20 ml NMS
223 with the NO_3^--N concentrations of 3, 8, 10, 15, 30, 40, 50 or 80 mgN l^{-1} was added the
224 serum bottles containing granular sludge samples previously washed with the medium
225 having corresponding NO_3^--N concentrations. The serum bottles were sealed and
226 injected with O_2 and CH_4 to obtain the O_2 concentrations of 21% or 10% and the CH_4
227 concentration of 8%.

228 Sterilized sludge was used as control for each treatment. All treatments were
229 performed in triplicate. The serum bottles were incubated at 30 °C and 130 rpm for 0.5-
230 3 h. Gas and liquid samples were withdrawn to detect the concentrations of CH_4 and
231 NO_3^--N at the beginning and the end of incubation. The CH_4 oxidation activity and
232 NO_3^--N removal rate was calculated by a zero-order decrease (Wang et al., 2011) and
233 expressed as milligram per gram volatile solid per hour (mg gVS^{-1} h^{-1}). The differences
234 in the CH_4 oxidation activity and NO_3^--N removal rate of the sludge samples from the
235 O_{21} and O_{10} systems were analyzed by ANOVA (one-way analysis of variance) using
236 SPSS 19.0 software.

237

238 *2.5. Exogenous carbon sources test*

239 Approximately 0.1 g (dry weight) of granular sludge from the O_{21} and O_{10} systems
240 was added into 80-ml serum bottles containing 20 ml NMS medium with the NO_3^--N
241 concentration of 30 mgN l^{-1} and a series of concentrations between 10 and 500 mg l^{-1}
242 of sodium acetate, methanol, sodium citrate, sodium formate and formaldehyde in
243 triplicate and incubation as described above in section 2.2. These metabolite
244 concentrations were selected based on the hypotheses that the conversion rate of
245 methane-to-methanol was 64% (Duan et al., 2011). Liquid samples were withdrawn to
246 detect the NO_3^--N concentration at the beginning and the end of incubation, and then
247 the NO_3^--N removal rate was calculated.
248
249 *2.6. Metagenomic analyses*
250 Sludge samples were taken from the O_{21} and O_{10} systems at the end of the
251 experiment. Genomic DNA was extracted from the samples using E.Z.N.A.TM Soil
252 DNA Kit (Omega Bio-Tek, Inc., Norcross, USA), verified using gel electrophoresis,
253 and quantified using a Qubit Fluorometer (Thermo, USA). Shotgun metagenomic
254 analysis was conducted by BGI (Shenzhen, China). Specifically, after fragmentation,
255 paired-end fragment library with the insert size of 350 bp was constructed. Adaptor-
256 appended fragments were sequenced on Illumina HiSeq 4000 platform. Reads were
257 excluded from further analysis if they had 10% or more ambiguous bases, contained 15
258 bp or more overlapping regions with adapter sequences, had 40% or more low quality
259 (Q<20) bases.
260 Qualified sequencing data that produced by Illumina platform were preprocessed
261 and then assembled *de novo* with SOAP denovo2 (Luo et al., 2012) and Rabbit (You et
262 al., 2013). MetaGeneMark (version 2.10, default parameters) was used to predict open
263 reading frames based on assembly results (Zhu et al., 2010; Guo et al., 2016). Genes

from different samples were combined together and clustered using CD-HIT. CD-HIT (version 4.6.1) was used to remove redundant sequences (sequence identity threshold 95% and alignment coverage threshold 90%) and to determine gene abundance and statistics among the samples (Guo et al., 2016).

The gene catalogs were blasted against public databases including nr, Swiss-Prot, COG, KEGG, GO and eggNOG with an e-value cutoff of 10^{-5} (Guo et al., 2016). Reads mapping to multiple genes were then reassigned to a gene using Pathoscope v1.0 (Francis et al., 2013), which used a Bayesian framework to examine each read's sequence and mapping quality within the context of a global reassignment. The gene abundance of each sample was calculated. Taxonomic classification of nr BLAST output files was assigned to NCBI taxonomies with MEGAN (version 5.3) by using the lowest common ancestor algorithm. After that, we summed the relative abundance of each taxonomy level from the same taxonomy. Significance analysis of gene abundance was performed as described by Audic and Claverie (1997).

The taxonomic affiliation of the genes was estimated the correlation of their abundances between the O_{21} and O_{10} systems with ccrepe (version 1.7.0). The species network of the top 300 correlation coefficient was visualized by Cytoscape 3.4.0 (Shannon et al., 2003). The phylogenies and relative abundance of the top 500 taxonomically assigned species were constructed using GraphlAn (Asnicar et al., 2015). The difference in the gene abundance of nitrogen metabolism and CH_4 oxidation between the two groups was analyzed by ANOVA (one-way analysis of variance) using SPSS 19.0 software.

The metagenomic data sets were deposited in NCBI Short Read Archive under the accession number of SAMN08813215.

3. Results

3.1. Nitrogen removal in the AME-D systems

Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% with the CH_4 concentration of 8% (i.e., O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the O_{10} system, reaching about 100% after day 169, followed by the O_{21} system, while the O_5 and $O_{2.5}$ systems had the lowest NO_3^--N removal (Fig. 1a). The CH_4 removal efficiency increased with the increasing O_2 concentration and the highest was observed in the O_{21} system (Fig. 1b). From day 76, the CH_4 removal efficiency in the O_{21} system reached 100%, while it was kept stable at 12%-70% in the other systems. At the end of experiment, the sludge formed granules, likely due to the secretion of extracellular polymeric substances, microbial self-immobilization, or the growth of filamentous microorganisms. Compared with the other sludge samples, the sludge in the O_{10} system was slightly black at the end of the experiment (Fig.1c).

3.2. Fate of CH_4-C and NO_3^--N

Based on the NO_3^--N removal in the AME-D systems, we selected the granular sludge samples in the O_{21} and O_{10} systems to further characterize the AME-D process. The fate of CH_4-C and NO_3^--N in the AME-D systems was investigated by applying isotopically labeled $K^{15}NO_3$ and $^{13}CH_4$. After CH_4 was completely consumed at 13 h, the NO_3^--N concentration decreased slowly over time in the O_{21} system (Fig. 2). $^{15}NO_3^-$-N was mainly converted into $^{15}N_2O$-N and sludge-^{15}N (mainly biomass-N) in the O_{21} system, accounting for 50.3% and 35.8% of the $^{15}NO_3^-$-N loss, respectively. In the O_{10} system, after O_2 was used up at 13 h, NO_3^--N was still removed quickly between 13 and 18 h. $^{15}NO_3^-$-N was mainly converted into $^{15}N_2$-N, $^{15}N_2O$-N and sludge-^{15}N, accounting for 57.1%, 25.3% and 5.2% of the $^{15}NO_3^-$-N loss, respectively, in the O_{10} system. After

314 a 24-h cycle, the NO_3^--N removal efficiency was 45.2% in the O_{21} system, while it
315 reached 88.3% in the O_{10} system.

316 As CH_4 was consumed, the amount of CO_2-C and sludge-C (mainly biomass-C)
317 increased in the AME-D systems. Compared with the O_{10} system, more CO_2-C and
318 sludge-C were produced in the O_{21} system. Moreover, the conversion of $^{13}CH_4$-derived
319 carbon to $^{13}CO_2$-C and sludge-^{13}C was also higher in the O_{21} system, accounting for
320 70.8% and 22.6%, respectively, while they were 48.7% and 9.3%, respectively, in the
321 O_{10} system.

322

323 *3.3. Kinetics of CH_4 oxidation and denitrification*

324 When the CH_4 concentration was below 8%, the CH_4 oxidation rate increased with
325 the increasing CH_4 concentration and no significant difference was observed in the CH_4
326 oxidation rate at the two experimental O_2 concentrations (P=0.136-0.783) (Fig. 3a). The
327 CH_4 oxidation rate reached 60.1 mg gVS^{-1} h^{-1} at the CH_4 concentration of 15% and then
328 kept stable at higher concentrations in the O_{21} system. However, when the CH_4
329 concentration was above 8%, the CH_4 oxidation rate did not change significantly and
330 fluctuated around 36.1 mg gVS^{-1} h^{-1} in the O_{10} system. These data suggested that the
331 CH_4 concentration was a limiting factor for the CH_4 oxidation rate at concentrations
332 below 15% CH_4 for the O_{21} system and below about 8% for the O_{10} system. Above
333 these concentrations, the CH_4 oxidation rate did not increase, indicating saturation.
334 When the CH_4 concentration ranged from 1% to 30%, the kinetics of CH_4 oxidation of
335 the AME-D systems fit the Michaelis–Menten model well (R^2=0.932-0.974, P<0.001).

336 When the NO_3^--N concentration was less than 40 mgN l^{-1}, the NO_3^--N removal rate
337 increased with the influent NO_3^--N concentration (Fig. 3b). Compared with the O_{21}
338 system, the NO_3^--N removal rate was higher in the O_{10} system. When the NO_3^--N

concentration was between 40 and 80 mgN l^{-1}, the NO_3^--N removal rate in both systems showed no obvious variation. When the NO_3^--N concentration was 3-80 mgN l^{-1}, the Michaelis–Menten model fit the NO_3^--N removal rate in the AME-D systems well (R^2=0.892-0.975, P<0.001). Compared with the O_{21} system, the maximum NO_3^--N removal rate was higher in the O_{10} system, which was about 3.7 times of the former. And, the affinity of denitrifiers for NO_3^--N was higher with the K_m value of 6.9 mgN l^{-1} in the O_{10} system relative to the O_{21} system.

3.4. Trophic linkage in AME-D

In this study, we investigated organic compounds that may serve as trophic linkages between methanotrophs and denitrifiers in the AME-D systems. Ion chromatography analysis showed that acetate and oxalate were the only organic acids detected in the AME-D systems (Fig. 4a and Fig.4b). The highest oxalate concentration was determined at 7-13 h with 311.2 and 611.0 ng l^{-1} in the O_{10} and O_{21} systems, respectively. Compared with oxalate, the concentration of acetate was lower, with the highest concentration of 3.5 ng l^{-1} in the O_{10} system at 13 h and 5.6 ng l^{-1} in the O_{21} system at 7 h, respectively. At 3, 18 and 23 h, acetate was undetectable in the two systems with the detection limit of ~0.4 ng l^{-1}.

In order to examine the possible carbon sources for denitrification in the AME-D systems, exogenous carbon sources including methanol, sodium acetate, sodium citrate, sodium formate, sodium oxalate and formaldehyde were added to the AME-D systems. Little or no NO_3^--N removal was observed with the addition of sodium citrate, sodium oxalate and sodium formate as carbon sources (Fig. S1). When the methanol concentration was below 25 mg l^{-1}, almost no NO_3^--N was removed in the O_{21} system (Fig. 4d). An obvious increase in the NO_3^--N removal rate occurred in the O_{21} system

364 at the methanol concentration of 30 mg l^{-1}. The NO_3^--N removal rate rapidly increased
365 to 2.71 mgN gVS^{-1} h^{-1} when the methanol concentration was 200 mg l^{-1}, and then kept
366 steady at the methanol concentration between 300 and 500 mg l^{-1}. However, in the O_{10}
367 system, the NO_3^--N removal rate increased with the increasing methanol concentration,
368 and reached 12.13 mgN gVS^{-1} h^{-1} at the methanol concentration of 500 mg l^{-1}, which
369 was about four times of that at the acetate concentration of 500 mg l^{-1} (Fig. 4e and Fig.
370 4f).

371 In the O_{21} system, when the concentration of sodium acetate increased to 20 mg l^{-1}
372, the NO_3^--N removal rate increased rapidly to 1.46 mgN gVS^{-1} h^{-1} and then remained
373 steady (Fig. 4c). In the O_{10} system, when the concentration of sodium acetate increased
374 to 150 mg l^{-1}, the NO_3^--N removal rate increased rapidly to 3.21 mgN gVS^{-1} h^{-1} and
375 then kept steady (Fig. 4e). Compared with methanol, the addition of acetate had lower
376 NO_3^--N removal in the O_{10} system. There was no significant difference in the NO_3^--N
377 removal between acetate and methanol treatments in the O_{21} system at the O_2
378 concentration of ～0. However, the NO_3^- removal rate increased quickly to the
379 maximum with the acetate addition of 20 mg l^{-1}, while there was a lag phase (little or
380 no NO_3^--N removal) with the methanol addition in the O_{21} system. When the O_2
381 concentration was decreased to 10% and ～0, no lag phase was observed in the O_{21}
382 system. Of the two exogenous carbon sources, the K_m value of Michaelis-Menten model
383 was lower for acetate treatments at the O_2 concentrations of 21% and 10%, while it was
384 lower for methanol treatments at the O_2 concentration of ～0 (Table S1).
385

386 *3.5. Taxonomic profiling of the metagenomes*

Average contig lengths were 1674 and 1973 bp for the metagenomic assemblies from the O_{21} and O_{10} systems, respectively (Table S2). Taxonomic assignment for the gene catalogue using nr annotation and Megan showed that there was no significant difference in the total gene number and the annotated gene number in the two experimental treatments (P=0.667-0.852).

Proteobacteria, Chloroflexi, Bacteroidetes, Planctomycetes, Firmicutes, Verrucomicrobia and Cyanobacteria predominated in the two AME-D systems, accounting for 56.4% and 58.5% of the metagenomic reads in the O_{21} and O_{10} systems, respectively (Fig. 5a). Compared with the O_{10} system, higher microbial diversity was observed in the O_{21} system (Figure S2). In the top 30 genera of the two AME-D systems, methanotrophs, methylotrophs and denitrifiers accounted for about 50% (Fig. S3). Among the top 30 genera, 18 genera had significant difference in the relative abundance between the O_{21} and O_{10} systems (Fig. 5b). Methanotrophs including *Methylocaldum*, *Methylomicrobium*, *Methylomonas*, *Methylobacter*, *Methylococcus* had similar relative abundance in the two treatments, but *Methylosarcina* and *Methylocystis* were more abundant in the O_{21} system than in the O_{10} system. Methylotrophs including *Methylophilus*, *Methylovorus, Methyloversatilis* and *Methylotenera* were abundant in the O_{21} system with the relative abundance of 1.7%, 0.6%, 0.3% and 0.5%, respectively. The relative abundance of *Methylophilus*, *Methylovorus* and *Methylotenera* was significantly lower in the O_{10} system than in the O_{21} system, while the relative abundance of *Methyloversatilis* was significantly higher in the former. Denitrifiers including *Azoarcus*, *Pseudomonas*, *Thauera* and *Thiobacillus* dominated in the two treatments. Among them, *Azoarcus*, *Thauera* and *Thiobacillus* were more abundant in the O_{10} system than in the O_{21} system.

The top 300 species associations in the two AME-D systems showed that many

412 microorganisms had significantly higher relative abundance in the O_{21} system,
413 including methanotrophs *Methylosarcina fibrate*, *Methylocystis* sp. SB2, *Methylocystis*
414 *rosea*, *Methylocystis* sp. SC2, *Methylocystis parvus*, *Methylosinus trichosporium* and
415 methylotrophs *Methylophilus methylotrophus*, *Hyphomicrobium* sp. MC1,
416 *Hyphomicrobium* sp. 802, *Bacillus mycoides*, *Methylibium petroleiphilum*, *Thiobacillus*
417 *prosperus* (Fig. 6). Moreover, complicated associations occurred between aerobic
418 methanotrophs and non-methanotrophs in the O_{21} system. In the O_{10} system, fewer
419 microorganisms had obviously higher relative abundance than in the O_{21} system and the
420 associated species mainly belonged to methylotrophs and denitrifiers.
421
422 *3.6. Differences in gene abundances of CH_4 oxidation, denitrification and chemotaxis*
423 CH_4 is first converted to methanol by methane monooxygenase (MMO) during
424 CH_4 oxidation, which has two forms, soluble MMO (sMMO) and particulate MMO
425 (pMMO). The genes of *pmo*A and *mmo*X encode key subunits of pMMO and sMMO,
426 respectively. Compared with *mmo*X, *pmo*A was more abundant in the AME-D system
427 accounting for 0.0131%-0.0135% of the total genes. The relative abundance of *mmo*X
428 in the O_{21} system was 0.00084%, which was two orders of magnitude higher than in
429 the O_{10} system, while the relative abundance of *pmo*A was similar in the two AME-D
430 systems (Fig. 7a and Fig. 7c). The gene *mxa*F encoding key subunit of methanol
431 dehydrogenase was more abundant in the O_{21} system. In the Kyoto Encyclopedia of
432 Genes and Genomes (KEGG) pathway of CH_4 oxidation, there are three pathways
433 involved in formaldehyde oxidation with the first stage catalyzed by enzymes, i.e.,
434 glutathione-independent formaldehyde dehydrogenase (*fdhA*), S-hydroxymethyl
435 glutathione synthase (*gfa*) and 5,6,7,8-tetrahydromethanopterin hydro-lyase (*fae*). *fae*
436 was the most abundant gene, accounting for 81.8% and 88.3% of the total abundance

437 of the three genes. *fae* was mainly associated with methanotrophs, accounting for 50.3%
438 in the O_{21} system, while it was less abundant in the O_{10} system (Table S3). In addition,
439 the gene encoding formate dehydrogenase (FDH) was also more abundant in the O_{21}
440 system.

441 NO_3^- can be removed by assimilatory NO_3^- reduction, dissimilatory NO_3^- reduction
442 and denitrification. The metagenomic data revealed that *nar*B and *nas*A associated with
443 assimilatory NO_3^- reduction and *nor*B and *nor*C denitrification genes were significantly
444 more abundant in the O_{21} system than in the O_{10} system (Fig. 7b and Fig. 7d). Genes
445 involved in dissimilatory NO_3^- reduction including *nar*G, *nar*J, *nap*A and *nap*B, and
446 *nir*S were significantly more abundant in the O_{10} system than in the O_{21} system. Some
447 methanotrophs have genes involved in nitrogen metabolism such as *haoAB*, *nirS*, *nirK*
448 and *norCB* (Stein and Klotz, 2011). In the two AME-D systems, some *nas*A, *nar*G,
449 *nar*H, *nar*I, *nar*J, *nir*B, *nir*D, *nir*K, *nor*B and *nor*C were affiliated with methanotrophs
450 (Table S4). Among them, *nar*G, *nar*H, *nar*I, *nar*J, *nir*B, *nir*K, *nor*B and *nor*C associated
451 with methanotrophs were all significantly more abundant in the O_{21} system.

452 Since the relative abundance of genes involved in bacterial chemotaxis appeared
453 in the top 10 list of genes with significantly different abundances in the two AME-D
454 systems (Fig. S4), the chemotactic genes and associated with microorganisms were
455 analyzed. Genes involved in chemotaxis including MCP, Aer, CheA, CheB, CheR and
456 CheV, CheX and FliG were significantly more abundant in the O_{10} system (Fig. 8a).
457 Methylotrophs and denitrifiers were the main two type microorganisms associated with
458 the chemotactic genes with the average relative abundance of 28.9-35.9% and some
459 even reaching the relative abundance of 81.9% in the two AME-D systems. Except for
460 CheV, the genes involved in bacterial chemotaxis that were associated with
461 methylotrophs were significantly more abundant in the O_{21} system than in the O_{10}

system (Fig. 8b). However, the genes involved in bacterial chemotaxis associated with denitrifiers were significantly more abundant in the O_{10} system (Fig. 8c).

Based on these data, we hypothesized proposed schemes for the AME-D process at different O_2 levels (Fig. 8d). In this study, methanol and acetate might serve as the trophic linkage between aerobic methanotrophs and denitrifiers. Under the O_2-sufficient condition (the O_2 concentration of 21% and O_2/CH_4 ratio of 2.62 with the O_2 concentration kept at above 8% within the cycle), methylotrophs could outcompete denitrifiers for CH_4-derived carbon, such as methanol and acetate, thus resulting in the low NO_3^- removal in the AME-D process. The competition of denitrifiers and methylotrophs for CH_4-derived carbon could be influenced by chemotaxis. Under the O_2-sufficient condition, methylotrophs had a competitive advantage in obtaining CH_4-derived carbon, while they might not outcompete denitrifiers in the O_2-limited environment (the O_2 concentration of 10% and O_2/CH_4 ratio of 1.25 with the O_2 concentration lower than the detection limit for half a cycle). Additionally, methanotrophs might participate in incomplete denitrification and produce N_2O.

4. Discussion

The data presented in this study suggested that O_2 level played an important role in regulating the AME-D process. Among the four experimental O_2 levels of 21%,10%, 5% and 2.5% at a CH_4 concentration of 8% (i.e., O_2/CH_4 ratios of 2.62, 1.26, 0.63 and 0.31), the highest NO_3^--N removal occurred in the O_{10} system, followed by the O_{21} system, while the O_5 and $O_{2.5}$ systems had the lowest NO_3^--N removal. This indicated that the nitrate removal in the AME-D system highly depended on the carbon sources generated by CH_4 oxidation that correlated well with O_2 concentrations and O_2/CH_4

ratios. Similarly, Sun et al. (2013) reported that an apparently improved NO_3^- removal rate when the O_2/CH_4 ratio ranged from 1.0 to 1.5, due to the suitable O_2 levels for the biomass mixture, by which sufficient organic substrate could be generated from CH_4 oxidation for denitrification in the AME-D system.

The relative abundance of the genes involved in bacterial chemotaxis (Bonnie et al., 2006; Bi and Sourjik, 2018) differed significantly between the experimental AME-D systems with the O_2 concentration of 21% and the O_2/CH_4 ratio of 2.62 (where the O_2 concentrations were kept at above 8% within the cycle, referred to as "the O_2-sufficient condition") and the O_2 concentration of 10% and the O_2/CH_4 ratio of 1.25 (where the O_2 concentrations were lower the detection limit for half a cycle, referred to as "the O_2-limited condition") (Fig. S4). The genes associated with methylotrophs were significantly more abundant under the O_2-sufficient condition, while the genes associated with denitrifiers were significantly more abundant in the O_2-limited environment. This might be attributed to the difference in aerotaxis between methylotrophs (most are strongly positively aerotactic) and denitrifiers (usually are attracted by low O_2 concentration (Grishanin and Bibikov, 1997), which resulted in the flow of methane-derived carbon into different heterotrophic species. In the experimental AME-D systems, more methane-derived carbon flowed into methylotrophs under the O_2-sufficient condition, while more methane-derived carbon was used for denitrification in the O_2-limited environment (Fig. S3).

Organic metabolites generated from aerobic CH_4 oxidation including methanol, citrate, acetate, formaldehyde and formate have been reported to be the possible carbon sources for coexisting denitrifiers in the AME-D process (Zhu et al., 2016). In this study, acetate and oxalate were detectable in the AME-D systems. Between 13 and 23 h into a cycle, the oxalate concentration was high in the experimental AME-D systems (Fig.

4b), yet little NO_3^- was removed (Fig. 2a). Additionally, NO_3^- removal was negligible in the AME-D systems with the addition of exogenous oxalate. This demonstrated that oxalate was unlikely the direct trophic linkage between aerobic methanotrophs and denitrifiers in the AME-D systems. The highest concentration of acetate was observed at 7 h under the O_2-sufficient condition when CH_4 was rapidly consumed, while it occurred at 13 h in the O_2-limited environment when O_2 was consumed (Fig. 4a). This might be attributed to the highly efficient pyrophosphate-mediated glycolytic pathway of aerobic methanotrophs under the O_2-limited condition, which converted less CO_2 but produced more organic compounds, such as formate, acetate and lactate in the environment (Kalyuzhnaya et al., 2013). After O_2 was depleted, acetate was quickly consumed and not detected in the O_2-limited environment at 18 h. Meanwhile, a high NO_3^--N removal occurred in the O_2-limited environments. It suggested that acetate was an important trophic linkage between aerobic methanotrophs and denitrifiers in the AME-D system under the O_2-limited condition.

Although methanol was not detectable in the AME-D systems (the detection limit was about 200 ng l^{-1} in this study), the addition of exogenous methanol enhanced the denitrification capacity in the AME-D systems. This suggested that methanol might work as the trophic linkage between aerobic methanotrophs and denitrifiers in the two AME-D systems. Among the possible carbon sources, methanol is considered as a critical intermediate for the cooperative AME-D process performed by the microbial consortium (Zhu et al., 2016). In this study, the wastewater was simulated with NMS medium including phosphate and ethylenediaminetetraacetic acid ferric sodium salt. Aerobic methanotrophs could excrete methanol to adjust their metabolic pathways for survival under specific environments such as phosphate, NaCl and sodium formate (Mehta et al., 1987; Duan et al., 2011; Hur et al., 2017). There was a lag phase in the

536 NO_3^- removal efficiency in the O_{21} system at the O_2 concentration of 21% with the
537 addition of methanol, while it was absent when the O_2 concentration decreased to 10%
538 and ～0. This might be attributed to two causes: 1) some bacteria such as
539 methylotrophs competed with denitrifiers for methanol under the O_2-sufficient
540 condition (Veillette et al., 2011); 2) some denitrifiers could use both NO_3^- and O_2 as
541 electron acceptors and preferentially utilize O_2 over NO_3^- at high O_2 concentrations (Ji
542 et al., 2015).

543 Methylotrophs were observed to dominate in various methanotrophic
544 environments such as lake sediments and landfill cover soils (Hernandez et al., 2015;
545 He et al., 2020). Among the two AME-D systems, a higher relative abundance of
546 methylotrophs was observed in the O_{21} system. Compared with the O_{10} system,
547 *Methylophilus* were more abundant in the O_{21} system, which was in agreement with
548 previous studies (Oshkin et al., 2015; Hernandez et al., 2015). Oshkin et al. (2015)
549 observed that *Methylophilus* was more competitive at higher dissolved O_2 concentration
550 of 150 mM and resembled the organism was not capable of respiratory denitrification
551 and possessed MxaFI type methanol dehydrogenase, while *Methylotenera* dominated
552 at low dissolved O_2 concentration of 15 mM, which could potentially denitrify and
553 utilized a more efficient variant of the ribulose monophosphate pathway, likely due to
554 a lack of an MxaFI methanol dehydrogenase (Beck et al., 2014). The occurrence of
555 specifc Methylophilaceae types was considered as oxygen-dependent (Hernandez et al.,
556 2015). Methylotrophs such as Methylophilaceae also have been found to dominate in a
557 methanol-denitrifying bioreactor (Osaka et al., 2006). Compared with the O_{21} system,
558 *Methyloversatilis* was significantly more abundant in the O_{10} system. It was plausible
559 that *Methyloversatilis* can utilize several C1 and multicarbon compounds (Kalyuzhnaya
560 et al., 2006). A similar result was observed by Baytshtok et al. (2008) who showed that

561 *Methyloversatilis* dominated in methanol and ethanol-fed denitrifying bioreactors. In
562 the top 30 genera, *Azoarcus*, *Thauera*, *Acidovorax* and *Dechloromonas* were
563 significantly more abundant in the O_{10} system and have also been reported to dominate
564 in methanol or acetate-fed denitrifying bioreactors (Hallin et al., 2006; Baytshtok et al.,
565 2008; Osaka et al., 2008). These findings further confirmed that methanol and acetate
566 might be the trophic linkage between aerobic CH_4 oxidation and denitrification in the
567 AME-D systems.

568 In addition to denitrifiers, some genes such as *nas*A, *nar*G, *nar*H, *nar*I, *nar*J, *nir*B,
569 *nir*D, *nir*K, *nor*B and *nor*C were associated with methanotrophs in the AME-D systems.
570 Many methanotrophs have been reported to encode partial denitrification pathways and
571 have the ability to reduce NO_3^- and/or NO_2^- to N_2O (Nyerges et al., 2010; Campbell et
572 al., 2011). The methanotrophs having *nar*G, *nar*H, *nar*I, *nar*J, *nir*B, *nir*K, *nor*B and
573 *nor*C were all significantly more abundant in the O_{21} system than in the O_{10} system.
574 This might be attributed to methanotrophs with denitrifying capacity outcompeting
575 other methanotrophs under aerobic conditions. Nyerges et al. (2010) also found that
576 methanotrophs with denitrifying capacity might outcompete other methanotrophs in
577 ecosystems with high nitrogen loading as they have the capacity to withstand NO_3^-
578 and/or NO_2^- stress and may even derive a growth benefit in the presence of both oxygen
579 and nitrite. However, it cannot be ruled out that the high O_2 concentration might result
580 in high numbers of methanotrophs with denitrifying capacity in the O_{21} system. Further
581 investigation is needed to understand the effects of oxygen and NO_3^- on the denitrifying
582 activity of methanotrophs.

583 NO_3^--N was mainly converted into N_2O-N and sludge-N (mainly biomass-N) in the
584 O_{21} system, while the majority was converted into N_2-N in the O_{10} system. Compared
585 to the O_2-limited condition, microorganisms grew more quickly under the O_2-sufficient

586 condition and more NO_3^--N was converted into biomass-N. Denitrification can proceed
587 under both aerobic and anaerobic conditions. In anaerobic respiratory NO_3^- reduction,
588 O_2 is toxic to anaerobic denitrifiers. However, most aerobic denitrifiers, such as
589 *Hyphomicrobium*, *Methylotenera* and *Pseudomonas stutzeri* TR2 can utilize both O_2
590 and NO_3^- or NO_2^- as their terminal electron acceptors (Meschner and Hamer, 1985;
591 Kalyuhznaya et al., 2009; Ji et al., 2015). Under aerobic condition, O_2 can inhibit N_2O
592 reductase and result in the production of N_2O, an important greenhouse gas with a
593 global warming potential of 298 times of CO_2, rather than N_2 (Thomson et al., 2012).
594 Under the O_2-limited condition, aerobic denitrifiers might utilize NO_3^- or NO_2^- as their
595 terminal electron acceptors and convert the majority of NO_3^- into N_2. Additionally,
596 aerobic methanotrophs have the capacity for denitrification, but they only reduce NO_3^-
597 and/or NO_2^- to N_2O (Nyerges et al., 2010; Campbell et al., 2011). Thus, an optimal O_2
598 concentrations is needed to control the AME-D process to mitigate nitrogen pollution
599 and the emission of greenhouse gases such as CH_4 and N_2O.
600
601 **5. Conclusion**
602 O_2 level was an important factor influencing the performance of AME-D process
603 and the microbial communities and metabolic pathways involved. Acetate and
604 methanol could serve as the key compounds trophically linking aerobic methanotrophs
605 to methylotrophs and denitrifiers in the AME-D process. The chemotactic abilities of
606 the bacterial community might influence the trophic link of aerobic methanotrophs to
607 methylotrophs and denitrifiers in the AME-D systems at different O_2 levels. Under the
608 O_2-sufficient condition, methylotrophs dominated the assimilation of CH_4-derived
609 carbon, while more methane-derived carbon was used for denitrification in O_2-limited
610 environments. These findings may help in future efforts to evaluate the distribution and

contribution of AME-D and develop strategies for mitigating CH_4 emission and nitrogen pollution in natural and engineered ecosystems.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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