Letter

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Biological Bromate Reduction Driven by Methane in a Membrane Biofilm Reactor

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Abstract

As a potent greenhouse gas with a greenhouse warming potential 28 times that of carbon dioxide over a 100-year timescale, methane has been proven to be utilized as electron donor to remove various of contaminants, e.g. nitrate, nitrite, perchlorate, and chromate from contaminated water. However, microbial bromate reduction supported by methane has not been reported so far. Here, a lab-scale membrane biofilm reactor (MBfR) was set up to explore the feasibility of bromate reduction driven by methane under oxygen-limiting condition. Long-term operational performance demonstrated that a complete bromate (BrO_3^-) reduction to bromide (Br^-) could be achieved, with 100% of bromate removal efficiency under a volume loading of 1 mg Br L^{-1} d^{-1}. Volatile fatty acids (VFAs) were produced in the reactor (ranging from 1.81 to 27.9 mg/L) under oxygen-limiting condition. High-throughput 16S rRNA gene sequencing indicated that *Methanosarcina* became the only dominate methane-oxidizing microorganism and the abundance of *Dechloromonas* increased from 0.9% to 18.0% after feeding bromate. It is hypothesized that under oxygen-limiting conditions methane was oxidized into VFAs, which might be used to reduce bromate by dissimilatory

bromate-reducing bacteria (likely *Dechloromonas*). This study offers a potential technology for bromate removal by using the methane-based MBfR.

Introduction

Bromate (BrO_3^-) contamination has been detected in various water environments, including drinking water, surface water and groundwater.[1, 2] The occurrence of bromate contamination in drinking water or groundwater could pose serious threats to public health (e.g. kidney effects, nervous system effects and hearing loss under exposure of high bromate concentrations), as it has been classified as Group 2B carcinogen by World Health Organization,[3] thus attaching great significance to develop efficient bromate removal technologies.

Bromate removal from contaminated water could be achieved via physical (e.g. filtration and ultraviolet irradiation), chemical (e.g. coagulants and zero-valent iron) or biological processes.[4] Microbial bromate reduction has been proved to be one of the most effective processes for remediation of bromate-contaminated groundwater.[1] A variety of reactor configurations including biologically active carbon filters[5] and fixed-bed reactors[6] have been utilized for microbial bromate remediation. However, the supplement of external organic carbons (e.g. ethanol[6] or acetate[7]) as electron donors could potentially increase operational costs or incur a secondary pollution. Autotrophic bromate reduction using hydrogen as electron donor has also been demonstrated in hydrogen-based hollow fiber membrane biofilm reactors (MBfR).[8, 9] Compared to hydrogen, methane is a much widely available carbon source,[10] thus could be as a promising electron donor for bromate removal. To date, various contaminants including nitrate, nitrite, chromate and perchlorate have been proved to be removable from synthetic groundwater or wastewater using methane-supported MBfR.[11-13] However, microbial bromate reduction driven by methane has not been reported so far.

Microbial methane oxidation can take place in both aerobic and anaerobic environments. Under aerobic conditions, methane is activated by methanotrophs through a mono-

oxygenation step, and organic intermediates such as methanol, acetate or formaldehyde is subsequently generated, providing electron and carbon sources for heterotrophic microorganisms.[14, 15] Recently, the aerobic methane oxidation process has been successfully applied to remove nitrate and chromate from wastewater through separated methane and oxygen supply.[16, 17] Under anaerobic conditions, methane oxidation is mediated by anaerobic methanotrophic archaea (known as ANME including ANME-1, ANME-2 and ANME-3). Previously, sulfate was the only confirmed electron acceptor for anaerobic methane oxidation process, in which a consortium of ANME archaea and sulfate reducing bacteria coupled methane oxidation to sulfate reduction.[18] Very recently, denitrifying anaerobic methane oxidation (DAMO) processes (nitrate or nitrite as electron acceptors) have been discovered. A novel member of ANME-2d lineage (*Candidatus* 'Methanoperedens nitroreducens', known as DAMO archaea) is able to oxidize methane by reverse methanogenesis, where methane is activated by methyl-coenzyme M reductase (MCR).[19] Moreover, *Candidatus* 'Methylomirabilis oxyfera' (known as DAMO bacteria) belonging to the NC10 phylum could oxidize methane through utilizing intracellular oxygen produced by the NO dismutation.[20] In recent studies, the DAMO processes have been successfully applied to remove nitrate from synthetic wastewater. [11, 21] Although aerobic or anaerobic methane oxidation processes have been proved practically useful in removing various contaminants, bromate removal driven by methane still remains unexplored regardless the presence of oxygen. Considering drinking or groundwater typically contains dissolved oxygen (DO, concentration up to 9 mg/L in a shallow groundwater),[22] methane oxidation under oxygen-limiting condition appears more practically feasible than anaerobic for bromate removal from contaminated drinking or groundwater.

The aim of this work is to explore the feasibility of bromate reduction using methane as electron donor under oxygen-limiting condition. A mixed culture enabling to couple bromate

reduction to methane oxidation was adopted and enriched by changing the feed of a lab-scale MBfR performing DAMO from nitrate to bromate. The reactor was initially inoculated with the enriched co-culture containing DAMO archaea and DAMO bacteria. After 250-day operation by feeding nitrate (start-up stage), synthetic groundwater containing bromate and dissolved oxygen was continuously fed to the reactor at hydraulic retention time of 1 day (operational stage for more than 100 days). Bromate removal rate was monitored to evaluate the reactor performance. The shift of microbial community was analysed based on high-throughput 16S rRNA gene sequencing.

Materials and Methods

Reactor setup

A laboratory-scale MBfR with 1 L working volume was set up in this study, as described previously.[23] Briefly, the reactor had eight bundles of composite hollow fibre membranes and membrane surface/reactor volume ratio was 181 m^2/m^3. The methane pressure inside all hollow fibres was controlled at 150 kPa using a gas-pressure regulator (Ross Brown, Australia). The bulk liquor in the MBfR system was mixed by a magnetic stirrer at 500 rpm (Labtek, Australia). A peristaltic pump (Masterflex, USA) and Tygon E-Lab tubing (internal diameter 3.1 mm, Masterflex, Cole-Parmer) was employed to recirculate the liquid at a flow rate of 100 mL/min. The MBfR was operated for about 360 days at 22±2 ^oC. The pH in the reactor was controlled between 7 and 8 by manual injection of 1 M NaOH.

Operational conditions

In order to obtain biofilm growth on the hollow fibre membranes, the MBfR was inoculated with 150 mL enriched co-culture dominated by DAMO archaea and DAMO bacteria.[19] Two stages, namely start-up (Stage I, 250 days) and operational stage (Stage II, 113 days), were

involved in the entire experimental period. In the start-up stage, nitrate stock solution (40 g N/L) was manually dosed into the reactor, giving an initial nitrate concentration of 40-160 mg N/L after each dosage. During the operational stage, the influent (composition as shown in Supporting information) with a bromate concentration of ~1 mg Br/L was continuously fed into the reactor at hydraulic retention time (HRT) of 1 day. As nitrogen was not used to flush influent to remove oxygen, dissolved oxygen of 7-9 mg/L could be detected in influent.

Every week 3 liquid samples were regularly collected to monitor the bromate and bromide concentrations. In addition, volatile fatty acids (VFAs) in effluent were measured to monitor possible intermediates from methane oxidation under oxygen-limiting condition.

Analytical methods

The effluent of 2 mL was sampled to determine the concentrations of bromate species and VFAs after 0.22 μm-filtration. Bromate and bromide concentrations were measured by ion chromatography (Dionex ICS-2100).[24] A gas chromatography (7890A, Agilent) with a polar capillary column (DB-FFAP) and a flame ionisation detector (FID) was employed to determine VFAs. The pH level in the reactor was monitored by a pH meter (Oakton, Australia). DO concentration in influent was measured using a DO meter (HACH, USA).

For microbial community analysis, biofilm samples of 5 mL were collected from membrane surfaces at the end of start-up phase (Day 250) and on Day 335 of operational phase when a stable bromate removal efficiency was achieved. DNA was extracted from the biomass samples using the FastDNA SPIN for Soil kit (MP Biomedicals, USA) according to the manufacture's instruction. The 16S rRNA gene was amplified, sequenced and analyzed based on the procedures shown in Supporting Information.[25]

Results and Discussion

Performance of bromate bioreduction in the methane-based MBfR reactor

In order to enrich biofilm for methane oxidation, 150 mL of inoculum harvested from a suspended reactor performing DAMO was seeded into the methane-based MBfR. [19] After the inoculation, nitrate was supplied in the liquid phase as the sole electron acceptor, while methane was delivered from the hollow fibre membranes as electron donor. A steady nitrate reduction rate of 3.7-5.0 mg N/L/d without nitrite accumulation was achieved at the end of start-up phase (Fig. S1). In parallel, a layer of biofilm gradually attached to the surface of hollow fiber membranes after 250 days of enrichment. On Day 251, the MBfR was switched into continuous operation feeding with synthetic contaminated water containing bromate and DO (7-9 mg/L), in order to test if microbial community shaped by nitrate feeding could reduce bromate under oxygen-limiting conditions. Interestingly, after switching electron acceptor from nitrate to bromate, the reactor achieved 100% of bromate removal efficiency (Fig. 1a). Purified nitrate reductase was previously reported to be potentially involved in bromate reduction process and denitrifying organisms also reduced bromate (BrO_3^-) to bromide (Br^-) after a complete nitrate reduction,[26, 27] indicating that some given microorganisms might be able to perform bromate reduction in the reactor after immediate switching of electron acceptor from nitrate to bromate. However, the removal percentage of bromate kept decreasing to 43% after 10 days. The possible reason is that microbial communities in the reactor were shifted after bromate introduction (see details later). The bromate removal efficiency was recovered to 66% on Day 274 and then 100% on Day 278, suggesting that biofilm community has eventually adapted to reduce bromate. The bromide concentration in effluent was stable at 0.8-1 mg/L during the whole experiment period (Fig.

1a), but other intermediates (e.g. bromite and hypobromite) were not detected, indicating that bromate in influent might be completely reduced to bromide.

Production of VFAs by methane oxidation

DO concentration of 7-9 mg/L in influent could be consumed by the biofilm, thus no DO could be detected in the MBfR during the entire Stage II, leaving the system a quasi-anaerobic condition (named as oxygen-limiting conditions in this study). Interestingly, VFAs were produced under oxygen-limiting conditions (Fig. 1b). During the initial 36 days of Stage II, total VFAs were much higher (22.2-27.9 mg/L), afterwards decreased to 1.81-13.6 mg/L, possibly due to its ongoing consumption by microorganisms as carbon sources. Considering VFAs were not observed under anaerobic condition in Stage I, the oxygen introduction from the influent in Stage II could likely induce the VFAs production, which is consistent with our previous study.[24] In this study, total VFAs were dominated by acetate (>75%), followed by propionate (approximately 2-10%). Differently, previous studies documented that other soluble organic matters, e.g. methanol, formate, lactate, formaldehyde or citrate were dominant intermediates under micro-aerobic or aerobic conditions. [28-30]

Microbial community structure shaped by bromate reduction

The shift of microbial community structure under nitrate-feeding (Stage I) and bromate-feeding conditions (Stage II) was investigated based on 16S rRNA gene sequencing. Surprisely, it was found that DAMO archaea and DAMO bacteria that dominated in the inoculum became relatively minor (< 1%) in the biofilm at the end of start-up phase, which explained the relatively slow nitrate removal rate (3.7-5.0 mg N/L/d) in Stage I compared to the seeding sludge reactor (average 25 mg N/L/d). A possible reason is that oxygen was

accidentally introduced into the reactor when replacing a recirculation tube on Day 150. Previous studies also reported that DAMO microorganisms are very sensitive to oxygen.[31] Compared to Stage I, the abundance of class *Methanomicrobia* and *Betaproteobacteria* in the biofilm, significantly increased from 0.62% to 5.2% and from 11.2% to 22.9%, respectively at the end of Stage II (Fig. 2). Further analysis of the community composition at the genus level indicated that the class of *Methanomicrobia* and *Betaproteobacteria* were dominated by the genus of *Methanosarcina* and *Dechloromonas*, respectively. In comparison with the biofilm of Stage I, the abundance of *Methanosarcina* increased from 0.18% to 4.0% and became the only dominating methane-related archaea in bromate-shaped biofilm. In addition, the abundance of *Dechloromonas* increased significantly from 0.9% to 18.0% after bromate was supplied as the only electron acceptor, indicating *Dechloromonas* was potentially responsible for bromate reduction using VFAs as carbon and electron sources.

Mechanisms of bromate reduction coupled to methane oxidation

It has been reported that nitrate, nitrite, perchlorate and chromate could be reduced by using methane as sole electron donor in CH_4-based MBfRs.[12, 13, 32] To the best of our knowledge, this study is the first report that a complete bromate reduction could also be achieved in the methane-based MBfR. The bromate removal seems to be achieved via synergistic interactions between multiple microorganisms. In order to elucidate bromate reduction driven by methane under oxygen-limiting conditions, two batch tests were conducted at the end of Stage II (Supporting information). In the first abiotic control without microorganisms, no bromate was reduced with methane and fresh medium (Fig. S2), ruling out the possibility of bromate reduction via chemical reactions. In the second test without methane supply for the reactor, no bromate reduction and bromide production could be observed as well (Fig. S2),

195 indicating methane plays an important role in the bromate reduction process in the MBfR. It
196 is assumed methane was partially oxidized to VFAs under oxygen-limiting conditions, in
197 terms of the fact that methane was the only carbon source fed to this reactor. The VFA
198 production was likely through aerobic methane oxidation, although aerobic methanotrophs
199 were not identified by 16S rRNA gene sequencing. Interestingly, *Methanosarcina,* as a
200 known methanogen, became the only methane-related microorganism. It has been reported
201 that *Methanosarcina barkeri* could mediate methane oxidation and produce methanol and
202 acetate as oxidation product,[33] indicating *Methanosarcina* is probably responsible for VFA
203 production in Stage II. Simultaneously, *Dechloromonas*, whose abundance increased by 17%
204 after the input of bromate, might play a role in utilizing VFAs as electron donor to reduce
205 bromate. It has been documented that *Dechloromonas* is able to reduce chlorate, perchlorate
206 or nitrate using acetate as electron donor.[34] The perchlorate reducing bacterium
207 *Dechloromonas* sp. PC1 was also reported to reduce bromate without measurable growth.[35]
208 In addition, bromate was reduced to bromide via mediation of nitrate reductase in
209 denitrifying *Pseudomonas* spp. [36] Given that nitrate was not provided in Stage II, unique
210 conditions (e.g. oxygen-limiting and only bromate fed as electron acceptor) in the MBfR
211 might select a specialized bromate-reducing bacterium, which warrants further studies. Based
212 on long-term performance, batch tests and microbial community structure results, a
213 hypothesis was proposed for the bromate reduction in the methane-based MBfR (Fig. 3). It is
214 assumed methane was oxidized into VFAs by *Methanosarcina* or unknown methanotrophs
215 under oxygen-limiting conditions, then the generated VFAs served as electron donors for
216 dissimilatory bromate-reducing bacteria (like *Dechloromonas*). The detailed pathway and the
217 responsible microorganisms should be elucidated by isotope, metagenomics and
218 metatranscriptomics in the future.

219 ***Practical implications***

This study provided the first proof of concept that the complete bromate removal is feasible in the methane-based MBfR under oxygen-limiting conditions. A bromate removal rate of 1 mg Br L^{-1} d^{-1} (5.5 mg/m^2/d) was achieved in this work. Compared to the reported rates (Table S1), bromate removal rate achieved in this work is lower than the typical rate in hydrogen-based MBfR (15.6-232 mg/m^2/d), while it is comparable to the rates achieved in reactors using ethanol (0.4-1.0 mg/L/d) or glucose as carbon source (1.5-3.0 mg/m^2/d). The reactor operation should be further optimized to increase the bromate removal rate by regulating operation conditions, e.g. methane partial pressure and oxygen flux rates. In addition, high nitrate concentrations are commonly expected in most groundwater[37] and may inhibit bromate removal as nitrate and bromate are competing terminal electron acceptors.[38] The effect of nitrate on bromate removal efficiency remains further exploration.

Although the bromate removal rate achieved in this study is relatively limited, methane supported bromate removal technique might be an alternative process in bromate-containing water treatment as it has several advantages over other technologies. Firstly, methane is inexpensive and widely available compared to organic carbon sources (e.g. methanol) or hydrogen. In addition, residue organic matter could be detected in effluent to cause secondary pollution due to excess addition of soluble organic carbon, while methane solubility is much lower and will not remain in effluent. Furthermore, for aerobic methane oxidation, great potential safety hazard could be involved when mixing flammable methane with oxygen in membrane lumens. The MBfR configurations used in this study, in which methane is supplied through membranes and oxygen is provided via liquid, avoids the safety hazard.

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Conflict of interest: The authors declare that they have no conflict of interest.

Supporting Information Available: Additional method details and supporting data, results in figures and tables.

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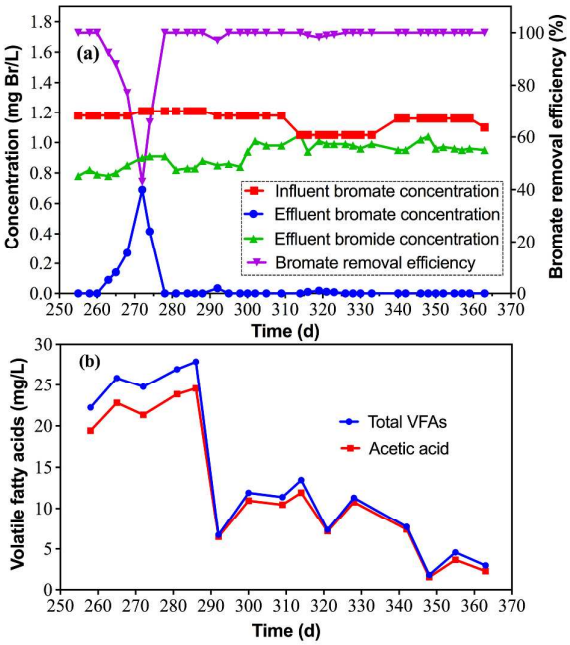

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349 Fig. 1 Long-term performance of bromate reduction during Stage II in the MBfR (a) and
350 variations of residual volatile fatty acids (VFA) produced in the reactor (b) under oxygen-
351 limiting conditions (Stage II).

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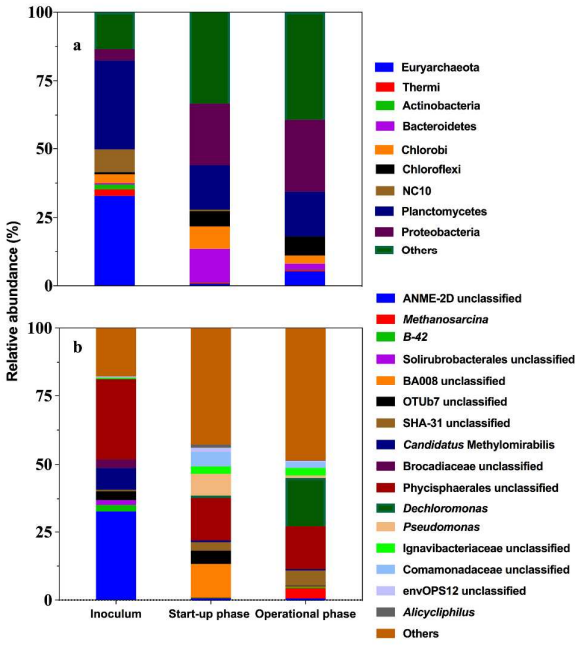
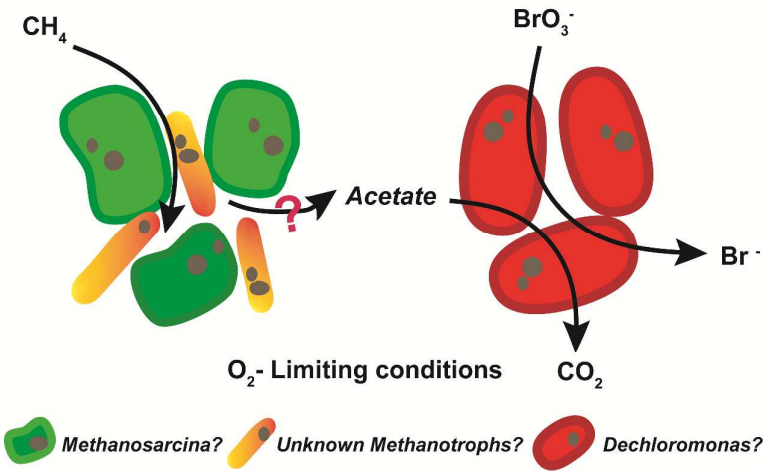


Fig. 2 Relative abundances of microbial communities at different stages: (a) phylum, and (b) genus. The relative abundance is defined as a percentage in total microbial sequences in a sample. Phylum or genera that account for ≥ 1% of at least one 16S rRNA gene sequence are shown, while phylum or genera with an abundance of less than 1% in all sequences are grouped into Others.



363

364 Fig. 3 The proposed pathway of bromate reduction coupled to methane oxidation under
365 oxygen-limiting conditions. *Methanosarcina* or unknown methanotrophs might convert
366 methane into VFAs under oxygen-limiting conditions, and then the produced VFAs would be
367 utilized by potential bromate reducers (e.g. *Dechloromonas*) to reduce bromate into bromide.

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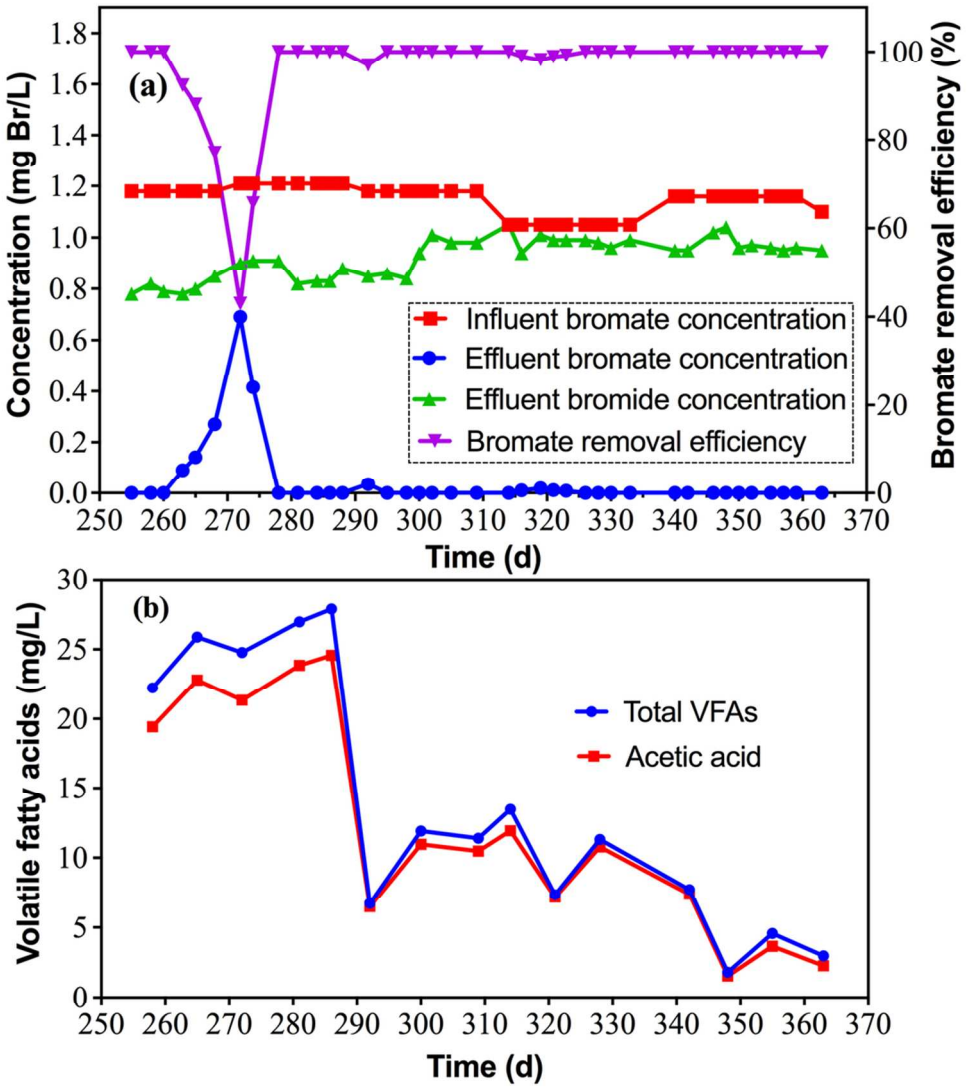


Fig. 1 Long-term performance of bromate reduction during Stage II in the MBfR (a) and variations of residual volatile fatty acids (VFA) produced in the reactor (b) under oxygen-limiting conditions (Stage II).

95x109mm (300 x 300 DPI)

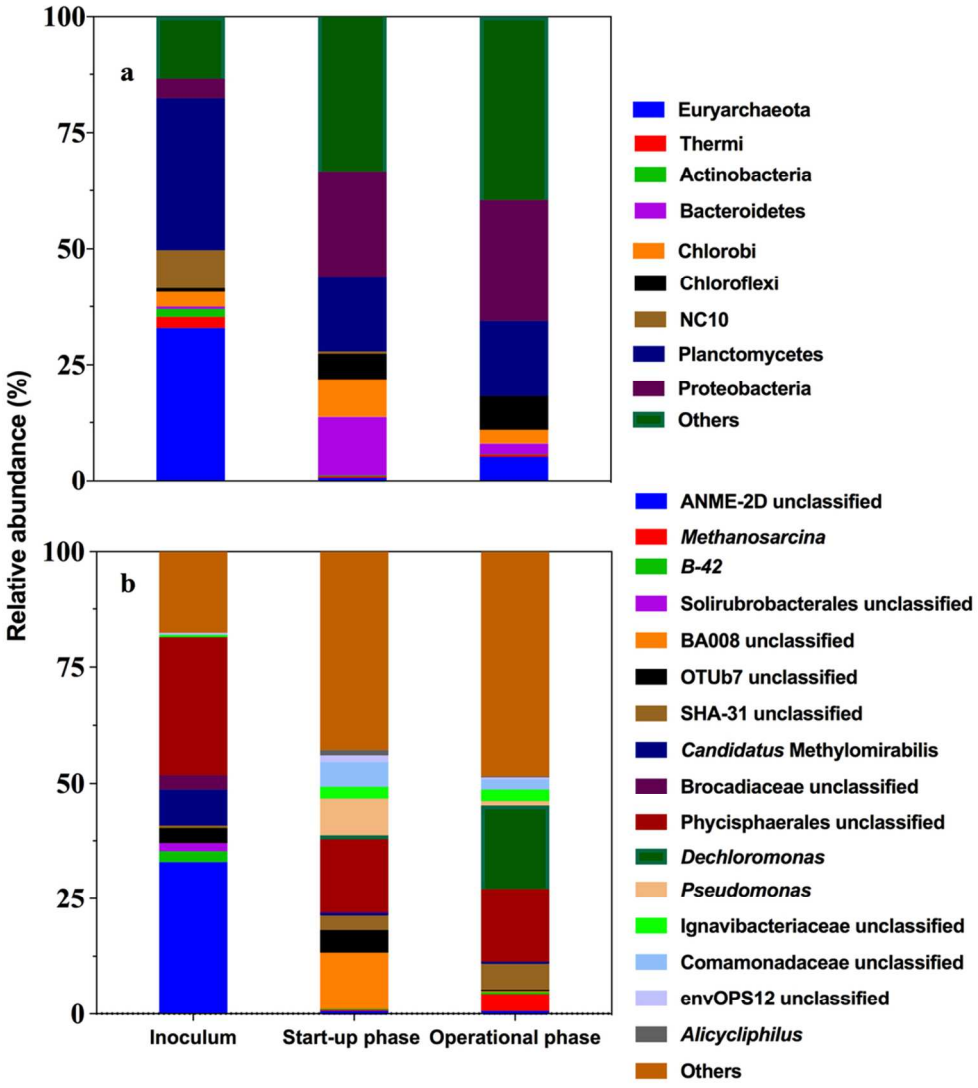


Fig. 2 Relative abundances of microbial communities at different stages: (a) phylum, and (b) genus. The relative abundance is defined as a percentage in total microbial sequences in a sample. Phylum or genera that account for ≥ 1% of at least one 16S rRNA gene sequence are shown, while phylum or genera with an abundance of less than 1% in all sequences are grouped into Others.

93x106mm (300 x 300 DPI)

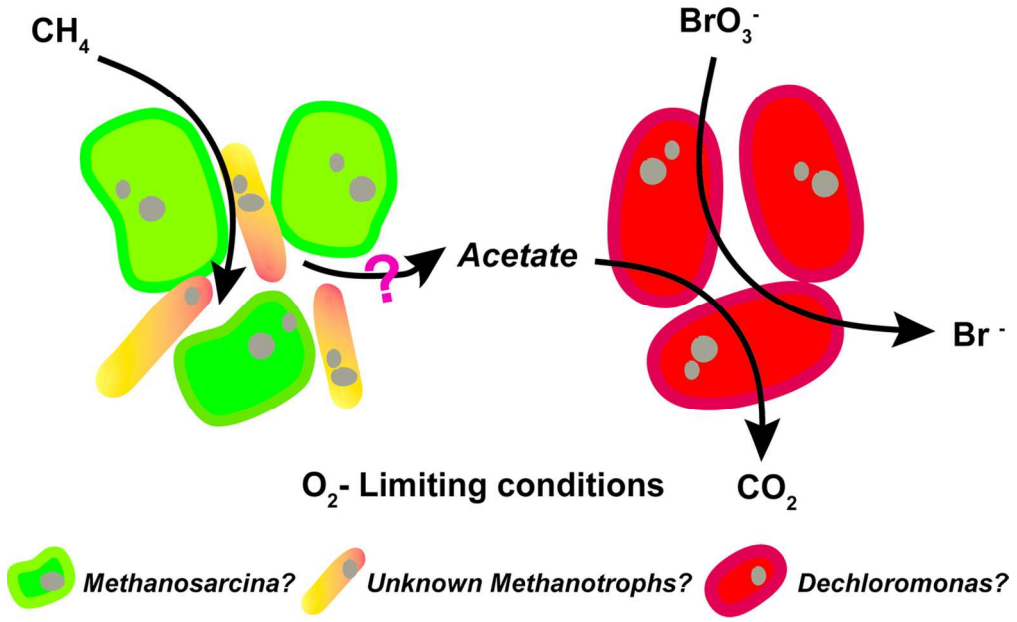
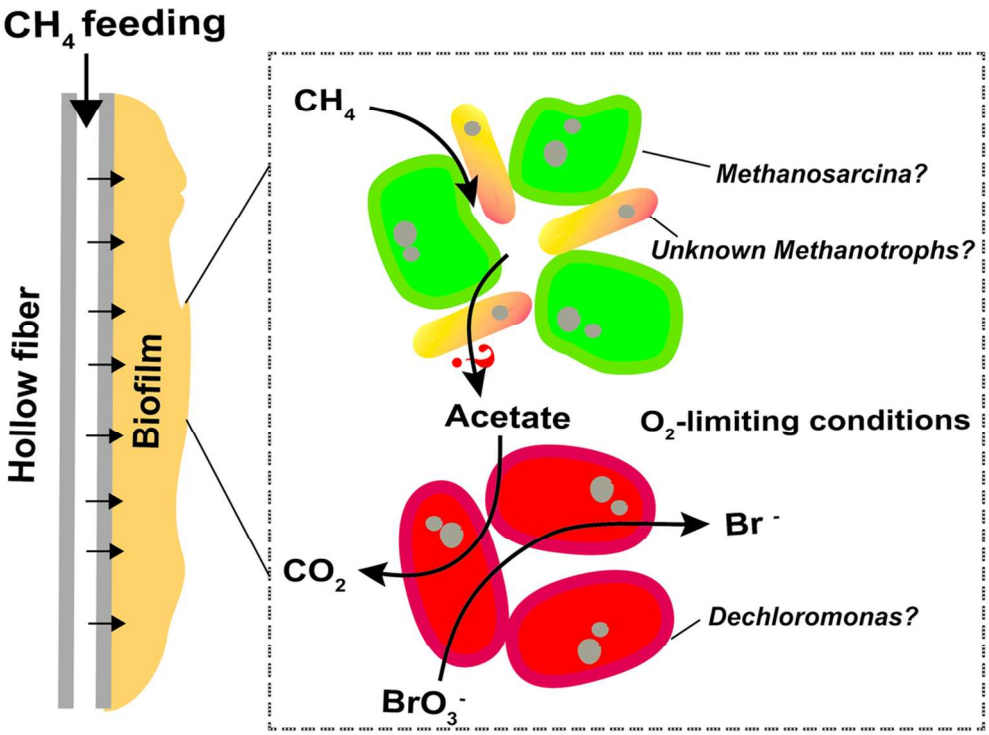


Fig. 3 The proposed pathway of bromate reduction coupled to methane oxidation under oxygen-limiting conditions. Methanosarcina or unknown methanotrophs might convert methane into VFAs under oxygen-limiting conditions, and then the produced VFAs would be utilized by potential bromate reducers (e.g. Dechloromonas) to reduce bromate into bromide.

119x74mm (300 x 300 DPI)



102x76mm (300 x 300 DPI)