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Distribution of ammonia-oxidizing *Betaproteobacteria* community in surface sediment off the Changjiang River Estuary in summer

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Abstract
The spatial distribution of ammonia-oxidizing *Betaproteobacteria* (βAOB) was investigated by FISH (fluorescence in situ hybridization) and DGGE (denaturing gradient gel electrophoresis) techniques in the sediment off the Changjiang River Estuary. Sediment samples were collected from eight stations in June before the formation of hypoxia zone in 2006. The abundance of βAOB ranged from 1.87×10^5 to 3.53×10^5 cells/g of sediment. βAOB abundance did not present a negative correlation with salinity, whereas salinity was implicated as the primary factor affecting nitrification rates. The DGGE profiles of PCR-amplified *amo*A gene fragments revealed that the βAOB community structure of sample S2 separated from other samples at the level of 40% similarity. The variations in composition of βAOB were significantly correlated with the salinity, temperature, absorption ability of sediments and TOC. The statistical analysis indicates that the βAOB abundance was a main factor to influence nitrification rates with an influence ratio of 87.7% at the level of 40% biodiversity similarity. Considering the good correlation between βAOB abundance and nitrification estimates, the abundance and diversity of βAOB community could be expected as an indirect index of nitrification activity at the study sea area in summer.

Key words: ammonia-oxidizing *Betaproteobacteria* (βAOB), diversity, abundance, nitrification, surface sediment, Changjiang River Estuary (CRE)

1 Introduction

Human activity has increased the flux of nitrogen from land to the oceans by twofold globally over the past 40 years (Howarth et al., 2002). The increasing loads of nitrogen have caused serious anthropogenic eutrophication which would produce series of environmental problems and even threat to human economy and health (Łysiak-Pastuszak et al., 2004; Pruell et al., 2006). As a critical link in nitrogen cycle, nitrification, the oxidation of ammonia to nitrite and then to nitrate, is thought to connect biological N fixation and anaerobic N losses (Webster et al., 2002; Francis et al., 2005). Moreover dissolved oxygen in the water column and the sediments can be depleted by intense nitrification, leading to the formation of hypoxic zone at continental shelves (Álvarez-salgado and Gilcoto, 2004). Thus there is considerable concern on the nitrification process influenced by pollution, for example, global warming, contamination with recalcitrant organic compounds, and nitrogen overloading.

Nitrification is the microbial mediated process performed by ammonia-oxidizers and nitrite oxidizers. Ammonia-oxidizing bacteria (AOB), and newly discovered ammonia-oxidizing Archaea (AOA), are chemoautotrophic microorganisms carried out the first and rate-limiting step (Santoro et al., 2010). The majority of AOB in estuaries form a monophyletic lineage within *Betaproteobacteria*, including clusters of *Nitrosomonas* and *Nitrosospira* species. Recent advances in DNA-based techniques for direct whole microbial community analysis have made it possible to study βAOB communities by using probes or PCR primers target *amo*A, a functional gene coding for the active subunit of the enzyme ammonia monooxygenase (*AMO*) (Rotthauwe et al., 1997). By the application of culture-independent methods, researchers have found that community structure of βAOB can be shaped by

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several environmental variables including temperature, oxygen, ammonium, salinity, nitrite, sand content and so on (Francis et al., 2003; Bernhard et al., 2005; Freitag et al., 2006; Dang et al., 2010). The current evidence from estuarine showed a complex relationship between characteristics of βAOB community and nitrification process (see review by Bernhard and Bollmann, 2010). Although AOA are far more abundant than βAOB, Di et al. (2009) provided an evidence that nitrification might be driven by bacteria rather than archaea in nitrogen-rich environment. So far, despite many studies were conducted to investigate how those microorganisms are distributed in the environment and how they influence nitrogen cycling, the importance and contribution of βAOB to nitrification is not well understood (Cetecioğlu et al., 2009).

As one of the largest continental shelves in the world, the Changjiang River Estuary (CRE) is subject to a greater impact of human activities. According to Gong et al. (2006), the primary production off the CRE, as high as 2 079 mg/(m^3·d), was induced mostly by riverine nutrient input. NO_3^- concentration off the CRE, in average of 17.6 μmol/L in 2003–2006, was about 1.5-fold of that in the 1980s (Chen et al., 2010). Serious eutrophication leads to frequent occurrence of toxic algal blooms. The *Prorocentrum dentatum*, a dominant species of toxic algal blooms off the CRE, was increased from 12.5% of the whole phytoplankton cell density in the 1980s to 36% in 2002. This abnormal phenomenon closely related to the nitrogen transmission (Li et al., 2007). Excess nitrogen loading, associated by water column stratification, can shape hypoxia off the CRE in summer (Chen et al., 2007). However, there are few publications describing the hypoxic conditions off the CRE and very little is known about the biogeochemical cycling of nitrogen which may be the mainly causes of ecological consequences (Chen et al., 2007; Li et al., 2002; Li and Dag, 2004).

The present study of βAOB off the CRE was carried out prior to hypoxic events (June) in 2006. We examined the abundance and composition of sediment βAOB population at eight sampling stations, and investigate potential links between βAOB community characteristic parameters and environmental variation or nitrification estimates. It aimed to reveal the controlling factors of nitrification process of surface sediment off the CRE.

2 Materials and methods

2.1 *Site location and sample collection*

The research area is located off the CRE that belongs to the East China Sea (ECS), where low dissolved oxygen (DO) zone is documented in the bottom water from July to September (Li et al., 2002). This study was carried out from 12 to 24 June 2006 aboard the *Dongfanghong* II Research Vessel. Eight stations were selected in two sections that crossed at Site S4 (Fig. 1). One section, consisting of Sites S1, S4, S6, S7 and S8, was almost parallel to the coastline. Another was perpendicular to the coastline extending from the Changjiang River mouth including Sites S2, S3, S4 and S5. Sites S4 and S8 are the centers of two hypoxia zones in summer of the study area according to the data of 1999 (Li et al., 2002).

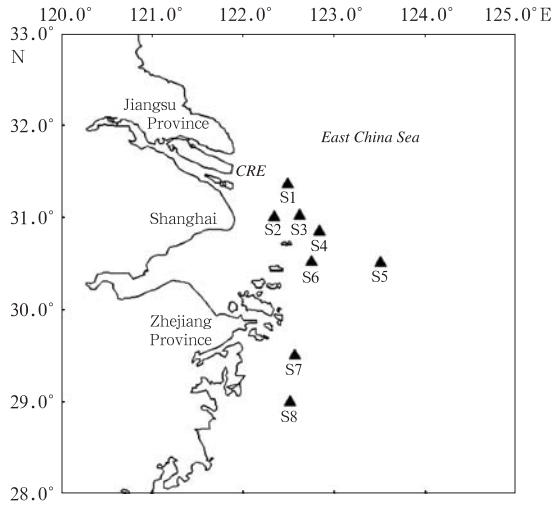

Fig.1. Map of the sampling locations.

Temperature, salinity and depth were recorded throughout the water column with a SeaBird CTD (Sea-bird Electronics, Washington, America). NH_4^+ and NO_3^- were measured using standard colorimetric techniques on a Bran-Luebbe Auto-analyzer-III (SEAL Analytical GmbH, Germany). Sediment core sample (10 cm diameter and 2 cm depth) collected in sterilized Perspex and stored in sterilized plastic bags for measuring quantity and diversity of AOB. Information on the nitrification estimates at those sites can be found in Li et al. (2009).

2.2 *Quantification of βAOB*

Fluorescence in situ hybridization (FISH) technique was used to investigate the βAOB abundance. Three replicated samples (1.0 g) were suspended in 5 ml sterile filtered Milli-Q water that had been filtered through a sterile 0.2 μm filter, then ultrasonicated for 2 min to disperse and centrifuged for 15 min at 4 000 r/min. The water phase was fixed in 4% freshly paraformaldehype solution and immediately frozen at –20 °C until the analysis (Pollard, 2006). Ten microlitres above fixed samples were placed on a slide

coated with 0.1% gelatin in the presence of 0.01% chromium potassium sulfate and dried overnight. The specimen was dehydrated by successive 50%, 80% and 98% ethanol washes and air dried. The following FAM labeled oligonucleotide probe Nso1225 was used for detecting βAOB, which is a special probe targeting all known βAOB except *N. mobilis* with few mismatches (Junier et al., 2008; Kim et al., 2006; Purkhold et al., 2000). Hybridization was performed according to Manz et al. (1992). The slides were examined with Leica DMLA equipped with He/Ne lasers (excitation wave length 550 nm).

2.3 *DGGE analysis of ammonia-oxidizing bacteria*

DNA was extracted from sediment samples (0.5 g) using the freeze-thaw method described by Powell et al. (2003). The bacteria *amo*A gene were amplified on an iCycle Thermal Cycler (Bio-Rad laboratories, USA) using the primers *amo*A-1F-GC and *amo*A-2R targeting 490 bp of the open reading frame of the ammonia monooxygenase subunit A gene (*amo*A) (Sahan and Muyzer, 2008). PCR reactions and conditions were similar to those described by Rotthauwe et al. (1997) and Coolen et al. (2004). The quantity of the extracted DNA was analyzed by electrophoresis on a 1.0% agarose gel. For DGGE, 6% polyacrylamide gels with a denaturant gradient of 40%–60% for the bacteria *amo*A gene fragments were used. Electrophoresis was run at a constant voltage of 60 v for 16 h at 60 °C using the Dcode universal mutation detection system (Bio-Rad Laboratories, USA). Subsequently, the gel was stained in ethidium bromide solution, destained for 30 min in water, and photographed under UV illumination using the Gel Doc XR System (Bio-Rad Laboratories, USA). DGGE profiles were analyzed using Quantity One 4.0 (Bio-Rad Laboratories, USA) to obtain a matrix containing the band percentage values of samples.

The interested bands were excised and incubated overnight in 20 μl TE buffer to elute DNA. Reamplification of eluted DNA by PCR was carried out by primers *amo*A-1F and *amo*A-2R. Purified PCR products were used for DNA sequence determination. The obtained sequences were edited and searched against the NCBI GenBank database by using the BLAST program.

2.4 *Statistical analysis*

Univariate statistical and linear regression analyses were performed by SPSS 13.0 for Windows to test specific difference within samples and determine the correlation between data sets. All data analyses on the DGGE profile were performed using the software program Primer 6.0 to elucidate samples similarity, and BIO-ENV procedure in the software package was used to relate environment variables to βAOB community composition (Clarke and Gorley, 2006).

3 Results

3.1 *Environmental parameters and nitrification estimates*

Environmental parameters and NE values were in Table 1. Dissolved oxygen concentrations were consistently higher than 3 mg/L (defined as DO critical level of hypoxia) which had a similar distribution trend to those during the hypoxic period in CRE region. Overlying water of S2 was characterized as obvious lowest salinity (16.96) and remarkable highest turbidity (122.07), which indicated that the bottom layer could exchange well with the Changjiang River diluted water at this site. Despite S3 is close to S2, there are significantly different results of salinity and turbidity between them; it maybe indicate that the water column would be stratified to isolate the bottom water from exchange with low-salinity overlying surface water (Chen et al., 2007). The nitrogen nutrients showed

Table 1. Overview of the environmental parameters and NE of the different sites in the CRE[1)]

Site	DO/ $mg\cdot L^{-1}$	S	T/ °C	Turbidity/ FTU	TOC/ $mg\cdot g^{-1}$	K	NH_4^+/ $\mu mol\cdot L^{-1}$	NO_3^-/ $\mu mol\cdot L^{-1}$	Nitrification/ $\mu mol\cdot m^{-2}\cdot h^{-1}$
S1	5.95	33.31	18.74	37.38	26.5	3.47	3.60	2.85	116.0
S2	7.32	16.96	22.89	122.07	38.6	2.85	26.30	10.96	514.3
S3	3.31	30.06	20.46	23.61	51.6	3.10	18.72	5.07	175.3
S4	4.05	31.72	20.18	94.76	51.6	4.54	6.92	3.21	130.5
S5	6.23	33.91	20.39	7.54	19.6	7.88	6.70	1.90	100.3
S6	5.85	33.89	20.47	26.36	39.8	4.38	11.18	6.46	127.0
S7	5.47	34.18	18.21	13.73	41.2	3.63	10.30	2.64	111.5
S8	3.58	34.42	18.92	4.79	48.5	2.52	7.71	4.79	103.8

Note: [1)] Data are from Li et al. (2009).

a trend with a decrease from the estuary mouth to the offshore area. The nitrification estimate of S2 was significantly higher than other sites, which may be explained by the moderate salinity environment accompanied by the sufficiently high NH_4^+ and DO concentration. TOC concentrations and absorption ability for NH_4^+ of sediments (K) ranged between 19.6–51.6 and 2.52–7.88 respectively.

3.2 *βAOB abundance distribution*

βAOB quantities varied from 1.87×10^5 to 3.53×10^5 cells/g of surface sediment at the study area (Fig. 2). In general, their abundance was relatively higher at S2 and S8, and generally reduced from estuary mouth to offshore sea. The concentration of NH_4^+ had a positive relationship with βAOB quantities (r=0.792, α <0.05). Compared with the significant maximum nitrification estimate, there is no significant higher value of βAOB quantities at Site S2.

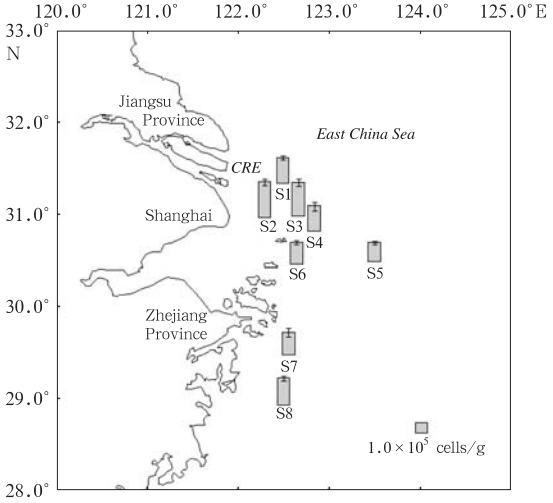

Fig.2. Distribution of βAOB quantity.

Although nitrification rates generally decrease as salinity increases, βAOB abundance did not show a negative correlation with salinity in our study (Bernhard et al., 2007).

3.3 *Diversity of βAOB*

The part of *amo*A gene was amplified by PCR technology from the eight sediment samples and an ammonia-oxidizing pure culture (*N. europaea*) (Fig. 3).

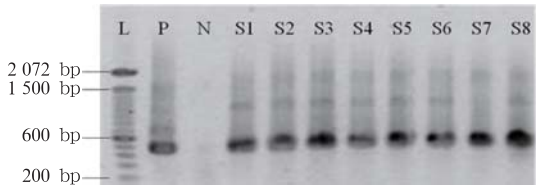

Fig.3. PCR amplification of the *amo*A fragment from environmental DNA. Lane L is DNA size standard (100 bp DNA ladder plus), and Lanes P and N are positive and negative samples.

The βAOB community structure was analyzed by DGGE profiles (Fig. 4). Differences of eight sediment samples could be observed with bands appearing or disappearing with sites. The number of the DGGE bands, as an indicator of richness (S), ranges from 2 (S4) to 9 (S1 and S2). Statistical analysis of the DGGE data showed, at a level of 40% similarity, the S2 sample was clearly separated from other samples. At a level of 60% similarity, S5 formed another cluster (Fig. 4b). According to dendogram cluster results, three different assemblages could be distinguished, which located at intermediate salinity, high-salinity near shore and high-salinity offshore sea area.

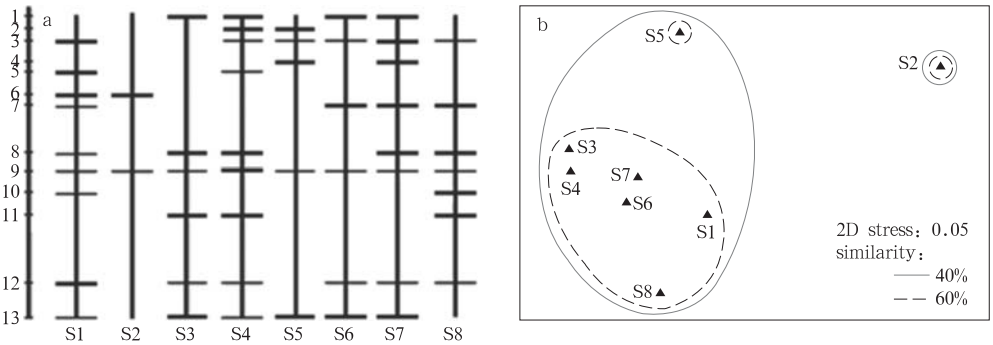

Fig.4. The stucuture of βAOB community for part of derived from *amo*A gene derived from sediments of CRE. a. Community patterns identified from DGGE gel by Quantity One; b. Multi-Dimensional Scaling (MDS) ordination with cluster analysis by Primer 6.0.

Six bands were excised from the DGGE profiles for phylogenetic analysis (Table 2). Most of the *amo*A sequences were uncultured AOB closely related to the *Nitrosospira* group. Band 9 appeared at all sites. Its sequences was similar to the member of *Nitrosospira*, which were frequently found in the freshwater and ocean (Sahan and Muyzer, 2008; Lage et al., 2010; Allen et al., 2010).

Table 2. Aligment results of *amo*A sequences detected by PCR-DGGE

DGGE Band No.	Closest match by FASTA search (assession number)	Sequence identity (%)	Alignment length/bp
3	Uncultured *Betaproteobacterium* clone (EU651365)	99	395
6	*Nitrosovibrio tenuis* (U76552)	96	423
9	Uncultured *Nitrosospira sp.* clone Nt3 (AY445619)	94	357
10	Uncultured *Betaproteobacterium* clone (EF551028)	100	403
12	Uncultured *Betaproteobacterium* clone (EF551022)	95	405
13	Uncultured bacterium (EU244546)	99	434

4 Discussion and conclusions

This study used FISH and DGGE techniques to quantify and cluster βAOB in sediment and investigated their dynamics with environmental variations and biogeochemical functions. FISH were developed to detect specific bacteria independent of culturing in many areas of microbial ecology. We should be aware of the possible limitations of technique such as background auto-fluorescent non-target cells or debris that makes it more difficult to recognize the target cells (Hug et al., 2005). We also recognize that using fluorescence techniques to enumerate βAOB will always lead to an underestimate of the total cell concentration of any physiological type. However, as a specific identification and enumeration method, FISH can quantitatively detect βAOB with a genetic similarity and approximately inform tendency of βAOB distribution (Ward and Carlucci, 1985).

The abundance of βAOB measured in this study was similar to those in soil which typically contains 10^4–10^6 βAOB cells/g and in Lake Taihu sediments which had 10^5 βAOB cells/g at most sites (Nicol and Schleper, 2006; Wu et al., 2010). In the study of Dang et al. (2010) there were much more βAOB detected in the sediments of the Jiaozhou Bay. High abundance of βAOB provided evidence of their potential importance in nitrification. It is known that AOA widely distributed in sediments off the CRE (Dang et al., 2008), but no report has provided the data of AOA abundance, making it incapable to study the relative abundance of βAOB to AOA.

Although significant variation was detected in βAOB quantity, none of the environmental parameters measured can be implicated in controlling the patterns of variation in βAOB. This result could be due to a complex interaction of multiple parameters, lacking either a dominant cause-and-effect relationship or lacking adequate data to resolve the dominant relationship. With long generation times, βAOB community should be changed slowly as a response to dynamic environmental factors. For example, a kinetic response to the experimental addition of ammonia for cultured βAOB strains can be detected, but this phenomenon was typically not observed for natural βAOB community (O'Mullan and Ward, 2005).

To compare the difference of βAOB diversity among eight samples, PCR-DGGE was chosen to analyze the βAOB community. By BIO-ENV procedure in the software package Primer 6.0, main environmental factors was related to bacteria communities. This analysis consists of the similarity matrix obtained from DGGE profiles to the resulting matrix of Euclidean distances obtained after normalization of the environmental data (Sahan and Muyzer, 2008). The variations in species composition of βAOB was significantly correlated and best explained by changes in salinity, temperature, K and TOC (r=0.685, $\alpha < 0.01$). Figure 5 shows the relationship of βAOB community composition with strong correlated environmental data in two-dimensional plot. It's obviously observed that variations in temperature and salinity separated the βAOB communities of sediment sample collected at Site S2 from other samples. The spatial variability of βAOB by temperature and salinity has also been shown in the studies of Sahan and Muyzer (2008). There are few studies showing the impact of TOC and K on the community structure of βAOB. It was hypothesized that high concentration of organic matter should increase competitive ability of heterotrophic bacteria for DO to limit the growth of βAOB (Konneke et al., 2005). As for K, there should be more research to explain the mechanism of effect. In our study, no effect of inorganic nitrogen species on βAOB distribu-

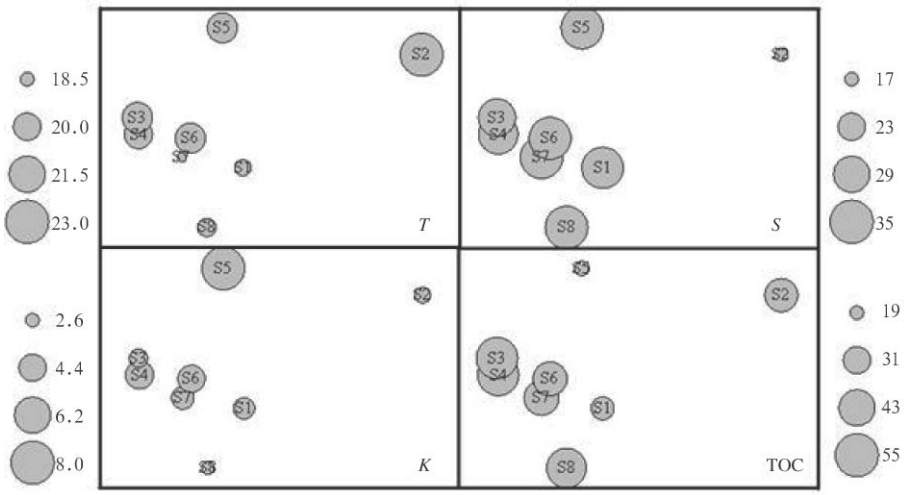

Fig.5. Multi-Dimensional Scaling (MDS) ordination with superimposed circles, the area is proportional to the selected environmental parameters obtained from the DGGE profiles.

tion was observed, which was similar to the study of Sahan and Muyzer (2008) in the Westerschelde estuary, and Wuchter et al. (2006) with cultured strains from the North Sea.

The step-by-step evaluation of multivariate regression analysis identifying βAOB abundance was a main factor to affect NE of high-salinity sea area (S2 excluded), with an influence ratio of 87.7%, at a level of 40% biodiversity similarity. Although we did find strong correlations between them (Fig.6), it is normally thought that potential nitrification rates roughly represent the abundance of βAOB (Henriksen, 1980). The reasonable explanation for the observed relationship is the similar temperature and high ammonium concentrations of all seven sites. The sediment βAOB abundance, considering its positive relationship to NE, is expected to be used as an indirect index of nitrification activity at the study sea area. It should be noted that our estimation of abundance based on gene targets only the AOB within *Betaproteobacteria* (Bernhard et al., 2005), however there would be other microorganisms contributing to nitrification process, for example methane-oxidizing bacteria and ammonia-oxidizing Archaea (Dang et al., 2008). This reinforces the need to evaluate the explicit contribution of AOB or AOA to nitrification.

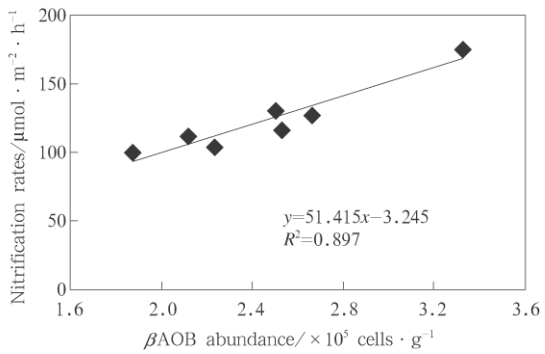

Fig.6. Correlation between AOB abundance and nitrification.

Understanding the link between microbial community and ecosystem processes is a fundamental goal of microbial ecology, however conclusive results could not be ascertained. Our work was focused on studying the βAOB community off CRE by FISH and PCR-DGGE methods, trying to set a direct and quantitative link between community and process in a natural system. However, we are aware of the possible limitation of those two molecular biology technique. Future studies should include a more complete accounting of contributing populations with other techniques, such as quantitative PCR, stable isotope probing and measurements of potential nitrification rates.

In summary, we investigate the abundance and structure of sediment βAOB community off the CRE on June in 2006. Correlations between nitrification estimates and βAOB were detected within the similar phylogenic affiliation of dominant members of the communities. Salinity, temperature, K and TOC, co-determined the community composition of βAOB. The relative abundance of βAOB and its contribution to nitrification need further investigation.

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