

1 SEDIMENTS, SEC 2 • PHYSICAL AND BIOGEOCHEMICAL PROCESSES • RESEARCH
2 ARTICLE

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4 **An overlooked nitrogen loss linked to anaerobic ammonium oxidation in estuarine sediments in**
5 **China**

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27 **Abstract**

28 *Purpose* Despite its importance, anammox (anaerobic ammonium oxidation) in estuarine sediment
29 systems remains poorly understood, particularly at the continental scale. This study aimed to
30 understand the abundance, diversity, and activity of anammox bacteria and to determine the main
31 factors influencing the anammox process in estuarine sediments in China.

32 *Materials and methods* Estuarine sediments were collected from 18 estuaries spanning over 4,000
33 kilometers. Experiments using an ^{15}N -tracer, quantitative PCR, and clone library construction were
34 used to determine the activity, abundance, and diversity of anammox bacteria. The impact of
35 environmental factors on anammox processes were also determined.

36 *Results and discussion* The abundance of the anammox-specific hydrazine synthase (*hzsB*) gene ranged
37 from $1.8 \times 10^5 \pm 3.4 \times 10^4$ copies g^{-1} dw to $3.6 \times 10^8 \pm 7.5 \times 10^7$ copies g^{-1} dw. *Candidatus Scalindua*,
38 *Brocadia*, *Kuenenia*, *Jettenia*, and two novel unidentified clusters were detected, with *Scalindua*
39 dominating the anammox population. Additionally, the abundances of *Scalindua*, *Kuenenia*, and
40 *Brocadia* were found to be significantly correlated with latitude. The anammox rates ranged from
41 0.29 ± 0.15 to 13.68 ± 3.98 nmol N g^{-1} dw h^{-1} and contributed to 2.39-82.61% of total N_2 production.
42 Pearson correlation analysis revealed that the anammox rate was positively correlated with total
43 nitrogen, total carbon, and temperature, and was negatively correlated with dissolved oxygen (DO).
44 The key factors influencing the *hzsB* gene abundance were ammonium concentration, salinity, and DO.
45 Ammonium concentration, pH, temperature, and latitude were main variables shaping the
46 anammox-associated bacterial community.

47 *Conclusions* Our results suggested that anammox bacteria are ubiquitous in coastal estuaries in China
48 and underline the importance of anammox resulting in N loss at a continental scale.

49

50 **Keywords** Activity • Anammox • Estuarine sediments • N loss • Spatial variation

51

52 **1 Introduction**

53 Estuarine environments are partially enclosed coastal water bodies with rivers or streams and a free
54 connection to the open sea, resulting in potential anthropogenic pollutants, particularly inorganic
55 nitrogen. Anaerobic ammonium oxidation (anammox) couples ammonium oxidation with nitrite
56 reduction under anaerobic conditions. Based on marine geochemical data, Richard first proposed the
57 hypothesis that a group of unknown microorganisms might exist in anoxic marine sediments that could
58 carry out anammox (Richard 1965). Over a decade later, the existence of anammox bacteria was also
59 predicted via chemical reaction thermodynamic calculations (Broda 1977). Heterotrophic
60 denitrification was considered to be the only known pathway for the loss of fixed nitrogen to the
61 atmosphere for decades until the discovery of anammox bacteria in a wastewater treatment plant
62 (Mulder et al. 1995). Subsequently, anammox bacteria were detected with broad biogeographic
63 distribution in various natural ecosystems, including marine sediments (Thamdrup and Dalsgaard 2002;
64 Trimmer and Nicholls 2009; De Brabandere et al. 2014; Shao et al. 2014; Dang et al. 2016), estuarine
65 sediments (Dale et al. 2009; Dang et al. 2010; Li et al. 2011; Wang et al. 2012; Dang et al. 2013), and
66 paddy soils (Zhu et al. 2011; Nie et al. 2015; Yang et al. 2015). The relative contribution of anammox
67 to dinitrogen production can vary widely, with >80% of total N₂ production observed in the eastern
68 tropical South Pacific oxygen minimum zone (OMZ) off the coast of northern Chile (De Brabandere et
69 al. 2014).

70 The anammox process is mediated by bacteria belonging to the Candidate Brocadiales order, which
71 are affiliated with the Planctomycetes phylum (Jetten et al. 2010). Five *Candidatus* genera of
72 anammox bacteria have been described: '*Brocadia*' (Kartal et al. 2008), '*Kuenenia*' (Schmid et al.
73 2000), '*Scalindua*' (Schmid et al. 2003; Kuypers et al. 2005), '*Anammoxoglobus*' (Kartal et al. 2007)
74 and '*Jettenia*' (Quan et al. 2008). Each of these genera have been detected in coastal estuaries (Zhu et
75 al. 2015). The anammox genotype is associated with hydrazine synthase, an intermediate step in the
76 anammox pathway that synthesizes hydrazine from nitric oxide and ammonium (Kartal et al. 2011). We
77 used the *hzsB* gene that encodes one of the hydrazine synthase subunits as a molecular marker (Wang et
78 al. 2012; Zhu et al. 2013; Yang et al. 2015) in order to determine the abundance and diversity of
79 anammox bacteria.

80 China is the largest rice producer in the world, stably consuming ~20 Tg of N based chemical
81 fertilizers per year (21.6 Tg N in 2005, 23.8 Tg N in 2011; China Agricultural Yearbook, 2012). It was

82 estimated that nearly 20% of the total nitrogen loss, mostly in the form of nitrate, was transported into
83 estuarine and coastal ecosystems in the past three decades through riverine discharge and atmospheric
84 deposition (Cui et al. 2013), resulting in water pollution (e.g., coastal eutrophication, hypoxia, harmful
85 algae blooms) (Deegan et al. 2012). Thus, estuaries are thought to be a potential sink of nitrogen,
86 especially nitrate, which is the substrate for many nitrogen cycling processes (e.g., denitrification and
87 anammox). Several studies have reported anammox processes in estuarine sediments in China (Dang et
88 al. 2010; Li et al. 2011; Hu et al. 2012; Dang et al. 2013; Zhu et al. 2013), but understanding of N
89 cycling and the release of fixed N as dinitrogen gas (N₂) is lacking due to the high heterogeneity of the
90 anammox activity in different estuarine and coastal sediments. Therefore, it is crucial to estimate the
91 contributions of N₂ from anammox in different estuarine environments and discover the key factors
92 governing activity and microbial diversity at a large scale.

93 On the basis of previous studies (Dang et al. 2010; Li et al. 2011; Hu et al. 2012; Zhu et al. 2013;
94 Hou et al. 2015), we hypothesize that coastal estuaries are hotspots of anammox processes. Therefore,
95 we sampled estuarine sediments from 18 rivers (from north to south China) in order to: (i) determine
96 anammox abundance *via* qPCR of the hydrazine synthase (*hzsB*) gene and investigate the community
97 composition of anammox bacteria; (ii) evaluate the contribution of denitrification and anammox to N₂
98 production using an ¹⁵N-tracer technique; and (iii) elucidate the impact of environmental factors on
99 anammox process in estuarine sediments along the coastal zone in China.

100

101 **2 Materials and methods**

102 **2.1 Sample collection**

103 Surface sediment samples (0-10 cm) of five replicates (*ca.* 500 g each subsample) were collected
104 from 18 estuaries during July 2013 (total of 90 subsamples), spanning over 4,000 kilometers of the
105 coastline and covering a range of climatic and geological zones. The study area and sampling sites are
106 presented in Table S1. The overlaying water was also collected and kept on ice during transportation.
107 Sediment samples were stored in sterile plastic bags, sealed and transported to the laboratory on ice.
108 Each replicate sample was partitioned into three subsamples. One subsample was incubated to
109 determine denitrification and anammox activities immediately after arrival and another subsample was
110 used for analysis of chemical properties. The remainder was stored at -80 °C for genomic DNA
111 extraction and further molecular analysis.

112

113 **2.2 Chemical properties of sediment samples**

114 Sediment pH was determined at a soil/MilliQ water ratio of 1:2.5 with a pH analyzer (XL60, Fisher,
115 USA). Water temperature, dissolved oxygen (DO,) and salinity were measured *in situ* using a Hydrolab
116 DS5 multiparameter water quality analyzer (Hach, Loveland, CO, USA). The total N (TN) and total C
117 (TC) were analyzed by using dry combustion in a C/N analyzer (Vario MAX C/N, Germany). NO_x^- -N
118 and NH_4^+ -N were measured using an ion chromatograph (ICS-3000, USA) after 2M KCl extraction at a
119 soil/KCl ratio of 1:10 and filtration through a 0.22 μm membrane filter. All analyses were performed in
120 triplicate for each sample.

121

122 **2.3 Anammox and denitrification activity measurement with ^{15}N labeled ammonium and nitrate**

123 The rates of anammox and denitrification were measured using the ^{15}N -tracer technique (Thamdrup
124 and Dalsgaard 2002; Risgaard-Petersen et al. 2004) with slight modifications, and their relative
125 contributions to N_2 production were then calculated based on the rates. Briefly, ~3.5 g sediment (wet
126 weight) was transferred to a 12.0 mL glass vial (Extainer, Labco, High Wycombe, Buckinghamshire,
127 UK) and filled with N_2 -purged water. The resulting sediment slurries were pre-incubated for 24 h to
128 remove intrinsic NO_x^- and oxygen. After the pre-incubation step, both NO_2^- and NO_3^- were under the
129 detection limit of ion chromatograph (0.05~0.1 ppm and 0.075~0.1 ppm for NO_2^- and NO_3^- ,
130 respectively). Subsequently, the vials were portioned into three treatments, which were spiked through
131 the stopper of each vial with 100 μL of N_2 -purged stock solution of (1) $^{15}\text{NH}_4^+$ (^{15}N - $(\text{NH}_4)_2\text{SO}_4$, ^{15}N
132 at. %: 99.14), (2) $^{15}\text{NH}_4^+ + ^{14}\text{NO}_3^-$, and (3) $^{15}\text{NO}_3^-$ (^{15}N - KNO_3 , ^{15}N at. %: 98.15%), resulting in a final
133 concentration of about 100 μM N. The incubations were performed at a temperature of $25 \pm 1^\circ\text{C}$ and
134 blocked at schedule time intervals (0, 3, 6, 12, 24 h) by injecting ZnCl_2 solution (200 μL , 7 M) to stop
135 microbial activity. Concentrations of the produced $^{29}\text{N}_2$ and $^{30}\text{N}_2$ were measured by continuous flow
136 isotope ratio mass spectrometry (MAT253 with Gasbench II and autosampler (GC-PAL), Bremen,
137 Thermo Electron Corporation, Finnigan, Germany). The rate and potential contribution to N_2
138 production of either anammox or denitrification were calculated as described before (Thamdrup and
139 Dalsgaard 2002; Trimmer et al. 2003).

140 **2.4 DNA extraction and clone library construction**

141 Approximately 0.5 g soil was used for genomic DNA extraction using a FastDNATM SPIN Kit for

142 Soil (MP Biomedicals, USA) according to the protocols provided by manufacturer's instructions. A
143 nested PCR approach was used to amplify the anammox 16S rRNA genes. In the first round of this
144 PCR, the primer set Pla46f-630r was used to amplify the Planctomycetales 16S rRNA genes
145 (Juretschko et al. 1998; Schmid et al. 2005). In the next round, anammox 16S rRNA genes were
146 amplified using Amx368f-Amx820r as the primer set (Schmid et al. 2000; Schmid et al. 2003) and
147 amplicons of Planctomycetales 16S rRNA genes as templates. The PCR reactions and thermal cycles
148 were performed as previously described (Zhu et al. 2011). The amplified products were verified by
149 electrophoresis in a 1.0% agarose gel, and then purified using a Universal DNA Purification Kit
150 (Tiangen, Beijing China). The purified products were cloned into a pMD19-T vector (TaKaRa, Bio Inc.,
151 Shiga, Japan) according to the manufacturer's instruction. At least 40 positive clones of each clone
152 library were randomly selected for sequencing (MajorBio LTD., Shanghai, China). The quality of
153 sequence was examined using the Chromas LITE (version 2.01, Technelysium Pty, QLD, Australia)
154 program and the existence of chimeric sequence was further checked using QIIME (version 1.8)
155 (Caporaso et al. 2010). After that, the sequences were aligned with the MEGA (version 6.0) (Tamura et
156 al. 2013) software and manually checked and trimmed.

157

158 **2.5 Quantitative PCR (qPCR) assay**

159 The abundance of *hzsB* gene was determined in triplicate using a Light Cycler 480 with the primer
160 set HSBeta396F-HSBeta742R (Wang et al. 2012). The 20 μ L qPCR reaction contained 10 μ L
161 2 \times TransStart[®] Top Green qPCR SuperMix (AQ131, Transgen biotech, Beijing, China), 0.25 μ M each
162 primer, 0.8 μ L bovine serum albumin (BSA, 20 mg mL⁻¹) and 2 μ L of 5-fold diluted DNA as a template.
163 The standard curve was obtained using 10-fold serial dilutions of plasmid DNA with target-gene of
164 *hzsB*. Three non-template controls were carried out for each quantitative assay. The PCR was
165 performed in triplicate with the following thermal profile: 95°C 3 min, followed by 40 cycles of 95°C
166 for 15 s and 62°C for 34 s. Melting curves showed only one peak at 86°C. Only the reactions with
167 efficiencies between 90% and 110% (Malte et al. 2015), and standard curves with correlation
168 coefficient above 0.99 were employed in this study.

169

170 **2.6 Phylogenetic analysis**

171 The sequences from each clone library were identified by blasting in NCBI GenBank database.

172 Operational taxonomic units (OTUs) were defined using 97% similarity in the nucleotide sequences by
173 Mothur (version 1.34.0) (Schloss et al. 2009). The related reference sequences and our representative
174 sequences were aligned, and the neighbor-joining phylogenetic tree was constructed by MEGA
175 (version 6.0). A bootstrap analysis based on 1,000 replicates was applied to assess the cluster stabilities.
176 The diversity, community composition of anammox, and redundancy analysis (RDA) were performed
177 using R (version 2.14.0) software (<https://www.r-project.org/>). The plots in this study were created
178 using Origin program (version 9.0).

179

180 **2.7 Statistical analysis**

181 Pearson correlation analyses were used to test the correlations among the anammox bacterial activity,
182 abundance and different environmental factors, using the SPSS 20.0 (SPSS, Chicago, Illinois, USA).

183

184 **2.8 Nucleotide sequence accession numbers**

185 The sequences obtained in this study are available in GenBank under accession numbers
186 KU987935 - KU990864.

187

188 **3 Results**

189 **3.1 Physicochemical properties of estuarine sediments**

190 The locations and physicochemical properties of the sampling sites can be found in Table S1. The
191 physicochemical properties exhibited great heterogeneity among samples from northern and southern
192 China. The pH ranged from 8.57 ± 0.59 in LN-LH in the north to 6.49 ± 0.03 in GD-LJ in the south. The
193 TC varied from 0.53 ± 0.33 to 1.84 ± 0.87 g kg⁻¹ dw, while TN ranged from 0.03 ± 0.01 to 0.17 ± 0.08 g kg⁻¹
194 dw, resulting in the C/N ratio ranging from 7.34 ± 1.7 to 26.84 ± 3.63 . Concentration of NH₄⁺-N ranged
195 between 0.18 ± 0.1 mg kg⁻¹ dw (ZJ-QTJ) and 12.54 ± 9.59 mg kg⁻¹ dw (TJ-YDXH). NO_x⁻-N
196 concentration varied from 0.03 ± 0.01 mg kg⁻¹ dw (ZJ-JJ) to 5.40 ± 4.72 mg kg⁻¹ dw (LN-LH), while not
197 detected in ZJ-QTJ, GX-NLJ, GX-QJ and GX-FCJ.

198

199 **3.2 Anammox rates and contributions to N₂ production**

200 No significant accumulation of $^{15}\text{N}_2$ -labeled gas ($^{29}\text{N}_2$ and/or $^{30}\text{N}_2$) was detected in any of the sample
201 slurries amended with only $^{15}\text{NH}_4^+$ (Fig. S1A, Supplemental material), indicating that all residual
202 $^{14}\text{NO}_x^-$ had been consumed during pre-incubation. When both $^{15}\text{NH}_4^+$ and $^{14}\text{NO}_3^-$ were added, only $^{29}\text{N}_2$
203 accumulated in each soil (Fig. S1B, Supplemental material), indicating that anammox must have been
204 occurring. When amended with $^{14}\text{NH}_4^+$ and $^{15}\text{NO}_3^-$, significant production of both $^{29}\text{N}_2$ and $^{30}\text{N}_2$ were
205 detected, as a result of both anammox and denitrification (Fig. S1C, Supplemental material). Anammox
206 rates ranged from 0.29 ± 0.15 to 13.68 ± 3.98 $\text{nmol N g}^{-1} \text{dw h}^{-1}$, with the lowest and highest rate detected
207 in QTJ in ZheJiang province and ZJ in GuangDong province, respectively (Table 1). Denitrification
208 rates varied substantially from 0.31 ± 0.06 to 56.63 ± 17.29 $\text{nmol N g}^{-1} \text{dw h}^{-1}$. The contribution to N_2
209 production was calculated based on these rates, with anammox contributing between 2.39 % (GX-FCJ)
210 and 82.61% (ZJ-OJ) of total N_2 production (Table 1), with the remaining production attributed to
211 denitrification.

212

213 3.3 Abundance and composition of anammox bacteria

214 The presence of anammox bacteria was confirmed by qPCR in all samples from 18 estuaries (Fig.1).
215 The abundance of *hzsB* genes in the sediments was from $1.8\times 10^5 \pm 3.4\times 10^4$ copies $\text{g}^{-1} \text{dw}$ to $3.6\times 10^8 \pm$
216 7.5×10^7 copies $\text{g}^{-1} \text{dw}$. The ratio of *hzsB* gene copies to total bacterial 16S rRNA gene copies ranged
217 from 0.005% (ZJ-YJ) to 3.72% (GD-ZJ).

218 A total of 2,930 sequences were retrieved from the 18 estuarine sediment samples and clustered
219 into 223 operational taxonomic units (OTUs). The phylogenetic analysis of the 16S rRNA gene
220 showed that 80.17% of sequences were affiliated with the following known anammox bacterial genera:
221 *Candidatus Brocadia*, *Candidatus Scalindua*, *Candidatus Kuenenia*, *Candidatus Jettenia*, and an
222 additional 19.83% belonged to the unclassified Planctomycetes phylum (4 Clusters, I-IV) (Fig. S2).
223 The most abundant genus was *Candidatus Scalindua*, followed by *Candidatus Brocadia*, *Candidatus*
224 *Kuenenia* and *Candidatus Jettenia* (Fig. S2). The distribution of each cluster in 18 estuarine sediments
225 was visualized in Fig. 2. Among them, *Candidatus Scalindua* was found to be prevalent in all sites
226 and occupied up to 43.07% of the relative abundance (1262 sequences). *Candidatus Brocadia* was
227 detected in most of the sites with the exception of GX-FCJ, FJ-HTX, ZJ-OJ, ZJ-JJ and LN-LH, and
228 accounted for 19.32% (566 sequences). *Candidatus Kuenenia* was found to be present in all sediments
229 except LN-LH, accounting for 16.18% (474 sequences). Sequences belonging to the *Candidatus*

230 *Jettenia* genus contributed 1.6% (47/2930) overall, but up to 20% of the likely anammox bacteria in
231 TJ-YDXH were assigned to *Candidatus Jettenia*. The remaining sequences (577 sequences) were
232 affiliated to unknown Clusters and up to 60% of these unclassified taxa were found in TJ-YDXH. The
233 ratio of *Candidatus Scalindua* to total anammox bacteria decreased from north to south China based
234 on the latitude, with the exception of GX-FCJ and TJ-YDXH (Fig. 2, Fig. 3). In contrast, the
235 abundance of *Candidatus Brocadia* and *Candidatus Kuenenia* exhibited a general pattern which
236 increased from north to south (Fig. 2, Fig.3). Additionally, the ratio of the genus of *Candidatus*
237 *Scalindua* to total anammox bacteria was found to be positively correlated with the latitude, whereas
238 the proportions of genera of *Candidatus Brocadia* and *Candidatus Kuenenia* were both negatively
239 correlated with the increasing latitude (Fig. 3).

240

241 **3.4 Influence of environmental factors on anammox rate, abundance, and diversity**

242 Pearson correlation analysis was used to illustrate the effects of the environmental factors on the
243 anammox rate and abundance (Fig. 4). Results showed that anammox rates were positively correlated
244 with TC ($P<0.01$), Temperature ($P<0.01$), NO_3^- concentration ($P<0.05$), *hzsB* abundance ($P<0.05$),
245 *hzsB/16S rRNA* ($P<0.05$), and salinity ($P<0.05$), whereas negatively correlated with DO ($P<0.01$). The
246 *hzsB* abundance was positively correlated with salinity ($P<0.01$), NO_3^- concentration ($P<0.05$), and
247 negatively with NH_4^+ concentration ($P<0.01$). As revealed by RDA analysis, NH_4^+ concentration, pH,
248 latitude, and temperature were found to be the main factors affecting the anammox bacterial diversity
249 (Fig. 5).

250

251 **4 Discussion**

252 In the present study, the occurrence of anammox in coastal estuarine sediments in China was
253 corroborated by using both molecular and isotope-tracing experiments. Four known genera of
254 anammox bacteria (*Candidatus Scalindua*, *Candidatus Brocadia*, *Candidatus Kuenenia*, and
255 *Candidatus Jettenia*) were identified, illustrating a relatively diverse set of anammox bacteria in the
256 selected estuarine sediments. Similarly, diverse anammox bacteria have been observed in other
257 estuarine and coastal wetlands (Dale et al. 2009; Hong et al. 2014; Lisa et al. 2014). This indicates that
258 the fresh-seawater interface may provide diverse habitats and eco-niches for a higher diversity of
259 anammox bacteria. In contrast, anammox community diversity in oceans (Schmid et al. 2007), rivers

260 (Zhang et al. 2007; Hu et al. 2012), and lakes (Hamersley et al. 2009) was low, limited primarily to
261 *Scalindua* or *Brocadia*. Specifically, *Candidatus Scalindua* species were reported to be dominant in
262 marine (Schmid et al. 2007; Dale et al. 2009; Cao et al. 2011) and fresh water ecosystems (Schubert et
263 al. 2006). Furthermore, the microbial diversity of anammox bacteria exhibited a latitudinal gradient
264 along the coastal wetlands of China, which was consistent with previous results (Hou et al. 2015). This
265 implies that temperature is a key environmental factor shaping the distribution and diversity of
266 anammox bacteria in the coastal estuaries of China, and was further supported with the RDA analysis
267 (Fig. 5). Therefore, the distribution pattern based on latitude underlines the significance of temperature
268 in regulating the biogeographical distribution of anammox bacterial community structure and diversity
269 over a large spatial scale.

270 Our results exhibited a clear group specific biogeographical distribution (Fig. 5, Fig. S1).
271 *Candidatus Scalinduas* was detected at all sites and had the highest relative abundance (up to 43%)
272 among all anammox genera. *Candidatus Scalinduas* has been found in both marine (Schmid et al. 2007)
273 and fresh water ecosystems (Schubert et al. 2006) and this flexibility could be the reason for its high
274 abundance in most of our estuarine sites. *Candidatus Brocadia* accounted for 19.32% of the total
275 anammox bacteria across all sites. It was reported that *Brocadia* possess a diverse metabolism (Gori et
276 al. 2011). This may explain its ubiquitous distribution, where high organic loading is imported from
277 river water. Interestingly, four novel clusters were also detected in our samples, suggesting that
278 unknown anammox bacteria are yet to be discovered and investigated.

279 A wide range of *hzsB* copy numbers from anammox bacteria was detected (ranging from 1.80×10^5
280 $\pm 3.4 \times 10^4$ copies g^{-1} dw to $3.6 \times 10^8 \pm 7.5 \times 10^7$ copies g^{-1} dw), indicating that the overall abundance of
281 anammox bacteria was highly variable in sediments at a continental scale. To our knowledge, the
282 highest anammox abundance (2×10^9 copies per gram dry weight) recorded in natural environments was
283 detected in riparian sediments of the Pearl River Estuary in winter (Wang et al. 2012). The *hzsB*
284 abundance in our study, measured in the summer, was comparable to the Pearl River Estuary in
285 summer (1.3×10^6 - 1.2×10^7 copies g^{-1} dw) (Wang et al. 2012), China coastal wetlands (1.17×10^7
286 -4.25×10^7 copies g^{-1} dw) (Hou et al. 2015) and interface sediments (8×10^6 - 2×10^7 copies g^{-1} dw) (Zhu et
287 al. 2013). Additionally, the abundance of the *hzsB* gene was significantly related to salinity, and it was
288 positively but not significantly correlated with temperature (Fig. 4). One should expect copy numbers
289 of anammox functional genes will be a significant factor in controlling anammox activity; however,

290 here the anammox activity was not positively correlated with the *hzsB* gene copy number. This was
291 probably due to quantification based on DNA rather than RNA. Similar results by Etchebehere et al.
292 (2005) and Metz et al. (2003) demonstrated that no correlation was observed between copy numbers of
293 functional gene and relative functions. Additionally, the abundances of anammox-related genera were
294 significantly correlated with the latitude (Fig. 5), indicating that temperature was likely a key
295 environmental factor shaping the biogeographical distribution and diversity of the anammox bacterial
296 community.

297 Our results suggest a ubiquitous distribution of anammox bacteria in estuarine sediments along
298 4,000 kilometers of coast in China. On average, it was estimated that the anammox process contributed
299 15.94 % to the total N loss from the coastal wetland sediments of China. This approximate value was
300 comparable to that reported in rivers (Zhao et al. 2013), lakes (Schubert et al. 2006; Wenk et al. 2014),
301 paddy soils (Zhu et al. 2011; Yang et al. 2015) and other estuaries (Risgaard-Petersen et al. 2004;
302 Rysgaard et al. 2004; Engström et al. 2005), but lower than that generally detected in marine
303 ecosystems (Tamdrup and Dalsgaard 2002; De Brabandere et al. 2014). Based on our results, N loss
304 attributed to anammox was estimated to reach an average value of 9.70×10^5 t N per year on the basis
305 of sediment weight, study area, and anammox average rate obtained from slurry incubations. This
306 removal suggests approximately 40.4% of the total average terrigenous inorganic nitrogen (2.4×10^6 t N
307 per year) transported into the coastal wetlands of China. However, our results from the slurry
308 incubations in lab might overestimate the *in situ* anammox activity for three reasons. First, the
309 anammox activity might be enhanced since excess substrates were amended. Second, labile organic
310 carbon could be depleted after the prolonged pre-incubation, leading to favorable conditions for
311 anammox rather than denitrification. Finally, co-denitrification may be an additional pathway for N₂
312 production in sediments. Co-denitrification can generate ²⁹N₂ by reducing ⁴⁵N₂O, which is produced by
313 using ¹⁴NH₄⁺ and ¹⁵NO₃⁻/¹⁵NO₂⁻ in ¹⁵N isotope pairing experiments (Long et al. 2013). Nevertheless, the
314 ubiquitous detection of anammox bacteria indicated that anammox must play an important role in N₂
315 production. Therefore, our current study on the roles of anammox bacteria on N cycling at a continental
316 scale is an important step for the estimation N loss in the vast coastal estuaries which span over 4,000
317 kilometers of coastline and cover a range of climatic and geological zones. Furthermore, this study also
318 emphasizes the importance of protecting coastal estuaries due to their non-negligible removal capacity
319 of terrigenous inorganic nitrogen via both denitrification and anammox.

320

321 **5 Conclusions**

322 In conclusion, our results suggested that anammox bacteria are ubiquitous in estuarine sediments
323 across coastal China. The anammox community was dominated by the genera of *Candidatus Scalindua*,
324 *Candidatus Kuenenia* and *Candidatus Brocadia* and showed a clear biogeographic variation pattern
325 from north to south. The anammox activity contributed 2.39-82.61% of total N₂ production, suggesting
326 the important role of anammox in controlling N cycling across ecosystems.

327

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332

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488 **Figure legends**

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490 **Fig. 1** Abundance of anammox bacteria in the 18 estuarine sediments in China. The base map used is
491 from the National Fundamental Geographic Information System of China. The columns in the box
492 chart represent the log number of *hzsB* gene copy (copies g⁻¹ dry soil). For all boxplots, black center
493 lines represent the median and box edges are the first and third quartiles.

494 **Fig. 2** The relative abundance of each cluster in the Planctomycetes phylum in the 18 estuarine
495 sediment samples. The colors indicate different taxa. Horizontal axis is the relative abundance and the
496 vertical axis is the sampled estuaries.

497 **Fig. 3** The locations of the 18 sampling sites (a) and the correlation analysis between relative
498 abundance of the predominant genera (b) *Candidatus Scalindua*, (c) *Candidatus Brocadia*, (d)
499 *Candidatus Kuenenia* to the Planctomycetes phylum and the latitude of sampling sites

500 **Fig. 4** Pearson correlation analyses of anammox rate, *hzsB* gene abundance, *hzsB/16S rRNA* and the
501 soil properties (including TN, TC, C/N, pH, NH₄⁺, NO₃⁻, temperature, dissolved oxygen and salinity) in
502 the collected paddy soils. Red and blue denote positive, and negative correlation, respectively. * denotes
503 a P value of <0.05 and ** denotes a P value of <0.01. Tem=temperature; DO= dissolved oxygen;
504 sal=salinity; TN=total nitrogen; TC=total carbon.

505 **Fig. 5** Redundancy analysis (RDA) to measure the relationship between the anammox community
506 composition, physicochemical properties, and relative abundance of *Candidatus Scalindua*, *Candidatus*
507 *Brocadia*, *Candidatus Kuenenia* and *Candidatus Jettenia*. Only those factors that significantly describe
508 variance in the composition of the anammox bacterial community determined by variation inflation
509 factors (VIFs) calculated during RDA are shown here.

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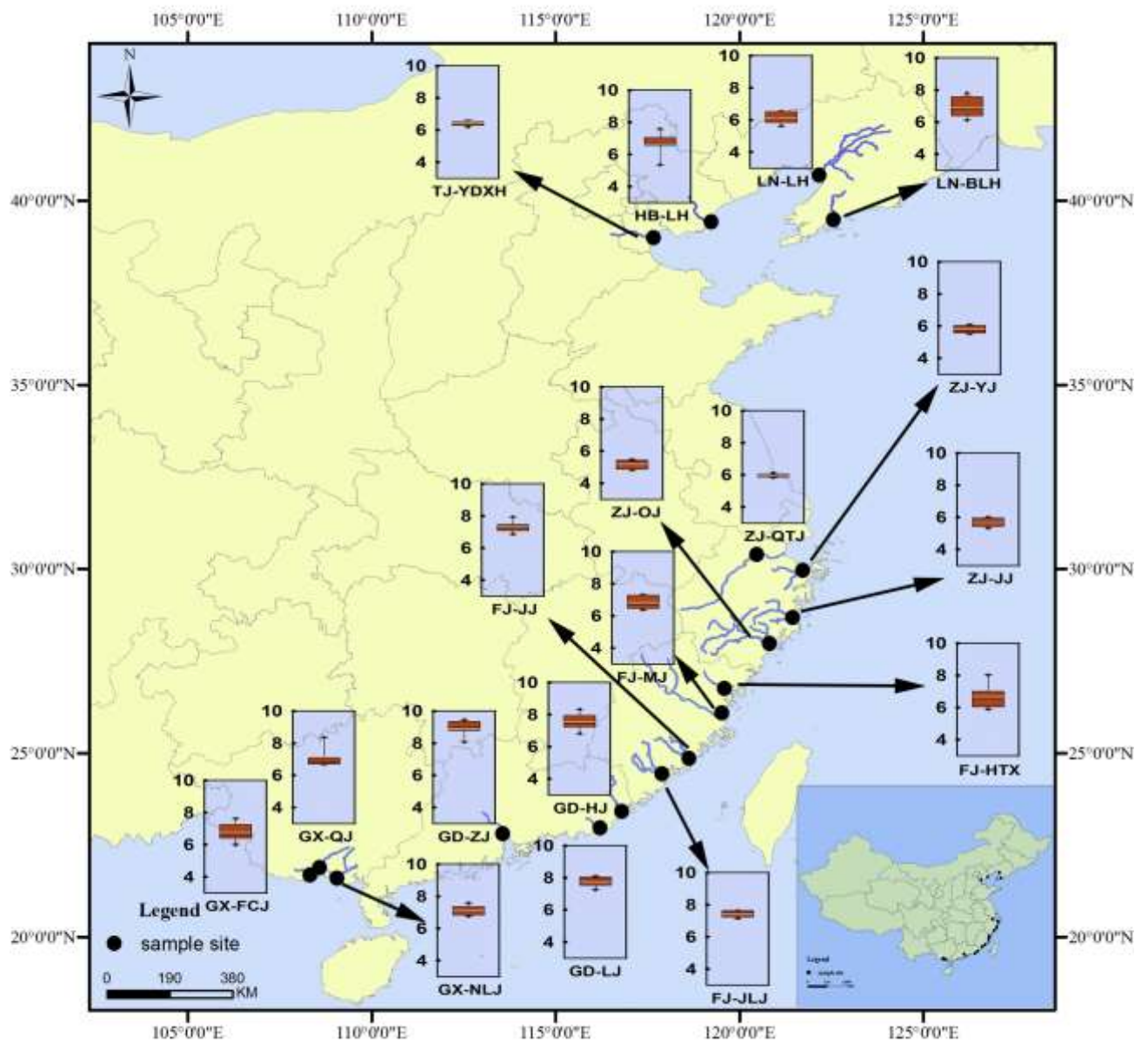
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519 Fig.1

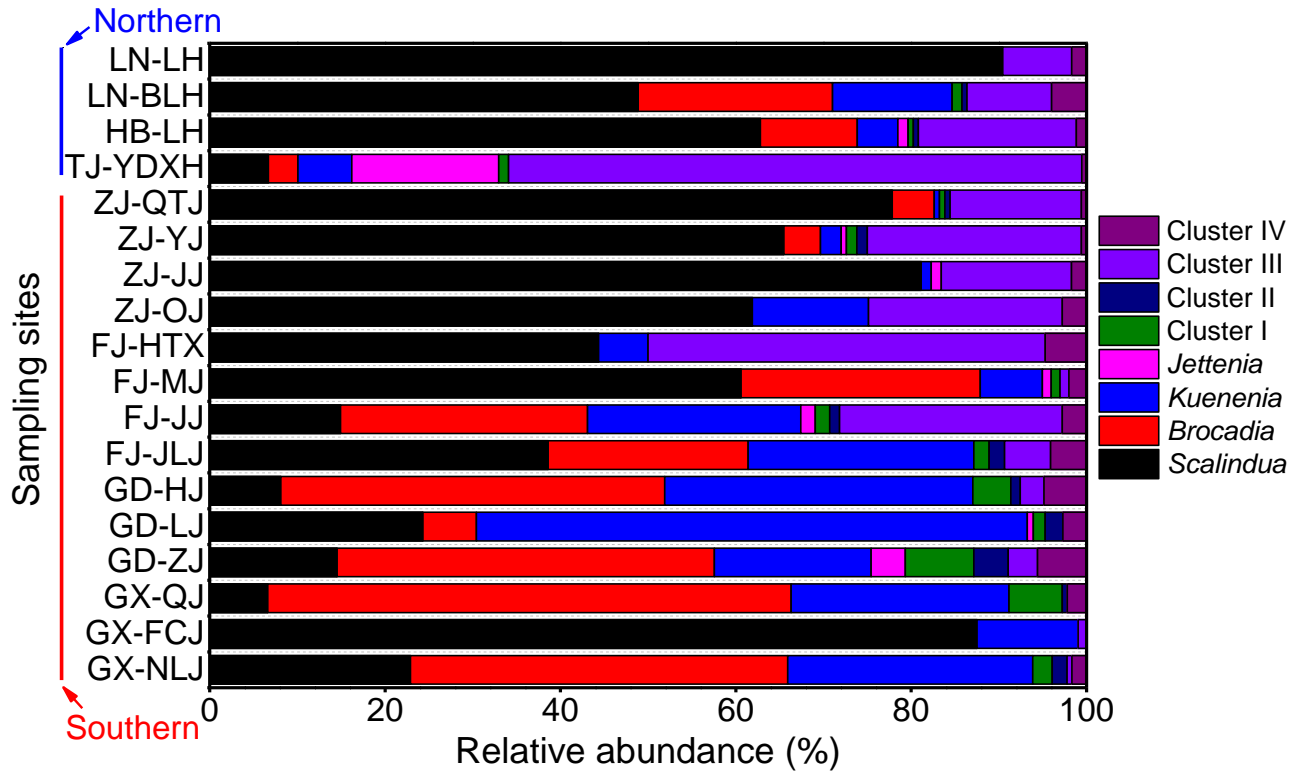
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526 Fig. 2

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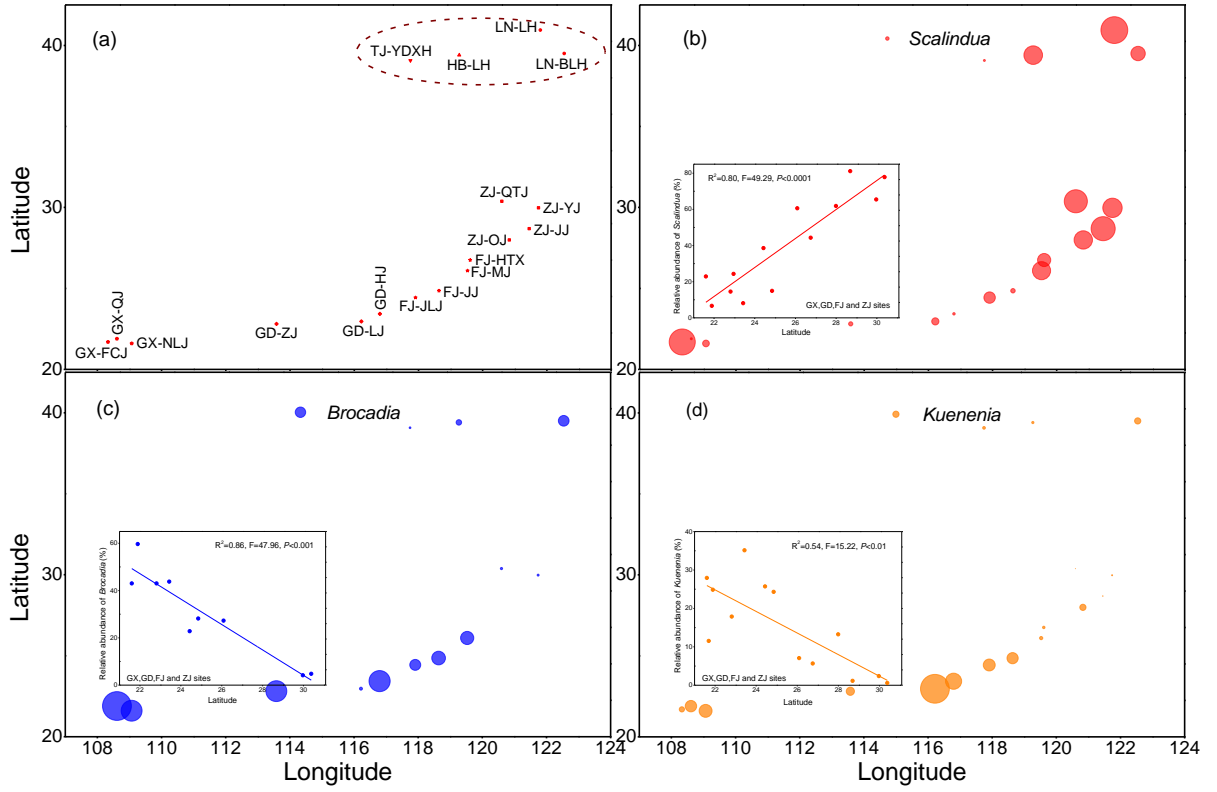
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545 **Fig. 3**

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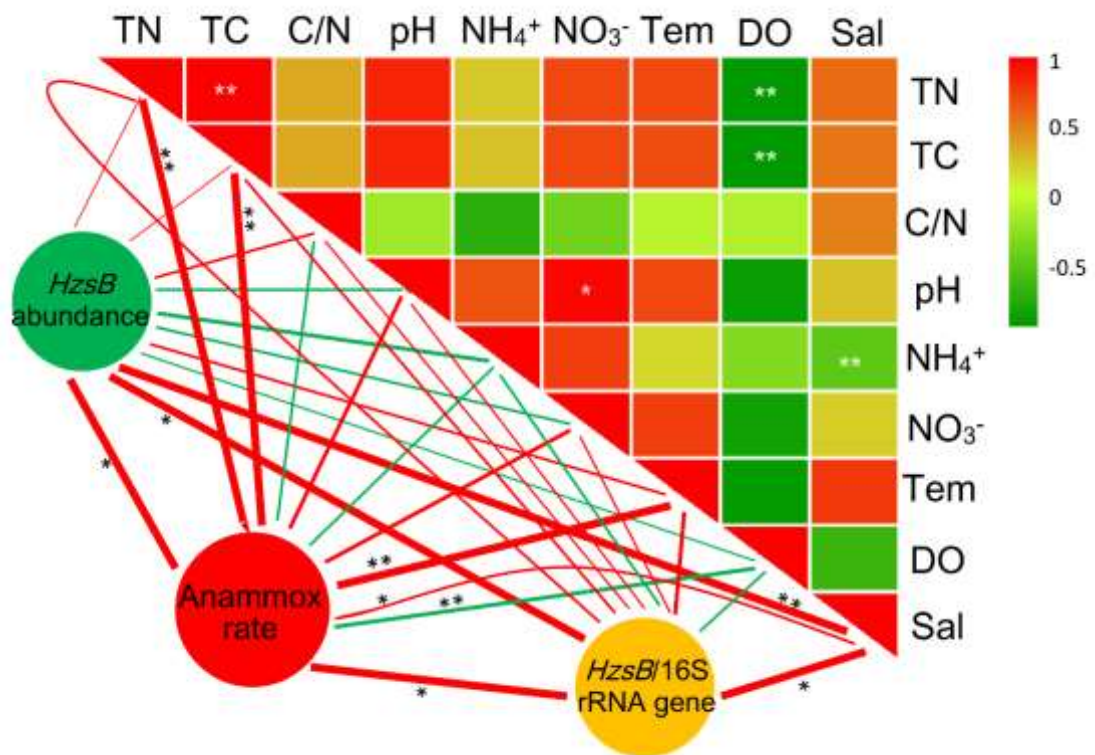
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560 Fig. 4

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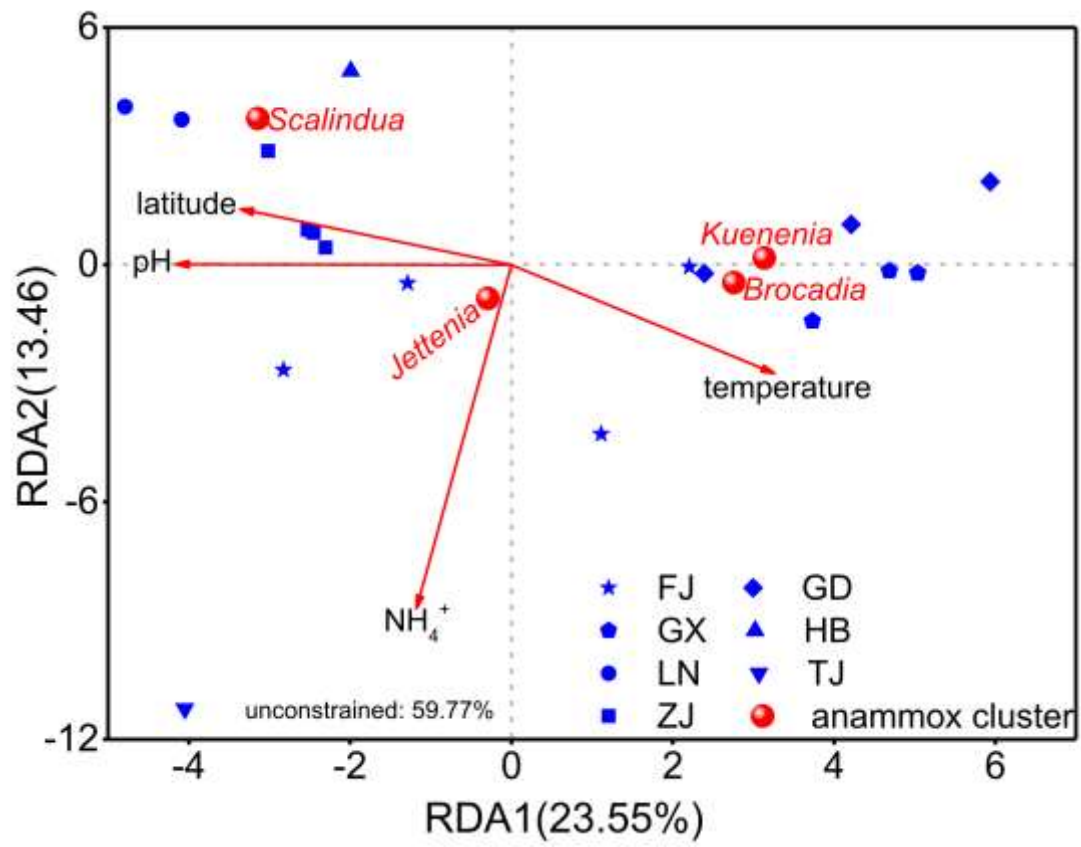
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573 Fig. 5

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575 **Table 1** Rates of anammox and denitrification measured by ¹⁵N tracing technique and the relative
 576 contribution to total dinitrogen production

Sites	Anammox rate (nmol N g ⁻¹ dw h ⁻¹)	Denitrification rate (nmol N g ⁻¹ dw h ⁻¹)	Anammox contribution (%)
LN-LH	1.72±0.97	3.25±0.46	34.61
LN-BLH	1.35±0.32	17.98±1.56	6.98
HB-LH	0.57±0.03	15.63±9.58	3.52
TJ-YDXH	1.32±0.23	25.61±3.40	4.90
ZJ-QTJ	0.29±0.15	0.31±0.06	48.33
ZJ-YJ	1.55±0.31	30.00±1.97	4.91
ZJ-JJ	1.10±0.51	15.31±1.88	6.70
ZJ-OJ	2.09±0.48	0.44±0.02	82.61
FJ-HTX	4.18±1.86	22.85±3.04	15.46
FJ-MJ	2.51±0.46	36.11±9.53	6.50
FJ-JJ	0.97±0.97	26.95±2.11	3.47
FJ-JLJ	3.24±0.45	56.63±17.29	5.41
GD-HJ	1.09±0.46	33.81±4.01	3.12
GD-LJ	1.11±0.70	21.52±2.70	4.90
GD-ZJ	13.68±3.98	50.9±2.46	21.18
GX-NLJ	1.66±0.43	4.76±1.02	25.86
GX-QJ	2.83±0.43	43.3±8.59	6.13
GX-FCJ	1.15±0.12	47.01±4.93	2.39