Different Driving Factors for Potential Activity of Ammonia-Oxidizing Archaea and Bacteria in Coastal Wetlands

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Abstract

This study aimed to evaluate which environmental factors and genetic groups were important in explaining measured activity of Ammonia-oxidizing archaea (AOA) and bacteria (AOB), which play important roles in global nitrogen cycle, providing a new insight into the mechanism of archaeal and bacterial ammonia oxidation. We sampled 62 soil/sediment samples from coastal wetlands of the Bohai area of China and assessed the abundance of functional genes involved in the nitrogen cycle, soil/sediment characteristics and the potential activity of AOA (PAOA) and AOB (PAOB) using specific inhibitors. At last, we introduced the structure equation model (SEM) to infer direct and indirect effects of variables on potential activities. The results indicated that the change in AOA-*amoA* gene abundance may be more independent, while AOB-*amoA* was closely associated with the change in abundance of *amx* and denitrifier. PAOA was mainly defined by AOA-*amoA* abundance and partially influenced by the *norA* gene, suggesting coupling of archaeal ammonia oxidation with nitrite oxidation. PAOB was significantly defined by the abundance of *amx* and denitrifier, indirectly mediated by AOB-*amoA*. The activity of AOA seemed to be more independent of other microbial activities, while the activity of AOB varied closely with fluctuations of other microbial species. PAOA was mediated directly by the C/N ratio and indirectly by nitrite concentration and TOC value, while PAOB was mediated directly by ammonium concentration and TOC value and indirectly by C/N ratio. The activity of AOB may be determined by several other functional gene groups and had little correlation with AOB abundance while the activity of AOA was mostly controlled by itself.

1 Introduction

Increasing consumption of nitrogen fertilizer all around the world leads to global nitrogen overload and had been identified as a main emerging environmental issue in this century (Zheng et al. 2013). A large-scale production of ammonia has been rising steadily (+ 1.5 %/year) to over 150 mT every year and most of them are used to the production of ammonium fertilizer (Wendeborn 2019). Only 30–50% of nitrogen is assimilated by crop plants while the remaining 50–70 % goes into biological processes. In the processes mostly dominated by microorganisms, ammonia must be oxidized at least once before returning into atmosphere as N_2 or N_2O (Wang et al. 2014).

Ammonia oxidation is the first and rate-limiting step in nitrification as well as an essential part of the global nitrogen cycle (Kowalchuk and Stephen 2001; Pester et al. 2012). This process is important to nitrogen availability, nitrate leaching and nitrous oxide emission (Godde and Conrad 1999; Levy-Booth et al. 2014). For a long time, the process was thought to be driven only by AOB (Kowalchuk and Stephen 2001; Prosser and Nicol 2008). However, in the past decade, AOA (Hatzenpichler et al. 2008; Könneke et al. 2005; Venter et al. 2004) has been confirmed to be jointly responsible for ammonia oxidation along with AOB under aerobic conditions (Jia and Conrad 2009).

AOA and AOB had been found to co-exist in most ecosystems and may be influenced by various environmental factors (Erguder et al. 2009; Gleeson et al. 2010). The abundance and community of AOA

and AOB vary in different ecosystems and are easily influenced by soil conditions (Bouskill et al. 2012; Caffrey et al. 2007; Wuchter et al. 2006). Many previous studies have demonstrated the influence of environmental factors on ecological niche differentiation between AOA and AOB using cultivation-independent molecular methods. Researchers found that salinity (Caffrey et al. 2007), DO (Abell et al. 2011), pH value (Nicol et al. 2008; Wang et al. 2014; Ying et al. 2017) and N fertilizer (Glaser et al. 2010) had great influence on the abundance and community structure of AOA and AOB in different soil/sediment environments. However, most studies focused only on the gene abundance, expression or community structure of AOA and AOB. Only a few researches reported the weak correlation between ammonia oxidation activity and *amoA* abundance for AOA and AOB (Hou et al. 2013). To date, there is no study reporting the quantitative response of AOA and AOB activity to multiple environmental factors and the driving factors for AOA and AOB activity remained unknown.

In addition, many studies showed that potential nitrogen cycling activities were controlled by the combination of various genetic groups and environmental factors (Guo et al. 2011; Siles et al. 2017; Zhang et al. 2017). For instance, the nitrification gene ratio and free ammonium may well explain nitrite and nitrous oxide production in urea-amended soils (Breuillin-Sessoms et al. 2017). And the complex genetic drivers are thought to be responsible for nitrogen removal in tidal flow constructed wetlands, which was the result of analysing the quantitative relationships between the rate of denitrification and nitrogen cycling gene groups (Zhi and Ji 2014). However, the associations between AOA/AOB and other nitrogen cycling microorganisms are poorly understood. It is very important for us to explore the driving factors, both genetic groups and environmental factors, for the activity of AOA and AOB to understand the archaeal and bacterial ammonia oxidation and the important step in the nitrogen cycle.

In this study, 62 samples from four typical wetlands along the Bohai area were collected. We measured the abundance of functional genes involved in the nitrogen cycle and the potential activities of AOA and AOB using antibiotic inhibitors. We used structure equation model (SEM) to infer direct and indirect effects of variables on potential activities to evaluate which environmental factors and genetic groups were important in explaining measured activity of AOA and AOB.

2 Materials And Methods

2.1 Sampling

A total of 62 soil/sediment samples across four typical wetlands were collected from coastal wetlands of the Bohai area in June 2012 (Fig. 1). The four wetlands are paddy fields (PF), estuary wetlands (EW), reed wetlands (RW) and shallow wetlands (SW). Paddy fields are the wetlands people farm and fertilize, which are influenced most by human activity. Estuary wetlands are the wetlands near estuary where rivers and the inside pollutants going into the sea. The other two wetlands are influenced less by human activity, reed wetlands covered by reeds while shallow wetlands with little vegetation but shallow seawater. The specific information of the 62 samples were listed in Table 1. At each sampling site, triplicate samples were collected from the top 10 cm with PVC tubes and then composited into one per site. The samples

were placed in sterile plastic bags, which were sealed and transported to the laboratory on ice. Each sample was divided to three subsamples, one subsample incubated to determine the potential ammonia oxidation activity of AOA and AOB while another processed through a 2.0-mm sieve for subsequent analysis of the physical-chemical components. The remainder was stored at -80°C for DNA extraction and molecular analysis.

2.2 Sediment physiochemical characteristics

Approximately 10.0-g samples were extracted using 100 mL of 2 M KCl. The solution after filtration was used to analyse nitrate nitrogen (NO_3^- -N), nitrite nitrogen (NO_2^- -N) and ammonium nitrogen (NH_4^+ -N) by a continuous flow analyser (TRRACS, Bran + Luebbe, Norderstedt, Germany). The pH of the soil/water suspensions was measured (1/2.5 w/v) (Hou et al. 2013), and the total organic carbon (TOC) was determined using an elemental analyser (2400II CHNS/O, PerkinElmer, USA).

2.3 Potential ammonia oxidation activity

The ammonia oxidation rates of AOA and AOB were measured in three replicates with two sets of experiments (groups A and B). The homogenized, field-moist soil/sediment samples (10.0 g) were weighed into 150-mL incubation flasks, and 80 mL of solution (0.4 g/L MgCl_2 , 0.5 g/L KCl, 0.2 g/L KH_2PO_4 , 1 g/L NaCl, 0.1 g/L CaCl_2 , and 10 mM KClO_3 ; Fisher Scientific) was added to each replicate. Additionally, a final concentration of 100 mg/L streptomycin sulfate was added to group B to inhibit the activity of bacteria. After one-day pre-incubation, a final concentration of 0.5 mM ammonium chloride was added to all groups. The flasks were incubated at 30°C in an orbital shaker, and the mud was sampled every 24 hours to define the total ammonia oxidation rate (group A) and the archaeal ammonia oxidation rate (group B) through analyses of nitrite concentration changes. The bacterial ammonia oxidation rate was determined by subtracting group B from group A.

2.4 DNA extraction and quantitative PCR

DNA was extracted from 0.5 g of each soil/sediment sample using a Fast DNA SPIN Kit for Soil (MP Biomedicals, Santa Ana, CA) in accordance with the manufacturer's instructions. The three extracts from the same replicate plot were pooled before further analysis. DNA concentration and quality were measured using a NanoDrop ND-1000 spectrophotometer (Thermo Scientific, Wilmington, DE, USA).

Several genes were used to assess the abundance of different functional groups of N cycling microorganisms. The AOA-*amoA*, AOB-*amoA* and *norA* genes were used to quantify archaeal and bacterial ammonia oxidizers and nitrifiers, respectively. The *amx* gene, biomarker of anaerobic ammonia oxidation process, was used to quantify anaerobic ammonia oxidizers. The membrane-bound nitrate reductase gene (*narG*) and periplasmic nitrate reductase gene (*napA*) were used to quantify nitrate reducers. The cytochrome cd1-containing nitrite reductase gene (*nirS*), Cu-containing nitrite reductase gene (*nirK*), nitric oxide reductase gene (*qnorB*) and nitrous oxide reductase gene (*nosZ*) were used to quantify groups of denitrifiers. Amplification was performed in triplicate with an ABI PRISM 7300 (Applied Biosystems, USA) using a 20-μL reaction mixture including 10 μL Power SYBR Green Mixture (Applied

Biosystems, USA), 4 µL primer pairs, 1.0 µL template DNA and 1 µL PCR grade MQ-water (MP Biomedicals). Specific primer combinations and thermal cycling conditions used for each reaction are listed in Table 2 and Table 3. Standard curves were obtained using serial dilutions of linearized plasmids containing targeted genes. Sterile water was used as a negative control to detect and exclude any possible contamination or carryover.

2.5 Statistical analyses

The structure equation model (SEM) was introduced to infer direct and indirect effects of variables on potential activities (Fox 2006). To determine whether the further addition or removal of paths would improve model accuracy, we used modification indices in the R package. The model fit was evaluated using the Chi-square goodness-of-fit (χ^2), Tucker-Lewis non-normed fit index (NNFI), Akaike information criterion (AIC), and root mean square error of approximation (RMSEA) (Hershberger 2001). A non-significant *P*-value (*P* > 0.05) for the Chi-square statistic indicates no significant difference in the covariance pattern predicted by the SEM and from the observed covariance, which indicates good fit of the data. We fitted the SEMs as linear models and reported the standardized coefficient for each path. The importance of an explanatory variable was based on its total effect, which was the sum of direct and indirect effects on the response variable (Wootton 1994). Data processing and analysis were performed with SPSS Statistics 20 (IBM, USA). A priori *P*-value of *P* < 0.05 was defined to test significant difference.

3 Results

3.1 Abundance of nitrogen cycling functional genes

The abundances of the ten tested functional genes involved in the nitrogen cycle (namely, *amx*, AOA-*amoA*, AOB-*amoA*, *norA*, *narG*, *napA*, *nirS*, *nirK*, *nosZ* and *qnorB*) in all 62 wetland samples are shown in Fig. 2. The average abundance of the *amx* gene was about 5.0×10^8 copies/g. AOA-*amoA* was numerically dominant over AOB-*amoA*, with one order of magnitude higher abundance. The abundance of *nirK* was close to that of *nirS*, at 1.15×10^8 and 2.97×10^8 copies/g, respectively. The abundances of *nosZ* and *qnorB* varied insignificantly among all samples (approximately 10^7 copies/g). The abundance of *napA* was higher than that of *narG*.

All functional genes could be divided into six genetic groups, which presented six different nitrogen transferring processes. The *amx*, AOA-*amoA*, AOB-*amoA*, and *norA* genes represented anaerobic ammonia oxidation, archaeal ammonia oxidation, bacterial ammonia oxidation and nitrite oxidation, respectively. The summation of *narG* and *napA* represented nitrate reduction, and the summation of *nirK*, *nirS*, *qnorB* and *nosZ* represented denitrification. The gene abundance of each process is shown in Fig. 3. The gene abundances of anaerobic ammonia oxidation, denitrification and nitrate reduction were relatively higher, at 2 ~ 3 orders of magnitude, than archaeal ammonia oxidation, bacterial ammonia oxidation and nitrite oxidation.

3.2 Cluster analysis based on functional gene groups involved in nitrogen cycle

All samples could be divided into six clusters (clusters A, B, C, D, E and F) according to heat map analysis based on the abundance of functional gene groups involved in different nitrogen cycle processes, and the results are shown in Fig. 4. The samples in each cluster mean that they have the most similarity of gene abundance composition, and each cluster had its characteristic gene abundance composition. Cluster A contained 3 samples from EWs and SWs, all with relatively higher abundance of the *norA* gene than others. Cluster B contained 21 samples mainly from EWs, all with relatively higher abundance of the AOA-*amoA* gene than others. Cluster C contained 17 samples mainly from EWs and SWs, all with relatively higher abundance of denitrifier (represented by accumulation of *nirK*, *nirS*, *nosZ* and *qnorB* gene) than others. Cluster D contained 7 samples mainly from EWs, all with relatively higher abundance of the AOB-*amoA* gene than others. Cluster E contained 3 samples from EWs and SWs, all with relatively higher abundance of nitrate-reducing communities (represented by accumulation of *narG* and *napA* gene) than others. Cluster F contained 11 samples mainly from EWs and RWs, all with relatively higher abundance of both the *amx* and *norA* genes than others.

The abundance of functional gene groups in each cluster is shown in Fig. 5. Cluster A has the highest abundance of *norA* gene, with an average value of 1.88×10^7 copies/g, which was 2 orders of magnitude higher than that of other clusters. In cluster B, the average abundance of AOA-*amoA* gene was 7.50×10^7 copies/g, which was approximately 6- to 20-fold greater than that of other clusters. Cluster C has the highest abundance of gene groups responsible for denitrification, with an average value of 8.59×10^8 copies/g. In cluster D, the average abundance of AOB-*amoA* was 1.15×10^7 copies/g, which was approximately 2- to 15-fold greater than that of the other clusters. Cluster E has the highest abundance of nitrate-reducing genes, with an average value of 1.09×10^9 copies/g, which was 1 ~ 2 orders of magnitude greater than that of the other clusters.

3.3 Potential ammonia oxidation activity

The potential ammonia oxidation activity of AOA (PAOA) ranged from 0.01 to $6.26 \mu\text{g N g}^{-1} \text{d}^{-1}$, while the potential activity of AOB (PAOB) ranged from 0.01 to $26.24 \mu\text{g N g}^{-1} \text{d}^{-1}$. AOA contributed approximately 0.46–83.21% to ammonia oxidation across the landscape, while the remainder was attributed to AOB.

The activity of ammonia oxidation driving by AOA was markedly higher in samples from cluster A, cluster B and cluster E, at 2.27 , 1.32 and $2.23 \mu\text{g N g}^{-1} \text{d}^{-1}$, respectively (Fig. 6a). The rate of AOB was significantly higher in cluster C, with a value of $8.67 \mu\text{g N g}^{-1} \text{d}^{-1}$ (Fig. 6b). The average activities of AOA and AOB were both the maximum in EW with the values of 6.14 and $7.22 \mu\text{g N g}^{-1} \text{d}^{-1}$. The average contributions of AOA in were close to a half in EW, SW and RW, meanwhile the average contribution of AOA was only 25.40% in PF.

3.4 Group sorting of the observed N cycling microbial community

The ordination of non-metric multidimensional scaling (NMDS) showed a clear sorting of samples according to the abundance of functional gene groups clustered with 95% confidence levels (Fig. 7). The NMDS analysis can more intuitively reflect the distribution characteristics of gene groups involved in the nitrogen cycle. The coordination of each point in the figure indicated comprehensive standardized assessment values of gene abundance in the tested soils/sediments (Liang et al. 2017). The distribution value is related to each element, where proximity to a certain element indicates a greater contribution of gene abundance to the comprehensive standardized assessment value. With the increase in AOA-*amoA* gene abundance, these samples clearly separated from other samples. However, samples with relatively higher AOB-*amoA* gene abundance still coupled with other samples. The group with relatively higher AOB-*amoA* gene abundance overlapped with groups with a higher abundance of *amx*, denitrifier, *norA* and even AOA-*amoA*. The activity of archaeal ammonia oxidation has a strong relationship with samples with relatively higher abundance of AOA-*amoA*, while the activity of bacterial ammonia oxidation has a relationship with samples with higher abundance of AOB-*amoA*, *amx* and denitrification genes. In addition, the activity of archaeal ammonia oxidation was related to nitrite concentration, while the TOC value had a significant effect on the activity of bacterial ammonia oxidation. The distribution of archaeal and bacterial ammonia oxidation activity among all samples suggested that most samples with higher PAOA gathered in cluster B with a higher abundance of AOA-*amoA*, in cluster A with a higher abundance of *norA* and in cluster E with a higher abundance of nitrate-reducing gene groups. Meanwhile, most samples with higher PAOB gathered in cluster C with a higher abundance of denitrification groups.

3.5 Driving factors for potential archaeal and bacterial ammonia oxidizing activities

We fitted a SEM to infer the direct and indirect effects of biotic and abiotic factors on potential activities and to advance our quantitative understanding of the molecular mechanism responsible for ammonia oxidation. The results showed that the control system for PAOA was different and simpler than that for PAOB. The results suggested that *norA* and AOA-*amoA* were the two most important driving microbial factors for PAOA (Fig. 8a). Meanwhile, C/N was the most important driving environmental factor for PAOA. Furthermore, AOB-*amoA* acted directly on AOA-*amoA*. The TOC value acted directly on both *norA* and C/N, and nitrite concentration had a directly negative effect on C/N (-0.250). However, the most important driving microbial factors for PAOB were *amx* and denitrifier groups (Fig. 8b). The most important driving environmental factors for PAOB were TOC value and ammonium concentration. In addition, C/N had a directly negative effect on ammonium and a positive effect on TOC. Furthermore, the nitrate reducing group and AOB-*amoA* affected PAOB indirectly via *amx*, while the nitrate reducing group had another indirect effect on PAOB via the denitrifier group.

4 Discussion

A few literatures reported archaeal ammonia oxidation activity using inhibitors. (Zheng et al. 2013) suggested that archaeal ammonia oxidation activity ranged from 1.4 to 47.6 $\mu\text{g N g}^{-1}$ sediment d^{-1} in Yangtze Estuary, while (Zhou et al. 2016) reported that archaeal ammonia oxidation activity ranged from 29.1 to 58.1 $\mu\text{g N g}^{-1}$ sediment d^{-1} in sediments from ancient canal. Our results were comparable to the rates from Yangtze Estuary, but lower than that of sediments from ancient canal. It may be caused by higher ammonia concentration in canal sediments which can promote ammonia oxidation activity.

There were significant differences in AOA and AOB activities in different types of wetlands. The AOB activities in EW, PF, RW and SW were decreasing, while the AOA activities decreased in EW, RW, PF and SW in turn. However, except for in PF, the average contributions of AOA and AOB were almost equal. In SW, the activities of AOA and AOB were significantly lower than other wetlands. It may be because the lowest nitrogen level in SW (Fig. 9). AOB was more competitive under high ammonia nitrogen conditions (French et al. 2012), while AOA was better under low ammonia nitrogen conditions (Martens-Habbena et al. 2009), thus the AOA contributed much more than AOB in PF where a great deal of nitrogenous fertilizer release for a good harvest.

Besides the influence of nitrogen concentration, pH, TOC, C/N ratio and other environmental factors were also found to be important factors affecting the activity of AOA and AOB. Environmental conditions had a great effect on microbial gene abundance and may cause changes in real-time process rates (Jusselme et al. 2016; Pester et al. 2012; Shrewsbury et al. 2016). The analysis of environmental effects on ammonia oxidation activity suggested that archaeal activity had a strong correlation with nitrate concentration. The positive relationship suggested that a higher abundance of AOA-*amoA* gene led to greater accumulation of nitrate. Meanwhile, bacterial activity had a strong correlation with ammonium concentration and TOC value (Fig. 8). As organic C and ammonium provided substrate and promoted nitrification for AOB (Forbes et al. 2009). In further SEM analysis, PAOA was mediated directly by the C/N ratio and indirectly by nitrite concentration and TOC value, while PAOB was mediated directly by ammonium concentration and TOC value and indirectly by C/N ratio.

The NMDS analysis reflected that AOB has close relationship with microbes involved in other nitrogen cycle processes, which is consistent with previous studies (Chen et al. 2012; Third et al. 2001). The samples in cluster B, with a relatively higher abundance of the AOA-*amoA* gene than other clusters, obviously separated from other samples, except for partial overlap with cluster D with a relatively higher abundance of AOB-*amoA* gene (Fig. 7), which suggested that the increase of AOA-*amoA* abundance may lead to a significantly different composition of functional genes in soil/sediment samples. However, samples in cluster D with a relatively higher abundance of AOB-*amoA* gene overlapped more closely with other samples, which suggested that the increase in AOB-*amoA* abundance had only slight effect on the composition of functional genes for most samples. The results indicated that the change in AOA-*amoA* gene abundance may be more independent, while AOB-*amoA* may associate closely with the changes in the abundance of *amx* and denitrifier.

In the six clusters, higher bacterial ammonia oxidation activities occurred in cluster C, with a higher abundance of denitrifier, than in cluster D, with a higher abundance of AOB-*amoA* gene (Fig. 10b). This suggested that the activity of AOB may be easily influenced by other microorganisms. As for the distribution of PAOA, although some samples with higher archaeal ammonia oxidation activities occurred in cluster A with a higher abundance of *norA* and cluster E with a higher abundance of nitrate reducing groups, most samples were distributed in cluster B with a higher abundance of AOA-*amoA* gene (Fig. 10a), suggesting that the higher activity of AOA was strongly correlated with a higher abundance of the AOA-*amoA* gene. Previous results indicated that one nitrogen cycling process was comprehensively influenced by gene groups involved in other nitrogen cycle processes (Zhang et al. 2016, 2017). For instance, N₂O production was also influenced by *narG* and *napA* which were responsible for nitrate reduction. This study also demonstrated the coupling mechanism in nitrogen cycle. The activity of AOB may be determined by several other functional gene groups and had little correlation with AOB abundance. However, the activity of AOA was mostly controlled by itself, presenting a more stable, simpler regulation for the activity of AOA had little correlation with other functional gene groups.

Several studies reported the quantitative response of N transformation rate to the functional gene abundances. For instance, Wang et al. (2015) reported that NH₄⁺-N transformation rate was jointly determined by several functional genes, including *amoA*, *nirS*, *nirK*, *anammox* 16S rRNA and archaea 16S rRNA (Wang et al. 2015; Wang et al. 2015), and Pang reported that NH₄⁺-N transformation rate was influenced by *amoA*, *nirS* and *anammox* 16S rRNA (Pang et al. 2015). This study further demonstrated the complex relationship between activity of AOA/AOB and functional gene groups and the results of the SEM analysis showed the significant difference between the responses of AOA and AOB. The pathway analysis indicated that PAOA was mainly correlated with absolute abundance of AOA-*amoA* and *norA* genes, while PAOB was mainly correlated with absolute abundance of denitrifier group and *amx* genes. The result of PAOA indicated that the activity of AOA was mostly defined by AOA-*amoA* abundance and was only slightly influenced by the abundance of *norA* gene. However, the strong association between PAOB and *amx* indicated close coupling of partial nitrification and ANAMMOX processes (Third et al. 2001; Zhu et al. 2011). The activity of AOA appeared to be more independent of other microbes, while the activity of AOB varied closely with the fluctuations of other microbes.

The different effects of functional genes on PAOA and PAOB provide evidence for varying relative strengths of different microbial processes on their potential activities. The influence of *norA* on PAOA indicated that the archaeal ammonia oxidation potential was mainly limited by the assumption of nitrite through nitrite oxidation, consistent with the negative effect of nitrite concentration on PAOA via C/N ratio (Fig. 8a). The strongest impacts of the abundance of denitrifier groups on PAOB suggested a limited role for nitrite reduction, which involves the complete reduction of nitrite into dinitrogen. The abundance of *amx* also exhibited a positive relationship with PAOB. The strong impacts of the abundance of *amx* and the denitrifier group on PAOB suggested coordination of these three nitrogen processes (ANAMMOX, denitrification and bacterial ammonia oxidation). AOB-*amoA* abundance had only a weak effect on PAOB

via *amx*, which further demonstrated that AOB activity may rely on the abundance of other microbes rather than AOB.

5 Conclusions

Aerobic ammonia oxidation is driven by both AOA and AOB, but no clear results have been published about the response of AOA and AOB in terms of activity levels. This study reveals the differential driving factors for activity of ammonia-oxidizing archaea and bacteria. PAOA was mediated directly by the C/N ratio and indirectly by nitrite concentration and TOC value, while PAOB was mediated directly by ammonium concentration and TOC value and indirectly by C/N ratio. In addition, this study also demonstrated the coupling mechanism in nitrogen cycle. The activity of AOB may be determined by several other functional gene groups and had little correlation with AOB abundance while the activity of AOA was mostly controlled by itself.

Declarations

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Code availability Not applicable.

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Data Availability The authors guarantee the availability of data and material.

Declarations

Conflicts of interest/Competing Interests The authors declare no conflict of interest.

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Tables

Table 1 Basic properties of the samples

	Wetlands	NH ₄ ⁺ -N (mg/kg)	NO ₂ ⁻ -N (mg/kg)	NO ₃ ⁻ -N (mg/kg)	pH	TOC (%)	TN (mg/kg)
1	SW	1.24	2.11	6.67	8.27	0.015	10.02
2	SW	0.21	1.20	0.38	7.91	0.006	1.79
3	EW	0.90	0.74	0.23	7.91	0.008	1.87
4	EW	1.75	1.55	0.55	8.44	0.014	3.86
5	SW	1.58	1.23	0.64	8.17	0.011	3.45
6	PF	14.79	2.12	4.37	8.24	1.730	21.28
7	SW	0.18	1.30	1.21	7.68	0.004	2.69
8	EW	2.68	2.72	0.54	8.68	0.011	5.93
9	SW	2.62	1.97	2.48	7.79	0.012	7.07
10	EW	5.36	0.67	0.46	7.77	0.016	6.49
11	RW	1.04	1.69	0.40	8.57	0.018	3.14
12	EW	2.42	1.54	1.38	8.71	0.011	5.34
13	SW	0.37	1.31	0.17	8.76	0.017	1.84
14	PF	3.36	2.46	0.00	8.25	1.720	5.82
15	RW	1.40	1.91	2.49	8.55	0.008	5.80
16	RW	2.13	1.29	1.99	8.67	0.013	5.41
17	RW	0.91	0.73	0.26	8.67	0.013	1.90
18	SW	6.82	1.35	1.65	8.64	0.013	9.82
19	SW	3.29	1.34	2.23	7.79	0.031	6.86
20	EW	6.84	1.27	0.11	8.13	0.013	8.22
21	PF	46.37	1.33	0.38	7.73	0.521	48.07
22	SW	2.99	1.00	0.14	8.25	0.027	4.13
23	EW	0.59	1.32	0.42	8.36	0.005	2.33
24	PF	34.48	1.78	0.27	7.38	1.535	36.53
25	PF	13.23	1.49	0.52	8.27	1.340	15.24
26	EW	1.19	1.88	0.21	8.16	0.007	3.28
27	RW	5.09	1.63	0.44	8.47	0.024	7.16

28	EW	1.54	0.57	/	8.76	0.005	2.11
29	EW	0.71	1.15	0.21	8.09	0.011	2.07
30	EW	1.70	1.11	0.74	7.93	0.006	3.55
31	EW	19.07	1.24	2.21	8.94	0.019	22.51
32	EW	11.54	3.47	2.89	8.28	0.011	17.90
33	RW	1.67	1.52	2.91	8.36	0.014	6.10
34	RW	6.64	3.03	1.66	8.02	0.011	11.33
35	PF	10.68	1.83	1.54	7.89	2.210	14.05
36	SW	1.68	1.38	1.04	8.01	0.015	4.11
37	EW	1.77	1.02	0.42	8.53	0.016	3.21
38	EW	39.21	1.59	4.25	7.83	0.038	45.05
39	SW	0.83	0.36	0.14	8.23	0.018	1.33

Table 1 Basic properties of the samples

	Wetlands	NH ₄ ⁺ -N (mg/kg)	NO ₂ ⁻ -N (mg/kg)	NO ₃ ⁻ -N (mg/kg)	pH	TOC (%)	TN (mg/kg)
40	EW	0.27	0.03	0.12	8.63	0.025	0.42
41	RW	5.90	1.76	0.43	7.94	0.035	8.09
42	EW	2.91	2.11	/	8.86	0.019	5.02
43	RW	0.98	1.34	3.70	8.95	0.017	6.02
44	SW	1.28	1.41	1.71	8.95	0.019	4.40
45	EW	1.41	1.45	/	9.06	0.026	2.86
46	EW	4.94	12.90	8.51	8.17	0.020	26.36
47	RW	1.58	0.44	3.83	8.29	0.018	5.85
48	SW	0.90	0.51	1.28	7.87	0.015	2.69
49	RW	1.74	0.32	0.50	8.33	0.026	2.56
50	EW	0.74	0.07	/	8.40	0.022	0.81
51	EW	5.41	0.52	0.69	8.60	0.019	6.62
52	EW	1.11	1.49	0.00	8.65	0.017	2.60
53	EW	1.11	1.28	0.92	8.34	0.015	3.30
54	EW	0.09	1.25	0.77	8.29	0.012	2.11
55	EW	6.24	0.62	0.16	8.25	0.017	7.02
56	EW	1.17	1.40	/	8.07	0.006	2.58
57	EW	9.07	1.03	0.31	8.10	0.021	10.42
58	EW	0.17	1.52	1.08	8.39	0.007	2.77
59	EW	31.63	1.38	3.19	7.70	0.012	36.20
60	EW	3.21	2.42	0.35	7.65	0.008	5.97
61	SW	1.47	1.64	1.67	8.13	0.005	4.78
62	SW	0.63	1.27	0.71	7.78	0.004	2.61

Table 2 Primers of target genes used in the qPCR analysis

Target gene	Prime name	Primer sequence(5'-3')	Reference
<i>AOA-amoA</i>	<i>Arch-amoAF</i>	STA ATG GTC TGG CTT AGA CG	Francis et al.,2005
	<i>Arch-amoAR</i>	GCG GCC ATC CAT CTG TAT GT	
<i>AOB-amoA</i>	<i>amoA-1F</i>	GGG GTT TCT ACT GGT	Rotthauwe et al.,1997
	<i>amoA-2R</i>	CCC CTC KGS AAA GCC TTC	
<i>norA</i>	F1norA	CAGACCGACGTGTGCGAAAG	Poly et al., 2008
	R1norA	TCYACAAGGAACGGAAGGTC	
<i>Amx</i>	AMX809F	GCCGTAAACGATGGGCACT	Tsushima et al.,2007
	AMX1066R	AACGTCTCACGACACGAGCTG	
<i>napA</i>	napAV17m	TGGACVATGGGYTTYAAYC	Bru et al., 2007
	napA4r	ACYTCRCGHGCVGTRCCRCA	
<i>narG</i>	narG1960m2f	TA(CT)GT(GC)GGGCAGGA(AG)AAACTG	Lopez-Gutierrez et al., 2004
	narG2050m2r	CGTAGAAGAAGCTGGTGCTGTT	
<i>nirK</i>	nirK876	ATYGGCGGVAYGGCGA	Henry et al.,2004
	nirK1040	GCCTCGATCAGRTRTGTT	
<i>nirS</i>	nirSCd3aF	AACGYSAAGGARACSGG	Kandeler et al.,2006
	nirSR3cd	GASTTCGGRTGSGTCTTSAYGAA	
<i>qnorB</i>	qnorB2F	GGNCAYCARGGNTAYGA	Baker et al.,1998
	qnorB5R	ACCCANAGRTGNACNACCCACCA	
<i>nosZ</i>	nosZ1527F	CGCTGTTCHTCGACAGYCA	Scala and Kerkhof, 1998
	nosZ1773R	ATRTCGATCARCTGBTCGTT	

Table 3 Protocols and parameters of target genes used in qPCR analysis

Target gene	Programs
<i>AOA-amoA</i>	5 min at 94 °C, 40 cycles of 30 s at 94 °C, 60 s at 53 °C, and 60 s at 72 °C (plate read)
<i>AOB-amoA</i>	5 min at 94 °C, 40 cycles of 30 s at 94 °C, 56 s at 60 °C, and 60 s at 72 °C (plate read)
<i>norA</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 58 °C, and 30 s at 72 °C (plate read)
<i>Amx</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 45 s at 56 °C, and 30 s at 72 °C (plate read)
<i>napA</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 50 s at 56 °C, and 30 s at 72 °C (plate read)
<i>narG</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 45 s at 58 °C, and 30 s at 72 °C (plate read)
<i>nirK</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 40 s at 64 °C, and 30 s at 72 °C (plate read)
<i>nirS</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 57 °C, and 30 s at 72 °C (plate read)
<i>qnorB</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 30 s at 56 °C, and 30 s at 72 °C (plate read)
<i>nosZ</i>	10 min at 95 °C, 40 cycles of 15 s at 95 °C, 50 s at 58 °C, and 30 s at 72 °C (plate read)

Figures

Figure 1

Sampling sites in coastal wetlands around Bohai area. Note: The designations employed and the presentation of the material on this map do not imply the expression of any opinion whatsoever on the part of Research Square concerning the legal status of any country, territory, city or area or of its authorities, or concerning the delimitation of its frontiers or boundaries. This map has been provided by the authors.

Figure 2

Abundance of *amx*, *AOA-amoA*, *AOB-amoA*, *napA*, *narG*, *nirK*, *nirS*, *norA*, *nosZ* and *qnorB*. The upper and lower boundaries of each box indicate the 75th and 25th percentile, respectively, and the mid-line marks the median values, while dots indicate outliers.

Figure 3

Abundance of functional gene groups involved in each nitrogen cycle process.

Figure 4

Heat map and cluster analysis of all samples based on the abundance of functional gene groups involved in each nitrogen cycle process. All samples could be divided into 6 clusters, with relatively higher abundance of certain groups. Clusters A, B, C, D, E and F stand for relatively higher abundances of *norA*, AOA-amoA, denitrifier, AOB-amoA, nitrate-reducer and amx, respectively.

Figure 5

Abundance of functional gene groups in different clusters. (a) *norA*, (b) AOA-*amoA*, (c) Denitrifier, (d) AOB-*amoA* gene, (e) Nitrate-reducer, (f) *amx*. The upper and lower boundaries of each box indicate the 75th and 25th percentile, respectively, and the mid-line marks the median values, while dots indicate outliers. Clusters A, B, C, D, E and F stand for samples with relatively higher abundances of *norA*, AOA-*amoA*, denitrifier, AOB-*amoA*, nitrate-reducer and *amx*, respectively.

Figure 6

Potential activity of archaeal ammonia oxidation (PAOA) (a) and bacterial ammonia oxidation (PAOB) (b) in 6 clusters. Clusters A, B, C, D, E and F stand for samples with relatively higher abundances of *norA*, AOA-*amoA*, denitrifier, AOB-*amoA*, nitrate-reducer and *amx*, respectively.

Figure 7

Non-metric multidimensional scaling (NMDS) analysis based on various functional gene groups involved in nitrogen cycling. The significance of vector fits was determined using permutation tests ($n = 1000$) at the $P = 0.05$ level. Ellipses indicate 95% confidence interval for replicates. Different colours represent different groups dominated by certain gene groups.

Figure 8

Structure equation model (SEM) describing the direct and indirect contributions of biotic and abiotic factors to potential activity of AOA (PAOA) (a) and AOB (PAOB) (b). Single-pointed arrows indicate causal paths. Path coefficients are standardized, and all solid arrows indicate significant paths at a significance level of 0.05.

Figure 9

The concentrations of NH_4^+ , NO_2^- and NO_3^- and the pH value and TOC of four wetlands. The data are presented as the mean $\pm$ standard error ($n=3$). The different letters above the columns indicate a significant difference ($P<0.05$) based on the analysis of variance.

Figure 10

Relative activity of AOA (a) and AOB (b) in all samples. The circle size represents relative value of corresponding activity in all samples.