The short- and long-term effects of environmental conditions on anaerobic methane oxidation coupled to nitrite reduction

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ABSTRACT

Anaerobic oxidation of methane coupled to nitrite reduction (n-damo) plays an important role in global carbon and nitrogen cycles and also is a potential bioprocess in wastewater treatment. In this work, the effects of environmental conditions — temperature, pH and salinity — on the metabolic activity and growth rate of n-damo bacteria were investigated by short-term batch test and long-term bacterial incubation. Quantitative PCR and 16S rRNA and pmoA gene sequencing were applied to detect the microbial community in the long-term incubation. The results indicated that all the three environmental factors significantly affected the metabolic activity and growth rate of n-damo bacteria and the optimum temperature, pH and salinity were 35°C, 7.6 and 0 g NaCl L⁻¹, respectively. Notably, salinity adaption of n-damo bacteria was first observed under salinity stress of 20 g NaCl L⁻¹. It's predicted that n-damo process might occur in saline environments and future work could focus on this.

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1. Introduction

Anaerobic oxidation of methane (AOM) coupled to denitrification is a novel bioprocess (Raghoebarsing et al., 2006) and includes two sub-bioprocesses, namely AOM coupled to nitrite reduction (NIR) (Eq. (1)) and AOM coupled to nitrate reduction (NAR) (Eq. (2)). AOM coupled to NIR was widely named as nitrite-dependent anaerobic methane oxidation (n-damo) in previous literature, and it is mediated by ‘Candidatus Methylomirabilis oxyfera’ (Methylomirabilis oxyfera) bacteria in affiliation with the uncultured ‘NC10’ phylum (Ettwig et al., 2009, 2010). Recently, it was demonstrated that archaea from ANME-2d population is responsible for AOM coupled to NAR, which anaerobically oxidizes methane through reverse methanogenesis using nitrate as the terminal electron acceptor (Eq. (2)) (Haroon et al., 2013; Shi et al., 2013). M. oxyfera-like bacteria have been detected in some natural habitats and contribute largely to the removal of methane (the second most important greenhouse gas) (DeutzmannSchink, 2011; Hu et al., 2014b; Shen et al., 2014b). Moreover, it was deemed that n-damo is a potential process to treat nitrogenous pollutants in wastewater (Hu et al., 2014a; Luesken et al., 2011; Shen et al., 2012) or dissolved methane in anaerobic digestion effluent (Kampman et al., 2012). However, for applying this process to treat actual wastewater, there are a lot of challenges should be...
overcome, such as shortening enrichment period, improving biological activity and strengthening process robustness. At present, optimizing culture conditions is an important work to improve n-damo activity and reduce the doubling times of n-damo bacteria.

\[
\begin{align*}
3\text{CH}_4 + 8\text{NO}_3^- + 8\text{H}^+ &\rightarrow 3\text{CO}_2 + 4\text{N}_2 \\
+ 10\text{H}_2\text{O} &\quad \Delta G^\circ = -928\text{kJ mol}^{-1}\text{CH}_4.
\end{align*}
\]

(1)

\[
\begin{align*}
\text{CH}_4 + 4\text{NO}_3^- &\rightarrow 4\text{NO}_2^- + \text{CO}_2 + 2\text{H}_2\text{O} \\
&\quad \Delta G^\circ = -503\text{kJ mol}^{-1}\text{CH}_4.
\end{align*}
\]

(2)

The environmental conditions are critical to microbial metabolism and growth, and the effects of environmental conditions (including temperature, pH and oxygen) on n-damo bacteria was referred in several previous works (Hu et al., 2009; Luesken et al., 2012; Zhu et al., 2012), but still not well understood. Enrichment culture containing M. oxyfera-like bacteria could be obtained at 22°C and 35°C, but failed at 45°C (Hu et al., 2009). Zhu et al. (2012) investigated the effect of pH (<7.4) on n-damo enrichment culture, and the activity tests showed that the culture preferred circumneutral conditions, but they didn’t test the effect of pH above 7.4. In addition, M. oxyfera has an intra-aerobic methane oxidation pathway (Ettwig et al., 2010), but both activities of AOM and NIR of n-damo culture were instantly inhibited when adding 2% and 8% of oxygen in headspace (Luesken et al., 2012). It is generally recognized that AOM coupled to sulfate reduction occurs in marine and n-damo in freshwater, but 16S rRNA genes of ‘NC10’ phylum and pmoA genes were also detected in saline water, including saline lake (Yang et al., 2012), estuary (Shen et al., 2014b) and the South China Sea (Chen et al., 2014). The findings of the gene sequences implied that n-damo process may also occur in saline water, but further evidences were needed, like n-damo activity in saline water.

To clarify the effects of environmental conditions and to identify the optimal conditions of n-damo bacteria, short- and long-term experiments were performed at varying temperature, pH and salinity. The short- and long-term periods were set according to the doubling times of n-damo bacteria, 14–25 days (He et al., 2013; Raghoebarsing et al., 2006). Three models – the extended Arrhenius equation, the modified Antoniou equation and Han-Levenspiel model – were applied to describe the short-long effects of temperature, pH and salinity, respectively, and the results could be the basis of the further modeling studies. Quantitative PCR (qPCR) and 16S rRNA and pmoA gene sequencing were applied to illustrate the change of microbial community in the long-term experiments. The effects of environmental conditions on n-damo bacteria were then clarified by combining the short- and long-term experimental results.

2. Materials and methods

2.1. Biomass and medium

The biomass was taken from a sequencing batch reactor (SBR) that had been reported in a previous literature (He et al., 2013). The original inoculum of the biomass was freshwater sediment from Tiesha river (30°15′59″N, 120°11′01″E) in the east of China. The 16S rRNA genes sequences of the M. oxyfera-like organisms in the initial biomass had identities of 96.0–96.6% to that of M. oxyfera. The relative abundance of M. oxyfera-like organisms in the initial biomass was about 60% of the population, estimated from the fluorescence in situ hybridization (FISH) image reported by He et al. (2013). The normal medium contained (per litre): 0.5 g KHCO₃, 0.2 g KH₂PO₄, 0.3 g CaCl₂, 2H₂O, 0.2 g MgSO₄.7H₂O, 0.5 ml of an acidic trace element solution and 0.2 ml of an alkaline trace element solution. The acidic and alkaline trace element solutions were prepared as Ettwig et al. (2009), and the initial pH was kept at 7.0 ± 0.1.

2.2. Short-term experimental setup

To assess the short-term effects of temperature, pH and salinity on the metabolic activity of n-damo bacteria (characterized by the conversion rate of nitrite), the biomass was incubated at 15, 20, 25, 30, 35, 40 and 45 °C; a pH of 6.0, 6.5, 7.0, 7.5, 8.0, 8.5 and 9.0; and a NaCl salinity of 0, 2, 5, 10, 15 and 20 g NaCl L⁻¹. The certain conditions were obtained by following modifications based on the normal medium and the standard protocol (30 °C, pH 7.0 and 0 g NaCl L⁻¹). The needed temperatures were obtained using temperature-controlled shaking tables. The pH was adjusted by adding 1.0 M NaOH or 1.0 M HCl solution until the needed pH was reached. The pH was jointly buffered by nutrients, KHCO₃ and KH₂PO₄ in the medium, and the acid-base titration curve of the medium was shown in Fig. S1. The certain salinities were prepared by proportionately mixing the normal medium and a NaCl added medium with a salinity of 50 g NaCl L⁻¹.

The short-term batch experiments were carried out in 76-ml serum bottles in triplicate. 50 ml biomass was directly taken from the SBR, loaded into each serum bottle, rinsed three times with the corresponding medium (containing 0.5 mM nitrite), and the final volume was controlled at 50 ml. Subsequently, the serum bottles were flushed with pure argon (99.999%) for 15 min, sealed with stoppers, and then the headspace was replaced with 19.5 ml pure methane (99.99%). The serum bottles were shaken at the corresponding temperatures (15, 20, 25, 30, 35, 40 and 45 °C) and 150 rpm. After 2.0 h of pre-incubation, 0.5 ml of liquid was sampled by syringes, centrifuged at 7440 × g for 5 min, and nitrite and nitrate were then measured in the supernatant liquid every 2.0 h. The short-term experimental period was set at 10 h that was much shorter than the doubling times of n-damo bacteria (14–25 days). In addition, control groups without methane were set up under same conditions.

2.3. Long-term incubation procedure

To assess the long-term effects of temperature, pH and salinity on the incubation of n-damo bacteria, the biomass was incubated for 90 days (much longer than the doubling times of n-damo bacteria) at 30 °C, pH 7.0 and 0 g NaCl L⁻¹ (group CK); 35 °C, pH 7.0 and 0 g NaCl L⁻¹ (group T); 30 °C, pH 7.6 and 0 g NaCl L⁻¹ (group pH); and 30 °C, pH 7.0 and 20 g NaCl L⁻¹ (group S) in duplicate. Eight 180-ml serum bottles were taken and each was loaded with 120 ml biomass from the
SBR. The serum bottles were deoxygenated and sealed as the short-term experiments above, added with pure methane of 45 ml, and then incubated at 150 rpm and 30 °C or 35 °C. Every three days (one cycle), 20 ml supernatant liquid was exchanged with the corresponding fresh medium, nitrite and nitrate were measured before and after liquid exchange, and 45 ml methane was replaced in the headspace. The biomass was settled 4 h for liquid exchange. The concentration of nitrite in the liquid was controlled at 0–1.0 mM by adding stock solution with the nitrite concentration of 120 mM in the whole experimental period. The initial pH (before cycles) of the medium in the serum bottles was adjusted at 6.7–7.0 (for groups CK, T and S) or 7.3–7.6 (for group pH) using 1.0 M NaOH or 1.0 M HCl solution, the final pH values (after cycles) fell into 7.0–7.3 or 7.6–7.9, for base production, and the average pH values were kept close to 7.0 or 7.6 (see Fig. S2 for detailed information). The procedure of the long-term incubation was expressed by a flow chart, Fig. S3. Before and after the 90-day incubation, 2 ml sludge was collected for molecular analysis.

To determine the activities of n-damo and heterotrophic denitrification precisely in the long-term incubation, activity tests (activity tests 1, 2 and 3) were carried out in the original 180-ml serum bottles before, during and after the 90-day incubation (indicated in Fig. 2). The nitrite stock solution (120 mM nitrite) was added to final 1.0 mM nitrite concentration. The bottles were deoxygenated, sealed and incubated as the description above. After 6 h of pre-incubation, 0.5 ml of liquid was sampled to determine the nitrite and nitrate concentrations every 12 h. After 36 h of incubation without methane, 1.0 ml pure methane was injected into the bottle (the final methane partial pressure was about 2.8 kPa). After methane addition, the methane concentration in headspace was measured in triplicate every 12 h.

2.4. Chemical and microbial analysis

Nitrite, nitrate and volatile soluble solid (VSS) were measured according to the APHA standard methods (APHA, 2005). The methane in the headspace was determined by Agilent 6890 gas chromatography (Agilent, United States) equipped with a flame ionization detector (FID) and a Carbonplot capillary column (Ø 0.53 mm, 30 m length) as previously described (Liebner Wagner, 2007). The pH was measured with an FE20 pH meter (Mettler-Toledo, Shanghai). The abundance of the 16S rRNA genes of ‘NC10’ bacteria was determined by qPCR using primer set qP1F/qP1R as previously described (Ettwig et al., 2009). 16S rRNA and pmoA gene sequencing and phylogenetic analysis were performed as described by Hu et al. (2014a).

2.5. Calculation and estimation methods

The nitrite conversion rate without methane in headspace was assumed to be the activity of heterotrophic denitrification, and that with methane in headspace was deemed to be the sum of the activities of heterotrophic denitrification and n-damo
The activity of n-damo bacteria in the short-term batch experiment was estimated from the nitrite conversion rate in experimental group (with methane of about 75 kPa) minus that in the corresponding control group (without methane). The activity of n-damo bacteria in the long-term activity test was calculated by subtracting the decrease rate of nitrite in the methane-free phase from that in the methane phase (with 75 kPa or 2.8 kPa methane in headspace). The n-damo activity of group S was also calculated from the consumption rate of methane in headspace and its value was corrected with the increase of headspace volume due to liquid sampling. The experimental data were plotted and fitted by Origin 8.0 (OriginLab, United States).

3. Results

3.1. Short-term effects of temperature, pH and salinity

The n-damo activity was observed at a temperature from 15 °C to 45 °C, pH from 6.0 to 9.0 and NaCl salinity from 0 to 15 g NaCl L⁻¹, but almost no n-damo activity was observed at NaCl salinity of 20 g NaCl L⁻¹. The maximum n-damo specific activity was 4.1 ± 0.3 μmol NO₂⁻ h⁻¹ g⁻¹ VSS obtained at 35 °C, pH 7.0 and 0 g NaCl L⁻¹ among all the tested environmental conditions.

The n-damo specific activity at varying temperature was plotted in Fig. 1a. The specific activity increased exponentially at low temperatures (<35 °C), and sharply decreased at high temperatures (>35 °C). The correlation between the n-damo specific activity and temperature was fitted by the extended Arrhenius equation, Eq. (3) (derived in supplementary material), and the Adj. R-Square was 0.988. The parameters in Eq. (3), k_a, k_d, E_a, and E_d, can be determined by the best fitting of the data in Fig. 1a, and their values were 33 ± 3, 157 ± 1, 80 ± 7 kJ mol⁻¹ and 410 ± 1 kJ mol⁻¹, respectively. Based on these parameter values, the optimum temperature was calculated by Eq. (4) (derived in supplementary material) and its value was 35.5°C. Moreover, Q₁₀ value (proportional change in specific activity per 10 °C change) was calculated to be 2.8 ± 0.2 from the first 5 data in Fig. 1a.

\[
 r = \frac{r_{\text{unit}} \exp(k_a - E_a/RT)}{1 + \exp(k_d - E_d/RT)}; \tag{3}
\]

where \( r \) is the n-damo specific activity (μmol NO₂⁻ h⁻¹ g⁻¹ VSS), \( r_{\text{unit}} \) is the unit of specific activity (1 μmol NO₂⁻ h⁻¹ g⁻¹ VSS) that is introduced to make the equation dimensionally consistent, \( R \) is the universal gas constant (8.314 J K⁻¹ mol⁻¹), \( T \) is the Kelvin thermodynamic temperature (K), \( k_a \) and \( k_d \) are dimensionless constants (⁻), and \( E_a \) is the activation energy of n-damo bioreaction (kJ mol⁻¹), and \( E_d \) is the activation energy about enzyme inactivation (kJ mol⁻¹).

\[
 T_{\text{opt}} = \frac{E_d}{k_d + \ln(E_d/E_a - 1)}; \tag{4}
\]

where \( T_{\text{opt}} \) is the optimum temperature at which the specific activity reaches its maximum value (K).

Fig. 1b presents the n-damo specific activity at varying pH (from 6.0 to 9.0). As the pH increased, the n-damo specific activity increased at low pH (<7.5), but decreased at high pH (>7.5), and reached its maximum value at 7.5 among the tested pH values. The data were fitted by the modified Antoniou equation, Eq. (5) (derived in supplementary material), and the

\[
 r = \frac{r_{\text{unit}} \exp(k_a - E_a/RT)}{1 + \exp(k_d - E_d/RT)}; \tag{5}
\]
The total nitrite conversion rates of the long-term incubation are shown in Fig. 2. In overall, the total nitrite conversion rates of groups CK, T and pH increased during the 90-day operation period. Group T had the highest nitrite conversion rate and was followed successively by groups pH, CK and S. Group S went through three phases: stability at low level (day 0–6), linear increase (day 6–30) and stability at high level (day 30–90). The long-term activity tests 1, 2 and 3 are marked in Fig. 2 and their results are presented in Table 1. The activities of total denitrification and heterotrophic denitrification were obtained by linear fitting of data from activity tests 1, 2 and 3, and the activities of n-damo were calculated from the activities of total and heterotrophic denitrification. The n-damo activities increased consistently in all the groups during the 90-day incubation. Group T had the highest n-damo activity and was followed by group pH and group CK. For group S, the n-damo activity could hardly be detected at the very start, but it was significant (0.69 ± 0.10 μmol L⁻¹ h⁻¹) at the end of the 90-day incubation. The activities of heterotrophic denitrification were stable in group CK and group pH, but increased significantly in group T and group S. The heterotrophic denitrification activity in group T was much higher than those in group CK and pH, and that in group S increased sharply in the first 45 days (from activity test 1–2) and decreased slightly in the next 45 days (from activity test 2–3).

Fig. 3 shows the results of the activity tests S1 and S2 of group S. The methane consumption rates were 0.002 μmol CH₄ h⁻¹ in activity test S1 and 0.029 μmol CH₄ h⁻¹ in activity test S2. The nitrite conversion rates of heterotrophic and total denitrification in activity test S2 were 0.215 and 0.282 μmol NO₂⁻ h⁻¹, respectively. Thus, the nitrite conversion rate of n-damo in activity test S2 was 0.067 μmol NO₂⁻ h⁻¹, while no n-damo activity was detected in activity test S1. The ratio of methane consumption rate to nitrite conversion rate for n-damo in activity test S2 was calculated to be 3.46:8, close to the theoretical stoichiometric ratio of 3:8 (Eq. (1)).

The total biomass concentration (characterized by VSS) and the abundance of n-damo bacteria in the biomass (characterized by copies of 16S rRNA genes of ‘NC10’ bacteria) were quantified before and after 90-day incubation (Table 2). The results showed that both of the total biomass and the n-damo bacteria increased significantly (Table 2).
damo bacterial abundance of groups CK, T and pH increased after 90 days, but those of group S decreased. Group T produced more biomass than group CK, but the abundance of n-damo bacteria was lower. For both of the total biomass and the abundance of n-damo bacteria, group pH went near to group CK.

Phylogenetic trees of the ‘NC10’ phylum 16S rRNA gene and the pmoA gene sequences from the initial biomass (before 90 days) and group S (after 90 days) were constructed (Fig. 4a and b), and it was confirmed that M. oxyfera-like bacteria existed in the biomass of group S. The sequence identities of 16S rRNA gene and the pmoA gene sequences between M. oxyfera (FP565575) and the M. oxyfera-like bacteria obtained from group S were 96.2–97.4% and 84.7–88.2%, respectively. Further, no significant divergences occurred between the sequences from the initial biomass and group S (identities of 97.9–99.8% for 16S rRNA gene and 86.6–99.7% for pmoA gene). Moreover, phylogenetic trees of the gene sequences from groups CK, T and pH were constructed in Fig. S5, and there were also no significant divergences between the sequences obtained before and after 90-days incubation.

4. Discussion

4.1. Effect of temperature on n-damo process

The n-damo activity of the short-term experiments was optimal at around 35 °C, which implied that n-damo bacteria were mesophilic bacteria. The results of the long-term experiments also showed that the n-damo bacteria performed better at 35 °C than at 30 °C. Hitherto n-damo culture was most enriched at 22–30 °C (Luesken et al., 2011; Raghoebarsing et al., 2006; Shi et al., 2013), but 35 °C might be a better temperature of n-damo bacterial incubation according to this work. Although n-damo bacteria are still active at 45 °C with a specific activity of 0.54 ± 0.05 μmol NO₂⁻ h⁻¹ g⁻¹ VSS, Hu et al. (2009) failed to obtain n-damo biomass at 45 °C, probably because one or more essential cell components, such as a key enzyme, became so damaged at a high temperature of 45 °C that the cells lost the ability of growth (Madigan et al., 2010).

The apparent activation energy of n-damo bioreaction was 80 ± 7 kJ mol⁻¹ (Q10 value was 2.8 ± 0.2) from Fig. 1a, which showed n-damo process were much more temperature dependent than heterotrophic denitrification. The activation energy of heterotrophic denitrification growth on nitrite was reported as 70.5–71.9 kJ mol⁻¹ (Isaka et al., 2012). Methane is one of the least reactive organic molecules, and aerobic methanotrophs should overcome its high barrier energy by a reaction with molecular oxygen (Ettwig et al., 2010), which leads to a high activation energy. From a view of the valence bond theory, methane oxidation has a high activation energy because of a high dissociation energy of C–H bond of methane (439.3 ± 0.4 kJ mol⁻¹), and that of methanol, a typical substrate of heterotrophic denitrification, was only 401.9 ± 0.6 kJ mol⁻¹ (Bond dissociation energies were taken from ‘CRC Handbook of Chemistry and Physics’ (Haynes, 2011)).

These results suggested a high temperature (like 35 °C) benefited n-damo bacteria within their adapted temperature range. But a high temperature also promoted the activity of heterotrophic denitrification, leading to a lower n-damo percentage at 35 °C (Table 1). On one hand, a high temperature (35 °C) directly activated heterotrophic denitrifying bacteria, like n-damo bacteria mentioned above. On the other hand, a high temperature increased the decay rate of biomass and then increased the concentrations of decay productions in the systems, and the biomass decay productions were the possible carbon and energy sources for heterotrophic denitrifying bacteria, which would lead to the increase of heterotrophic denitrification activity in the long-term group T (Table 1). Frankly, the apparent activation energy was underestimated for the decrease of methane solubility in water as temperature increase. But the error was negligible, because
the influence of this aspect was estimated to be only about 4% (temperature changes from 15 to 45 °C), calculated based on Henry’s constant of methane (Haynes, 2011) and methane affinity of n-damo bacteria (He et al., 2013).

4.2. Effect of pH on n-damo process

The optimum pH was around 7.6 from the best fitting in Fig. 1b with the modified Antoniou equation, although the biomass was cultivated at pH 7.0–7.2 for a long time (He et al., 2013). N-damo bacteria might be favored at weakly alkaline conditions, like most heterotrophic denitrifying bacteria (optimum pH was 7.0–9.0 (Tang et al., 2011)). A previous work of pH effect on n-damo enrichment culture had been carried out by Zhu et al. (2012), but they only investigated a narrow pH range of 5.9–7.4 in which the n-damo activity increased continuously and optimum pH value wasn’t obtained, while this work supplied complete information (pH 6.0–9.0) including the part of activity decrease at high pH values. Previous n-damo cultures were most incubated in a pH range of 6.8–7.6 (Ettwig et al., 2009; Luesken et al., 2011; Shi et al., 2013), and the results of this work suggested pH around 7.6 was better than 7.0. In addition, the different pH (7.0 and 7.6) didn’t change the percentage of n-damo activity (Table 1) and the abundance of 16S rRNA genes (Table 2), probably because of the similar effect of pH on n-damo and heterotrophic denitrification. A pH range of 7.0–8.0 is suitable to incubate n-damo bacteria according to the results in Fig. 1b.

Both of n-damo and heterotrophic denitrification associated with base production (see Eq. (1) and Eq. S17), and our previous research (unpublished) showed that the pH value of culture solution reached to about 9.0 after several weeks of batch operation without pH-control. The activity of n-damo bacteria would be inhibited significantly by a high pH of 9.0, so it is necessary to adjust the pH back to neutral values. There are three candidate methods to control pH in bioreactors: to
add a certain HCl in the influent to balance the base production; to automatically add HCl or NaOH (or NaHCO₃) stock solution in the bioreactor directly with a pH sensor in the culture; and to add CO₂ gas in the bioreactor to control the pH with carbonic acid (Tang et al., 2011). As to n-damo process, the third one, adding CH₄–CO₂ mix gas into the bioreactor, should be a perfect way to control the pH gently. The added CO₂ could be used not only to lower the pH value, but also as a direct carbon source of n-damo bacteria that had been proved to fix CO₂ via the Calvin-Benson-Bassham cycle very recently (Rasigraf et al., 2014).

4.3. Effect of salinity on n-damo process

There was no surprise that the optimum salinity was 0 g NaCl L⁻¹, since the n-damo culture was enriched from freshwater sediment. The n-damo activity was inhibited at high NaCl salinities, and this was probably due to high osmotic pressure caused by high salt concentration (Jin et al., 2012). The metabolic enzymes in the periplasm, like nitrite reductase (Nit), NO dismutase (NOD) and methanol dehydrogenase (MDH) (Wu et al., 2011), could also be damaged directly for the salting-out effect (Zhao et al., 2013). Moreover, the methane solution was lower in saline water than in freshwater, which aggravated the nutritional conditions and further reduced the n-damo activity. In general, salinity was an important impact factor of n-damo bacteria, as it strongly inhibited the n-damo activity and decreased n-damo bacterial abundance.

Unexpectedly, the biomass re-obtained n-damo activity after 90 days’ incubation under NaCl salinity stress of 20 g NaCl L⁻¹, in spite of a very low n-damo activity of 0.69 ± 0.10 μmol L⁻¹ h⁻¹. Before this work, there were no reports of n-damo activity under salinity stress condition, to our knowledge. Fig. 2 and Table 1 showed that heterotrophic denitrification activity began to recover after 6 days, mainly because of the gradual salinity adaption of the microorganisms (Osaka et al., 2008) and the biomass decay productions may be reused as their carbon and energy sources. Subsequently, the heterotrophic denitrification activity stopped increase after 30 days, and this was mainly due to the deficiency of organic substrate as no any organic matter added in the influent (the biomass decay productions might be no longer enough to support the fast growth of heterotrophic denitrifying bacteria). Similarly, n-damo bacteria may adapt gradually to the environment of high salinity and presented a feeble activity after 90-day incubation. Compared with heterotrophic denitrifying bacteria, n-damo bacteria were more salinity sensitive for their lower activity and slower recovery. The results of the ‘NC10’ phylum 16S rRNA gene and pmoA gene sequences confirmed that M. oxyfera-like bacteria also existed in group S after 90 days and had no significant divergences with those in the initial biomass (before 90 days) (Fig. 4). Therefore, the increase of n-damo activity could be attributed to the acquisition of salinity resistance of M. oxyfera-like organisms in group S, and these salt-adapted M. oxyfera-like bacteria should be responsible for the n-damo activity in group S after 90 days. To our knowledge, it’s the first observation and discussion of salinity adaption of n-damo bacteria.

Although M. oxyfera-like bacteria were most discovered in freshwater ecosystems (DeutzmannSchink, 2011; Hu et al., 2014b; Shen et al., 2012, 2014a), several 16S rRNA and pmoA genes of M. oxyfera-like bacteria were also detected in saline habitats (Chen et al., 2014; Shen et al., 2014b; Yang et al., 2012). Combined with the previous researches, this work suggested that M. oxyfera-like bacteria could adapt to a certain extent of salinity (like 20 g NaCl L⁻¹) and n-damo process may occur in saline environments, in spite of growth better in freshwater. For biotechnological application, the salt-tolerant M. oxyfera-like bacteria have promising potential in the treatment of brine wastewater containing nitrogenous pollutants, like pickle wastewater and seafood processing wastewater, although there is a long way to go to apply n-damo process to treat these wastewaters.

5. Conclusion

- This work indicated that n-damo bacteria were significantly influenced by temperature, pH and salinity, and their short-term effects could be described well by the extended Arrhenius equation (derived in this work), the modified Antoniou equation and Han-Levenspiel model, respectively.
- The optimum temperature, pH and salinity were 35 °C, 7.6 and 0 g NaCl L⁻¹, respectively, which were estimated from the short-term experiments and subsequently validated in the long-term experiments.
- At a salinity of 20 g NaCl L⁻¹, salinity adaption of n-damo bacteria was first observed in this work. The results suggested that n-damo process probably occurred in saline ecosystems, combined with the previous ecological researches.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2014.09.055.

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