A missing link in the estuarine nitrogen cycle?: Coupled nitrification-denitrification mediated by suspended particulate matter

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In estuarine and coastal ecosystems, the majority of previous studies have considered coupled nitrification-denitrification (CND) processes to be exclusively sediment based, with little focus on suspended particulate matter (SPM) in the water column. Here, we present evidence of CND processes in the water column of Hangzhou Bay, one of the largest macrotidal embayments in the world. Spearman’s correlation analysis showed that SPM was negatively correlated with nitrate ($\rho = -0.372$, $P = 0.018$) and marker genes for nitrification and denitrification in the water column were detected by quantitative PCR analysis. The results showed that $\text{amoA}$ and $\text{nir}$ gene abundances strongly correlated with SPM (all $P < 0.01$) and the ratio of $\text{amoA}/\text{nir}$ strongly correlated with nitrate ($\rho = -0.454$, $P = 0.003$). Furthermore, aggregates consisting of nitrifiers and denitrifiers on SPM were also detected by fluorescence in situ hybridization. Illumina MiSeq sequencing further showed that ammonia oxidizers mainly belonged to the genus $\text{Nitrosomonas}$, while the potential denitrifying genera $\text{Bradyrhizobium}$, $\text{Comamonas}$, $\text{Thauera}$, $\text{Stenotrophomonas}$, $\text{Acinetobacter}$, $\text{Anaeromyxobacter}$, $\text{Sulfurimonas}$, $\text{Paenibacillus}$ and $\text{Sphingobacterium}$ showed significant correlations with SPM (all $P < 0.01$). This study suggests that SPM may provide a niche for CND processes to occur, which has largely been missing from our understanding of nitrogen cycling in estuarine waters.

The amount of reactive nitrogen (N) ultimately ending up in estuarine and coastal ecosystems, which stems from human activity has increased extensively (over 150%) in the 20th century. Excess N has resulted in increased eutrophication across numerous estuarine ecosystems and has consequently become a global matter of concern over the last several decades1. Estuarine eutrophication has resulted in a suite of environmental problems including oxygen depletion, algae blooms, loss of biodiversity, global acidification and even the establishment of invasive species2.5,6. Recently, the Bulletin of China’s Marine Environmental Status (2014) showed that anthropogenic pollution is of particular concern in China, with nearly 80% of Chinese estuaries subject to some level of eutrophication. Thus, understanding N transformation, as well as removal mechanisms in estuarine systems are critical for the maintenance and conservation of estuarine ecosystem health.

N biogeochemistry, which is almost exclusively reliant on reduction-oxidation (redox) reactions, is facilitated primarily by microorganisms8. Denitrification, the sequential reduction of nitrate (NO$_3^-$) to dinitrogen gas (N$_2$) via oxidized intermediates, is considered to be the dominant loss pathway for fixed N in shallow coastal and estuarine systems9, although anammox (the anaerobic oxidation of ammonium) has been recently identified as

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Figure 1. Map of Hangzhou Bay, China showing sampling sites on the west coast of the East China Sea. The Surfer 11 software (http://www.goldensoftware.com/products/surfer) was used to generate the map.

an alternative microbial pathway of N₂ production⁶. Two nitrite reductases—copper-containing and cytochrome cd, nitrite reductases (nirK and nirS) are key enzymes in the denitrification pathway⁷. Nitrification, the two-step conversion of ammonium (NH₄⁺) to NO₃⁻ via nitrite (NO₂⁻), is generally thought to play a critical role in the N cycle. Ammonia oxidation is considered to be the rate-limiting step of nitrification and is catalyzed by ammonia monooxygenase (AMO), which is encoded by the amoA gene from both archaea (AOA amoA) and bacteria (AOB amoA)⁸. When coupled with denitrification, nitrification partially mitigates the adverse effects of eutrophication, by removing bioavailable N from the water and releasing it to the atmosphere as N₂O or N₂.

In general, nitrification is an aerobic process and denitrification is an anaerobic one, and the two processes are often carried out in different ecological niches. Sediment, with substantial niche diversity (characterized by complex structural properties and sharp redox gradients), has been identified as the dominant sink for fixed N⁹. Coupled nitrification-denitrification (CND) processes in estuarine sediments contribute substantially to N loss, in some cases removing 10-80% of anthropogenic N pollution¹⁰. To date, there are a number of studies which have shown the importance of sediment-based CND in estuaries¹¹,¹²; however there may be additional sites with comparable niche diversity in estuaries that may facilitate CND and subsequent N removal.

Strong tidal currents often resuspend fine particulates from the benthos, and the resulting suspended particulate matter (SPM) is a universal component of estuarine waters, particularly in macrotidal estuaries¹³. Composed of nutrients, organic micro-pollutants and heavy metals, SPM can affect material exchange and biochemical processes in estuarine systems¹⁴. In the maximum turbidity zone (MTZ), where primary production tends to be light-limited, the accumulating SPM is a substrate for most bacterial activity¹⁵. While a number of studies have followed the effects of particle-attached heterotrophic bacteria on estuarine carbon circulation¹⁶,¹⁷, the fate of excess N, driven by microbially-mediated processes related to SPM in estuarine environments, remains unclear. Does SPM provide adequate conditions for CND processes to occur in estuarine waters?

Hangzhou Bay is located in the northern Zhejiang Province of China and is adjacent to the East China Sea (Fig. 1). Covering an area of approx. 8,500 km², it is one of the world’s largest macrotidal embayments. The tidal amplitude at the mouth is 3–4 m, and it exceeds 4–6 m further upstream. Tidal currents are mainly rectilinear and the maximal flood velocity exceeds 4.0 m/s¹⁸. Influenced by tidal currents and waves, Hangzhou Bay has a high carrying capacity for SPM, which is carried downstream from the Changjiang River and the Qiantang River¹⁹. The mean annual water discharge and sediment transport in Hangzhou Bay are 2.91 × 10¹⁰ m³ and 6.68 × 10⁶ tons, respectively²⁰. The purpose of this study is to investigate the potential for CND processes mediated by SPM in the water column of Hangzhou Bay and provide new insight into the role of SPM in estuarine N cycle.

Results

Environmental parameters and correlation analyses. Environmental parameters in the water column of Hangzhou Bay are shown in Supplementary Table S1, across a scale of ~55 km. Ranging from 0.02 to 8.07 g/L, SPM of the surface water was significantly lower than that of the bottom water (Table 1; F(1,39) = 26.869, P = 0.000) and the opposite result was seen for particulate organic carbon (POC) (Table 1; F(1,39) = 40.301, P = 0.000). NO₃⁻ accounted for 95.85–99.80% of the dissolved inorganic nitrogen (DIN) in the water column. However, NO₂⁻ of the surface water was significantly higher than that of the bottom water (Table 1; F(1,39) = 6.110, P = 0.018). The remaining environmental parameters, including temperature, salinity, dissolved oxygen (DO), pH, chlorophyll a (Chl a), NH₄⁺ and NO₂⁻, showed no significant differences between the surface and bottom water of Hangzhou Bay (Table 1).

Spearman's correlation analyses between SPM and other environmental parameters (pooled surface and bottom) were performed (Table 1; N = 40). Strong positive correlation was identified between SPM and depth (Table 1; rho = 0.760, P = 0.000) and strong negative correlations were identified between SPM and both temperature and POC (Table 1; rho = −0.436, P = 0.005 and rho = −0.706, P = 0.000). Furthermore, NO₃⁻ and NO₂⁻ showed weak correlations with SPM (Table 1; rho = 0.345, P = 0.029 and rho = −0.372, P = 0.018), while...
no relationship between SPM and NH$_4^+$ was observed. It is worth noting that no correlation was found between SPM and NO$_3^−$ in surface water (N = 20) or bottom water (N = 20) when compared alone (data not shown).

Abundances of nitrifying and denitrifying genes and correlation analyses. Quantitative PCR (qPCR) analysis was used to estimate the abundances of the key nitrifying (AOA amoA and AOB amoA) and denitrifying (nirK and nirS) genes in the water samples obtained from the study area. Among the nitrifying genes, at each site, the abundance of AOB amoA gene ($4.46 \times 10^5$ − $4.22 \times 10^8$ copies/L) was higher than that of AOA amoA gene ($1.74 \times 10^5$ − $7.43 \times 10^7$ copies/L), with the exception of sample H4_S (Supplementary Table S2). Among the denitrifying genes, nirS gene appeared to be more numerous, with copy numbers ranging from $2.64 \times 10^6$ to $5.90 \times 10^8$ per liter of water, while copy numbers of nirK gene ranged from $2.39 \times 10^5$ to $4.95 \times 10^7$ per liter of water (Supplementary Table S2). The relative nirS/nirK ratios ranged from 6.90 to 76.29 in all samples, showing the clear dominance of nirS gene in the water column of Hangzhou Bay. Moreover, along the SPM gradient, both nitrifying and denitrifying genes showed significantly higher abundance of the bottom water than that of the surface water (Fig. 2). In addition, the abundance of anammox bacterial 16S rRNA gene,
The most abundant phylum across all water samples, accounting for 28.56–35.32% of the total effective bacterial community patterns. Overall, the first two RDA axes explained 52.3% of the total variation, and RDA1 axes clearly distinguished the bacterial distributions of surface water from that of bottom water. Of all the environmental parameters measured, depth (Table 2; F = 5.492, P = 0.001), temperature (F = 3.836, P = 0.033) and POC (F = 10.413, P = 0.001) appeared to be the major drivers influencing bacterial distributions, while other variables (e.g., salinity, DO, pH, Chl a, NH₄⁺, NO₂⁻ and NO₃⁻) had weaker effects on bacterial assemblages (Fig. 4).

**Bacterial community structures and RDA analysis.** The Illumina MiSeq platform produced ~1,500,000 raw reads of the V4 amplicons. After removing the short and low-quality reads, 34,117–69,053 bacterial reads of each sample were available for further analyses. The numbers of OTUs, Chao 1 and Shannon’s indices at cutoff levels of 3% are summarized in Supplementary Table S3. On the basis of OTU numbers, the H7_B sample had the richest abundance, and declined to less than half that in H19_S sample. The comparison between surface and bottom water samples showed that the latter contained more bacterial richness than the former at most of the sampling sites (Supplementary Table S3). Very similar trends in the Chao 1 index were observed in comparison with OTU richness (Supplementary Table S3). Shannon diversity indices also indicated a higher bacterial diversity for the bottom water samples relative to the surface at all the sampling sites (Supplementary Table S3).

Cluster analysis at the phylum level revealed similarities of bacterial community structure in each sample of Hangzhou Bay (Fig. 3). In contrast to the surface water layer, bacterial communities in the bottom water layer were relatively stable and showed little variability, with almost all samples clustering together (Fig. 3). Proteobacteria was the most abundant phylum across all water samples, accounting for 28.56–35.32% of the total effective bacterial sequences. Within the Proteobacteria group, Deltaproteobacteria (6.37–15.52%, averaging 11.36%) was the most dominant class, followed by Gammaproteobacteria (4.44–12.19%, averaging 10.34%), Alphaproteobacteria (1.61–8.94%, averaging 5.41%) and Betaproteobacteria (1.73–7.19%, averaging 2.92%). The other dominant phyla were Planctomycetes (11.73–22.69%, averaging 14.10%), Bacteroidetes (3.86–9.82%, averaging 7.76%), followed by a few other major phyla (abundance >1% in each sample), including: Acidobacteria, Firmicutes, Chloroflexi, Chlamydiae and Verrucomicrobia. A few phyla (e.g. Actinobacteria, Spirochaetes, Cyanobacteria, Lentisphaerae, Gemmatimonadetes, Nitrospirae, Nitrospirae and Deinococcus-Thermus) were only major contributors (abundance >1%) to phyletic compositions of single samples.

Redundancy analysis (RDA) was conducted to identify the potential effect of environmental parameters on bacterial community patterns. Overall, the first two RDA axes explained 52.3% of the total variation, and RDA1 axis clearly distinguished the bacterial distributions of surface water from that of bottom water. Of all the environmental parameters measured, depth (F = 5.492, P = 0.001), temperature (F = 5.150, P = 0.005), POC (F = 3.836, P = 0.033) and POC (F = 10.413, P = 0.001) appeared to be the major drivers influencing bacterial distributions, while other variables (e.g., salinity, DO, pH, Chl a, NH₄⁺, NO₂⁻ and NO₃⁻) had weaker effects on bacterial assemblages (Fig. 4).

**Sequences belong to nitrifying and potential denitrifying genera.** Taxonomically, AOB are phylogenetically restricted to two lineages within the Proteobacteria: the Betaproteobacteria, including the genera Nitrosomonas, Nitrosospira, Nitrososybrio and Nitrosolobus¹⁰; and the Gammaproteobacteria, including the genus Nitrososoccus²¹. The Betaproteobacterial Nitrosomonas and Nitrosospira, and the Gammaproteobacterial Nitrososoccus were observed in this study. The Nitrosomonas and Nitrososoccus groups were the two dominant genera of AOB, accounting for 0.06–5.25% and 0.03–0.17% of the total sample sequences respectively.

Table 2. Spearman’s correlation coefficients (rho) between environmental parameters and gene abundances and the ratios of target genes across sampling sites (pooled surface + bottom samples). *Represents the sum of AOB amoA and AOA amoA gene abundances. **Represents the sum of nirK and nirS gene abundances. Data in bold indicate significant correlations, *P < 0.05, **P < 0.01.

| Parameter | Chla | DO | pH | SPM | POC | NH₄⁺ | NO₂⁻ | NO₃⁻ |
|-----------|------|----|----|-----|-----|------|------|------|
| Depth     |      |    |    |     |     |      |      |      |
| Temperature |    |    |    |     |     |      |      |      |
| Salinity  |     |    |     |      |      |      |      |      |
while the genus *Nitrosospira* was nearly absent in this study (Supplementary Figure S1a). In addition, the relative abundance of *Nitrosomonas* showed strong negative correlations with SPM, NO$_2^-$ and AOB amoA gene (Supplementary Table S4; all $P < 0.01$), and a weak positive correlation with NO$_3^-$ (Supplementary Table S4; $\rho = 0.442$, $P = 0.014$). While, the relative abundance of *Nitrosococcus* was only positively correlated with SPM (Supplementary Table S4; $\rho = 0.422$, $P = 0.020$).

Previous studies provided a list of ~100 denitrifying bacterial genera$^{23–26}$, among which 44 genera were detectable in the water column of Hangzhou Bay (Supplementary Figure S1b). In this study, the majority of the potential denitrifying genera belonged to Proteobacteria (31 genera; 0.47–1.61% of the total 16S rRNA sequences).
second largest group of denitrifiers was categorized as Bacteroidetes (4 genera; 0.17–0.83%), followed by the group of Firmicutes (5 genera; 0.08–0.46%) and Actinobacteria (4 genera; 0–0.05%) (Supplementary Figure S1b). Among the 44 denitrifying genera, many of them showed strong positive correlations with SPM ($P < 0.01$), which mainly included (i.e. average abundance >0.05%) the genera *Bradyrhizobium* of Alphaproteobacteria; *Comamonas* and *Thauera* of Betaproteobacteria; *Stenotrophomonas* and *Acinetobacter* of Gammaproteobacteria; *Anaeromyxobacter* of Deltaproteobacteria; *Sulfurimonas* of Epsilonproteobacteria; *Paenibacillus* of Firmicutes; and *Sphingobacterium* of Bacteroidetes (Supplementary Table S5).

**In situ** characterization of nitrifying and denitrifying bacteria. The presence of nitrifiers and denitrifiers in the bacterial consortia of the SPM was examined by fluorescence *in situ* hybridization (FISH) analysis. *In situ* hybridization clearly indicated that AOB and denitrifiers were not uniformly distributed within the SPM, and AOB were in greater abundance than acetate/methanol-denitrifying cells (Fig. 5a–c and Supplementary Figures S2a–c). Specifically, the majority of NSO190 probe-stained AOB and DEN124 probe-stained acetate-denitrifying cells formed irregularly shaped (2–10 μm), dense aggregates, with ammonia-oxidizing microcolonies found mainly in the outer part of the aggregate and acetate-denitrifying cells in the middle (Fig. 5d and Supplementary Figure S2d). Though fewer cells hybridizing with probe DEN67 were detected (Fig. 5c and Supplementary Figure S2c), the white signals in the figures indicated that the methanol-denitrifying cells also existed in inner sections of the aggregates.

**Discussion**

Although a previous study has shown that NH$_4$ is the primary anthropogenic nutrient entering Hangzhou Bay from the Qiantang River$^{27}$, NO$_3$ was found to be the most important form of DIN in the water column of Hangzhou Bay. Chl a concentrations (0.26–1.75 μg/L) in the water column were much lower than those measured in other estuaries$^{28,29}$, and is likely indicative of poor light availability which severely limits phytoplanktonic uptake of NH$_4$$^{+}$.$^{30}$ Furthermore, no negative relationship between Chl a and NH$_4$ or NO$_3$ was found (data not shown). It is likely that microbial nitrification is a key process for the fate of NH$_4$ in the water column. SPM was found to be negatively correlated with NO$_3$ and positively correlated with NO$_2$-N, potentially linking SPM-attached denitrifiers to nitrate reduction. We investigated these possibilities by quantifying the abundances of nitrifiers and denitrifiers in the water column and tested their relationships with the measured environmental parameters. High abundances of *amoA* and *nir* genes were detected in both the surface and bottom layers of the water column, with the abundances of AOB *amoA* and *nirK* genes and the ratio of *amoA/nir* significantly
correlating with NO$_3^-$, showing links between nitrifiers/denitrifiers and available N$^{31}$. Although correlations between SPM and indicators of denitrifying bacteria do not necessarily indicate causation, these results provide insight on the potential possibility of SPM to act as an important site for CND processes in the water column of Hangzhou Bay.

Crucial environmental parameters known to influence abundance and diversity of nitrifiers and denitrifiers mainly include temperature, salinity, oxygen and nutrient availability$^{32,33}$. Nevertheless, in this study, the abundances of nitrifying and denitrifying genes were strongly correlated with SPM concentration. Moreover, the results of the RDA suggested that SPM played an important role in shaping the bacterial communities in the water column. In particular, the phyla (or class) Bacteroidetes, Planctomycetes and Deltaproteobacteria were the dominant community members and have recently been shown to be associated with larger particle size fractions$^{33}$. Overall, these results suggest that SPM may provide a large area for nitrifiers and denitrifiers to co-exist, shaping nitrifying and denitrifying communities in estuarine water. Meanwhile, aggregates consisting of outer nitrifiers and inner denitrifiers detected by FISH analysis indicate that anoxic/low oxygen microsites probably exist inside the SPM. According to Jia et al. (2016), high O$_2$ influx around suspended particles and decreasing DO concentration suggested that oxygen was being consumed near the particle's surface by nitrification and/or microbial respiration$^{34}$. They reported that particles with diameters <20 μm had the largest overall increase in O$_2$ influx. Schramm et al. had also reported that the volumetric respiration rate was negatively correlated with floc size$^{35}$. Accordingly, SPM in Hangzhou Bay is predominantly composed of fine and medium silt, and the grain size of over 90% of the SPM is less than 20 μm$^{34,35}$. FISH analyses in the present study support this, showing that the size of most aggregates on SPM were far less than 20 μm (Fig. 5 and Supplementary Figure S2). Based on these results, we suggest that SPM could not only provide large areas for the co-existence of nitrifiers and denitrifiers, but may also provide redox conditions for CND processes to occur on SPM.

Numerous earlier studies suggest that AOA phylotypes are more abundant than AOB in estuarine ecosystems$^{37,38}$. However, our qPCR estimates indicated that the abundance of bacterial amoA gene was higher than that of archaeal amoA gene in all samples (with a single exception). Moreover, the abundance of AOB amoA gene significantly correlated with NO$_3^-$, while the abundance of AOA amoA gene did not. Thus, it is possible that nitrification may be driven by bacteria rather than archaea in the water column of Hangzhou Bay. Furthermore, the abundance of Nitrosomonas was positively correlated with NO$_3^-$ and negatively correlated with NO$_2^-$, suggesting it may be the predominant AOB during nitrification process. Similar to previous studies, members of the Nitrosomonas group appear to be dominant in most terrestrial and aquatic environments$^{39}$. Despite Nitrosomonas being the most dominant AOB in the water column, the genus Nitrospicoccus was dominant in the sediment (Supplementary Figure S3). This may partly explain why the abundance of Nitrosomonas was negatively correlated with SPM.

Since the concentration of Chl a in the water column was very low, it seemed unlikely that nitrate was assimilated by algae. Additionally, the abundance of nirK gene was negatively correlated with NO$_3^-$, while the nirS gene was not. Thus, it is possible that the decrease of nitrate concentration in the system is mainly caused by nirK-type denitrifiers on SPM. Though nirS gene appears to be more abundant in nature$^{40}$, the activity of cytochrome cd$_1$ nitrite reductases (NirS) was more likely to be repressed than that of copper-containing nitrite reductases (NirK) in the oxic water of Hangzhou Bay. As Körner and Zumft (1989)$^{41}$ pointed out, the threshold values for synthesis of copper-containing nitrite reductases (NirK) was 5 mg of O$_2$ per liter, and several studies from different environments have also shown that bacteria carrying the nirK gene were not particularly sensitive to oxygen$^{42,43}$. The higher abundance of nirS gene in the water column of Hangzhou Bay was probably a result of sediment resuspension, as the abundance of nirS-type denitrifiers (1.90 × 10$^{-4}$–4.69 × 10$^{-4}$ copies/g dry sediment) was much higher than that of nirK-type denitrifiers (1.21 × 10$^{-3}$–3.27 × 10$^{-3}$ copies/g dry sediment) in the sediment of Hangzhou Bay (Supplementary Figure S4). While not definitive, many of the denitrifying genera identified in this study showed strong positive correlations with SPM, and are potentially indicative of denitrification process, providing a new direction for inquiry into CND processes in aerobic water columns.

Recently, a series of $^{15}$N isotopic tracer studies have suggested that the nitrification/denitrification rates in oxic water are significantly affected by the abundances of nitrifiers/denitrifiers on the suspended particles$^{44,47}$. Although the current study did not make direct measurement of nitrification or denitrification rate, on the basis of results above, we assume that the abundances of nitrifiers/denitrifiers on SPM can also reflect (to some extent) nitrification/denitrification rates in the turbid water of Hangzhou Bay. The microbial mechanisms behind CND processes are potentially as follows: in the case of water column with high oxygen concentration, SPM can provide large areas for the co-existence of nitrifiers and denitrifiers, which can potentially cause a decrease in NO$_3^-$ removal. Meanwhile, the number of nirK-type denitrifiers significantly increased, and the increase of both low/anoxic microbes and denitrifiers within the SPM promotes denitrification, leading to the increase in NO$_3^-$ removal. In combination, both processes eventually resulted in a significant decrease in NO$_3^-$ (Supplementary Figure S5). Correspondingly, the decrease of POC concentration suggested an increasing consumption of POC by denitrification and/or heterotrophic bacterial respiration.

With the caveat that correlations between SPM and indicators of denitrifying bacteria do not necessarily indicate causation, the current study reports for the first time that SPM may provide the conditions for CND processes to occur in the water column of Hangzhou Bay. These preliminary insights on the coupling of nitrification and denitrification processes mediated by SPM, help to resolve our understanding of N cycle in estuarine ecosystems. High SPM concentration, caused by erosion and bottom sediment resuspension, could accelerate N
transformation and the subsequent removal processes in turbid estuaries and should be included in budgets of riverine N flux to coastal oceans. The findings shed further light on our conceptual views of N fluxes and transformation in estuarine ecosystems and provide another avenue of consideration with respect to CND processes. However, the paucity of direct measurements of CND rates of SPM hinders further interpretations of this current dataset. Quantifying the relationship between direct measurements of nitrification/denitrification rates of SPM and the associated microorganisms, through the application of 15N isotopic tracer technologies for example, may help to elucidate how these coupling processes respond to SPM variability in the turbid estuarine waters.

**Materials and Methods**

**Sampling.** Samples of surface (at 0.5 m depth) and bottom water (1 m above the benthos) were collected from twenty sites along Hangzhou Bay during a 7 day cruise in May 2014, when the phytoplankton quantity was quite low59 (Fig. 1). All water samples were collected with a 5-L Niskin bottle (Tianjin test center, Tianjin, China), and then sampled from the Niskin bottle with a plastic syringe. Standard oceanographic properties including water temperature, salinity, DO and pH were measured immediately using a Horiba U-52 water quality checker (Horiba, Kyoto, Japan). The concentrations of Chl a (µg/L), SPM (g/L), POC (g per g SPM) and nutrients (NH4+, NO3− and NO3−; µM) were measured following standard protocols49. Water samples for nucleic acids analysis and SPM samples for FISH analysis were collected onto 0.2-µm and 0.45-µm polycarbonate GTTP membranes (Millipore, Billerica, MA, USA) respectively and preserved at −80 °C.

**DNA extraction.** Total nucleic acids of the water samples were extracted directly from the 0.2-µm membranes using a FastDNA spin kit for soil (Qbiogene, Carlsbad, CA, USA), following the manufacturer’s instructions. Duplicate DNA extractions for each water sample were performed. DNA quality was detected through 1% agarose gel electrophoresis which was stained with SYBR Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA). The duplicate DNA extractions were then merged together, and stored at −80 °C for subsequent molecular analysis.

**QPCR analysis.** To examine the spatial variation of community size for nitrifying and denitrifying groups in the water column of Hangzhou Bay, the abundances of AOA amoA, AOB amoA, nirK and nirS genes were quantified using qPCR. Serial tenfold dilutions (10−1 to 10−5) of linearized plasmids containing cloned AOA amoA, AOB amoA, nirK and nirS genes were used to obtain standard curves. All sample and standard reactions were performed in triplicate using CFX 96 C1000™ Thermal Cycler (Bio-Rad, Hercules, CA, USA), and average values were calculated. Each reaction was performed in 20 µL containing 2 µL of total DNA template, 0.4 µL of each primer (10 mM) and 10 × PCR buffer. The specificity of amplification was checked by the observation of melt curves. PCR amplification efficiencies were 83–100.7% with correlation coefficients (R2) over 0.99 for all calibration curves.

**Illumina MiSeq sequencing and sequence analysis.** Bacterial communities were investigated at 15 sites for both surface and bottom layers of the water column of Hangzhou Bay, using high-throughput sequencing according to the protocols described by Caporaso et al.50. The V4 regions of bacterial 16S rRNA gene were amplified from the DNA extracts using primers 520 F (5’-barcode-AYTGGGYYTAAAGNG-3’) and 802 R (5’-TACNVGGGTATCCTAAATCC-3’). The barcode is a seven-base sequence unique to each sample. A 25 µL reaction contained 1.25 U of Q5 polymerase (Stratagene, La Jolla, CA, USA), 5 µL Q5 reaction buffer (5×), 5 µL Q5 GC high Enhancer (5×), 0.2 mM of dNTPs (TaKaRa), 0.4 mM of each primer and 40 ng of total DNA. PCR amplification was conducted under the following conditions: initial denaturation at 98 °C for 5 min; 27 cycles at 98 °C for 30 s, 50 °C for 30 s and 72 °C for 30 s; and a final extension at 72 °C for 5 min. PCR amplicons were purified using AxyPrep DNA Gel Extraction Kits (Axygen, Union City, CA, USA) and quantified on a FLx800 Fluorescence Microplate Reader (BioTek, Winooski, VT, USA). Amplicons from different water samples were then pooled to achieve equal mass concentrations in the final mixture, which was sent out for high-throughput sequencing on the Illumina MiSeq platform (PersonalBio, Shanghai, China). Sequences are available in the NCBI short-read archive database (Accession Number: SRP091596).

The sequencer generated 1,459,768 reads of 16S rRNA gene from 30 samples. Raw sequences were de-multiplexed and quality-filtered using the default parameters in Qiime version 1.7.051. Criteria used for the filtering step were recommended by Bokulich et al.52. The remaining PCR chimeras were removed using the uchime method in mothur version 1.31.253. Bacterial sequences were then clustered into operational taxonomic units (OTUs; 97% similarity) with ucldst in Qiime54. A bootstrap cutoff of 50% suggested by the Ribosomal Database Project (RDP) was applied for taxonomic assignment55. On the basis of OTU numbers, the alpha diversity measures (Chao 1 and Shannon index) were calculated in mothur56.

**FISH analysis.** SPM samples were fixed on ice for 3 h in freshly prepared 4% paraformaldehyde solution and subsequently rinsed with phosphate-buffered saline. For in situ hybridization, 4 µL of each fixed sample was spotted onto adhesion microscope slides. Hybridization of the SPM samples was performed according to the procedure described by Amann (1995)57. Three 16S rRNA-targeted oligonucleotide probes were used for in situ detection of nitrifying and denitrifying bacteria: (1) Cy3-labeled NSO190 probes: specific for ammonia-oxidizing β-subclass Proteobacteria (2) Cy5-labeled DEN67 probes: specific for methanol-denitrifying cluster and (3) FAM-labeled DEN124 probes: specific for acetate-denitrifying cluster. The specificities, sequences and hybridization conditions of all probes are shown in Supplementary Table S7. All hybridizations were performed at a
temperature of 50°C. Subsequently, fluorescent and phase-contrast images were recorded using a multiphoton confocal LSM 780 NLO microscope system (Carl Zeiss AG, Oberkochen, Germany).

Data analysis. To assess the variations in environmental parameters between surface and bottom water layers, we used a one-way analysis of variance (ANOVA). Most of the variables were non-normal, except Chl a and NO$_3^-$-N. Spearman's correlation analyses were used to test the correlations between SPM and other environmental parameters (depth, temperature, salinity, DO, pH, Chl a, POC, NH$_4^+$, NO$_3^-$ and NO$_2^-$); between gene abundances (or gene copy ratios) and environmental parameters (depth, temperature, salinity, DO, pH, Chl a, SPM, POC, NH$_4^+$, NO$_2^-$ and NO$_3^-$); between the abundance of nitrifying genera and SPM, NH$_4^+$, NO$_3^-$, NO$_2^-$ and AOB amoA gene abundance; and finally between the abundance of denitrifying genera and SPM. All calculations were performed using IBM SPSS statistics 20.0 software.

To compare the bacterial community in different samples, hierarchical clustering was performed in the program PAST™ based on Bray-Curtis similarity index and unweighted pair group method average (UPGMA) algorithm. To test the influences of environmental parameters (depth, temperature, salinity, DO, pH, Chl a, SPM, POC, NH$_4^+$, NO$_2^-$ and NO$_3^-$) on the distributions of bacterial phylotypes, redundancy analysis (RDA) was performed using the software Canoco 5.0™. To meet assumptions, all measured environmental parameters were standardized before analyses.

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Author Contributions
Z.W.J., and W.W.X. designed and conducted the experiments. Z.W.J. and W.C. wrote the main manuscript text. Z.W.J. and H.Y.Y. performed the experiments. W.W.X., M.Z.H., T.B.Y. and J.H. contributed to the data analysis and manuscript revision and language editing. All authors reviewed the manuscript and have given approval to the final version of the manuscript.

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