

Response of ammonia-oxidizing betaproteobacteria to short-term fertilization in a salt marsh in China*

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Abstract This study examines the impacts of short-term (6 months) fertilization on the community structure and abundance of ammonia-oxidizing betaproteobacteria (β -AOB) and the potential nitrification rate in sediment colonized by *Suaeda heteroptera* in a saltmarsh located in Shuangtai estuary, China. The sediment samples were collected from plots treated with different amounts of an N fertilizer (urea supplied at 0.1, 0.2, 0.4, and 0.8 g/kg (nitrogen content in dry sediment)), and with different forms of N fertilizers (urea, $(\text{NH}_4)_2\text{SO}_4$, and NH_4NO_3 , each supplied at 0.2 g/kg). The fertilizers were applied 1–4 times during the plant-growing season in May, July, August and September of 2013. Untreated plots were included as a control. As revealed in denaturing gradient gel electrophoresis of the 16S rRNA gene, the β -AOB community responded to both the amount and form of N. Real-time quantitative PCR indicated that both abundance and potential nitrification rate of β -AOB increased after N addition, regardless of concentration and form (except NH_4NO_3). These results provide evidence that short-term N application influences the sediment β -AOB community, β -AOB abundance and potential nitrification rate in a saltmarsh ecosystem.

Keyword: ammonia-oxidizing betaproteobacteria (β -AOB); denaturing gradient gel electrophoresis (DGGE); nitrification; fertilization; saltmarsh

1 INTRODUCTION

Coastal salt marshes provide a number of services to ecosystems, including the interception of derived nitrogen (N) before it reaches nearshore oceans (Nelson and Zavaleta, 2012). Human alteration of the N cycle has accelerated the transfer of N through rivers to coastal areas (Vitousek et al., 1997). By intercepting the excess nutrients, salt marshes mitigate eutrophication in coastal areas (Valiela and Cole, 2002; Vivanco et al., 2015).

One of the most important processes in the marine N cycle is nitrification, a two-step process that first oxidizes ammonia to nitrite via the intermediate hydroxylamine; then oxidizes the nitrite to nitrate. The first step, ammonia oxidation, is the rate-limiting step and is executed by autotrophic ammonia-oxidizing bacteria (AOB) and ammonia-oxidizing

archaea. AOB are restricted to two monophyletic clusters within the beta- and gamma-proteobacteria (β -AOB and γ -AOB) (Head et al., 1993; Purkhold et al., 2000). β -AOB are thought to be more abundant than γ -AOB in marine sediments (Nold et al., 2000). Environmental factors clearly influence the nitrification, abundance and composition of ammonia-oxidizers in salt marshes. Dollhopf et al. (2005) found that high concentrations of Fe (III) and macrofauna burrowing activity influenced the abundance of β -AOB and nitrification in a Georgia salt marsh dominated by *Spartina alterniflora*. In the

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Wequetequock-Pawcatuck tidal marsh of southeastern Connecticut, Moin et al. (2009) observed a significant salinity effect on β -AOB abundance at sites dominated by short or tall *S. alterniflora* or *S. patens*, but detected no γ -AOB 16S rRNA genes at these sites. Lage et al. (2010) and Peng et al. (2012) investigated the impact of fertilization on β -AOB. They identified the changes in β -AOB community after short-term (2 years) N addition in the saline Scarborough Marsh (which is dominated by *S. patens*), and after long-term (nearly 40 years) NPK application in the Great Sippewissett Marsh (which is dominated by *S. alterniflora*, *S. patens* or *Distichlis spicata*). The β -AOB abundance remained unaffected in both marshes. Conversely, in grassland soil systems, the β -AOB abundance reportedly increases after N addition (Shen et al., 2011; Chen et al., 2014; Tian et al., 2014).

The Shuangtai estuary wetland, located north of Liaodong Bay (which forms part of the innermost gulf of the Bohai Sea in northeast China), was listed as a wetland of international importance in 2004. Bai et al. (2010, 2011) found that nitrification was largely affected by ammonia N and total phosphorus content, and that soluble salt, total N and organic matter greatly influenced the quantity of AOB in sediments of the Liaohe estuary wetland. Moreover, high salinity significantly reduced the number and diversity of AOB in a reed wetland of Liaohe estuary (Zhong et al., 2013). The ecosystem of the Shuangtai estuary wetland receives increasing nitrogen inputs from anthropogenic sources, such as wastewater and N fertilizer application (Yi, 2013). However, how the β -AOB respond to N enrichment in this ecosystem has not been reported. In the present study, we investigated whether different levels and forms of N addition alter the composition and abundance of β -AOB in Shuangtai estuary, and the potential nitrification rate in a saltmarsh of this estuary, at a site dominated by *Suaeda heteroptera*.

2 MATERIAL AND METHOD

2.1 Experimental design

The experiment was conducted in a saltmarsh located in Shuangtai estuary, China. The dominant plant in the study area ($\sim 4\,800\text{ m}^2$) was *S. heteroptera*. We selected six fertilization treatments and an unfertilized control. Each treatment was applied to three replicate plots. At each plot, a polyvinyl chloride (PVC) plastic pipe (inner diameter 15 cm; height 20 cm) was driven into the sediment, leaving 2–3 cm

of pipe above the ground. Each pipe contained approximately 15 strains of seedlings. The plots were treated with (a) different amounts of a single N fertilizer (urea: 0.1 (A1), 0.2 (A2), 0.4 (A3), and 0.8 g/kg (A4)) and (b) different forms of N fertilizers (urea (B1=A2), $(\text{NH}_4)_2\text{SO}_4$ (B2), and NH_4NO_3 (B3), each applied at 0.2 g/kg). Treatments were applied 1–4 times during the plant-growing season (on May 15, July 4, August 14 and September 26 of 2013). Untreated plots (A0=B0) were included as controls. Each N amendment was blended in 50 mL of water and injected into the appropriate pipes using a spinal needle. An equivalent volume of deionized water was injected into the control pipes.

2.2 Sediment sampling

Before the nitrogen application, sediment samples (10–15 cm) were collected from each PVC pipe on July 4, August 14, September 26, and November 22 of 2013, placed in sealed plastic bags, and labeled. All samples were kept on ice in the dark during transportation to the laboratory, then stored at -20°C until required for denaturing gradient gel electrophoresis (DGGE) and real-time quantitative PCR (qPCR) analysis.

2.3 β -AOB community analysis by DGGE

The community DNA was extracted from 0.6 g of the pooled samples from the three replicate plots using a fast genomic DNA isolation kit for soils (Shanghai Sangon Biological Engineering Technology and Services Company Limited, Shanghai, China) following the manufacturer's instructions. The β -AOB 16S rRNA gene was detected by a nested PCR strategy. The first amplification was performed using β AMO primers (McCaig et al., 1994) in a 50- μL reaction mixture containing 5 μL of 10 \times PCR buffer, 3 μL of template, 200 $\mu\text{mol/L}$ of dNTP, 1.5 mmol/L of MgCl_2 , 0.4 $\mu\text{mol/L}$ of each primer and 0.5 U *Taq* of polymerase. The PCR procedure was 5 min of initial denaturation at 94°C , 30 cycles of denaturation (30 s) at 94°C , annealing (30 s) at 55°C and primer extension (90 s) at 72°C , followed by a final extension at 72°C for 5 min. The secondary amplification was performed using CTO189f-GC and CTO654r primers (Kowalchuk et al., 1997) in 50 μL of a reaction mixture with the above-described composition. The procedure was 5 min of initial denaturation at 94°C , 20 cycles of denaturation (1 min) at 94°C , 1 min of initial annealing at 65°C (decreasing by 0.5°C per cycle to a touchdown temperature of 55°C), and extension (1 min) at 72°C ; this was followed by 10 cycles of denaturation (1 min)

at 94°C, annealing (1 min) at 55°C, extension (1 min) at 72°C, and a final extension at 72°C for 10 min. The amplification products were analyzed by electrophoresis in 1% (w/v) agarose gel containing GoldView.

The sequence-specific separation of the PCR products was performed by a Dcode™ Universal Mutation Detection System (Bio-Rad Laboratories Inc., Hercules, CA, USA). The PCR samples were applied onto 6% (w/v) polyacrylamide gels in a running buffer (1% tris-acetate-EDTA). The gels were prepared with a denaturing urea and formamide gradient of 45% to 55%, and a polyacrylamide and bis-acrylamide ratio of 37.5:1. Electrophoresis was run at 70 V and 60°C for 16 h. Subsequently, the gels were stained with Genfinder according to the manufacturer's instructions and photographed under UV transillumination. The DGGE fingerprint patterns were analyzed by Quantity One Software (Bio-Rad Laboratories). The differences between the DGGE patterns and dendrograms were determined by presence-absence matrices constructed using the Dice coefficient and the unweighted pair-group method with arithmetic average algorithms. These analyses were performed in the NTSYS software package (version 2.10e, Exeter Software, Setauket, New York).

Bands selected from the DGGE gel were eluted and reamplified with the same primers but omitting the GC-clamp. The PCR products were sequenced by Shanghai Sangon Biological Engineering Technology and Services Company Limited (Shanghai, China). The sequences have been submitted to GenBank under NCBI accession numbers KT259283–KT259292, KT259294–KT259305, KT280472–KT280473 and KX819277–KX819292. They were aligned using the ClustalW program and compared with other AOB 16S rRNA gene sequences available in the NCBI database of GenBank (<http://www.ncbi.nlm.nih.gov/BLAST>). The neighbor-joining tree was constructed using MEGA 4 based on the Kimura-2-parameter distances and a bootstrap analysis of 1 000 re-samplings (Tamura et al., 2007).

2.4 Quantification of β -AOB *amoA* gene by qPCR

The genomic DNA was extracted from 0.3 g of sample using the fast genomic DNA isolation kit for soils (Shanghai Sangon Biological Engineering Technology and Services Company Limited, Shanghai, China) according to the instructions. qPCR was performed on a 7500 real-time PCR system (StepOne™, Applied Biosystems, Foster City, CA, USA) using Green-2-Go qPCR Mastermix. The

β -AOB *amoA* gene was amplified using an *amoA*-1F and *amoA*-2R primer set (Rotthauwe et al., 1997). The qPCR mixture (20 μ L) contained 10 μ L of Green-2-Go qPCR Mastermix (2 \times), 0.25 μ mol/L of each primer, and 2 μ L template DNA. All reactions were performed in 8-strip thin-well PCR tubes with ultraclean cap strips. The qPCR thermocycling parameters were as follows: initial denaturation at 95°C for 10 min, followed by 40 cycles of denaturation at 95°C for 15 s and primer annealing at 60°C for 60 s. The copy number of the standard plasmids carrying the *amoA* genes (constructed by cloning the sediment samples) ranged from 10^2 to 10^6 . The qPCRs of the standards and DNA from each sample were performed in triplicate. The specificity of the qPCR amplification was determined from the melting curve. In all experiments, possible contamination was detected by qPCR of negative controls containing no template DNA. Gene abundance was normalized per gram of wet sediment.

2.5 Potential nitrification rates

Potential nitrification rate experiments were set up within 2 h of transportation to the laboratory. Samples of ~10 g wet sediment were transferred to 250 mL flasks containing 100 mL of NH_4Cl solution (25 mg N/L, salinity 5). All samples were incubated at 25°C with continuous shaking at 150 r/min. Nitrogen (nitrate-N+nitrite-N) in the subsamples was measured every 4 h from 0 to 24 h using an AutoAnalyzer 3 (Bran Luebbe, Norderstedt, Germany). Potential nitrification rates were calculated from the exponential phase of the increase in N (nitrate-N+nitrite-N) per gram of wet sediment over time.

2.6 Statistical analyses

Multiple comparisons and Pearson's correlations among the quantitative variables were performed using SPSS version 22.0. The quantitative differences between treatments were examined by Tukey's test. The β -AOB *amoA* abundance data were logarithm-transformed prior to analyses to alleviate heteroscedasticity. If the normality criteria were not met, the data were analyzed by nonparametric tests.

3 RESULT

3.1 Responses of β -AOB community to fertilization

The different amounts and forms of N treatment yielded different banding patterns (Fig.1). Forty bands

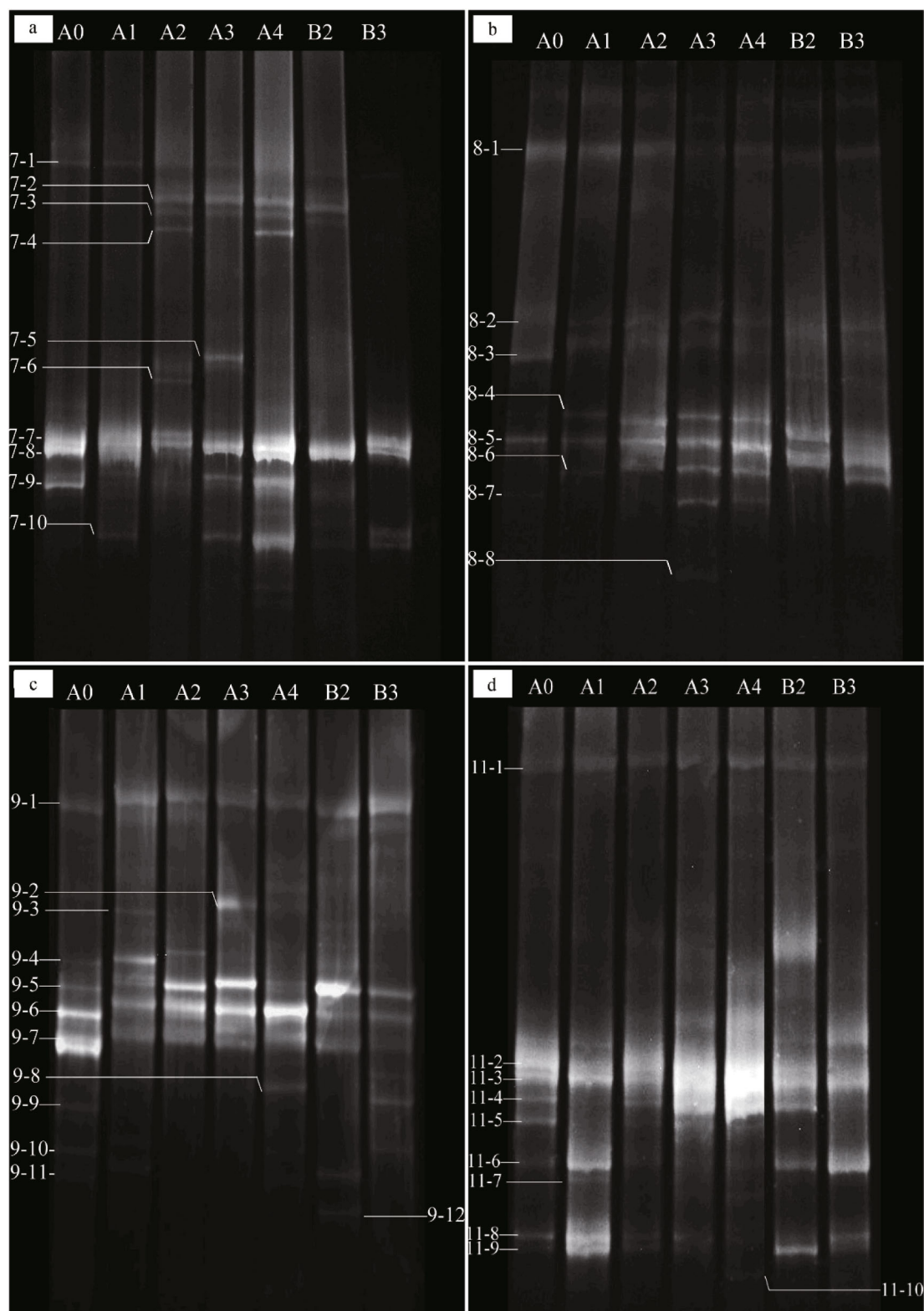


Fig.1 DGGE profile of nested PCR-amplified CTO fragments

Profiles a, b, c and d are derived from sediment samples pooled from three replicate plots collected on July 4, August 14, September 26, and November 22 of 2013, respectively. The marked bands were excised and sequenced.

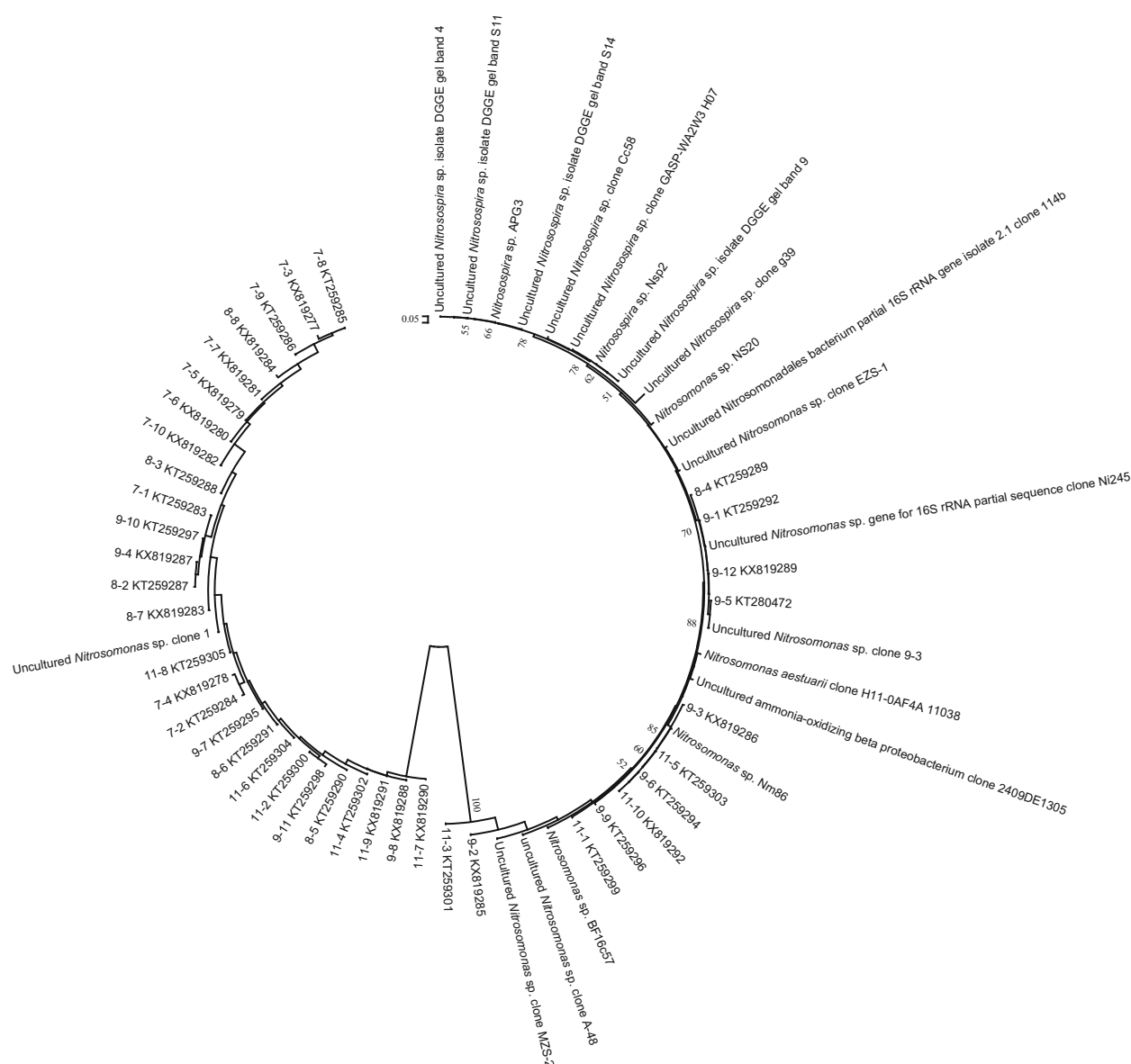


Fig.2 Phylogenetic relationships among the β -AOB 16S rRNA gene nucleotide sequences recovered from the saltmarsh plots

The tree construction was based on the β -AOB 16S rRNA gene sequences of the excised DGGE bands using the neighbor-joining algorithm with the Kimura two-parameter correction.

were excised from the DGGE profiles, sequenced and subjected to phylogenetic analysis (Fig.2). The sequences of 11 bands (7-2, 7-3, 7-4, 7-5, 7-6, 7-7, 7-8, 7-9, 7-10, 8-3 and 8-8) were highly similar to those of members of the genus *Nitrospira*, while the sequences of the other 27 bands (excluding 8-1 and 8-6) were closely related to *Nitrosomonas* bacteria (Table 1). Bands 7-2, 7-3, 7-4, 7-5, 7-6, 7-10, 8-6, 8-8, 9-2, 9-3, 9-8, 9-12, 11-7 and 11-10 were detected only in the N fertilizer treatments (Fig.1). Bands 9-8 and 9-12 were unique to the 0.8 g N and $(\text{NH}_4)_2\text{SO}_4$ treatments, respectively (Fig.1). Nitrogen treatment effects were verified by the unweighted pair group method with arithmetic average clustering (Fig.3).

3.2 Responses of abundance of β -AOB and potential nitrification rate to fertilization

Across all sampling dates except September, the β -AOB *amoA* gene abundance was significantly higher in samples treated with 0.2 g N than in the control samples (Table 2, $P < 0.05$). In samples treated with 0.1, 0.4 and 0.8 g N, the β -AOB *amoA* gene abundance was significantly higher than the control abundance only in July and August (Table 2, $P < 0.05$). Across all sampling dates, no significant differences in β -AOB *amoA* gene abundance were observed among different amounts of N application (Table 2, $P > 0.05$). Thus, the β -AOB *amoA* gene abundance

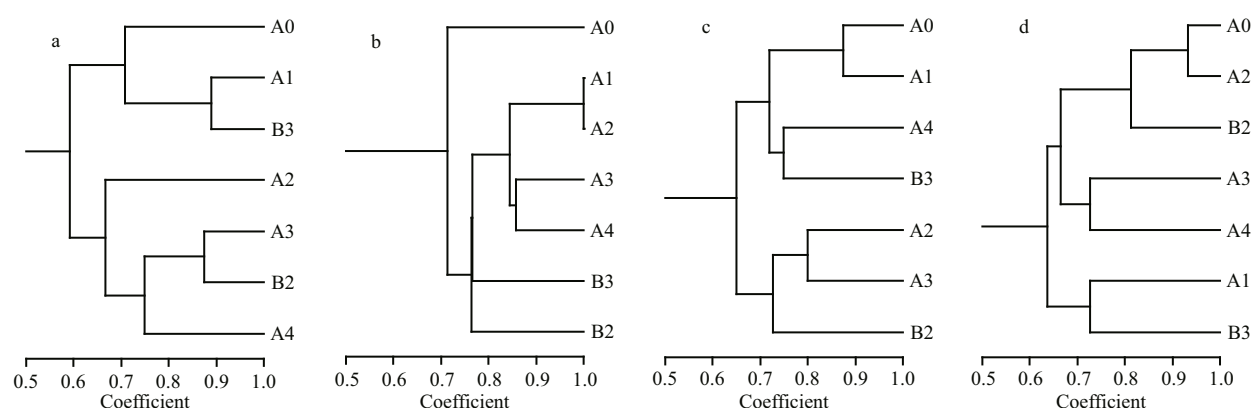


Fig.3 Dendrograms comparing the similarity levels of the β -AOB communities in different sediment samples

a, b, c and d are derived from the sediment samples pooled from three replicate plots collected on July 4, August 14, September 26, and November 22 of 2013, respectively.

Table 1 Blast analysis of the AOB 16S rDNA sequences derived from DGGE bands

	Closest <i>Nitrosomonas</i> / <i>Nitrospira</i> sequences (accession No.)	Similarity (%)	Origin	Reference
7-1; 8-2; 9-3; 9-4; 9-6; 9-10; 11-5; 11-10	<i>Nitrosomonas</i> sp. Nm86 (AY123798)	98	River Elbe	Purkhold et al., 2003
7-2	Uncultured <i>Nitrospira</i> sp. clone Cc58 (EF529682)	98	Marine sponge	Bayer et al., 2008
7-3; 7-7	<i>Nitrospira</i> sp. APG3 (KC477402)	99	Sandy lake sediment	Urakawa et al., 2015
7-4	<i>Nitrospira</i> sp. Nsp2 (AY123802)	98	Soil	Purkhold et al., 2003
7-5	Uncultured <i>Nitrospira</i> sp. clone GASP-WA2W3_H07 (EF072966)	99	Soil	Jangid et al., 2008
7-6	Uncultured <i>Nitrospira</i> sp. DGGE gel band 9 (JX862548)	98	Paddy soil	Liu et al., 2012 (unpublished)
7-8	Uncultured <i>Nitrospira</i> sp. DGGE gel band S14 (EU169049)	98	Landfill cover soil	Yu et al., 2009 (unpublished)
7-9	Uncultured <i>Nitrospira</i> sp. DGGE gel band S11 (EU169046)	99	Landfill cover soil	Yu et al., 2009 (unpublished)
7-10; 8-3	Uncultured <i>Nitrospira</i> sp. DGGE gel band 4 (JX862543)	99	Paddy soil	Liu et al., 2012 (unpublished)
8-1	Uncultured DGGE gel band b1-2 (JN977403)	100	Jiaozhao Bay sediment	Zhao, 2011 (unpublished)
8-4; 9-1; 9-5; 9-11; 9-12; 11-2; 11-9	Uncultured <i>Nitrosomonas</i> sp. clone: Ni245 (AB232659)	98	Aquaculture system filter materials	Itoi et al., 2006
8-5; 9-8; 11-4	Uncultured <i>Nitrosomonas</i> sp. clone EZS-1 (DQ002466)	97	Intertidal rocky areas overlain by macroalgal canopies of <i>Enteromorpha</i> spp.	Magalhães et al., 2007
8-6	Uncultured ammonia-oxidizing beta proteobacterium clone 2409DE1305 (JQ725980)	99	Mangrove soil	Laanbroek et al., 2012
8-7	Uncultured <i>Nitrosomonas</i> sp. clone 1 (AY683480)	99	Aquarium biofilter	Grommen et al., 2005
8-8	Uncultured <i>Nitrospira</i> sp. clone g39 (DQ167057)	98	Salt marsh	Shuang et al., 2005 (unpublished)
9-2; 11-3	<i>Nitrosomonas</i> sp. BF16c57 (AF386746)	98	Freshwater aquaria enrichments	Burrell et al., 2001
9-7	<i>Nitrosomonas aestuarii</i> clone H11-0AF4A_11038 (KF228156)	98	Aerated compost tea from grape marc compost	Marin, 2013 (unpublished)
9-9; 11-1	Uncultured <i>Nitrosomonas</i> sp. clone MZS-2 (DQ002465)	97	Intertidal muddy sediments	Magalhães et al., 2007
11-6	Uncultured <i>Nitrosomonas</i> sp. clone A-48 (EU155069)	97	Prawn farm sediment	Ma and Wang, 2007 (unpublished)
11-7	<i>Nitrosomonas</i> sp. enrichment culture clone Cb9 (KC706457)	98	North Sea nitrifier enrichment culture	Haaijer et al., 2013
11-8	<i>Nitrosomonas</i> sp. NS20 (AB212171)	99	Marine sediment	Urakawa et al., 2006

Table 2 Effects of varying the amount and form of N treatment on β -AOB *amoA* gene abundance

Treatment	β -AOB <i>amoA</i> gene abundance (lg copies/g wet sediment)			
	July	August	September	November
A0	5.83±0.05 ^b	5.87±0.02 ^b	5.73±0.11 ^a	5.78±0.07 ^b
A1	6.07±0.1 ^a	6.01±0.03 ^a	5.89±0.03 ^a	5.84±0.05 ^{ab}
A2	6.14±0.07 ^a	6.05±0.04 ^a	5.94±0.02 ^a	5.93±0.03 ^a
A3	6.13±0.07 ^a	6.01±0.04 ^a	5.92±0.02 ^a	5.88±0.05 ^{ab}
A4	6.11±0.05 ^a	6.01±0.03 ^a	5.92±0.01 ^a	5.88±0.06 ^{ab}
Significance of N amount	0.002	0.001	0.139	0.072
B0	5.83±0.05 ^b	5.87±0.02 ^c	5.73±0.11 ^b	5.78±0.07 ^{ab}
B1	6.14±0.07 ^a	6.05±0.04 ^a	5.94±0.02 ^a	5.93±0.03 ^a
B2	6.06±0.09 ^a	5.97±0.01 ^b	5.87±0.03 ^{ab}	5.85±0.01 ^a
B3	6.00±0.04 ^{ab}	5.97±0.04 ^b	5.79±0.07 ^{ab}	5.77±0.01 ^b
Significance of N form	0.002	0.001	0.023	0.003

Different letters following the means ($n=3$) in the same column indicate significant differences ($P<0.05$) between corresponding treatments.

was significantly affected by the N amount only in July and August (Table 2, $P<0.05$).

Relative to the control, the β -AOB *amoA* gene abundance was significantly increased by urea application across all sampling dates except November (Table 2, $P<0.05$), by $(\text{NH}_4)_2\text{SO}_4$ addition in July and August alone (Table 2, $P<0.05$), and by NH_4NO_3 addition in August alone (Table 2, $P<0.05$). Moreover, the β -AOB *amoA* gene abundance significantly differed between the urea- and NH_4NO_3 -treated samples in August and November (Table 2, $P<0.05$), between the urea- and $(\text{NH}_4)_2\text{SO}_4$ -treated applications in August alone (Table 2, $P<0.05$), and between the NH_4NO_3 - and $(\text{NH}_4)_2\text{SO}_4$ -treated applications in November alone (Table 2, $P<0.05$). Thus, the β -AOB *amoA* gene abundance was significantly affected by the N form across all sampling dates (Table 2, $P<0.05$).

Over 6 months' application of 0.1, 0.2 and 0.4 g N, the potential nitrification rate was significantly higher than the control rate (Table 3, $P<0.05$). However, the addition of 0.8 g N significantly increased the potential nitrification rate only in November (Table 3, $P<0.05$). Moreover, the potential nitrification rate after 0.2 g N addition was significantly higher than after 0.1 and 0.4 g N addition in August and November (Table 3, $P<0.05$), and after 0.8 g N addition at all sampling dates except September (Table 3, $P<0.05$). The potential nitrification rates after 0.4 g N and 0.8 g N

Table 3 Effects of varying the amount and form of N on potential nitrification rate

Treatment	Potential nitrification rate (mg N/kg wet sediment/d)			
	July	August	September	November
A0	16.43±3.37 ^c	21.74±1.77 ^c	19.39±0.97 ^b	17.74±2.17 ^d
A1	26.71±1.86 ^{ab}	36.09±3.9 ^b	35.85±2.84 ^a	27.39±1.83 ^{bc}
A2	33.91±2.86 ^a	46.05±3.34 ^a	45.44±4.21 ^a	36.83±0.96 ^a
A3	26.44±3.81 ^{ab}	36.56±3.25 ^b	33.17±2.49 ^a	30.09±1.77 ^b
A4	22.62±4.33 ^{bc}	30.03±3.11 ^{bc}	31.6±6.75 ^{ab}	24.2±2.98 ^c
Significance of N amount	0.001	0.000	0.004	0.000
B0	16.43±3.37 ^b	21.74±1.77 ^c	19.39±0.97 ^b	17.74±2.17 ^c
B1	33.91±2.86 ^a	46.05±3.34 ^a	45.44±4.21 ^a	36.83±0.96 ^a
B2	24.52±5.66 ^{ab}	34.31±3.49 ^b	38.22±5.68 ^a	29.54±3.86 ^b
B3	24.42±3.04 ^{ab}	38.67±3.04 ^{ab}	40.10±4.37 ^a	28.94±3.23 ^b
Significance of N form	0.004	0.000	0.000	0.000

Different letters following the means ($n=3$) in the same column indicate significant differences ($P<0.05$) between corresponding treatments.

addition were significantly different only in November (Table 3, $P<0.05$). No significant differences in potential nitrification rate were observed between 0.1 g N and 0.4 g N application, or between 0.1 g N and 0.8 g N addition, across all sampling dates. Thus, the potential nitrification rate was significantly affected by the N amount at all sampling dates (Table 3, $P<0.05$).

Urea, $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3 addition significantly increased the potential nitrification rate over the control (Table 3, $P<0.05$) across all sampling dates except July (in which the rates of the $(\text{NH}_4)_2\text{SO}_4$ - and NH_4NO_3 -treated samples were statistically similar to the control rate). Furthermore, significant differences in the potential nitrification rate were observed between the urea- and $(\text{NH}_4)_2\text{SO}_4$ -treated samples in August and November alone (Table 3, $P<0.05$), and between the urea- and NH_4NO_3 -treated samples in November alone (Table 3, $P<0.05$). Finally, no differences in potential nitrification rate were observed between the $(\text{NH}_4)_2\text{SO}_4$ - and NH_4NO_3 -treated samples at any sampling date. Thus, the potential nitrification rate was significantly affected by the N form across all sampling dates (Table 3, $P<0.05$).

The potential nitrification rates were significantly correlated with β -AOB abundance across all sampling dates (Fig.4, $P<0.05$), suggesting that the resident β -AOBs are active ammonia oxidizers.

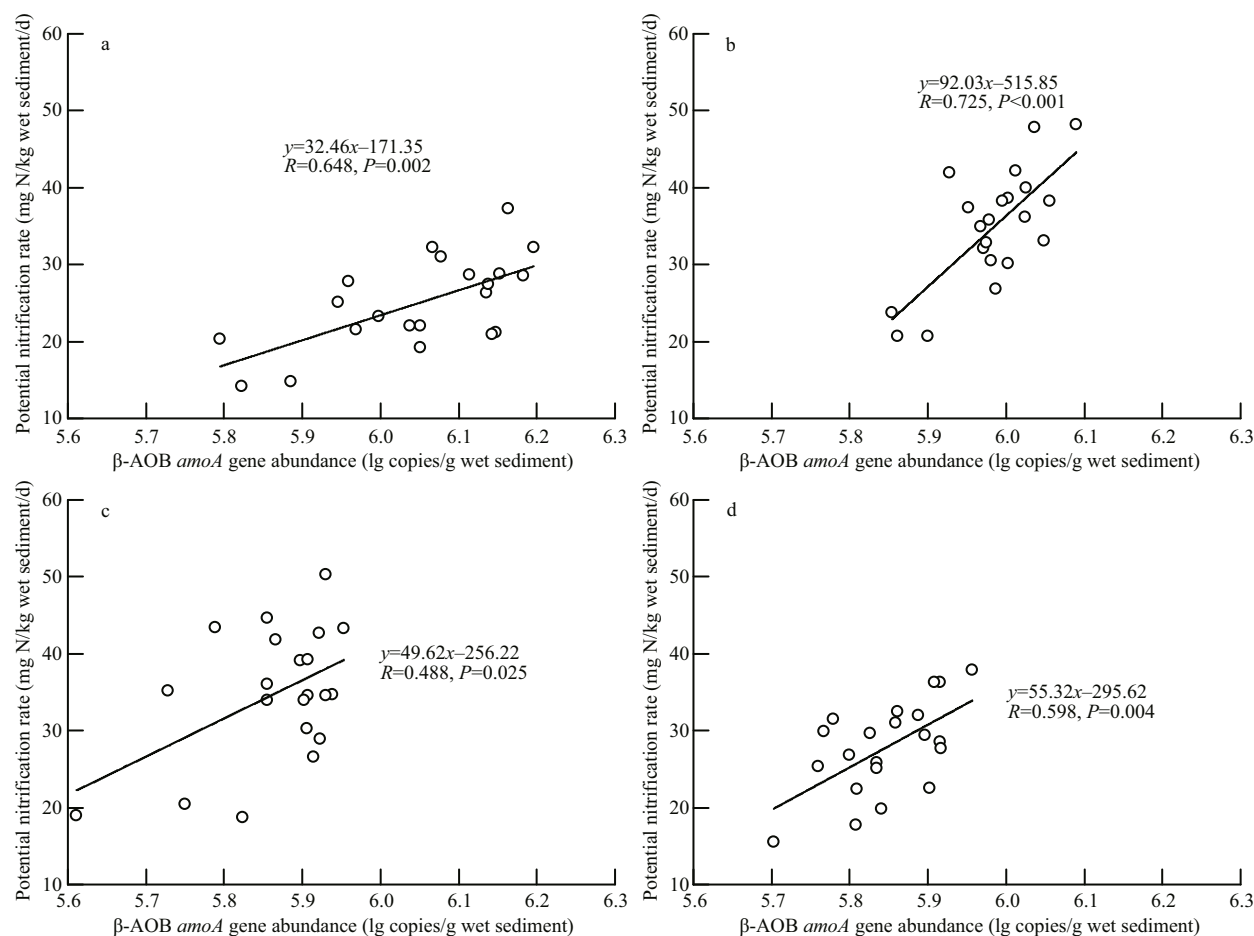


Fig.4 Relationship between potential nitrification rate and *amoA* gene abundance of β -AOBs

a, b, c and d are the results of sediment samples pooled from three replicate plots collected on July 4, August 14, September 26, and November 22 of 2013, respectively.

4 DISCUSSION

In previous experiments on salt marsh sediments, the β -AOB community composition shifted after 2 years' application of single nutrients (N), and after nearly 40 years of mixed fertilizer (NPK) application (Lage et al., 2010; Peng et al., 2012). In the present study, the β -AOB community responded to short-term (2–6 months) fertilization with different amounts of a specific N source (urea) and with different forms of N (urea, $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3). The AOB community composition might actually be linked to N availability (Hynes and Germida, 2012). Similarly, the β -AOB community in fertilized soils is affected by urea, $(\text{NH}_4)_2\text{HPO}_4$ and NH_4NO_3 applied at different amounts (Shen et al., 2011; Chen et al., 2013; Xiao et al., 2014; Wang et al., 2015). Sequences related to *Nitrosospira* and *Nitrosomonas* were detected in the present study of a saltmarsh in Shuangtai estuary. Eleven *Nitrosospira* sequences were recovered in July and August. In the Wequetequock-Pawcatuck

tidal marsh (dominated by *S. alterniflora* or *S. patens*) and the saltmarsh in Scarborough Marsh (dominated by *S. patens*), almost all of the β -AOB *amoA* sequences form a *Nitrosospira*-like clade (Moin et al., 2009; Lage et al., 2010). The same sequence type predominates in the Great Sippewissett Marsh, which is mainly populated by *S. alterniflora*, *S. patens* or *D. spicata* (Peng et al., 2012). The present study found 27 *Nitrosomonas* sequences in July, August, September and November. The intensities of bands 8-4 and 9-5 increased after N addition, band 9-8 was restricted by 0.8 g N treatment, and band 9-12 was detected only after $(\text{NH}_4)_2\text{SO}_4$ application. These findings were partly supported by Peng et al. (2012), who found that sequences related to *Nitrosomonas* spp. dominated in systems treated with high-to-excessive levels of mixed NPK fertilizer. *Nitrosomonas*-related sequences also predominate in fertilized or polluted sediments (Beman and Francis, 2006; Dang et al., 2010; Cao et al., 2012). Moreover, most of the 16S rRNA gene sequences were closely

related to uncultured AOB. By cultivating these culturally recalcitrant AOBs and characterizing their ecophysiology, we can potentially identify specific AOB populations that consistently respond to fertilization (Peng et al., 2012).

In this study, the abundances of the β -AOB *amoA* genes ranged from 4.1×10^5 to 1.6×10^6 copies/g sediment, within the abundance ranges reported in other estuarine and saltmarsh studies (Moin et al., 2009; Bernhard and Bollmann, 2010; Peng et al., 2012; Marton et al., 2015). Specifically, the abundance of β -AOB *amoA* genes ranged from 3.6×10^3 to 2.6×10^7 copies/g sediment in the Wequetequock-Pawcatuck tidal marsh (Moin et al., 2009), from 1.2×10^6 to 1.63×10^8 copies/g wet sediment in a mid-tidal saltmarsh in Scarborough Marsh (Lage et al., 2010), and from 4.5×10^3 to 1.3×10^7 copies/g sediment in the Great Sippewissett Marsh (Peng et al., 2012). In our study, the β -AOB abundance significantly increased in the presence of various amounts of a specific N source (urea), and in the presence of various N sources (urea, $(\text{NH}_4)_2\text{SO}_4$ and NH_4NO_3). Nitrogen addition positively affects the β -AOB abundance in agricultural soils (Glaser et al., 2010; Xu et al., 2012; Ai et al., 2013), forest soils (Long et al., 2012; Wertz et al., 2012) and grassland soils (Di et al., 2009; Shen et al., 2011; Tian et al., 2014; Chen et al., 2014). Consistent with our results, Kastl et al. (2015) found that N amounts affects the β -AOB abundance at the root-soil interfaces of plants with different N-uptake strategies, and Tian et al. (2014) reported that β -AOB abundance in fertilized grassland soil responds differently to different forms of N. However, Lage et al. (2010) and Peng et al. (2012) reported no effect of fertilization on β -AOB abundance in saltmarshes. The contrasting responses may arise from differences in the duration and amount of the added N, the form in which the N was applied, the plant species and the environmental factors of the investigated sites. In the present study, the β -AOB abundance may have positively responded to the increased N availability (Bai et al., 2011), as N provides energy for ammonia oxidizers. In addition, different amounts and forms of N fertilizers can alter the salinity, which may also affect β -AOB abundance (Moin et al., 2009).

The range of potential nitrification rates was higher in the saltmarsh in Shuangtai estuary than in other saltmarsh sediments (Dollhopf et al., 2005; Moin et al., 2009). Consistent with previous studies (Di et al., 2009; Wertz et al., 2012; Ai et al., 2013; Chen et al., 2013, 2014), we found that N fertilization increases

the sediment potential nitrification rate, indicating that short-term N application greatly promotes the sediment nitrification function. Moreover, the potential nitrification rate was significantly correlated with β -AOB abundance during the plant-growing season, suggesting that nitrification is driven by β -AOB in the saltmarsh in Shuangtai estuary. Dollhopf et al. (2005) reported similar findings in saltmarsh sediments.

5 CONCLUSION

In summary, N addition altered the β -AOB community composition, increasing both β -AOB abundance and potential nitrification rate. Moreover, the microbial parameters responded differently to varying amounts of a specific nitrogen source (urea) and to different forms of nitrogen applied at the same concentration. To our knowledge, we present the first demonstration that short-term N application influences the sediment β -AOB community, β -AOB abundance and potential nitrification rate in a saltmarsh ecosystem.

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