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RESEARCH ARTICLE

Effects of salinity on simultaneous reduction of perchlorate and nitrate in a methane-based membrane biofilm reactor

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Abstract This study builds upon prior work showing that methane (CH_4) could be utilized as the sole electron donor and carbon source in a membrane biofilm reactor (MBfR) for complete perchlorate (ClO_4^-) and nitrate (NO_3^-) removal. Here, we further investigated the effects of salinity on the simultaneous removal of the two contaminants in the reactor. By testing ClO_4^- and NO_3^- at different salinities, we found that the reactor performance was very sensitive to salinity. While 0.2 % salinity did not significantly affect the hydrogen-based MBfR for ClO_4^- and NO_3^- removals, 1 % salinity completely inhibited ClO_4^- reduction and significantly lowered NO_3^- reduction in the CH_4-based MBfR. In salinity-free conditions, NO_3^- and ClO_4^- removal fluxes were 0.171 g N/m^2-day and 0.091 g/m^2-day, respectively, but NO_3^- removal fluxes dropped to 0.0085 g N/m^2-day and ClO_4^- reduction was completely inhibited when the medium changed to 1 % salinity. Scanning electron microscopy (SEM) showed that the salinity dramatically changed the microbial morphology, which led to the development of wire-like cell structures. Quantitative real-time PCR (qPCR) indicated that the total number of microorganisms and abundances of functional genes significantly declined in the presence of NaCl. The relative abundances of *Methylomonas* (methanogens) decreased from 31.3 to 5.9 % and *Denitratisoma* (denitrifiers) decreased from 10.6 to 4.4 % when 1 % salinity was introduced.

Keywords Methane-based membrane biofilm reactor · Nitrate · Perchlorate · Reduction · Salinity

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Introduction

Perchlorate (ClO_4^-) is widely used in rocket fuel, fireworks, and explosives due to its high energetic value as a strong oxidant and its chemical stability (USEPA 2005). Its release into water resources causes contamination. Perchlorate can interfere with the synthesis and secretion of thyroid hormone, which affects human metabolism and hinders human growth and development (USEPA 2008). Though the US Environmental Protection Agency (USEPA) has not yet established a nationwide maximum contaminant level (MCL) for ClO_4^-, some states have set up cleanup target levels from 2 to 18 μg/L in drinking water (Gu and Coates 2006). Nitrate (NO_3^-) is a co-contaminant with ClO_4^- in groundwater (USEPA 2001). Nitrate has been regulated by the USEPA at 10 mg N/L in drinking water (USEPA 2009).

Commonly used technologies for removing ClO_4^- and NO_3^- from water include membrane filtration, ion exchange, chemical reduction, and biological reduction (Coates and Achenbach 2004; Matos et al. 2008; Zhao et al. 2014). Membrane filtration and resin regeneration in ion exchange produce highly saline ClO_4^- and NO_3^- brines that need to be further treated or disposed of. Biological reduction can be used to directly treat ClO_4^-- and NO_3^--contaminated water or brines produced during membrane filtration or ion exchange. Compared to chemical reduction, biological reduction consumes less energy and requires less chemical addition because enzymes in microbes are used as the

catalysts (Ye et al. 2012). During biological reduction, the perchlorate reductase (*pcr*ABCD) reduces ClO_4^- to chlorite (ClO_2^-) (Kengen et al. 1999), and chlorite dismutase (*cld*) catalyzes the disproportionation of chlorite (ClO_2^-) to chloride (Cl^-) and oxygen (O_2) (Van Ginkel et al. 1996), which is subsequently reduced to H_2O. During the NO_3^- reduction, reductions of nitrate to nitrite (*nar*GHJI), nitrite to nitric oxide (*nir*SJFD/GH/L), NO to N_2O (*nor*Z), and N_2O to N_2 (*nos*ZDFY) are performed by various reductases (Ettwig et al. 2010).

Perchlorate-reducing bacteria (PRB) and denitrifiers can use a variety of electron donors to gain energy (Miller and Logan 2000; Sahu et al. 2009; Son et al. 2006; Yu et al. 2006). When the electron donor is a gas, such as hydrogen and methane, membrane biofilm reactors (MBfRs) are one of the most widely used reactors because they have close to 100 % gas utilization efficiency and high NO_3^- and ClO_4^- removal rates (Zhao et al. 2013; Tang et al. 2012a, b; Luo et al. 2015; Chen et al. 2016).

Compared with CH_4-based MBfRs, H_2-based MBfRs have been extensively studied and used at the field scale for NO_3^- and ClO_4^- removal. The effects of salinity on nitrate and perchlorate reduction in H_2-based MBfRs are well understood. H_2-based MBfRs reach simultaneous reduction of nitrate and perchlorate, but the reduction rate decreases as the salt concentration increases. Chung et al. (2007) reported that nitrate and perchlorate reduction was not affected by 2 % salinity (20 g/L NaCl) in a H_2-based MBfR, while 4 % salinity (40 g/L NaCl) decreased reduction by 40 %. Van Ginkel et al. (2008) investigated the kinetics of ClO_4^- and NO_3^- reduction in a H_2-based MBfR fed with brines. They found that the NO_3^- removal fluxes and H_2 pressure were roughly first order but roughly zero order with NO_3^- concentration in H_2-based MBfR. NO_3^- and ClO_4^- were reduced simultaneously in a CH_4-based MBfR (Luo et al. 2015), but the effects of salinity on NO_3^- and ClO_4^- reduction in CH_4-based MBfRs are unknown.

CH_4-based MBfRs have been much less studied compared to their H_2-based counterparts. Previous research has demonstrated the potential of using CH_4-based MBfRs to simultaneously remove NO_3^- and ClO_4^- from water (Luo et al. 2015; Chen et al. 2016); however, these studies were all conducted using synthetic fresh waters. Therefore, it is of interest to investigate how salinity affects biological perchlorate and nitrate reduction in a CH_4-based MBfR. The overall objective of this work is to investigate the effects of salinity on the simultaneous reduction of NO_3^- and ClO_4^- in a CH_4-based MBfR.

Materials and methods

MBfR setup

A two-column MBfR system similar to Zhao et al. (2011) was used in this study. The MBfR had composite hollow fibers (hydrophobic microporous polyethylene fiber, 280-μm o.d., 180-μm i.d., and 0.1–0.15-μm pore size) manufactured by Mitsubishi Rayon (Model MHF-200TL, Mitsubishi, Ltd., Japan). One column contained 32 fibers, and the other contained 20 fibers as "coupon fibers" for sampling purposes. Each fiber ran all the way through the column, with one end glued into a CH_4 supply manifold and the other end sealed to avoid gas leakage. The total volume of the MBfR was 65 mL, and the total membrane surface area was 90.24 cm^2. The MBfR was completely mixed via recirculation with a peristaltic pump (Longer Pump, Model 1515X, Longer Precision Pump Co, Ltd., China) at 100 mL/min.

Inoculum source and culture medium

We inoculated the MBfR with a 10-mL culture from a CH_4-based MBfR (Luo et al. 2015). The medium contained the following mineral salts (analytical grade or purer) per liter of deionized water: 1 mg $CaCl_2$, 0.3 g $NaHCO_3$, 5 mg $MgSO_4.7H_2O$, 0.2 g KH_2PO_4, 0.4 g $NaHPO_4.12H_2O$, 1-mL acid trace element solution (100 mM HCL, 2.085 g $FeSO_4 \cdot 7H_2O$, 68 mg $ZnSO_4 \cdot 7H_2O$, 14 mg H_3BO_3, 120 mg $CoCl_2 \cdot 6H_2O$, 500 mg $MnCl_2 \cdot 4H_2O$, 320 mg $CuSO_4$, 95 mg $NiCl_2 \cdot 6H_2O$ per liter), and 1-mL alkaline trace element solution (10 mM NaOH, 67 mg SeO_2, 50 mg $Na_2WO_4 \cdot 2H_2O$, 242 mg $Na_2MoO_4 \cdot 2H_2O$ per liter). The medium was degassed with N_2 for 15 min to maintain anaerobic conditions. The medium pH was adjusted to 7.5 ± 0.2 with hydrochloric acid. The influent feeding rate was 0.5 mL/min (hydraulic retention time (HRT) of 130 min), the CH_4 pressure was 10 psig (0.69 bar), and the temperature was 29 ± 1 °C for all experiments.

Start up and continuous operation of the MBfR

After inoculation, the MBfR was fed with NO_3^- and ClO_4^- at ~15 and ~ 10 mg/L, respectively, and maintained for more than 20 days to allow sufficient biomass to accumulate. After the startup stage, we fed the reactor with 2 mg/L of perchlorate from stage 1 to stage 5. To investigate the effects of salinity on ClO_4^- and/or NO_3^- reduction, we set up salinity at 1 % (10 g/L of NaCl) in stage 2 and 0.2 % (2 g/L of NaCl) in stages 5 and 6. To further investigate if nitrate inhibition plays a role in the inhibition mechanisms, we varied the nitrate concentrations in different stages: 10 mg NO_3^-/L in stages 1, 2, and 3 and 5 mg NO_3^-/L in stage 6. We allowed each stage to reach steady state, which was defined as when effluent concentrations were stable (<10 % variation) at least three times the HRT.

Analyses

Liquid samples were taken from the MBfR with 5-mL syringes and filtered immediately through 0.2-μm membrane filters

(LC + PVDF membrane, Shanghai Xinya, China). NO_3^- and NO_2^- were assayed using ion chromatography (Metrohm 833 Basic IC plus, Switzerland) with an A-Supply-5 column and an eluent containing 3.2 mM $NaHCO_3$, 1.0 mM Na_2CO_3, and 5 % acetone at a flow rate of 1 mL/min. ClO_4^- was measured using ion chromatography (DIONEX ICS-1000, USA) with an AS 16 column and AG 16 precolumn, an eluent concentration of 35 mM KOH, and a 1.0 mL/min flow rate. The pH values of the influent and effluent were measured using a pH meter (Seven Easy, Mettler Toledo, Switzerland) and were between 7.5 and 7.7 for all stages. The bacterial morphology on the surface of membrane at the end of each stage was analyzed using scanning electron microscopy (SEM).

Biofilm sampling and DNA extraction

When the reactor reached a steady state, biofilm was collected. N_2 gas was sparged at the sampling point to preclude O_2 exposure. We cut off one 5-cm-long section from a coupon fiber and then sealed the remaining fiber by tying the end into a knot. The DNA was extracted using a DNeasy Blood & Tissue Kit (Qiagen, USA) as previously described by Zhao et al. (2011).

Quantification of bacterial 16S rRNA and functional genes

Plasmids containing target fragments were used as positive controls and to produce calibration curves (Zhao et al. 2011). The primers and qPCR conditions were the same as previously described: *pcrA* for ClO_4^- reductase (Nozawa-Inoue et al. 2008), *nirS* for NO_2^- reductase (Throbaeck et al. 2004), *mcrA* for the formation of methane by most of methanogens and reverse methanogenesis (Steinberg and Regan 2008), *pMMO* for CH_4 monooxygenase (Paszczynski et al. 2011), and *16S* ribosomal RNA (*rRNA*) gene for bacteria (Maeda et al. 2003). We used the SYBR Premix Ex Taq Kit (Takara Bio Inc., Japan) and performed qPCR as previously described by Zhao et al. (2011). The copy numbers of each gene were calculated by comparison to standard curves. Negative controls included water instead of template DNA in the PCR reaction mix. We performed triplicate PCR reactions for all samples and negative controls. The functional genes (*pcrA*, *nirS*, *pMMO*, *mcrA*) and *16S rRNA* gene abundances in different stages are shown in Fig. 3.

Results and discussion

Perchlorate and nitrate reduction

CH_4 pressure was set at 10 psig throughout the experiment, which made CH_4 available in all stages (Table S1). The influent and effluent concentrations of ClO_4^- and NO_3^- for the MBfR are plotted in Fig. 1. The reactor was operated for 23 days in batch mode before stage 1 to accumulate sufficient biomass to reduce up to 5 mg/L of perchlorate and 15 mg/L of nitrate.

The NO_3^- and ClO_4^- surface loadings in stage 1 were 0.171 g N/m^2-day and 0.091 g/m^2-day, respectively. Without salinity, the nitrate was fully removed and 57.2 % of the ClO_4^- was reduced after the reactor reached steady state. When 1 % salinity was applied in stage 2, the NO_3^- removal percentage dropped to ~50 %, and ClO_4^- reduction was completely inhibited. Obviously, the perchlorate and nitrate reductions were both severely affected by the salinity in the CH_4-based MBfR. Van Ginkel et al. (2008) reported that in a H_2-based MBfR, NO_3^- removal was not affected at NaCl concentrations less than 20 g/L (i.e., 2 %). Chung et al. (2007) demonstrated that both nitrate and perchlorate were reduced in the presence of NaCl ranging from 10 to 40 g/L (i.e., 1–4 %) in a H_2-based MBfR. By comparison, microorganisms using methane as the electron donor are thus more sensitive to salinity than the microorganisms using H_2.

To investigate if the biofilm could be recovered after salinity shock, we removed salinity in stage 3 so that the operating conditions in stage 3 were the same as in stage 1. To our surprise, the perchlorate and nitrate removal reached only 28.3 and 64.4 %, respectively. This removal is slightly higher than that observed in stage 2 but much lower than that in stage 1. The biofilm was possibly irreversibly damaged by the high salinity. Park et al. (2001) reported that the activity of denitrifying bacteria decreased after adding salt to the system. Gingras and Batista (2002) reported that the ClO_4^- reduction rate declined to 10 % when 1–1.5 % salt concentrations were present in culture.

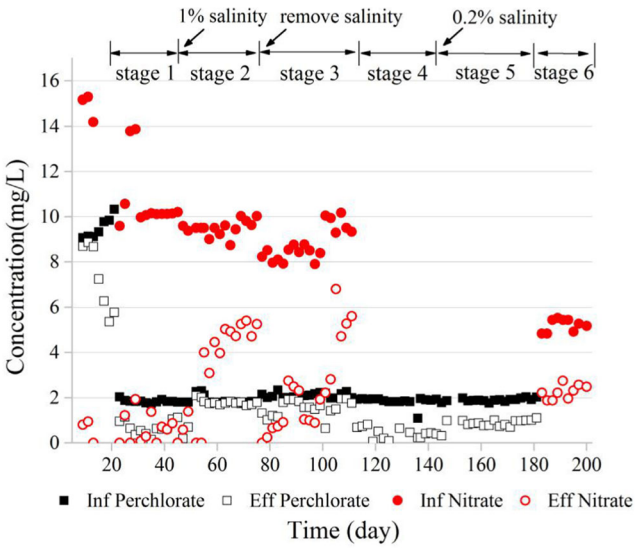

Fig. 1 The NO_3^- and ClO_4^- concentrations in the influent and effluent of the MBfR. Stage 2 and stage 5 were operated with 1 and 0.2 % salinity, respectively. Salinity was defined as the quantity of dissolved salt content of the water

To investigate if NO_3^- contributed to the inhibition of ClO_4^- reduction, we removed NO_3^- from the influent in stage 4. Without feeding NO_3^-, the ClO_4^- bioreduction improved to around 70 %. Therefore, it is possible that, in stage 2, the dual inhibitions of nitrate and salinity caused the absence of ClO_4^- reduction. In a model, Tang et al. (2012a, b) explained that in a H_2-MBfR simultaneously reducing NO_3^- and ClO_4^-, a high load of NO_3^- (>0.6 g N/m^2-day) strongly inhibits ClO_4^- reduction.

To test the perchlorate or nitrate reduction under lower salinity, we reduced the salinity to 0.2 % NaCl in stages 5 and 6. ClO_4^- was the only electron acceptor in stage 5, and NO_3^- was the only electron acceptor in stage 6. The ClO_4^- and NO_3^- removals were 53.3 and 59.2 %, respectively. This performance is much worse than that in stage 1, which can be explained by the 0.2 % salinity inhibition and the irreversible inhibition in stage 2. The inhibition mechanisms will be further explored in "Microbial morphology" section.

Microbial morphology

To investigate the bacterial morphological change, we analyzed biomass samples from each stage at steady state using SEM. Figure 2 compares the microbial morphologies at stages 1 (Fig. 2a), 2 (Fig. 2b), 3 (Fig. 2c), and 6 (Fig. 2d). Stage 2 had the highest salinity (1 %). Correspondingly, it has the most wire-like structures and the cell surfaces had the most wrinkles (Fig. 2b). These bacterial filaments may be a way for the microorganisms to accommodate the change in osmotic pressure and protect the cells from salt shock. The damaging effect of salinity on bacteria leads to cell content emission and the formation of extracellular polymeric substances (EPS)/soluble microbial products (SMP) (Di Bella et al. 2013; Jang et al. 2013; Reid et al. 2006). Some studies argue that the accumulation of cell contents and intermediate products and bacterial autolysis are the responses to salt toxicity (Sharghi et al. 2014; Pendashteh et al. 2011; Sun et al. 2010). However, stage 6 had a medium salinity (0.2 %) and the biofilm also had some wire-like structures. Similarly, Jin et al. (2007) observed that under higher osmotic stress, the structure of a nitrifying bacterial community was disrupted and microbial diversity decreased. Therefore, salinity inhibition likely played a role in the decrease of nitrate removal in stage 6.

Functional community structure

Figure 3 shows the functional and *16S rRNA* gene copy numbers of the microbial community at all stages. The bacterial community was very dense in stage 1, illustrated by the highest copy numbers of the *16S rRNA* gene (2.84×10^{12} copies/m^2 fiber). The functional respiration genes such as *pcrA* and *nirS* and methane oxidation gene *pMMO* reached the highest in stage 1, indicating a very good correlation between methane oxidation and nitrate/perchlorate reduction. When 1 % salinity was added in stage 2, the abundances of all tested genes dropped by three orders of magnitude and reached their lowest points, which was consistent with the

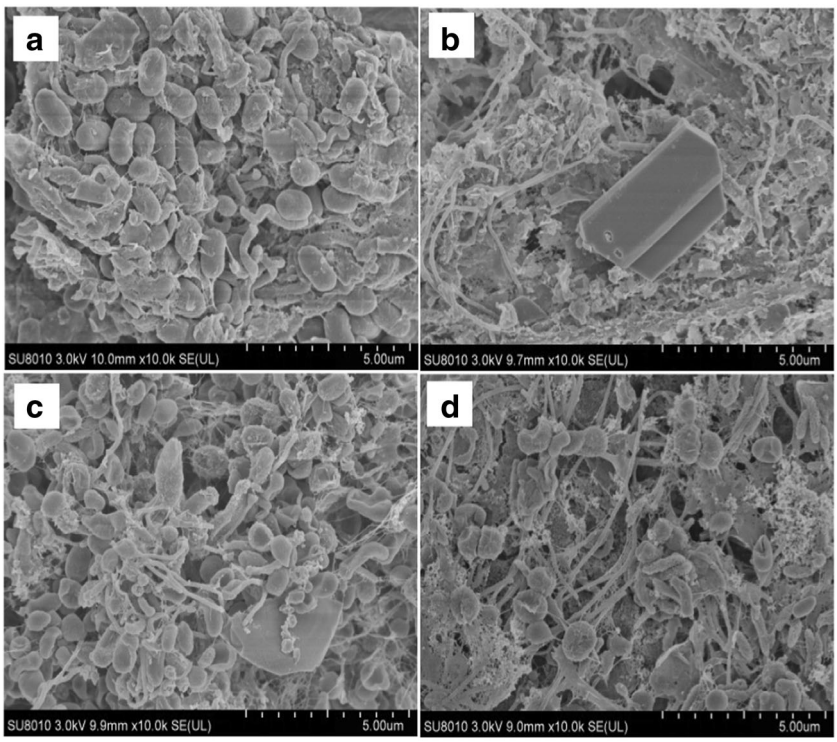

Fig. 2 The SEM images of biomass samples from different stages. **a** Stage 1 at 10,000 magnification (10 mg/L NO_3^-, 2 mg/L ClO_4^-). **b** Stage 2 at 10,000 magnification (10 mg/L NO_3^-, 2 mg/L ClO_4^-, and 10 g/L NaCl). **c** Stage 3 at 10,000 magnification (10 mg/L NO_3^-, 2 mg/L ClO_4^-). **d** Stage 6 at 10,000 magnification (5 mg/L NO_3^-, 2 g/L NaCl)

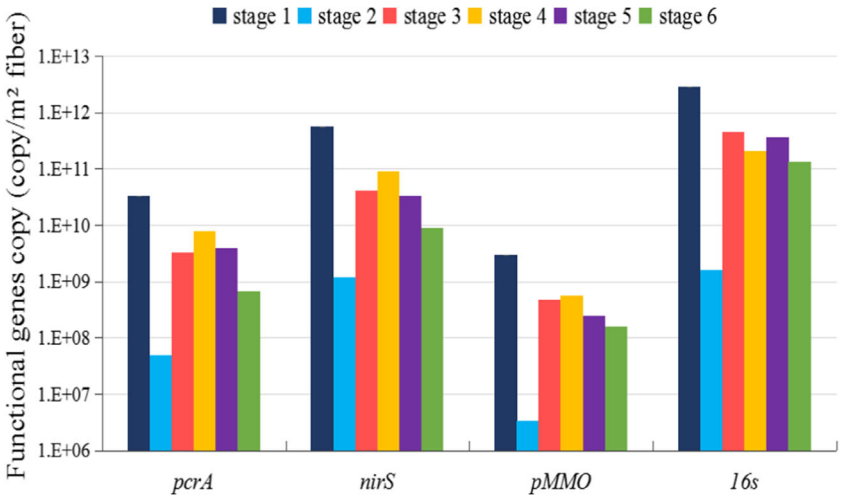

Fig. 3 The functional genes (*pcrA*, *nirS*, *pMMO*, *mcrA*) and *16S rRNA* gene abundances in different stages. Abundance refers to gene copy numbers per square centimeter fiber

drop in nitrate and perchlorate bioreduction. All the functional genes increased in stages 3 and 4, concurrent with the removal of NaCl, and decreased in stages 5 and 6 when 0.2 % salinity was added to the influent. Throughout the whole experiment, the functional gene abundance correlated very well with the salinity.

Microbial community structure

We analyzed the diversity and structure of the MBfR microbial communities using pyrosequencing of the 16S rRNA gene from the MBfR biofilm samples. Figure 4 shows the relative abundance of dominant microbial phylotypes at the genus level, while Fig. S1 shows the class level. *Methylomonas* was the dominant (31.3 %) bacterium in stage 1. Its abundance sharply decreased to 5.9 % in stage 2 when 1 % salinity was introduced and remained at low levels in the latter stages. *Methylomonas* are known methanotrophs and sensitive to salinity. For example, Sherry et al. (2016) reported the disappearance of *Methylomonas* when NaCl was higher than 1 g/L. Cunliffel et al. (2008) analyzed samples from surface (lower salinity) and subsurface (higher salinity) water of the Blyth estuary and found that *Methylomonas* was present in the surface samples but not in subsurface samples. In our MBfR, *Methylococcus* emerged in stage 2 (1.1 %), and its abundance continued to increase in the rest of stages. *Methylococcus* grows on CH_4 under low O_2 concentrations (Ward et al. 2004). Some studies show that *Methylococcus* is highly abundant in coastal saline soil (Mishra et al. 2012; Yousuf et al. 2012). Another methanotroph, *Methylocystis*, was present at a low abundance (2.1 %) in stage 1 but increased to ~10 % in stage 2 at 1 % salinity and further increased to 21.3 % in stage 3 when the salinity was removed. Its abundance decreased to ~10 % in stage 4 when nitrate was

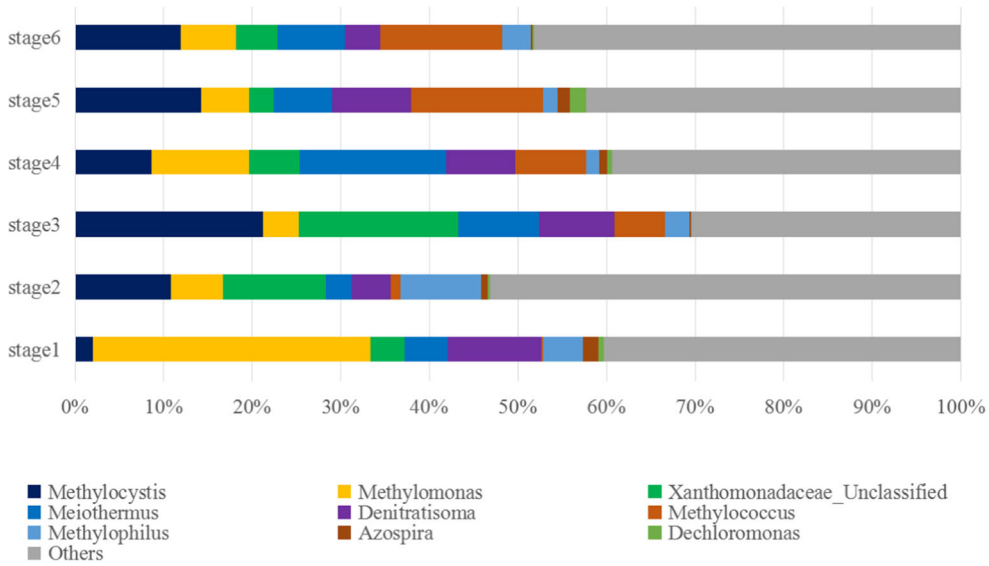

Fig. 4 Clustering based on relative abundance analysis of the microbial community structure at the genus level

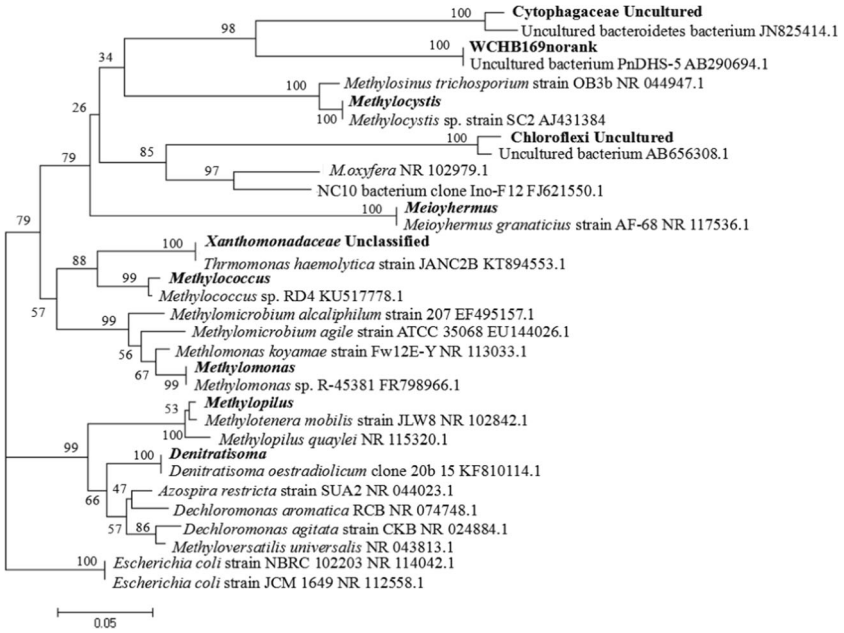

Fig. 5 Phylogenetic analysis of the dominant bacterial genera in the MBfR biofilm and two from outgroups

absent and perchlorate was the only electron acceptor. *Methylomonas* and *Methylocystis* reduce NO_3^- using O_2 to activate CH_4 (Dam et al. 2013; Kits et al. 2015).

Methylophilus (Betaproteobacteria) abundance reached ~9 % in stage 2 but remained at a low abundance in other stages. Giri et al. (2013) reported that *Methylophilus* sp. grow well at a NaCl concentration ≤200 mM. *Methylophilus* is phylogenetically close to known methanol assimilation denitrifiers. Figure 5 shows that *Methylophilus* is phylogenetically close to *Dechloromonas agitata*, *Dechloromonas aromatica*, and *Azospira restricta*, which are all known PRB. Therefore, *Methylophilus* probably played an important role in the reduction of NO_3^- and ClO_4^- under salinity stress. *Denitratisoma* abundance remained stable (~10 %) through all stages. It is phylogenetically close to the known denitrifier *Denitratisoma oestradiolicum*, which reduces NO_3^- to a mixture of N_2O and N_2 (Fahrbach et al. 2006). Unclassified *Xanthomonadaceae*, phylogenetically close to *Thermomonas haemolytica*, existed in all the stages and was significantly enriched in stages 2 and 3. It is possibly a denitrifier that can live under saline conditions. Mergaert et al. (2003) isolated two species of *Thermomonas* from a denitrifying reactor and reported that they could reduce NO_3^- and NO_2^-. Schmalenberger et al. (2013) reported the existence of *Xanthomonadaceae* in high salinity.

Figure S2 shows the PICRUSt analysis results of the functional genes. Genes encoding transporters (particularly ATP-binding cassette (ABC) transporters) and bacterial motility proteins were the most highly abundant. The abundance of these dominant genes decreased when the salinity increased in stage 2. Glycine betaine maintains osmotic pressure balance and bacterial morphology in salt environments, and ABC transporters are a member of the major betaine transporter family (Youssef et al. 2014; Silke et al. 2007). When NaCl appeared in stage 2, genes predicted to encode ABC transporters dropped to the lowest point. It is possible that the high salinity inhibited betaine absorption and caused changes in the microbial morphology and death, which explained the decreased NO_3^- and ClO_4^- reduction in stage 2.

Conclusions

In summary, salinity significantly affected the simultaneous ClO_4^- and NO_3^- reduction in a methane-based MBfR. Salinity at 1 % completely inhibited ClO_4^- reduction and significantly reduced NO_3^- reduction. Under saline conditions, the number of functional bacteria and total microorganisms decreased, wire-like cell structures became prevalent, and the cell surfaces developed wrinkles. In addition, the diversity of methanotrophs and denitrifying bacteria decreased significantly, and unclassified *Xanthomonadaceae* became more dominant when the salinity increased to 1 %. The saline environment changed cell osmotic pressure, which influenced the cell membranes and enzymes.

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