The potential rate and microbial communities of dissimilatory nitrate reduction to ammonium in Pearl River Estuary

Ran Jiang1,2,3, Fang Yang1,2, Wei Gou1, Rui He1 and Xinfeng Zhang1,2

1 The Pearl River Hydraulic Research Institute, Pearl River Water Resources Commission of the Ministry of Water Resources, Guangzhou, 510640, China; 2 Key Laboratory of the Pearl River Estuarine Dynamics & Associated Process, Ministry of Water Resources, Guangzhou, 510640, China

Abstract. Since nitrogen-related eutrophication has become a severe problem in the Pearl River Delta area in China, it is crucial to have a deep understanding of the nitrogen cycle. This study investigated the existence of dissimilatory nitrate reduction to ammonium (DNRA) in sediments of the Pearl River Estuary using the 15N isotope pairing method with continuous-flow systems. The results showed that the maximum \( N_{\text{NrA}} \) was 389.7 nmol/g in 216 h, while the maximum DNRA rate of 4.68 nmol/(g wet sediment h) occurred in 120 hours. The principal components analysis (PCA) based on community composition at the genus level was performed by the \( nrfA \) sequences distributed in the nine sites covering a salinity range of 25.3 - 30.1‰, and forming four distinct clusters. There was no significant relationship between the potential DNRA rates in the spatial distribution and salinity, but the highest potential DNRA rate occurred near the sewage discharge in the western coastal area. High contents of \( nrfA \) gene fragments were found at the same site, with values of 1159715 copies/g wet sediment, while very low contents of \( nrfA \) were found at other sites. Based on the \( nrfA \) gene pyrosequencing, Enterobacteriaceae accounts for 89.9 - 99.0% of the classified sequences.

1. Introduction
The Pearl River Estuary (PRE) is one of the most complex estuarine systems in the world, forming a transition zone between the Pearl River and the South China Sea [1]. Many environmental issues have subsequently emerged, such as the overloading of reactive nitrogen and hypoxic zones in this large perturbed estuary [2]. Huangmao Sea Estuary is one of the estuaries located in the western part of PRE (as shown in Figure 1), being the fastest-growing economic area in southern China. According to the marine quality bulletin of Jiangmen city, the reactive nitrogen (including \( \text{NO}_3^- \), \( \text{NH}_4^+ \) and \( \text{NO}_2^- \)) and reactive phosphorus were two major eutrophication pollutants in Huangmao Sea Estuary [3]. Since nitrogen-related eutrophication has become a severe problem in the Pearl River Delta area, it is important to have a full understanding of the nitrogen cycle, and not only of the biological processes of denitrification and nitrification in this area.

There are several processes by which inorganic nitrogen is removed from any aquatic ecosystem, including estuaries. Denitrification is regarded as a dominant process for nitrate reduction in the shallow marine sediments, during which nitrate is eventually converted to elementary nitrogen and released from the water body [4]. Unlike denitrification and anaerobic ammonium oxidation (anammox) [5], the transformation from nitrate to ammonium via dissimilatory nitrate reduction to
ammonium (DNRA) could prolong the residence time of fixed nitrogen, and thus may slow down the removal of bioavailable nitrogen from the ecosystem [6]. At present, most studies on the DNRA pathways relied on the correlations between the DNRA rates and environmental variables, such as salinity, temperature, concentrations of $\text{NO}_3^-$ and organic matter. However, as the potential microbial responses involve N transformations to the changing salinity and other environmental variables, it is necessary to further study in the microbial communities that promote DNRA.

There are some studies that focus on the organisms capable of DNRA in the coastal sediments, although it is difficult to determine the exact organisms involved. The functional gene assay by using a nitrate ammonification related gene - nrfA (named after nitrite reduction by a ferment, coding for another dissimilatory nitrite reductase containing cytochrome c552 (EC 1.7.2.2)) has been reported from anaerobic wastewater treatment reactors for the first time [7]. Some investigations have been also conducted for the sediment from different coastal areas in China. For example, one study showed that the N-loss via $N_2$ was the main pathway in the East China Sea (ECS), and DNRA accounted for 20–31% of benthic nitrate reduction [8]. The investigation on the sediments from Yellow River Estuary (YRE) indicated the variability in the activities and community structure of DNRA bacteria [9]. There were several reports showing the coexistence of denitrification and DNRA in YRE and Changjiang River in ECS. However, there are significant differences in both geographic location and climatic conditions among PRE, ECS and YRE. The PRE and ECS, as the two largest estuaries in China in terms of freshwater discharge, are located in the subtropical and temperate regions, respectively. YRE consists of typical turbid coastal waters that are influenced by the Yellow River which is the second largest river in the world in terms of sediment loadings and is located in a temperate region under significant influence of monsoons. To date, there is a lack of studies on the microbiologically mediated nitrate reduction pathways, especially DNRA that causes the longer presence of the reactive nitrogen in the PRE ecosystem. Therefore, it is necessary to conduct a genetic investigation based on the sediments collected from PRE areas in order to have a better understanding of DNRA pathway in PRE.

In this study, we chose Huangmao Sea Estuary, one of the PRE, as the typical area in the present investigation. The major focuses were: 1) to investigate the seawater, sediment characteristics and nitrogen pollution in PRE; 2) to examine the potential rates of DNRA in the sediments collected from the PRE continental shelf; 3) to develop the nrfA specific primer pairs for describing the distribution and phylogenetic status of the nitrate ammonifiers inhabited in the sediments.

2. Materials and methods

2.1. Study area

Huangmao Sea Estuary (HSE) (see Figure 1) is referred to as Huangmao Bay (HB) with an area of approximately 540 km$^2$. It is composed of a bay proper in the lower portion, a tidal river in the upstream and several island chains [10]. The average water depth in HB is approximately 4.5 m. Its mouth features a chain of rock islands (Gaolan, Hebao and Dajing Islands) that provides shelter from wave attack, resulting in an ideal place for port construction and tourism development. There exists a deep navigation channel extending from the mouth to the head of HB, with water depths ranging from 6 to 22 m. The studied sites are located in its mouth, around Dajing Islands. Sites C16-20 were near the west boundary of the PRE Chinese White Dolphin National Nature Reserve. The C3 was under the influence of the warm drainage pipe and domestic sewage discharge from a nuclear power plant.

Seawater and sediment characteristics were investigated at 20 stations (Figure 1). Twenty stations in the sites C1-C20 had been sampling quarterly for a one-year period during 2016. The national sea water quality standard in China (NO. GB3097-1997), approved by the Ministry of Environmental Protection, was used in assessing the sea water quality of PRE. The Standards classified the seawater quality into four categories, and each grade of water is served for different purposes. The fourth grade of seawater was suitable for marine development zones. The standard of dissolved inorganic nitrogen (DIN) is referred to the totality of $\text{NO}_3^-$, $\text{NO}_2^-$ and $\text{NH}_4^+$. 

2
2.2. Sample collection and preparation

Seawater was collected at twenty sites (C1-C20) quarterly (Figure 1). Nine sediment samples from the sites C1, C3, C5, C8, C11, C13, C15, C16, and C17 were collected (Figure 1) during a cruise from Jun 8 to 22, 2016. These sediment samples were used for measuring the potential DNRA rate. Each sampling series involved taking three sediment cores from every station [11]. A detailed description of these sites is listed in Table 1.

### Table 1. Water and sediment data in PRE.

| Site ID | Salinity S (%) | pH    | NH₄⁺ | PO₄⁻³ | NO₃⁻ | Corg | N mg/kg | P mg/kg |
|---------|----------------|-------|------|-------|-------|------|---------|---------|
|         |                | Surface water (0-50cm) |       | Sediment (0-5cm) |       |       |         |         |
| C1      | 0.3            | 7.76  | 1.3  | 42    | 633   | 1.70 | 1017    | 867     |
| C3      | 1.0            | 7.96  | 1.9  | 32    | 622   | 1.25 | 896     | 835     |
| C5      | 12.9           | 7.98  | 2.2  | 28    | 532   | 0.71 | 516     | 468     |
| C8      | 13.4           | 8.12  | 3.3  | 29    | 504   | 1.00 | 596     | 729     |
| C11     | 2.15           | 7.74  | 0.9  | 32    | 495   | 1.37 | 897     | 732     |
| C13     | 9.55           | 7.61  | 0.9  | 35    | 475   | 1.00 | 867     | 839     |
| C15     | 12.5           | 7.53  | 0.6  | 34    | 494   | 1.38 | 861     | 523     |
| C16     | 9.9            | 7.66  | 1.1  | 40    | 415   | 1.24 | 723     | 589     |
| C17     | 13.0           | 7.72  | 1.9  | 29    | 418   | 0.98 | 689     | 368     |

Temperature in the area: 22-26°C

At the laboratory, each intact core was installed into a continuous-flow system (Figure 2). After a day the systems were allowed to approach steady-state conditions, and 50 mg of K¹⁵NO₃ was added. The samples for ¹⁵NH₄⁺ analysis were collected at 0, 12, 24, 48, 72, 96, 120, 144, 168 and 216 h. DNRA rates were calculated as a mean from the incubation results obtained for 3 cores sampled from each station.

2.3. Chemical analysis

¹⁵NH₄⁺ was measured by membrane inlet mass spectrometry (Ultra Trace-PPT, EES, England) [12]. Environmental variables including temperature, pH and salinity were on-site measured by a pH metre (PhS-3C, Rex Electric Chemical, China) and a salinometer (5150, San-xin Instrument, China). DIN is the totality of NO₃⁻, NO₂⁻, NH₄⁺. The concentrations of NO₃⁻, NO₂⁻, NH₄⁺, reactive P i.e. orthophosphate (PO₄⁻³) in the water and organic carbon (Corg), and total P and N in the sediments were determined by using an ultraviolet-visible spectrophotometer (8453 UV-VIS, Agilent, USA) [13].

---

**Figure 1.** Location of sampling stations.
2.4. Rate calculations

The potential DNRA rate in sediment samples was calculated according to Silver et al. [14].

\[ \text{DNRA} = \left( [^{15}\text{NH}_4^+]_{t} - [^{15}\text{NH}_4^+]_{i} \right) \times \frac{[^{15}\text{NO}_3^-] \times t}{[^{15}\text{NH}_4^+]_{i}} \]

Here \( [^{15}\text{NH}_4^+]_{i} \) is initial atom % of \( ^{15}\text{NH}_4^+ \), \( [^{15}\text{NH}_4^+]_{f} \) is the final atom % of \( ^{15}\text{NH}_4^+ \), \( [^{15}\text{NO}_3^-] \) is the atom % of the added nitrate tracer, the extractable ammonium concentration \( [\text{NH}_4^+] \) is the initial sediment slurries (nmol/g dry soil), and “\( t \)” represents the incubation time. In our research the potential DNRA rates were calculated using the final incubation times of 12, 24, 48, 72, 96, 120, 144, 168 and 216 h in combination with the initial times of 0, 12, 24, 48, 72, 96, 120, 144 and 168 h.

The significance of the rate differences in the continuous-flow system was evaluated by the t-test or the Mann–Whitney Rank Sum test, using a statistical software package (SigmaStat 3.5).

2.5. Genetic analysis and DNRA communities

Using the alignment of 474 \( nrfA \) gene sequences, resulting from the September 2016 FUNGENE-DB search, primers (6F/5R) targeting \( nrfA \) genes (Table 2) were designed by examining nucleotide regions that were conserved in at least 75% of the \( nrfA \) sequences [15]. The other genetic analyses including \( nrfA \) gene sequence analyses and phylogenetic classification were performed following Tekeuchi’s research [16]. To compare the DNRA communities of nine sites, the principal components analysis (PCA) based on community composition at the genus level was performed [17].

| Primer | Sequence(5’ to 3’) |
|--------|------------------|
| 5R     | CGCCAYTGVGCTGRIGATATC |
| 6F     | GAYTGCADGATGACAAAGT |
| 4R     | GCATCCGCGCTTTATCCAT |

F: forward primer, R: reverse primer; Base Codes: K=T/G, R=A/G, Y=C/T, S=C/G

3. Results and discussion

3.1. Environmental variables

It was shown that 80% of \( \text{NO}_3^- \), DIN and \( \text{NH}_4^+ \) measurements for the sites C1-C17 exceeded the fourth grade of NO. GB3097-1997. The sites C18-20 had a somewhat better quality of water, being of the
third grade. The sites C18-20 are located near the PRE Chinese White Dolphin National Nature Reserve, and land pollutants were not so easily spread in the area.

As shown in Figure 3a, DIN concentration decreased sharply from the coast (~0.950 mg/L) to the outer shelf (~0.270 mg/L). The concentrations of DIN at about 80% of sites exceeded 0.5 mg/L. The maximum concentration of DIN appeared at the mouth of Yamen Gate, decreasing downstream. In general, the concentration of DIN was higher in the west and lower in the east, as shown in the profile figure. Figure 3b and Figure 3c showed that the major form of DIN was NO\textsubscript{3}\textsuperscript{-}. NO\textsubscript{3}\textsuperscript{-} ranged between 0.170 and 0.567 mg/L, which accounted for 60 - 80% of DIN. The high level of NO\textsubscript{3}\textsuperscript{-} was due to extensive anthropogenic activities and pH of seawater. As shown in Figure 4, the salinity was higher in the east than the west, and it is inclined to the west, especially in the flood season. The result of salinity characteristics was consistent with the hydrodynamic characteristics of the eastern tidal current in PRE. In flood season, the salinity was affected by fresh water runoff, so it was lower than in the dry period. The salinity in our studied area was near the salinity contour (22.5-30.6 ‰) of the bottom water of PRE at the maximum tide.
3.2. The potential rates of DNRA

$^{15}$NH$_4^+$ was detected at all sites, and after 12 hours incubation, it ranged from 5.62 to 38.9 nmol/g wet sediment, which showed that DNRA existed in the continuous-flow system. $^{15}$NH$_4^+$ ranged from 62.0 to 389.7 nmol/g after 216 hours. The potential rate of DNRA was different between all sampling sites. Moreover, the maximum potential DNRA rate was 4.66 nmol/(g wet sediment h) after 24 hours, $^{15}$NH$_4^+$ was 90.4 nmol/g at C3. The DNRA rate increased with incubation time, then the systems reached a stable DNRA rate in five days, with a maximum rate of about 4.68 nmol/(g h) after 120 hours. After five days, the rate slowed down to the range of 0.056 - 2.68 nmol/(g h). Low $^{15}$NH$_4^+$ (62.0 nmol/g and 79.64 nmol/g) were observed at C8 and C16, with the potential DNRA rates of 0.306 and 0.472 nmol/(g h) after 216 hours, respectively.

Sample sites have different hydrodynamic and pollutant characteristics, the sites C1 and C11 are situated near the urban runoff, exposed to anthropogenic pollutants from upstream. while the sites C5 and C7 were relatively near the coastline and the site C3 was near some domestic sewage pipes and warm drainage (below sea level). As shown in Figures 5a and 5b, there was no significant relationship between the potential DNRA rate, regarding the spatial distribution, and salinity. The highest potential DNRA rate occurred near the sewage discharge in the western coastal area.

3.3. nrfA sequences abundance

The abundances of DNRA bacteria based on nrfA gene quantification in the sediments of 9 sampling sites are presented in Table 3. The two nrfA primers (6F/4R) and (6F/5R) were used in high-throughput sequencing respectively (Table 2). Effective tags of 6F/5R nrfA primer are in the range of $11 \times 10^6$ to $7 \times 10^5$, while those of 6F/4R nrfA primer are in the range of $7 \times 10^5$ to $1 \times 10^1$. Therefore, the analysis of the DNRA community was based on 6F/5R nrfA primer. High contents of nrfA gene fragments were found at the site C3, with values of 1159715 copies/(g wet sediment). Correspondingly, the lowest contents of nrfA were found at C15, with values of 80407 copies/g.

![Figure 5a. Spatial distribution of Potential DNRA Rates in 24 hours.](image1)

![Figure 5b. Spatial distribution of Potential DNRA Rates in 48 hours.](image2)
### Table 3a. The abundances of DNRA bacteria based on nrfA gene quantification (Primer 6F/5R).

| Site ID | Qualified tags | Chimeras | Effective tags |
|---------|----------------|----------|----------------|
| C1      | 448912         | 12303    | 436609         |
| C3      | 1196456        | 36741    | 1159715        |
| C5      | 263778         | 16607    | 247171         |
| C8      | 141824         | 4343     | 137481         |
| C11     | 258689         | 10950    | 247739         |
| C13     | 139730         | 8824     | 130906         |
| C15     | 80407          | 6234     | 74173          |
| C16     | 272758         | 3040     | 269718         |
| C17     | 174853         | 16737    | 158116         |

### Table 3b. The abundances of DNRA bacteria based on nrfA gene quantification (Primer 6F/4R).

| Site ID | Qualified tags | Chimeras | Effective tags |
|---------|----------------|----------|----------------|
| C1      | 590263         | 33       | 590230         |
| C3      | 482256         | 98       | 482158         |
| C5      | 339795         | 169      | 339626         |
| C8      | 532520         | 187      | 532333         |
| C11     | 520037         | 425      | 519612         |
| C13     | 9              | 0        | 9              |
| C15     | 344623         | 315      | 344308         |
| C16     | 488368         | 0        | 488368         |
| C17     | 210657         | 102      | 210555         |

3.4. Spatial distribution patterns in the sediment

Via high-throughput sequencing, raw sequences were obtained from the sediment samples of PRE. After the removal of low quality reads, a total of 80407–119645 6 qualified sequences were produced. Then, a total of 74173–1159715 effective sequences for these samples were obtained via further filtering, which were clustered into operational taxonomic units (OTUs) ranging from 2541 to 8709.
The distribution pattern of the \textit{nrfA} gene sequences from the surface sediments (0 – 1 cm) was investigated. Figure 6 shows the spatial variation of nine samples with 54.36\% (PC1) and 18.15\% (PC2) of the variance explained. Figure 6 shows that the sites C5 and C15 were clustered together, and the community structures of these two sites were similar. The community composition of the site C11 also shared some similarities with those of the site C17. The sites C3 and C1 were divided into one group by some similarities. However, the community of the site C13 was the most distinct among all the samples. As shown in Figure 6, the \textit{nrfA} sequences distributed in the nine sites covering a salinity range of 25.3 - 30.1‰, formed four distinct clusters such as C1 and C3, C5 and C15, C11 and C17. This result suggests that the salinity per se was not likely to affect the selection of the \textit{nrfA} bacteria populations, whilst some factors such as coastal pollutant discharge might have had an impact on \textit{nrfA} bacteria.

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{figure6.png}
\caption{PCA analysis of DNRA communities.}
\end{figure}

3.5. Diversity of the \textit{nrfA} sequences in the sediments
In our study, a total of 7 bacterial phyla were identified according to modified OTUs. For bacterial phyla, \textit{Proteobacteria} was the most abundant phylum in all samples, accounting for 99.93 – 91.56\%. At the class level, a total of 8 classes at eight sites were obtained. \textit{Gammaproteobacteria} was the most abundant of all sites, accounting for 99.9 – 90.5\%. Other \textit{Proteobacteria} such as \textit{Alphaproteobacteria} and \textit{Deltaproteobacteria} existed but contributing less than 0.5\%. At the order level, \textit{Enterobacteriales} (89.9 - 99.9\%) which belong to \textit{Gammaproteobacteria} was the most abundant order. Of all filtered sequences, a total of 11 families were detected. \textit{Enterobacteriaceae}, which is a member of \textit{Enterobacteriales}, were more abundant than other families, accounting for 89.9 -99.0\% of the classified sequences.

A total of 63 sequences retrieved from the sequences found in the sediments were compared with those from known strains and environmental isolates. Figure 7 showed the diversity of the \textit{nrfA} partial nucleotide sequences and the other reference sequences obtained from the databases (EMBL, DDBJ and GenBank). Only one cluster belonged to the known group, as nitrate ammonifiers with the \textit{nrfA} sequences, comprised of fermentative bacteria (\textit{Escherichia coli}) and \textit{Shewanella}, which are known members of the nitrate ammonifiers [17]. A few of the sequences were affiliated with the \textit{nrfA} sequences retrieved from versatile anaerobes, such as sulphur-reducing nitrate ammonifier \textit{Sulfurospirillum deleyianum}. These microbes seem to be favoured to anaerobic marine sediments full of iron sulphide, involved in sulphur and iron cycles coupled with nitrate ammonification [18]. However, the available \textit{nrfA} sequences in the databases mentioned above were not consistent with most of the \textit{nrfA} sequences found in this study which are phylogenetically distant from some known \textit{nrfA} strains.
Figure 7. Dendrogram showing the diversity of the nrfA partial nucleotide sequences from the sediments, with reference sequences from the databases: *Escherichia coli*, *Shewanella*, *Sulfurospirillum*, *Dusulfovibrio* and so on. The bar indicates 10% nucleotide sequence divergence. Numbers near the branches represent the percentages of 1000 bootstrap repetitions. Confidence limits of less than 50% are not shown.

4. Conclusions
This study investigated the existence of DNRA using the $^{15}$N isotope paring method in continuous-flow systems, based on the 20 sites in PRE during a one-year period. The results showed that the maximum $^{15}$NH$_4^+$ was 389.7 nmol/g in 216 h, while the maximum DNRA rate of 4.68 nmol/(g h) occurred in 120 hours. High contents of nrfA gene fragments were found at the same site, with values of 1159715 copies/g. Correspondingly, very low contents of nrfA were found at other sites. Based on the nrfA gene pyrosequencing, *Enterobacteriaceae* (a member of *Enterobacteriales*) were more abundant than other families and accounted for 89.9 - 99.0% of the classified sequences. PCA, based on community composition at the genus level, was performed using the nrfA sequences distributed in the nine sites covering a salinity range of 25.3 - 30.1‰ and formed four distinct clusters. According to both, the continuous-flow systems incubation and PCA analysis, there was no significant relationship between the potential DNRA rates regarding the spatial distribution and salinity, whilst some factors such as coastal pollutant discharge might have an impact on nrfA bacteria. This study improves our understanding of DNRA in the PRE. However, further study is required to understand the contributions of anammox, denitrification and DNRA to nitrate reduction.
Acknowledgments
This study is funded by the Guangdong Province Natural Science Foundation of China (Grant no. 2017A030313329) and the National Natural Science Foundation of China (Grant no. 51409287).

Reference
[1] Pan J Y, Gu Y Z, Wang D X 2014 Res. Oceans. 119 2480
[2] Dai M, Wang L, Guo X, Zhai W L 2008 Biogeosciences. 5 1227-1244
[3] Bulletin of Marine Quality in Jiangmen City, China. 2016 http://hyyyj.jiangmen.gov.cn/zwgk/gb/201809/t20180928_1683848.html, last access:12 October 2018
[4] Herbert R A 1999 FEMS Microbiol Rev. 23 563
[5] Jetten M S M, Strous M, van de Pas-Schoonen K T, et al. 1998 FEMS Microbiol Rev. 22 421-37
[6] Gardner W S, McCarthy M J, An S 2006 Limnol. Oceanogr. 51 558
[7] Smith C J, Nedwell D B, Dong L F 2007 Appl. Environ. Microbiol. 73 3612
[8] Song G D, Liu S M, Marchant H, Kuyper M M M 2013 Biogeosciences. 10 6851
[9] Cuina B, Wang Y, Ge C, Ahmad H A 2017 Sci. Rep. 7 1
[10] Wei X, Wu X X 2011 China Earth Sci. 54 937-945
[11] An S, Gardner W S, 2002 Mar. Ecol. Prog. Ser. 237:41-52
[12] An S, Gardner W S, Kana T 2001 Appl. Environ. Microbiol. 67 1171
[13] State Administration for Market Regulation, China 2007 The specification for marine monitoring - Part 4: Seawater analysis (NO. GB 17378.4-2007)
[14] Silver W L, Herman, D J, Firestone, M K 2001 Ecology. 82 2410-2416
[15] Welsh A, Chee-Sanford J C, Connor L M 2014 Appl. Environ. Microbiol. 80 2010
[16] Takeuchi J 2006 Geomicrobiol. J. 23 75
[17] Hamady M, Lozupone C, Knight R 2010 ISME J. 4 17-27
[18] Eisenmann E, Beuerle J, Sulger K 1995 Arch. Microbiol. 164 180