Faunal Burrows Alter the Diversity, Abundance and Structure of AOA, AOB, anammox and n-Damo Communities in Coastal Mangrove Sediments

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Running title: AOA, AOB, anammox and n-damo bacteria in mangrove

Abstract

In the present work, the diversity, community structures and abundances of aerobic ammonia-oxidizing archaea (AOA) and bacteria (AOB), anaerobic ammonium-oxidizing (anammox) bacteria and denitrifying anaerobic methane oxidization (n-damo) bacteria were unraveled in the bioturbated areas of the coastal Mai Po mangrove sediments. Results indicated that the bioturbation by burrowing in mangrove sediments was associated with higher concentration of NH_4^+ but lower concentrations of both NO_2^- and NO_3^-, and increase in diversity and richness of both AOA and AOB, but relatively lower diversity and richness of n-damo bacteria. The phylotypes of anammox bacterial community were significantly increased while their phylogenetic lineages observed in the less bioturbated areas were also maintained. Infauna also showed a great impact on the composition of n-damo bacterial phylotypes and burrowing activity altered the n-damo community structure profoundly in the sampled areas. The communities of n-damo bacteria in the surrounding bulk sediments showed similar structures to marine n-damo communities, but those on the burrow wall and in the ambient surface layer had a freshwater pattern, which was different from previous findings in Mai Po wetland. On the other hand, the abundances of AOA, AOB and n-damo bacteria were greatly stimulated on burrow walls while the abundance of anammox bacteria remained unchanged. Infaunal burrows and mangrove roots affected the relative abundance of AOA and AOB. The benthic infauna stimulated the abundances of AOA, AOB, anammox and n-damo bacteria. Furthermore, NH_4^+ and NO_2^- were important environmental factors changing the structure of each group. The communities of anammox and n-damo bacteria in bioturbated areas showed a competitive relationship.

Keywords: N-damo; Anammox; Ammonia-oxidizing archaea; Ammonia-oxidizing bacteria; Bioturbation; Coastal sediment

Introduction

The intermediate disturbance hypothesis suggests that the highest diversity of the species composition of communities is maintained at intermediate scale of disturbances, which disrupt the ecosystem, change substrate availability and/or shift the physical environment [1]. The activities of benthic macrofauna introduces an ideal intermediate disturbance on the microbial communities involved in the nitrogen cycle in the coastal wetland via animal-microbe interactions of ecosystem perturbation, grazing and symbiosis [2].

Previous investigation indicated that the increase of benthic infaunal density resulted in the increase of ammonium and total inorganic nitrogen consumption rate [3]. Through particle reworking, burrow ventilation, irrigation and excretion, infauna in sediment may stimulate nitrogen cycling by extending the oxic-anoxic interfaces with more nutrients, where the nitrification-denitrification is particularly intense [4-6]. The burrowing activities of microfauna in coastal sediments can influence the redox in the sediments significantly and create oxidized zones within the sediments, which increases the diversity of aerobic ammonia oxidizing bacteria (AOB) and archaea (AOA) in the deep biosphere [7]. Macrobenthic irrigation could also promote microbial denitrification greatly [8]. It was shown that the biggest single influence on microbial community structure and diversity was the presence or absence of burrows in coastal marine sediments bioturbated by shrimps [6]. Additionally, bioturbation determines the response of benthic AOA and AOB to ocean acidification [9].

Nitrite-dependent anaerobic methane oxidation (n-damo) bacterium, *Ca.* Methylomirabilis oxyfera was discovered in recent years [10-13], which was shown important in coupling the nitrogen and carbon cycles [14, 15]. Subsequently, their distribution in the environments with

anaerobic ammonium-oxidizing (anammox) bacteria, another important contributor to anaerobic denitrification [8], were further studied together in the sediments of plateau saline lakes [16], freshwater wetland [17] and paddy fields [18, 19]. However, their coexistence in coastal sediments under bioturbation is poorly understood. Furthermore, the burrowing infauna provide dynamic oxic-anoxic lumen in the sediments [2]. Unfortunately, it has not yet been investigated together or documented on how the community structures and abundances of aerobic (AOA/AOB) and anaerobic (anammox and n-damo bacteria) species involved in nitrogen turnover respond to the bioturbance in the coastal ecosystem.

In our previous studies, the diversity and distribution of AOA, AOB, anammox and n-damo bacteria were successfully unraveled in the mangrove sediments of Mai Po coastal wetland of Hong Kong [20-26]. During the sampling, some areas with dense burrows were observed in the mangrove sediments (Fig. S1). It is important to study the overall communities of AOA, AOB, anammox and n-damo bacteria in the above bioturbated areas to understand the impacts of the bioturbance in the coastal wetland.

The objectives of the present work were to study (1) whether and how the bioturbation in mangrove sediments could result in significantly compositional changes in microbial communities of AOA, AOB, anammox and n-damo bacteria; (2) how the above microbes responded to the environmental factors in these specific bioturbated areas.

Materials and methods

Sample site, collection and physicochemical analyses

Sediment samples were collected at low tide in January 2014 in the intertidal mangrove of

Mai Po Nature Reverse of Hong Kong. This area is covered by mangrove trees with epifaunal communities of brachyuran crabs. Generally, brachyuran have a very high diversity with an estimation of 300 recorded species in Hong Kong [27] and 58 species were common in Hong Kong mangroves, most of which belong to the sub-family Sesarminae inside the mangrove habitats [28]. Several crabs were randomly collected in the study areas after digging the burrow and classified to be sesarmine crab *Perisesarma bidens.*

The sampling sites are shown in Fig. 1. Five sites harboring intense burrows (compared with the normal area harboring less than 5 burrows per square meter) were selected in the mangrove (Exact locations in Fig. S1 and a detailed schematic in Fig. S5). In one site, at least 15 burrows were sampled to collect the burrow-wall sediment layer at a depth of 15 cm from the top of each burrow using autoclaved stainless micro-spatula. Meanwhile, only the 1-3 mm burrow-wall sediment layers were carefully collected because it was suggested that the high redox zone under bioturbation by microfauna is within the several millimeters [7]. Due to the low yield of burrow-wall sediments at sites 1-3, the samples collected from the first three sites were pooled together to form a composite sample with more than 5 grams to provide enough amount for physiochemical and molecular analyses. The surface sediments (1-3 mm) near the burrow within each sampling site were also collected carefully using sterilized micro-spatula. In contrast, the bulk sediments surrounding the burrows at the same depth of 15 cm were sampled at each site and separated into black and yellow sediments, because the ambient sediments in some sampling grids were full of mangrove roots at the depth of 15 cm, which caused the yellow sediments rather than the dark ones in the other sampling lattices. Therefore, four types of the samples from Mai Po bioturbated areas in the mangrove were

collected, which were burrow-wall layer, ambient surface layer, surrounding bulk black and yellow sediments and labeled as 'Wall', 'Surface', 'Black' and 'Yellow', respectively. Samples were kept in ice-box after the collection, transported to the laboratory within two hours and stored at –20 °C for further analyses.

All the four sample types are regarded from Mai Po bioturbated areas, where intense faunal burrows were found. In the MP mangrove sediments without bioturbation by sediment fauna, AOA, AOB, anammox and n-damo bacteria had been investigated in our previous studies [20-22, 25, 26, 29, 30], of which the results were discussed with the findings in the present work.

Temperature, pH and redox were measured *in situ* using IQ180G Bluetooth Multi-Parameter System (Hach Company, Loveland, CO). Chemical analyses of ammonium, nitrite and nitrate in the samples were determined using an autoanalyzer (QuickChem, Milwaukee) according to Standard Methods of the American Public Health Association [31].

DNA extraction and PCR amplification

Genomic DNA from each sampling site was extracted independently using PowerSoil® DNA isolation kit (MO BIO, Carlsbad, CA, USA) according to the manufacturer's instruction. The DNA yields were determined using a biophotometer (Eppendorf AG 22331, Hamburg, Germany). After the extraction, DNA were pooled together based on the sample type, diluted using sterile ddH_2O to the appropriate range, and stored at –20 °C for further analyses.

PCR reaction mixtures (in a total volume of 50 µl with ddH_2O) contained 1 µl of dNTPs (10 mM; Promega, USA), 4 µl of $MgCl_2$ (25 mM; Promega, Hong Kong), 1 µl of forward primer

(20 µM), 1 µl of reverse primer (20 µM), template DNA (10-20 ng), 10 µl of GoTaq Flexi Buffer (Promega, Hong Kong), 2 µl of Bovine serum albumin (4 mg/ml; Roche), 0.25 µl of GoTaq Flexi polymerase (5 U/ml; Promega, Hong Kong).

For archaeal and bacterial *amoA*, n-damo *pmoA* and anammox 16S rRNA genes, different primer sets and procedures were used (details in Table S1). The selection of primer sets and combinations is stated in the supplemental materials with Table S1. The PCR products using primer A189_b and cmo682 were pooled together and diluted 10 times as the templates in the nest PCR step 2 using primer cmo182 and cmo568.

PCR products were electrophoresed on 1% agarose gels (Promega, Madison, WI, USA) using the GelDoc EQ system (Bio-Rad), purified using Takara MiniBEST Agarose Gel DNA Extraction Kit Ver.3.0 (Takara, Dalian, China), and stored at -20 °C.

Purified PCR products were ligated to pMD-18 T-vector (Takara, Otsu, Japan), and used to construct the clone libraries with *E. coli* competent DH5α cells (Takara, Dalian, China).

Sequencing and phylogenetic analyses

A maximum of about 75 clones per library were selected randomly for sequencing. Primer set M13F (5'-3': TGT AAA ACG ACG GCC AGT) and M13R (5'-3': CAG GAA ACA GCT ATG ACC) were used to verify the inserted DNA fragment through PCR amplification. Sequencing was performed using the Big Dye Terminator Kit (Applied Biosystems, Foster City, CA, USA) and an ABI Prism 3730 DNA analyzer (Applied Biosystems).

DECIPHER's Find Chimeras [32] was used to detect the chimeras in anammox 16S rRNA gene sequences. The sequences were checked using MEGA 6.0 software [33] and aligned by

MAFFT version 7 [34]. Reference sequences were retrieved from the GenBank database using nucleotide tool BLAST, Popset and Batch Entrez at NCBI (http://www.ncbi.nlm.nih.gov). Find Best DNA/Protein Models of MEGA was applied to determine the suitable Substitution Model as well as Rates and Patterns. Protein-coding *amoA* and *pmoA* nucleotide sequences were translated to amino acid sequences for phylogenetic analyses. Neighbor-Joining was used as the statistical method for phylogeny reconstruction using MEGA with the substitution models of Kimura 2-parameter for anammox 16S rRNA gene sequences and Jones-Taylor-Thornton for *amoA* and *pmoA* gene sequences, respectively. Gamma distribution with the parameter of 4 was selected for rates among sites. Gaps or missing data was treated as complete deletion. Some clusters of the reconstructed phylogenetic trees were compressed to subtrees in MEGA and edited in Adobe Illustrator CS3 (www.adobe.com) with the same topologies resulted in MEGA.

By defining the difference at 5%, the archaeal *amoA* gene sequences in the present work were analyzed with those from the references [21, 30] and the top hit sequences after performing Nucleotide BLAST of GenBank. The major clusters in Fig. 2-a were classified according to Wang and Gu [30]. This study adopted the classification methods of Hong, et al. [35] and Han, et al. [25] for anammox bacteria, in which they were divided into two major clades, the marine cluster-*Scalindua* and the remaining ones.

Real-time quantitative PCR analysis

The abundances of AOA, AOB, anammox and n-damo bacteria were quantified using the primer sets listed in Table S1. The quantitative analyses were conducted using Applied

Biosystems StepOne Plus™ Real-Time PCR System. The anammox bacterial 16S rRNA gene abundance, archaeal and bacterial *amoA* gene abundances were detected using primer sets of A438f + A684r, Arch-amoAF + Arch-amoAR and amoA-1F + amoA-2R, respectively. The methods were mainly based on a previous study [25] with some adjustments. Briefly, the modifications were that the annealing was 45 s at 53 °C for AOA-*amoA*, AOB-*amoA*, and anammox bacterial 16S rRNA gene, then followed by 1 min at 72 °C for elongation and finally ended with the melt curve stage of 15 s at 95 °C, 1 min at 60 °C and an temperature increment of 0.3 °C to 95 °C for 15 s. The 16S rRNA gene abundance of n-damo bacteria was determined using primers qP1F and qP2R according to another investigation [26]. Briefly, real time qPCR was performed with the following method: 5 min at 95 °C for holding stage; followed by 40 cycles of 15 s at 95 °C, 30 s at 54 °C, 45 s at 72 °C and 15 s at 78 °C; and at the end with the melt curve stage of 15 s at 95 °C, 1 min at 72 °C and an temperature increment of 0.8 °C to 95 °C for 15 s. Each run (20 µl) contained 10 µl iTaq^{TM} universal SYBR Green Supermix (Bio-Rad), 1 µl DNA, 0.3 µl primer forward (20µM), 0.3 µl primer reverse (20µM), 0.4 µl Bovine serum albumin (4 mg/ml; Roche) and 4.4 µl H_2O.

Statistical analyses

Mothur [36] was applied to analyze the operational taxonomic units (OTUs) by defining 3% difference for 16S rRNA and 5% for *amoA* and *pmoA* gene-PCR amplified sequences using the method of FURTHEST, and to generate the diversity index Shannon-Wiener [37] and richness index Chao1 [38].

The coverage was calculated according to the following. The coverage % = (No. of related

clones in sample X ÷ No. of total amplified sequences in sample X) * 100.

Fast Unifrac [39] was used to perform the Principal coordinate analyses (PCoA) and Jackknife Environmental Clusters of the n-damo bacterial communities in the present study together with those from other investigations in the environmental habitats (Table S2). Phylogenetic contexts and sample ID and mapping files were prepared following the Fast UniFrac tutorial.

Pearson moment correlation was calculated using PAleontological STatistics version 3.0 [40]. Detrended correspondence analysis (DCA) was conducted for microbial communities using Canoco for window 4.5 [41]. Linear model was applied and the constrained ordination method was Redundancy analysis (RDA) using Canoco. Monte-Carlo permutation test was examined under the reduced model (number of permutations: 499).

Nucleotide sequence accession numbers

Representative sequences in this work were submitted to GenBank database under the accession number KR132259 - KR132285 for archaeal *amoA* gene sequences, KR132286 - KR132344 for bacterial *amoA* gene sequences, KR132358 - KR132530 for anammox bacterial 16S rRNA gene sequences and KR132345 - KR132357 for n-damo bacterial *pmoA* gene sequences.

Results

Environmental variables in the bioturbated areas

Table 1 shows the main physicochemical characteristics of the sediment samples. Notably,

NH_4^+ concentrations were much higher that of NO_x^- in all four type samples. The highest NH_4^+/NO_x^- ratio was observed in the sediment type Black, while the burrow wall layer had the lowest. In addition, NO_2^- concentrations were lower than NO_3^- in all samples collected. The 'surface' contained the highest NH_4^+ and NO_2^-. On the other hand, redox data indicated the oxygen level of the burrow wall at the depth of 15 cm was similar to that in the ambient surface layer, and significantly different with the bulk sediments at the same depth. Meanwhile, it also showed that the bulk yellow sediments had a much higher redox potential than the dark ones. Generally, the Surface and Wall had lower salinity levels than the bulk sediments.

Phylogenetic analyses of target microbial communities

Using specific PCR primer sets (Table S1), the AOA, AOB, anammox and n-damo bacterial sequences were amplified from the samples with a recover range of 33.3-94.3% (Table 2). N-damo bacterial communities showed the lowest OTU numbers, alpha community diversity and richness indexes than AOA, AOB and anammox bacteria in all four sample types. Furthermore, OTU number, Shannon-Wiener and Chao1 of AOB in the sample type Wall, Surface and Black were generally higher than those of the anammox bacteria, while those of the AOA were lower even further. OTU number, Shannon-Wienner and Chao1 of all four microbial groups from the sample type Yellow were mainly the lowest. In addition, OTU numbers and Chao1 richness of AOA, AOB and anammox bacteria in sample Yellow were the same, respectively. In the sample Surface, OTU number, Shannon-wiener and Chao1 of AOB, anammox and n-damo bacteria were relatively higher than the other types. However,

these attributes of AOA were most favored in sample type-Wall rather than ‘Surface’.

The phylogenies of AOA, AOB, anammox and n-damo bacteria were reconstructed using PCR amplified sequences and are shown in Fig. 2. For AOA, archaeal *amoA* gene sequences retrieved from the bioturbated areas were distributed in Clusters 1, 2, 3 and 7, rather than observed in all clusters (Fig. 2-a). Several sequences recovered from sample type Wall, Surface and Black fell into Cluster 1 containing the partial *amoA* gene sequence of an ammonia-oxidizing marine archaeon, EU239959 *Nitrosopumilus maritimus* SCM1 [42]. Overall, the majority of archaeal *amoA* gene sequences retrieved in all four sample types showed a similar phylogenetic lineage and grouped within Cluster 7.

The phylogenetic tree of AOB is shown in Fig. 2-b, which was constructed with deduced amino acid sequences of the amplified bacterial *amoA* gene. A total of 164 bacterial *amoA* gene sequences were recovered in bioturbated areas. Almost all clones obtained in this work (162 out of 164) clustered into the *Nitrosomonas*-like genus, while only two sequences from sample type-Surface belonged to *Nitrosospira*-like genus. Generally, the sample type-Wall and Surface hosted a broader range of AOB phylotypes than the Black and Yellow. The sequences retrieved from black bulk sediment were mainly affiliated to Cluster 1 and other clades-Clusters sub. Fig. 2-b also indicated that sample type-Yellow had the narrowest range of phylotypes than the other three. Furthermore, some unique lineages were only observed in sample type-Wall, Surface and Black. Additionally, the top hit reference sequences in GenBank after BLAST analysis mostly came from the mangrove, estuarine or wetland sediments.

The phylogenetic tree of anammox bacteria is shown in Fig. 2-c. For the *Scalindua* clusters,

only subclusters zhenghei-II and wagneri contained the PCR amplified sequences from all four samples. Among them in this study, sequences of the surface layer fell into five known *Scalindua* subclusters and were observed in more sub-clusters than those from other samples. Secondly, sequences of sample-Wall belonging to *Scalindua* clusters were also phylogenetically more diverse than the Black and Yellow. Thirdly, a large portion of the sequences from sample-Yellow (42 out of 50) grouped within the zhenghei-I subcluster. For the *Kuenenia* cluster, it should be noted that the detected sequences of the four sample types clustered in this clade were much less than those grouped into the *Scalindua*, respectively (Wall: 9 and 31 of *Kuenenia* and *Scalindua*; Surface: 17 and 29; Black: 9 and 25; Yellow: 4 and 46, respectively).

Fig. 2-d shows the reconstructed phylogeny of n-damo *pmoA* gene sequences in the present work by containing most unique OTUs of n-damo *pmoA* gene sequences from different environmental habitats according to the previous investigation [26]. The Clusters SCS-1, SCS-2 and SCS-3 refer to the newly erected n-damo clades of *pmoA* gene sequences from marine sediments of the South China Sea [43]. Interestingly, all of the 35 retrieved sequences from Surface grouped with the freshwater clusters only. Surface clone 6 shared a very similar evolutionary history with the *pmoA* gene sequences of *Ca*. Methylomirabilis oxyfera [11]. Sequences amplified from Wall clustered within both marine clusters (SCS-1 and SCS-2) and freshwater one, but the majority of them (36 out of 42) fell into a freshwater lineage with n-damo *pmoA* sequences from NC10 enrichment culture [44] and wastewater treatment plant [45]. On the other hand, most recovered sequences from Black and Yellow grouped into Cluster SCS-1, in which the reference sequences were from either the sediments of the South

China Sea or Mai Po wetland [26, 43, 46]. In addition, Clusters SCS-2 and SCS-3 contained sequences only from the sample Wall and Black, respectively.

Community structures of n-damo bacteria

Fig. 3 reveals the results of Principal coordinate analyses (PCoA) for the n-damo bacteria in the bioturbated areas using Fast Unifrac metrics. Together with other 50 environmental habitats (Table S2), n-damo bacterial community structures of this work showed a clear difference between the niches where affected more by the activity of sediment infauna and those less influenced in the surrounding bulk sediments (Fig. 3). In agreement with the previous studies [26, 43], n-damo bacterial communities based on *pmoA* gene sequences could be generally divided into freshwater and marine groups based on either qualitative (Fig.3-a) or quantitative analysis (Fig.3-b). The PCoA scree plots are given in Fig. S2, showing the first 54 dimensions. An ideal 'elbow' was revealed in both Fig. S2-a and Fig. S2-b, suggesting that the first 4 and 5 principal components were enough to explain most of the variability in the results of qualitative and quantitative analyses, respectively.

When using the standard unweighted Unifrac algorithm to perform the calculations (Fig. 3-a), the first three principal axes could explain about 40% of the variance. On the community level, n-damo sequences of *pmoA* gene from sample Surface showed a strong freshwater pattern and were grouped closely to Lake Bosten and Lake Wuliangsuhai [47]. Meanwhile, Wall, Black and Yellow mainly grouped with the SCS and Mai Po n-damo communities [26, 43, 46]. However, when considering the abundance of each OTU number in the environments (Fig. 3-b), Wall scattered within the freshwater group and closely to Surface, the River

309 Pearl-summer and winter [47].

310 Taking account of the frequencies of certain OTU observed in a habitat (quantitative analysis)
311 or not (qualitative analysis) generated significantly different results for the samples in the
312 present work using Jackknife sample cluster of Fast Unifrac algorithm (Fig. S3). For the
313 unweighted Jackknife sample cluster analysis, samples collected in the bioturbated areas were
314 distinguished from all the other 50 environment samples, while samples from previous study
315 of Mai Po sediments distributed together with the SCS habitats under a very strong support of
316 red color (Fig. S3-a).

317 Principal coordinate analyses based on weighted Unifrac algorithm for the amplified
318 sequences in the present work were also conducted for AOA, AOB, Anammox bacteria and
319 n-damo bacteria, respectively. The plots are shown in Fig. S4.

320

321 **Quantification of AOA, AOB, anammox and n-damo bacteria**

322 The AOA abundances of detected *amoA* gene ranged from 2.48×10^{8} (Black) to 1.02×10^{10}
323 (Surface) copies per gram dry sediment, while that of AOB ranged from 3.89×10^{6} (Yellow) to
324 1.22×10^{10} copies/g (Surface) (Fig. 4). Anammox and n-damo abundances based on 16S rRNA
325 gene qPCR were comparatively much lower. The gene copy numbers of revealed anammox
326 bacteria in one gram of dry sediment were almost constant at the order of 7, but those of
327 recovered n-damo bacteria varied from 2.06×10^{6} (Wall) to 1.25×10^{7} (Surface).

328

329 **Correlation with the environmental factors**

330 Based on Pearson moment correlation, the linear *r* values between the environmental

variables and the targeting communities are given in Table S3. According to the significance test, NH_4^+ and NO_2^- significantly correlated with the n-damo OTU richness and the abundances of AOB and n-damo communities. Ammonium also significantly correlated with the alpha diversity of n-damo bacteria and the richness of anammox bacteria. Furthermore, nitrite significantly correlated with the AOA abundance.

All the community indexes negatively correlated with salinity except for the abundance of anammox bacteria; and positively correlated with temperature and nitrate except for the abundance of anammox bacteria as well (Table S3). The OTU number, alpha diversity, richness and abundance of n-damo bacteria positively correlated with all the environmental factors except for the salinity. The similar trend was also observed for anammox bacteria that their OTU number, Shannon-wiener and Chao1 positively correlated all environmental variables except for the salinity. It is also true for the OTU number, Shannon-wiener and abundance of AOB as well as the abundance of AOA. In addition, redox positively correlated with all community indexes except for the richness of AOB and the abundance of anammox bacteria. It is important to note that the abundance of anammox and n-damo bacteria had a directly opposite correlation pattern with all the parameters, but their OTU numbers, Shannon-wiener index and Chao1 shared the same correlation pattern with all environmental factors.

With the maximum lengths of gradient <3 after DCA analyses (archaeal *amoA*: 0.743; bacterial *amoA*: 0.614; anammox 16s rRNA: 1.640 and n-damo *pmoA*: 1.988), linear model was selected for the following constrained ordinate analysis-RDA considering the samples, species and environmental parameters (Fig. 5). Overall, all of the four microbial groups in

Surface positively correlated with NH_4^+, NO_2^- and pH, but those in sample Yellow had an opposite correlation pattern. In agreement with the Pearson analysis, salinity had an adverse effect on samples and species compared with NO_3^-, temperature and redox in all four plots as well as NH_4^+ and NO_2^- (Fig. 5-b and Fig. 5-c). Notably, the environmental factors correlated quite differently with the species in each plot, which were the clusters of each targeting microbial group classified according to the phylogenetic trees (Fig. 2). For example, NH_4^+ and NO_2^- had a positive correlation with species AOA Cluster 1 and 2 (Fig. 5-a), AOB Cluster 1 and Cluster *Nitrosospira* (Fig. 5-b), anammox zhanghei-I, zhenghei-III, clusters *Kuenenia* and *S. brodae* (Fig. 5-c), and n-damo freshwater clusters (Fig. 5-d). However, they correlated negatively with AOA Cluster 3, 7 amd M, AOB subclusters, anammox *S. wagner*i, *S. marina* and other unknown subclusters, and n-damo Clusters SCS 1, 2 and 3. Moreover, salinity favored the n-damo Clusters SCS-1 and SCS-3 (Fig. 5-d).

Discussion

In this study, NH_4^+ concentrations in the bioburbated areas (Table 1) were much higher than the samples collected in the less bioturbated mangrove sediments in Mai Po wetland [26]. However, NO_2^- concentrations were all significantly lower than either mangrove surface or subsurface sediments, while NO_3^- concentrations in burrow wall and ambient surface samples were lower than the mangrove surface sediments as well as those in the subsurface ambient sediments (Black and Yellow) when compared with mangrove subsurface sediments without apparent burrowing [26]. One explanation is that because sediment burrowing microfauna can introduce oxygen to stimulate benthic decomposition, nitrification and denitrification;

more actively nutrient cycling is accompanied, however, the release of NH_4^+ from sediments is enhanced more strongly than the sedimentary uptake of NO_3^- [2]. Meanwhile, the ventilation and excretion of many burrowing macrofauna species stimulated the solute exchange between water column and the sediment resulting in the net efflux of NH_4^+ and influx of NO_3^- because the rate of denitrification, NH_4^+ excretion and organic matter mineralization exceeded the rate of nitrification [3, 48-50]. Results suggested that excretion and accelerated mineralization caused higher NH_4^+ and might be dominant in the densely burrowed sediments in Mai Po wetland. On the other hand, the redox of burrow wall was similar to that of the ambient surface, and the ambient bulk sediments at the same depth of the burrow wall samples had a significant lower oxygen concentration and redox potential, suggesting the extension of oxic-anoxic interface and oscillation of oxygen in the burrow lumen due to periodic ventilation activity by the sediment infauna [7].

Results indicated the negative effect of burrowing infaunal activity on the diversity and richness of n-damo bacteria. The OTU numbers, alpha diversity and richness of n-damo bacteria were generally lower in the present work compared with the previous study on n-damo bacteria in Mai Po non-burrowed mangrove sediments [26]. This may be because of the ventilation-induced oxygen flux and fluctuation of dissolved inorganic nitrogen (DIN), which favored other microbes competing for the substrates. It is clear that AOA benefited from the burrowing activity, evident by the highest OTU numbers, Shannon-wiener and Chao1 indexes from burrow wall among all four sample types. AOA also showed a higher alpha diversity and richness in the ambient sediment sample Yellow than in the dark sediments surrounding burrows. Even so, those indexes in the Black were higher than in the

rarely bioturbated subsurface sediments of Mai Po wetland [21]. The possible explanation is that AOA oxidize ammonia under aerobic conditions, which can be enhanced by the higher NH_4^+ concentration and extended oxic-anoxic interface by sediment fauna as discussed in the above. For the AOB and anammox bacteria, their OTU numbers, Shannon-wiener and Chao1 indexes were significantly lower in the ambient yellow sediment, and were the highest in the ambient surface layer of the burrows. The communities of AOB and anammox were stimulated either on the burrow wall or in the ambient niches when taking the other studies on mangrove sediments into consideration [21, 30].

Burrowing activity of the mangrove sediment infauna had a great impact on the compositional phylotypes of n-damo bacterial communities in these bioturbated areas. It was unexpected that no n-damo *pmoA* gene sequence was amplified from the ambient surface layer fell into to the marine clusters (Fig.2-d). And the majority of those retrieved from the burrow wall were also freshwater phylotypes (36 out of 42). In the previous study examining n-damo bacteria in Mai Po mangrove sediment, recovered *pmoA* sequences from surface layer sediments of 0-2 cm grouped into marine clusters SCS-1 and SCS-2 [26]. And similar to the previous work [26], *pmoA* sequences from the surrounding bulk soils in this study showed similar phylogenetic lineages and grouped mostly into n-damo marine clusters, especially SCS-1.

The bioturbated burrows in Mai Po mangrove wetland might be an important factor in maintaining the phylogenetic diversity of anammox bacteria. '*Kuenenia*' anammox bacteria were only observed in the Mai Po mangrove sediments collected in the wet seasons [20, 22, 23, 25, 29]. However, *Kuenenia* lineages of anammox 16S rRNA gene sequences were

419 recovered not only in the dry season samples on the burrow walls, but also in the ambient
420 surface layers and surrounding bulk soils. This cannot simply be attributed to the higher
421 water content in the biotubated areas (Table 1) than in the previous investigation [26].
422 Because the seasonal variations of anammox bacterial community in Mai Po mangrove
423 sediments were mainly due to (1) the higher NH_4^+, NO_x^- and other factors in wet season than
424 in dry season; (2) the strong South China Coastal Current and less input of Pearl River and
425 Shenzhen River to the estuarine area with the marine anammox bacterium 'Scalindua' as the
426 dominant group in Mai Po coastal wetland during winter [22]. As discussed above, higher
427 NH_4^+, NO_3^- and NO_2^- in the burrow areas are likely the reason to result in a diverse
428 community. In agreement with the previous studies [20, 22, 23, 25, 29], phylogenetically
429 diverse sequences of amammox bacteria were retrieved within the *Scalindua* clusters and
430 there was also none belonging to the genera of *Jettenia*, *Brocadia* and *Anammoxoglous*.
431 Overall, the phylotypes of anammox bacterial community in the bioturbated areas in
432 mangrove sediments were significantly increased, while their phylogenetic features in the
433 mangrove sediments without apparent burrowing were also maintained.
434 Fig. 2-a contains all the unique OTUs in the previous AOA investigations in Mai Po
435 mangrove sediments (GQ331390-GQ331635), the sediments from another coastal mangrove
436 wetland and a constructed freshwater wetland in Hong Kong (JQ886237-JQ886313) [21, 30].
437 AOA communities obtained here in the bioturbated areas demonstrated a lower phylogenetic
438 diversity compared with those observed in freshwater wetland as previously reported [30].
439 However, it is consistent with the results of Shannon-Wiener and Chao1 tests that AOA
440 sequences (Fig. 2-a) not only fell into the main clusters where focusing on Mai Po mangrove

441 sediments [20, 21], but also introducing some novel lineages, especially from burrow wall
442 and ambient surface layers. Importantly, there was a new cluster formed by recovered
443 sequences from all four sample types with one sequence from the previous study in Mai Po
444 mangrove sediments [21]. Therefore, this new cluster is named as Cluster M in Fig. 2-a. In
445 addition, results implied the shift of the different AOA phylotype abundances in the
446 community from bioturbated areas because the majority of the amplified AOA sequences
447 grouped within Cluster 7, which was very different from the previous observations [20, 30].
448 Compared with other studies [20, 21, 25], AOB phylogenies of this work grouped similarly
449 within *Nitrosomonas*-like clusters, and very few sequences were classified within
450 *Nitrosospira*-like clade (Fig. 2-b). If only considering the four sample types in this
451 investigation, phylogenetic ranges of AOB on burrow walls and ambient surface layers were
452 broader than those in the surrounding sediments, suggesting that sediment infauna activity
453 positively contributed to the diversity of AOB community. On the other hand, reconstructed
454 phylogeny of AOB sequences of sample Yellow were the least diverse, suggesting the effect
455 of mangrove roots on microbial community. One explanation is that most AOB were not
456 adapted to acidic soils, but the Yellow had the lowest pH of 5.98 due to the oxidation of H_2S
457 from sulfate reduction under anaerobic condition, low-molecular-weight organic acids
458 released from mangrove roots and also possibly the produced protons when absorbing NH_4^+
459 by plants [20, 51-53]. Furthermore, the vegetated sediments contained lower NH_4^+ than the
460 other samples because of the strong absorption of the roots probably, which was in consistent
461 to the previous study [20].
462 Burrowing activity profoundly altered the n-damo community structure in Mai Po mangrove

463 wetland. The community structures of n-damo bacteria based on *pmoA* gene from various
464 habitats could be mainly explained as marine group-SCS and the freshwater one as they
465 responded and grouped quite differently with the environmental variables of the principal
466 components [43, 46]. Later, the investigation of the surface and subsurface sediments in the
467 dry season of Mai Po wetland showed that n-damo bacterial communities there based on
468 *pmoA* gene sequences had a marine n-damo community structure and scattered closely with
469 those unraveled in the sediment of the South China Sea in PCoA plots [26]. In agreement
470 with these findings, n-damo communities in surrounding sediments of the burrows also had a
471 very similar response toward the changes (Black and Yellow in Fig. 3). And they grouped
472 more closely to each other in quantitative analysis (weighted Unifrac) than in Fig. 3-a, which
473 implied that although the OTU compositions of each sample might vary, their abundant
474 OTUs were similar. On the other hand, it was reported that the difference between ambient
475 surface and subsurface bacterial communities were more pronounced within bioturbated
476 sediments than in the uninhabited sediments as a control [6]. Here, n-damo community in the
477 ambient surface showed a clear freshwater n-damo community structure as well as that on the
478 burrow walls in weighted Unifrac analysis, which was different from the previous findings in
479 the surface mangrove sediments of Mai Po wetland [26]. The possible reason is that although
480 the samples in the reference was collected in the dry season [26], they were from 0-2 cm
481 layer and in the areas without apparent burrowing, while those in the present work were
482 sampled from 1-3 mm layers of the burrow walls and ambient surface. Horizontal oxygen
483 concentration profiles suggested that the sediment infauna could increase O_2 in the surface
484 layer to the surrounding sediment significantly [7], and the burrowing activity might enhance

485 the exchange of substrates in the surface layer with the water column as well as introduced a
486 relatively rich O_2 and DIN zone [2]. These natures on the burrow wall and ambient surface
487 likely benefited the freshwater lineages of n-damo bacteria there. In addition, it is also
488 interesting to find that n-damo communities from the burrow wall and ambient surface
489 grouped with those recovered in the summer and winter sediments of Pearl River [47] either
490 in the quantitative PCoA or Jackknife environmental cluster analyses, where the main
491 freshwater source of Mai Po wetland comes from.
492 The abundances of AOA, AOB and n-damo bacteria were greatly enhanced in the bioturbated
493 areas, where the four type samples in the present work were collected. In previous studies, the
494 maximum of AOA abundance in Mai Po wetland regardless of the sample types was 10^7 gene
495 copies per dry sediment [20, 21, 25], which in the present study increased to 10^8 gene copies
496 per dry sediment on burrow walls and ambient black sediment, 10^9 in ambient vegetated
497 sediment (Yellow) and 10^{10} in the ambient surface layer. A similar phenomenon was found
498 for n-damo bacteria. Their abundances in surface and subsurface layers of Mai Po mangrove
499 sediments during dry season ranged within 10^5 gene copies with a higher value in subsurface
500 layer [26]. When examining the results of the bioturbated areas, the abundances of n-damo
501 communities increased two orders of magnitude in the ambient surface layer and one order of
502 magnitude on the burrow wall as well as in the surrounding sediments. Furthermore, AOB
503 abundances were one order of magnitude (Wall and Black) or three orders of magnitude
504 (Surface) higher than the previous findings [20, 21, 25]. Interestingly, the AOB abundance in
505 Yellow was significantly lower than that in Mai Po mangrove vegetated yellow sediment in
506 winter, but the same ten to the power of six with that in summer [20]. The rationale is yet to

be studied. But the reason for the lower AOB abundance in Yellow might be the same as that of AOB phylogenetic diversity (of sample Yellow) as discussed above. Another unexpected finding was that the abundances of anammox bacteria were at the same order of magnitude in all four sample types and also shared the same order with the previous observations [20, 25].

The relative abundance of AOA and AOB was influenced profoundly by the sediment infauna and mangrove roots. In the samples affected directly under the infauna activity, AOB accounted for a large portion, decreased to minor ones in the surrounding black sediment, and even became relatively negligible in ambient vegetated sediment-Yellow. Meanwhile, AOA showed an opposite trend. This finding was different from the previous investigation with Mai Po sediments, where AOB were more abundant in the vicinity of mangrove forest and decreased in inter-tidal mudflat without vegetation [21]. But the above trend was supported by another study on their distribution in mangrove sediment, showing that AOB were about 3 times more abundant than AOA in non-vegetated sediment of winter, and AOB/AOA ratio was <1 and even smaller in vegetated black and yellow sediments [20]. Results suggested that AOB were stimulated more than AOA from the burrowing activity and both of them contributed most to the nitrogen turnover among the four targeting groups in the bioturbated areas.

Ammonium and nitrite were suggested to be important environmental factors shaping the n-damo bacterial communities [26, 43, 54]. The results in the present work were also in agreement with this because n-damo bacteria require NO_2^- as electron acceptor and methane as electron donor [11]. Meanwhile, anammox bacteria also utilize NO_2^- as electron acceptor and NH_4^+ as electron donor instead of methane [55, 56], but they are proven to cooperate

529 with a novel lineage archaea to complete the denitrifying anaerobic methane oxidation
530 (n-damo) process, which use NO_3^- as electron acceptor and methane as electron donor [57].
531 Therefore, anammox bacteria were generally predicted as competitor for n-damo bacteria in
532 the environments. Still, there is no concrete evidence to support it. Here, it is exciting to find
533 that anammox and n-damo bacterial abundances had a directly opposite correlation pattern
534 with all the environmental parameters, while their OTU numbers, alpha diversity and richness
535 shared the same correlation pattern with all environmental factors (Table S3). Results
536 strongly suggested that the communities of anammox and n-damo bacteria in bioturbated
537 areas of this study required favorable environmental parameters similarly and had a
538 competitive relationship for the growth of their communities.
539 Salinity only positively correlated the abundance of anammox bacteria and negatively with
540 other microbial groups, which was in agreement with the previous study [51]. The reason was
541 probably that the majority of the phylotypes in each studied community were benefited from
542 certain saline range. For examples, (1) the anammox communities in all sample types were
543 dominant by the member of marine phylotype- *Scalindua* as discussed previously; (2) AOB
544 community structure shifted along salinity gradient [24] and AOB at medium and low salinity
545 sites had similar phylogentic pattern as unraveled in the present work [51, 58].
546
547 In summary, the activity of sediment infauna resulted in the increase of the NH_4^+
548 concentration and oxic-anoxic interfaces and the decrease of NO_2^+ and NO_3^+ concentrations
549 in Mai Po mangrove sediments. The results of this work indicated that bioturbation had a
550 great impact on the structures, compositions and abundances of the investigated communities.

(1) The alpha diversity, richness and abundance of AOA were increased by the burrowing activity and the mangrove root effect as well. The AOA abundance increased up to 3 orders in the bioturbated areas and they were relatively more abundant in the highly vegetated sediment. The phylogenetic composition of AOA shifted to be abundant in Cluster 7 and contained some novel lineages, when compared with previous studies. On the other hand, AOB showed a broader phylotype range in burrow wall and ambient surface layer. Their diversity and abundance were clearly stimulated by sediment infauna, but inhibited in the sample Yellow. (2) A small group of anammox bacterial phylotypes clustered within *Kuenenia* clade while the other large part belonged to *Scalindua* clusters, which was different with previous findings of only Scalindua clusters in winter samples and implied the bioturbation might be important for maintaining the phylogenetically diverse of anammox bacteria in Mai Po wetland. However, the abundance of anammox bacteria was not enhanced significantly by burrowing activity. In contrast, the abundance of n-damo bacteria increased up to two orders of magnitude in the bioturbated areas. The community structures of n-damo bacteria in burrow wall and ambient surface layer were altered profoundly and changed to freshwater pattern, when the surrounding bulk sediments hosted n-damo communities of marine phylotypes, similar to the previous investigation. Meanwhile, the burrowing infaunal activity was suggested to limit the alpha diversity and richness of n-damo bacteria, resulting in lower OTU numbers, Shannon-Wiener and Chao1 indexes when considering the results from the same sampling type in the previous investigation. In addition, results suggested that anammox and n-damo bacteria competed for their abundance and required beneficial environmental variables similarly.

Acknowledgments

This project was supported by Hong Kong PhD Fellowship (JC) and Hong Kong RGC GRF grant No. 701913 (J-DG). Additional financial support for this research project was from the laboratory fund. We thank Dr. Yong-Feng Wang for helping with the sampling, Dr. Kun Liu for assisting the real-time quantitative PCR and Ms. Kelly Lau for general laboratory assistance.

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