ATLANTIS PRESS

Advances in Engineering Research, volume 184

2nd International Conference on Sustainable Energy, Environment and Information Engineering (SEEIE 2019)

Bacterial Community Characteristics and Detection of Denitrifying Functional Genes *nirS*, *nirk* in the Coastal Water of Bohai Bay, China

Bacterial and denitrifying functional genes in Bohai

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***Abstract*—To understand the microbial community characteristics and denitrification status in coastal water ecosystem of Bohai Bay, China, the bacterioplankton from six representative stations were collected in September 2016, and the bacterial community and abundance of *nir*-encoding denitrifying bacteria were studied by 454-pyrosequencing and real-time quantitative PCR (qPCR), respectively. The results showed that the Shannon index of the bacterial community ranged from 4.86 to 5.56. The bacterial composition and their relative abundance varied significantly among the bacterial libraries from the six water samples. *Proteobacteria* was the largest phylum in the six samples varying between 63.19% and 77.34%. *α-proteobacteria* was the most abundant class in the W4 station with 46.09%, while *γ-proteobacteria* was the most abundant class in other five stations ranging from 36.39% to 60.58%. The qPCR results showed that the *nirS* gene abundance ranged from 2.26×10^7 copies/L to 9.63×10^7 copies/L, while *nirK* gene ranged from 1.01×10^6 copies/L to 2.09×10^7 copies/L, indicating that both of them played important roles during the denitrification of the local coastal water. Furthermore, the *nirS* abundance in each station was significantly higher than that of *nirK*, suggesting that the functional genes *nirS* played more important role than *nirK* in reduction process of nitrite (NO_2^-) to nitric oxide (NO). Canonical correspondence analysis (CCA) results indicated that petroleum, arsenic (As), chromium (Cr), lead (Pb) and cadmium (Cd) had significant effects on the distribution of bacterial community. In contrast, the key factors regulating the *nirS* and *nirK* gene abundances included nitrate (NO_3-N), phosphate (PO_3-P), chlorophyll a (Chla), As and Cd.**

Keywords—Bohai Sea; bacteria; denitrification; pyrosequencing; real-time quantitative PCR (qPCR)

I. INTRODUCTION

Microbes play key roles in the functioning of marine ecosystems, such as in the process of environmental detoxification, recycling of organic material to benthic food webs and biogeochemical cycles [1-3]. In order to understand microbial process underlying in coastal ecosystem, examining the entire microbial structure and diversity is an important step. Culturing methods have proven to be uncertain for the complete characterization of microbial population, since they yield only a small fraction of the microorganisms in the studied region [4-5]. Development of molecular techniques has made it possible to obtain information of the microbial population structure in ever more detail without the need to cultivate the microbial population. The microbial population composition can be determined by direct nucleic acid extraction and identification of the microorganisms by analysis of the 16S rRNA genes [6]. Next generation sequencing technology (454-pyrosequencing) has given more complete information about microbial communities due to its capacity to identify a greater number of sequences than traditional DNA approaches [7-8]. Recently it has been used to reveal the characterization of complete microbial communities in all kinds of environments [9-11].

Denitrification is the key process regulating the removal of bioavailable nitrogen (N) from natural and human-altered systems [12], and thus can potentially reduce the impacts of increased N loading in the coastal region [13-16]. The second step in denitrification, the reduction of NO_2^- to nitric oxide (NO), is catalyzed by two different types of nitrite reductases (NIR), either a cytochrome cd1 encoded by the *nirS* gene (*nirS* denitrifiers) or a Cu-containing enzyme encoded by the *nirK* gene (*nirK* denitrifiers). Since denitrifiers are widespread among taxonomically diverse microorganisms [17], NIR is used widely as a marker indicating the presence of denitrifying bacteria [18-19]. In the last decade, real-time quantitative PCR (qPCR) has become a powerful tool in microbial ecology as this approach allowed the quantification of selected functional genes in different environments [20-22].

Bohai Bay is located in the western region of the Bohai Sea, China, that is a typical semi-enclosed interior sea (38°00′-39°15′ N and 117°30′-119°15′ E). The surrounding Bohai Sea area has now become one of the most populous and economically developed regions. And it is now one of the regions with the developed culture and education, and the advanced science and technology and industrial base. To our knowledge, most of the investigations and studies for the coastal water in Bohai Bay have mainly focused on contaminants such as metal and persistent organic pollutants [23-24], and marine organisms such as plankton, macrobenthos [25-26]. The studies about microorganism in the Bohai Bay have been limited [27-28]. Moreover the entire microbial communities in coastal water of Bohai Bay have not been

studied, and only the investigation in coastal sediment has been performed [29]. The detection of predominant groups within bacterial population may be of distinct importance in ecological studies concerning biogeochemical cycles. The bacteria affiliated to different groups can express the peculiar activity with different degrees in a given ecosystem [30-32]. In addition, in order to highlight their potential biogeochemical functions, the investigation about the denitrifying bacteria based on NIR functional gene was performed since Bohai bay belongs to nitrogen enriched coastal ecosystems [33]. Thus the main objective of this work was to determine in depth the microbial community and detect the denitrifying bacterial abundance using *nirS* and *nirK* as gene markers in the coastal water ecosystem of Bohai Bay, China. Furthermore, we also tried to reveal the possible factors shaping the microbial community and *nir*-encoding denitrifying bacteria.

II. MATERIALS AND METHOD

A. Sample Collection and Environmental Condition Determination

The Bohai Bay is the second largest bay of the Bohai Sea covering an area of about 1.6×10^4 km^2 with an average water depth of 12.5 m. Fig. Ⅰ gives the location of the six representative sampling stations (same with Wang et al., 2015). W1(W4), W2(W5) and W3(W6) was at the 5 m, 10 m and 15 m depth contour, respectively. All of them located near the estuary of the Haihe River that was a main river flowing to this bay. Surface water samples (~0.5 m below the air-water interface) were collected from the six stations in September 18-19, 2016, and 500 ml water was filtered as soon as possible through 0.22-μm pore size cellulose acetate membrane (Millipore, Corp) to concentrate the suspended bacteria. The filters were transported on ice back to the lab and stored at -80 ℃ before analysis. The sampling positions were determined using a global positioning system. The temperature (T), dissolved oxygen (DO) and pH in each station were determined on-site using a Multi-parameter water quality meter (YSI, USA). And the salinity was measured in situ using a hand held salinity refractometer (ATAGO CO., LTD, Tokyo, Japan).

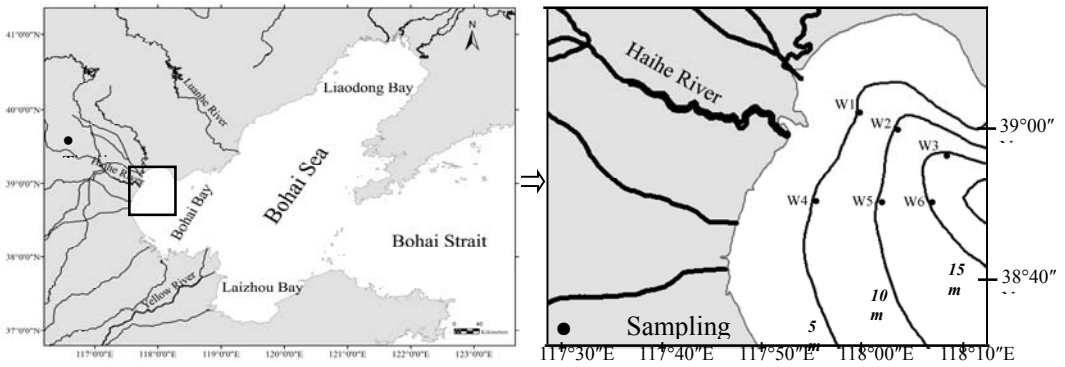

FIGURE I. THE STUDY AREA AND THE SAMPLING STATIONS. 5M, 10M AND 15M MEAN THE DEPTH CONTOUR

Replicate subsamples from the surface water (1000 ml) at each station were collected in sulfuric acid-washed (pH<2) plastic sample bottles and transported to the laboratory. The chemical oxygen demand (COD_{Mn}), total phosphorus (TP), phosphate (PO_3-P), total nitrogen (TN), nitrate (NO_3-N), ammonium (NH_4-N), and nitrite (NO_2-N) were determined using standard methods [34-35]. The trace metal concentrations including copper (Cu), lead (Pb), cadmium (Cd) and chromium (Cr) in water samples were analyzed by graphite furnace atomic absorption spectrometry (GFAAS) (Varian, USA), and the concentration of arsenic (As) in aqueous samples was determined by Atomic fluorescence spectrometry (AFS) (Jitian Company, China). Additional replicate subsamples analyzing the chlorophyll a (Chla) were collected by filtering known volumes of water into GF/F glass fiber filters, and then the filters were stored on ice until being frozen in the laboratory. The Chl-a concentration was measured using a spectrophotometric method [35].

All samples were collected in triplicates and processed for DNA extractions and environmental factors analysis within 2 days of collection.

B. DNA Extraction, PCR and Pyrosequencing

The total community DNA of suspended bacteria on the filter membranes was extracted using the PowerWater® DNA Isolation Kit (Mo Bio Laboratories, Inc., Solana Beach, California, USA) according to the manufacturer's instructions. The PCR, pyrosequencing and data analysis were performed according to Wang et al. (2013, 2015)[27, 29]. All of the sequences generated in this study can be downloaded from the NCBI Short Read Archive, submission number: SRA04770.

C. Real-Time Quantitative PCR

Real-time PCR was carried out in an ABI 7500 with Sequence Detection Software v1.4 (Applied Biosystems) in a reaction mixture of 20 μl. The mixture contained 0.25 μM of each primer, 10 μl of Power SYBR Green PCR master Mix (Applied Biosystems, Foster City, CA), 1 μl of the purified DNA template. Primer pairs modified-cd3aF and -R3cd [19] were used for *nirS* amplification, and nirK876F and nirK1040R [36] were used for *nirK* amplification. The double distilled water was used as the negative control. Agarose gel

electrophoresis and melting-curve analysis were employed to confirm qPCR specificities.

Standard curves were constructed with the plasmid containing insert of respective genes according to Wang et al (2014). Standards were prepared using serially diluted plasmid DNA with 10^3 to 10^8 gene copies/µl. Standard curves for the *nirS* and *nirK* assays were generated by plotting the threshold cycle values versus $\log_{10}$ of the gene copy numbers. The amplification efficiency (*E*) was estimated using the slope of the standard curve through the following formula: $E = (10^{-1/slope})-1$. The efficiency of the PCR should be between 90% and 110% [37].

D. Statistical Analysis

The similarity factor (Jaccard index by the Jest calculations, $C_{jaccard}$) was obtained by calculating the OTUs distribution structure within the six bacterial communities. The detail was provided in Wang et al. 2015[29].

FastTree software (http://www.microbesonline.org/fasttree/) with approximately-maximum-likelihood was used to construct the phylogenetic tree based on the sequences of the top 20 dominant genera in the six water samples. The relationship between the dominant phyla, the population diversity, the abundance of NIR function gene and environmental conditions were analyzed using Canonical correspondence analysis (CCA). Raw data of all the factors were log (X+1) transformed before the analysis. The detailed analysis steps were seen in the literature of Wang et al. 2015[29].

III. RESULTS

A. Environmental Conditions of the Sampling Sites

The various factors of the environmental conditions were measured at each sampling site. The T, Salinity, pH, DO in the six stations ranged from 22.5 to 23.6 ℃, 24.7 to 28.7‰, 7.98 to 8.08 and 6.23 to 8.29 mg/L, respectively. The smallest value of COD_{Mn} (2.75 mg/L) presented at W3 site, while the largest (3.79 mg/L) presented at W1 site. The TP, PO_3-P varied between 0.056 and 0.081 mg/L, 0.017 and 0.028 mg/L, respectively. The TN, NO_3-N, NO_2-N and NH_4-N ranged from 2.803 to 3.428 mg/L, 0.067 to 0.397 mg/L, 0.021 to 0.120 mg/L and 0.005 to 0.031 mg/L, respectively. The Chla varied between 2.896 and 9.551 µg/L. The five trace metals ranged from 2.97 to 5.47 µg/L for Cu, 3.36 to 5.66 µg/L for Pb, 0.72 to 1.63 µg/L for As, 0.09 to 0.13 µg/L for Cd and 0.25 to 0.91 µg/L for Cr, respectively.

B. Diversity of the Bacterial Communities

After quality filtering, a total of 74,850 high quality 454 reads corresponding to 7038 OTUs at 97% similarity threshold were obtained. Then these OTUs were used to calculate richness and diversity of the microbial communities (Table I). At 3% dissimilarity, the non-parametric richness indices of chao and ace were evaluated. And they showed similar comparative trends in predicting number of OTUs for each sample. Sample from W3 station had the highest richness (ace=2544, chao=2178), while sample from W1 had the lowest one (ace=1600, chao=1404). The Shannon diversity index provides not only the simply species richness (i.e. the number of species present) but how the abundance of each species is distributed (the evenness of the species) among all the species in the community. The most diverse bacterial population was observed in the water sample from station W6 (Shannon=5.56), and the least was from station W1 (Shannon=4.86). We obtained 892-1345 OTUs among the 6 samples at a 3% distance, respectively. The coverage ranged from 95.56 to 96.65%. The Simpson index varies between 0.0117-0.0371 with the largest value for W4 site and the lowest one for W6 site.

TABLE I. THE SUMMARY OF THE RICHNESS AND DIVERSITY OF BACTERIAL COMMUNITY.

Station	Reads	OTU	Ace	Chao	Shannon	Coverage(%)	Simpson
W1	9442	892	1600(1502,1718)	1404(1283,1563)	4.86(4.82,4.90)	95.91	0.0255(0.0244,0.0265)
W2	11801	1065	1692(1584,1817)	1547(1439,1685)	4.99(4.96,5.03)	96.39	0.0284(0.0270,0.0299)
W3	11891	1345	2544(2410,2695)	2178(2010,2389)	5.53(5.50,5.57)	95.56	0.0175(0.0165,0.0184)
W4	14305	1248	2115(2007,2554)	1832(1707,1991)	5.13(5.10,5.17)	96.65	0.0371(0.0351,0.0390)
W5	14764	1265	2406(2274,2554)	2133(1950,2365)	5.44(5.41,5.47)	96.38	0.0143(0.0136,0.0150)
W6	12647	1223	1831(1726,1958)	1875(1736,2052)	5.56(5.53,5.60)	96.13	0.0117(0.0111,0.0123)

Values in bracket are 95% confidence intervals calculated by MOTHUR.

At a 3% distance, the sequences from the six water samples were classified from phylum to species according to the Mothur program by the default setting [38]. The taxonomic results of the six stations at phylum, class, genus and species levels varied. Total 18, 18, 19, 22, 17, 14 phyla and 33, 33, 41, 40, 35, 31 classes were observed from the W1, W2, W3, W4, W5 and W6 sites, respectively. While 184, 199, 212, 211, 196, 201 genera and 181, 146, 294, 260, 260, 254 species were observed from the above six stations in order, respectively. In addition, a large proportion of unclassified representatives in the different phylum, class and genus were contained in the OTUs retrieved from the six samples. The abundances of the unclassified representatives were defined as the percentage of the unclassified species sequences in total effective bacterial sequences in the samples using SILVA databank. About 0.03-0.61% at phylum level, 1.09-4.57% at class level and 12.31-35.43% at genera level could not been classified successfully. The functions of these unclassified representatives in the investigated zones should been studied further.

The phylogenetic classification of sequences at phylum and class levels from the six coastal water samples was summarized in Fig. II. *Proteobacteria* (63.19~77.34%) was the largest phylum among all the six investigated samples, and other phyla including *Bacteroidetes*, *Cyanobacteria* and *Actinobacteria* was more than >1% in all of the six libraries (Fig. II a).

γ-proteobacteria was the most abundant class in W1, W2, W3, W5 and W6 stations with the percentage of 52.75%, 60.58%, 42.81%, 36.39% and 38.97%, respectively. In contrast, *α-proteobacteria* was the most abundant class in the W4 station with 46.09%. Other classes with percentage >1% in all the six libraries included *Flavobacteria* (Fig. II b). A phylogenetic tree was constructed based on the 16S rRNA gene sequences of the top 20 dominant genera at the six libraries (Fig. III). It showed that there were three main clusters which affiliated with *α-proteobacteria*, *Bacteroidetes* and *γ-proteobacteria*, respectively. Among the predominant genera, *Pseudoalteromonas* has the largest abundance in the W1, W2, W3 and W6 stations accounting for 23.02%, 31.71%, 16.84% and 10.34%, respectively. In contrast, *Rhodobacteraceae*_uncultured was the most abundant genus in the W4 and W5 stations (Fig. III).

C. *Detection of nirS- and nirK-encoding Denitrifying Bacteria*

The abundances of denitrifying bacteria were assessed by targeting the *nirS* and *nirK* functional genes of denitrification pathway. The concentrations of environmental DNA sample were calculated based on the standard curves. The efficiency of PCR amplification of *nirS* and *nirK* genes was 96% and 101%, respectively. The results showed that *nirS* gene abundance in the coastal water ranged from 2.26×10^7 copies/L to 9.63×10^7 copies/L, while *nirK* gene ranged from 1.01×10^6 copies/L to 2.09×10^7 copies/L (Fig. IV). The W4 station had the largest abundance for both *nirS* and *nirK* genes, which was slight larger than those in the W1 station. In contrast, W3 had the minimum of *nirS* and *nirK*.

D. *The Results of CCA*

Fig. V a was the CCA results between the dominant phylum abundance and the physicochemical parameters. It showed that axis 1 was affected significantly by petroleum, As and Cr with correlation coefficient (F) = 0.8392, 0.6804, and -0.7461, respectively. While axis 2 was affected obviously by Pb and Cd with F = 0.8565 and 0.8018, respectively. The CCA biplot showed that *Firmicutes* was located in a positive direction of axis 1, thus this organism was related positively to petroleum and As, and negatively to Cr. BD1-5 was located in a negative direction of axis 2, so these organisms were related negatively to Pb and Cd. Most of the other bacterial phyla were grouped close to the biplot center indicating relatively weak effect of the studied environmental factors on them. Fig. Ⅴb was the CCA results between the Shannon index, abundance of denitrifying functional genes and environmental variables. The variables that correlated most strongly with CCA 1 were NO_3-N, Chla, As, Cd and Cr (F=-0.8802, -0.6713, -0.8467, -0.7133, and 0.5231, respectively), whereas PO_3-P (F=0.7895) correlated best with CCA 2 (Fig. Ⅴb). The CCA biplot showed that *nirS* was located in a positive direction of axis 1, thus *nirS*-encoding denitrifiers were negatively to NO_3-N, Chla, As and Cd. *nirK* was located in a negative direction of axis 1, so *nirK*-encoding denitrifiers were related positively to NO_3-N, Chla, As and Cd. It also suggested that there was a significant correlation between Cr and *nirS* gene abundance (ANOVA, two-tailed test, p<0.01**).**

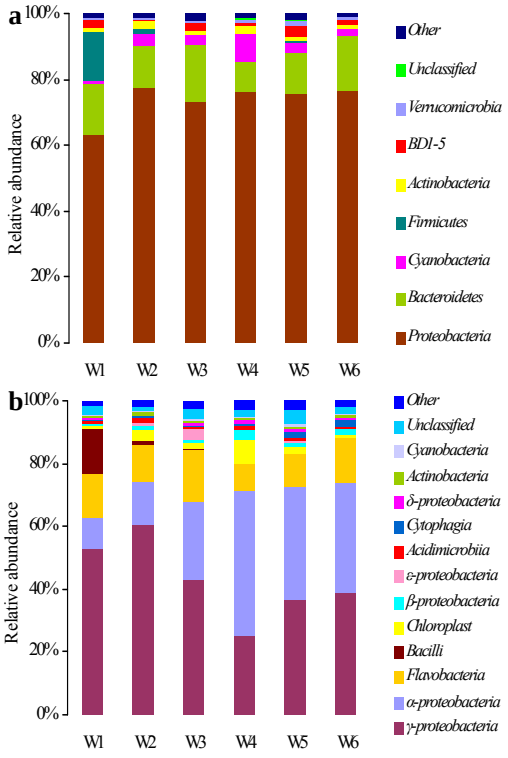

FIGURE II. BACTERIAL COMMUNITY COMPOSITIONS AT PHYLUM (A) AND CLASS (B) LEVELS REVEALED BY PYROSEQUENCING, THE RELATIVE ABUNDANCE WAS DEFINED AS THE PERCENTAGE OF THE SPECIES SEQUENCES IN TOTAL EFFECTIVE SEQUENCES IN SAMPLE, CLASSIFIED USING SILVA DATABANK. PHYLA/CLASSES MAKING UP LESS THAN 1% OF TOTAL COMPOSITION IN ALL OF THE SIX LIBRARIES WERE CLASSIFIED AS 'OTHER', RESPECTIVELY

IV. DISCUSSION

A. *Comparative Analysis of Bacterial Communities and Denitrifying Bacteria*

Not only the study of microbial ecology but also direct metagenomic detection in environmental samples have been revolutionized by pyrosequencing technology. High-throughput sequencing approach is unbiased and makes it possible to detect the bacterial community structure in depth [39]. Here we used pyrosequencing to detect the bacterial community in the coastal water of the Bohai Bay, China. The results showed that pyrosequencing allows the detection of microorganisms that are not part of the dominant community such as *Pseudobutyrivibrio* (only be observed in W2 station with one OTU) and *Pusillimonas* (only be observed in W4 station with one OTU). These minor microorganisms may contribute to some particular role in the biogeochemical process. The results indicated that a variety of population compositions were detected at the different taxonomic levels (Fig. II, Fig. III). Furthermore, a large of unclassified representatives was also detected and their percentages increased with the depth of classification (Fig. II). In the previous studies, pyrosequencing could obtain higher diversity at the phylum level comparing with the results based on Sanger

sequencing based analysis of 16S rRNA gene clone libraries [40]. Therefore pyrosequencing offered the ability to detect more unknown and low abundance sequences than traditional clone library approaches, which should assist in the discovery of new species. The sheer number of sequences generated by pyrosequencing was able to ensure that traces of microorganisms that composed only a minor portion of the population were not missed.

At the different taxonomic levels, distinct differences were observed in composition and their relative abundance among the six bacterial population (Fig. II, Fig. III). For instance, phylotypes belonging to the phylum *Firmicutes* accounted for 14.17% of the phylotypes in station W1, while in other five stations they accounted for only between 0.13% and 1.03%. *Bacilli* comprised 14.14% of the phylotypes at class level in station W1, while in other five stations they comprised less than 1.00%. Phylotypes belonging to the genus *Halomonas* accounted for 5.40% in station W6, whereas they accounted for only 0.09% in station W4, 0.29% in station W3, 0.41% in station W2, 0.78% in station W1 and 1.01% in station W5. In addition, the differences of six bacterial population compositions were identified using hierarchical cluster analysis. There were two clusters (Fig. VI). It showed that the bacterial communities in the W1, W2, W3 and W4 stations were clustered together, suggesting a similar population structure among the four samples. While the bacterial population at the W5 and W6 stations were clustered together, indicating a similar community structure between the two stations. The two clusters were obvious separated from each other, indicating that there were significant distinctions in the population composition between the two clusters. Furthermore, the results suggest that there were not significant relationship between the bacterial community and the depth contour (Fig. I and Fig. VI).

B. The Relationship between the Bacterial Communities and Environmental Factors

The bacterial community composition and their spatial distributions, and *nir*-encoding nitrite-reducing bacterial assemblages might be influenced by a variety of environmental factors, such as nutrients and some contaminants. CCA analysis of mainly dominant representatives and *nir*-encoding denitrifiers in response to environmental variables confirmed this suggestion (Fig. V). Here the key factors controlling the predominant species included petroleum, As, Cu, Pb and Cd. And the *Firmicutes* and BD1-5 were the more sensitive representatives (Fig. V a). The key factors controlling the abundance of *nir*-encoding denitrifying bacteria included NO_3-N, Chla, As, Cd and Cr. The *nirS*-encoding denitrifiers seem to be more sensitive to trace metal Cr than *nirK*-encoding denitrifiers. The results also suggested that *nirK*-encoding denitrifiers tended to be present in the surface water with higher NO_3-N concentration, while *nirS*-encoding denitrifiers tended to be present in the surface water with lower NO_3-N concentration (Fig. V b). The correlation between *nirK* gene abundance and NO_3-N was stronger than that between *nirS* gene abundance and NO_3-N. Therefore the *nirK* denitrifiers were more sensitive to the changes of NO_3-N concentration than *nirS* denitrifiers. The above results were in agreement with that of Kandeler et al. (2006) [19] and Bárta et al. (2010)[45]. These differences in the distribution of bacteria containing *nirS* and *nirK* confirmed the previous studies that both types of denitrifiers clearly occupy different ecological niches [46].

In CCA biplot, Shannon was distributed in one quadrant by itself, while all of the environment factors were not distributed in the same quadrant, indicating that Shannon has slight dependence on the investigated environmental variables (Fig. Ⅴb). The results suggested that the environmental pressure did not make bacterial population diversity occur a clear shift. In ecological systems, some species that is very sensitive to some environmental factors would occur a shift. However, population diversity has the potential to recover to that of an undisturbed state once the stressor disappears, although some population members may have changed [47]. Therefore in a long-term and cumulative polluted system, diversity has not been shown to be a good indicator of ecosystem stress since it can recover because of divergence and proliferation of tolerant species [48].

Coastal water contamination by petroleum and trace metals is a major environmental problem faced by many anthropogenically impacted aquatic environments. Microorganisms contact with their inhibiting environment intimately, and are very sensitive to contaminants [49-50]. So they can respond rapidly to environment perturbations. Some significant shifts of the composition and distribution in the microbial population that chronically exposed to petroleum have reported widely [51-52]. Some metals such as Cu and Zn may be toxic at higher concentrations for microorganisms, but they are also essential micronutrients [53]. Cu is also an important micronutrient, since some denitrifiers required copper for nitrite reductase [54]. Here Cu has the slightest effect (F= 0.4483) on the distribution of the predominant representatives among the concerned five trace metal, and followed by Cr (F= -0.7461). While Pb (F= 0.8565), Cd (F= 0.8018) and As (F= 0.6804) had significant effects (Fig.V a). In contrast, As (F=-0.8467), Cd (F= -0.7133) and Cr (F=0.5231) had stronger roles for *nir*-encoding denitrifying bacteria than Cu and Pb. In previous studies, the obvious influence of trace metals on bacterial community structure has been also widely reported in the literatures both in the fields [48,55] and under the laboratory conditions [56-57]. Magalhães et al. (2007) indicated that the addition of trace metals stimulated N_2O and NO_2^- accumulation in intertidal sandy (Cu, Cr and Cd) and muddy sediments (Cu), demonstrating a pronounced inhibitory effect on specific steps within the denitrification enzymatic system [58]. These results suggested that although some metals are important and essential trace elements, at high concentrations, such as those found in the present coastal water, most can be toxic to microbes. Microbes have adapted to tolerate the presence of metals or can even use them to grow. Thus, a number of interactions between microbes and metals have important significance in environmental implications and monitoring.

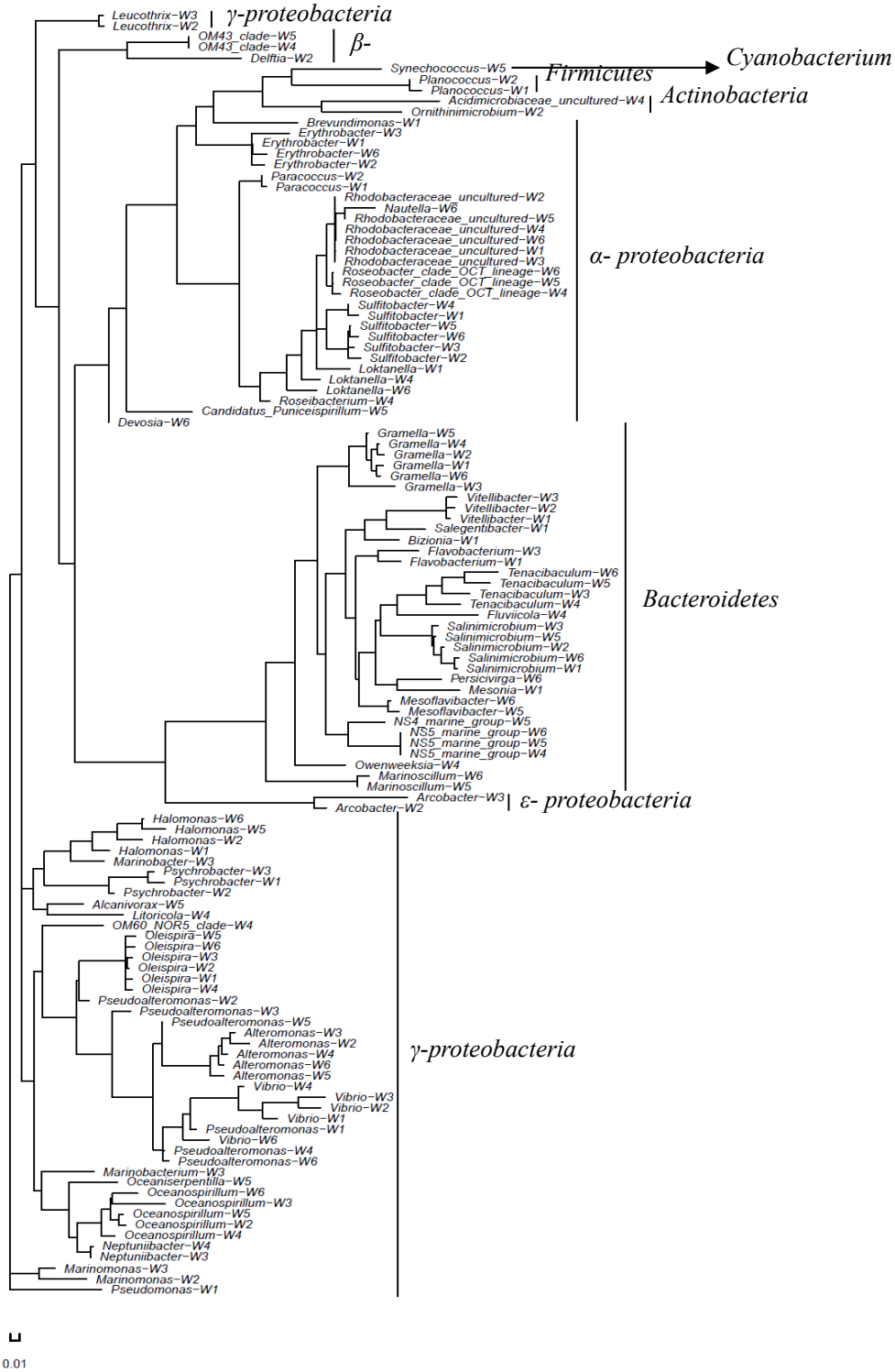

FIGURE III. PHYLOGENETIC TREE OF THE TOP 20 DOMINANT GENERA AT THE SIX WATER SAMPLES BASED ON 16S RRNA GENE SEQUENCES. BRANCH LENGTHS CORRESPOND TO SEQUENCE DIFFERENCES AS INDICATED BY THE SCALE BAR

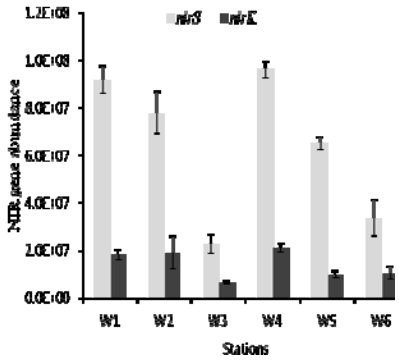

FIGURE IV. ABUNDANCES OF NIRS AND NIRK GENES

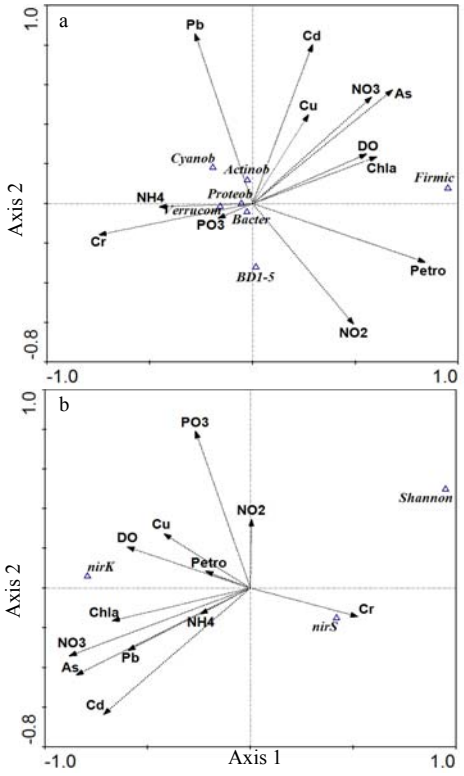

FIGURE V. CCA RESULTS BETWEEN THE ABUNDANCE OF THE MAINLY DOMINANT PHYLA (A), SHANNON OF BACTERIAL COMMUNITY, ABUNDANCE OF NIR FUNCTIONAL GENES AND THE PHYSICAL-CHEMICAL FACTORS. THE DOMINANT PHYLA: PROTEOB, PROTEOBACTERIA; BACTER, BACTEROIDETES; ACTINOB, ACTINOBACTERIA; CYANOB, CYANOBACTERIA; FIRMIC, FIRMICUTES; BD1-5 AND VERRUCOM, VERRUCOMICROBIA. THE ENVIRONMENTAL FACTORS: DO, DISSOLVED OXYGEN; PETRO, PETROLEUM; NO3, NO3-N; NO2, NO2-N; NH4, NH4-N; PO3, PO3-P; CHLA, CHLOROPHYLL-A

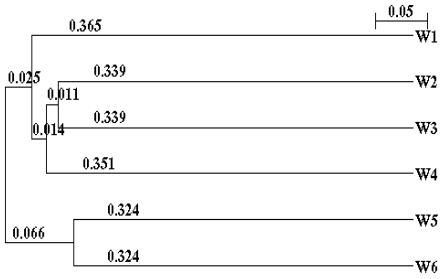

FIGURE VI. THE SIMILAR COMPARISON OF BACTERIAL COMMUNITY STRUCTURES AMONG THE SIX WATER SAMPLES. THE SHAREDTREE WAS MADE USING THE SOFTWARE PACKAGE MOTHUR 1.15.0 BASED ON THE CJACCARD. THE SCALE BAR REPRESENTS THE UNIT OF BRANCH LENGTH, AND THE LENGTH OF EACH BRANCH REPRESENTS THE DISTANCE OF THE DISSIMILARITY BETWEEN THE BACTERIAL COMMUNITIES

ACKNOWLEDGMENTS

This work was supported by the plan of the National Sci-Tech Major Special Item for Water Pollution Control and Management (No.2017ZX07107-004-004).

REFERENCES

[1] W. Schlesinger, Biogeochemistry: an analysis of global change. San Diego: Academic Press, 1997,pp588.

[2] P. Cabello, M.D. Roldán, C. Moreno-Vivián, Nitrate reduction and the nitrogen cycle in archaea. Microbiology, 2004, 150(11): 3527-3546.

[3] E.B. Barbier, S.D. Hacker, C. Kennedy, E.W. Koch, A.C. Stier, B.R. Silliman, The value of estuarine and coastal ecosystem services. Ecological Monographs, 2011, 81: 169-193.

[4] E. Ben-Dov, E. Kramarsky-Winter, A. Kushmaro, An in situ method for cultivating microorganisms using a double encapsulation technique. FEMS Microbiology Ecology, 2009, 68(3): 363-371.

[5] A. Shade, C.S. Hogan, A.K. Klimowicz, M. Linske, P.S. Mcmanus, J. Handelsman, Culturing captures members of the soil rare biosphere. Environmental Microbiology, 2012, 14(9):2247-2252.

[6] F. Armougom and D. Raoult, Exploring microbial diversity using 16S rRNA high-throughput methods. Journal of Computer Science & Systems Biology, 2009, 2(1): 074-092.

[7] S. Kwon, E. Moon, T.S. Kim, S. Hong, H.D. Park, Pyrosequencing demonstrated complex microbial communities in a membrane filtration system for a drinking water treatment plant. Microbes and Environments, 2011, 26 (2): 149-155.

[8] X.J. Zhang, S.Q. Yue, H.H. Zhong, W.Y. Hua, R.J. Chen, Y.F. Cao, L.P. Zhao, A diverse bacterial community in an anoxic quinoline-degrading bioreactor determined by using pyrosequencing and clone library analysis. Applied Microbiology and Biotechnology, 2011, 91: 425-434.

[9] S.E. Dowd, Y. Sun, P.R. Secor, D.D. Rhoads, B.M. Wolcott, G.A. James, W. Randall, Survey of bacterial diversity in chronic wounds using pyrosequencing, DGGE, and full ribosome shotgun sequencing. BMC Microbiology, 2008,8: 43.

[10] S.W. Roh, K.H. Kim, Y. D. Nam, H.W. Chang, E.J. Park, J.W. Bae, Investigation of archaeal and bacterial diversity in fermented seafood using barcoded pyrosequencing. ISME Journal, 2010, 4: 1-16.

[11] N.C.M. Gomes, D.F.R. Cleary, R. Calado, R. Costa, Mangrove bacterial richness. Communitative and Integrative Biology, 2011, 4: 419-423.

[12] S. Seitzinger, J.A. Harrison, J.K. Bohlke, F.B. Alexander, L. Richard, B.J. Peterson, C.R. Tobias, G.V. Drecht, Denitrification across landscapes and waterscapes: a synthesis. Ecol Appl 2006, 16: 2064-2090.

[13] C.P. Duncan and P.M. Groffman, Comparing microbial parameters in natural and constructed wetlands. Journal of Environmental Quality, 1994, 23: 298-305.

[14] W.J. Mitsch, J.W. Day, J.W. Gilliam, P.M. Groffman, H. Donald, G.W. Randall, N.M. Wang, Reducing nitrogen loading to the Gulf of Mexico from the Mississippi River Basin: strategies to counter a persistent ecological problem. Bioscience, 2001, 51: 373-388.

[15] .N. Galloway, J.D. Aber, J.W. Erisman, S.P. Seitzinger, R.W. Howarth, E.B. Cowling, B. Cosby, The nitrogen cascade. Bioscience, 2003, 53: 341-356.

[16] P.J. Mulholland, A.M. Helton, G.C. Poole, R.O. Hall, S.K. Hamilton, B.J. Peterson, et al., Stream denitrification across biomes and its response to anthropogenic nitrate loading. Nature, 2008, 452: 202-U246.

[17] L. Philippot, Denitrifying genes in bacterial and archaeal genomes. Biochimica et Biophysica Acta, 2002, 1557: 355–376

[18] G. Braker, J. Zhou, L. Wu, A.H. Devol, J.M. Tiedje, Nitrite reductase genes (nirK and nirS), as functional markers to investigate diversity of denitrifying bacteria in Pacific north-west marine sediment communities. Applied and Environmental Microbiology, 2000, 66: 2096-2104.

[19] E. Kandeler, K. Deiglmayr, D. Tscherko, D. Bru, L. Philippot, Abundance of narG, nirS, nirK, and nosZ genes of denitrifying bacteria during primary successions of a glacier foreland. Applied and Environmental Microbiology, 2006, 72: 5957-5962.

[20] B. K. Huic, K. Schauss, B. Hai, S. Sikora, S. Redzepović, V. Radl, M. Scholter, Influence of different Sinorhizobiummeliloti inocula on abundance of genes involved in nitrogen transformations in the rhizosphere of alfalfa (Medicago sativa L.). Environmental Microbiology, 2008,10: 2922-2930.

[21] F. M. Mrkonjic, M. Engel, A. Gattinger, U. Bausenwein, M. Sommer, J.C. Munch, M. Scholter, Factors influencing variability of proteolytic genes and activities in arable soils. Soil Biology & Biochemistry, 2008,40: 1646-1653.

[22] S. Schulz, A. Peréz-de-Mora, M. Engel, J.C. Munch, M. Schloter, A comparative study of most probable number (MPN)-PCR vs. real-time-PCR for the measurement of abundance and assessment of diversity of alkB homologous genes in soil. Journal of Microbiological Methods, 2010, 80: 295-298.

[23] Y. Wan, J.Y. Hu, J.L. Liu, W. An, S. Tao, Z.B. Jia, Fate of DDT-related compounds in Bohai Bay and its adjacent Haihe Basin, North China. Marine Pollution Bulletin, 2005, 50(4): 439-445.

[24] X.L. Gao, F.X. Zhou, C.T.A. Chen, Pollution status of the Bohai Sea: An overview of the environmental quality assessment related trace metals. Environment International, 2014, 62: 12-30.

[25] S.T. Peng, X.B. Qin, H.H. Shi, R. Zhou, M. Dai, D. Ding, Distribution and controlling factors of phytoplankton assemblages in a semi-enclosed bay during spring and summer. Marine Pollution Bulletin, 2012, 64(5): 941-948.

[26] W.Q. Cai, Á. Borja, L.S. Liu, W. Meng, I. Muxika, J.G. Rodríguez, Assessing benthic health under multiple human pressures in Bohai Bay (China), using density and biomass in calculating AMBI and M-AMBI. Marine Ecology, 2014, 35:180-192.

[27] [L.P. Wang, L.S. Liu, B.H. Zheng, Y.Z. Zhu, X. Wang, Analysis of the bacterial community in the two typical intertidal sediments of Bohai Bay, China by pyrosequencing. Marine Pollution Bulletin, 2013, 72: 181-187.

[28] H.P. Zhao, J.H. Tao, Q.X. Li, D.K. Yuan, Q.C. Gao, Microbial ecological characteristics in the Red Tide-Monitoring area of Bohai Bay. Journal of Hydro-environment Research, 2013, 7(2): 141-151.

[29] L.P. Wang, B.H. Zheng, K. Lei, Diversity and distribution of bacterial community in the coastal sediments of Bohai Bay, China. Acta Oceanologica Sinica, 2015, 34(10): 122-131.

[30] F. Azam and A.Z. Worden, Microbes, molecules, and marine ecosystems. Science, 2004, 303: 1622-1624.

[31] J.B. Martiny, B.J. Bohannan, J.H. Brown, R.K .Colwell, J.A. Fuhrman, L.G. Jessica, et al., Microbial biogeography: putting microorganisms on the map. Nature Reviews Microbiology, 2006, 4: 102-112.

[32] M.T. Cottrell and D.L. Kirchman, Natural assemblages of marine proteobacteria and members of the Cytophaga-Flavobacter cluster consuming low- and high-molecular-weight dissolved organic matter. Applied and Environmental Microbiology, 2003, 66: 1692-1697.

[33] M, Strokal, H, Yang, Y,C, Zhang, C, Kroeze, L.L. Li, S.J. Luan, H.Z. Wang, S.S. Yang, Y.S. Zhang, Increasing eutrophication in the coastal seas of China from 1970 to 2050. Marine Pollution Bulletin, 2014, 85(1):123-140.

[34] X.F. Huang, W.M. Chen, Q.M. Cai, Survey, observation and analysis of lake ecology. Beijing: Chinese Standardization Press, 1999.

[35] State Environmental Protection Bureau (SEPB), Methods of monitoring and analysis for water and wastewater. 4th edn. Beijing: China Environmental Science Press, 2002.

[36] S. Henry, E. Baudoin, J.C. López-Gutiérrez, F. Martin-Laurent, A. Brauman, L. Philippot, Quantification of denitrifying bacteria in soils by nirK gene targeted real-time PCR. Journal of Microbiological Methods, 2004, 59: 327-335 (Erratum, 61: 289-90).

[37] M.J. McPherson and S.G. Møller, PCR. 2nd edn. New York: Taylor & Francis, 2006,p 229.

[38] P.D. Schloss, S.L. Westcott, T. Ryabin, J.R. Hall, M. Hartmann, E.B. Hollister, et al., Introducing mothur: open-source, platform-independent, community-supported software for describing and comparing microbial communities. Applied and Environmental Microbiology, 2009, 75: 7537-7541.

[39] R.C. Novais and Y.R. Thorstenson, The evolution of Pyrosequencing for microbiology: from genes to genomes. Journal of Microbiological Methods, 2011, 86(1):1-7.

[40] Y. Zeng, Y. Yu, H. Li, J. He, H.L. Sang, K. Sun, Phylogenetic diversity of planktonic bacteria in the Chukchi Borderland region in summer. Acta Oceanologica Sinica, 2013, 32(6): 66-74.

[41] S.P. Seitzinger, Denitrification in freshwater and coastal marine ecosystems: ecological and geochemical significance. Limnology and Oceanography, 1988, 33: 702-724.

[42] S.P. Seitzinger, Scaling up: site-specific measurements to global-scale estimates of denitrification. In: Hobbie JE (ed) Estuarine Science: A Synthetic Approach to Research and Practice. Montague: Island Press, 2000, pp 211-241.

[43] B. Ogilvie, D.B. Nedwell, R.M. Harrison, A.D. Robinson, A.S. Sage, High nitrate, muddy estuaries as nitrogen sinks: the nitrogen budget of the River Colne estuary (United Kingdom). Marine Ecology Progress Series, 1997, 150: 217-228.

[44] C. Magalhães, W.J. Wiebe, S.B. Joye, A.A. Bordalo, Inorganic Nitrogen cycle dynamics in intertidal rocky biofilms and adjacent sediments biofilms: Douro River estuary (Portugal). Estuaries, 2005, 28: 592-607.

[45] J. Bárta, T. Melichová, D. Vaněk, T. Picek, H. Santruckova, Effect of pH and dissolved organic matter on the abundance of nirK and nirS denitrifiers in spruce forest soil. Biogeochemistry, 2010, 101:123-132.

[46] T.S. Kim, H.S. Kim, S. Kwon, H.D. Pack, Nitrifying bacterial community structure of a full-scale integrated fixed-film activated sludge process as investigated by pyrosequencing. Journal of Microbiology and Biotechnology, 2011, 21(3): 293-298.

[47] D. Ager, S. Evans, H. Li, A.K. Lilley, V.D.G. Christopher, Anthropogenic disturbance affects the structure of bacterial communities. Environmental Microbiology, 2010, 12: 670-678.

[48] D.C. Gillan, B. Danis, P. Pernet, G. Joly, P. Dubois, Structure of sediment associated microbial communities along a heavy-metal contamination gradient in the marine environment. Applied and Environmental Microbiology, 2005, 71: 679-690.

[49] V. Andreoni, L. Cavalca, M.A. Rao, G. Nocerino, S. Bernasconi, E. Dell'Amico, C. Milena, G. Liliana, Bacterial communities and enzyme activities of PAH polluted soils. Chemosphere, 2004, 57: 401-412.

[50] A.Y. Hu, X.Y. Yang, N.W. Chen, L.Y. Hou, Y. Ma, C.P. Yu, Response of bacterial communities to environmental changes in a mesoscale subtropical watershed, Southeast China. Science of the Total Environment, 2014, 472: 746-756.

[51] H. Ribeiro, A.P. Mucha, C.M.R. Almeida, A.A. Bordalo, Bacterial community response to petroleum contamination and nutrient addition in sediments from a temperate salt marsh. Science of the Total Environment, 2013, 458-460: 568-576.

[52] T.R. Silva, L.C.L. Verde, E.V. Santos Neto, Diversity analyses of microbial communities in petroleum samples from Brazilian oil fields. International Biodeterioration & Biodegradation, 2013,81: 57-70.

[53] B.J. Alloway and A.P. Jackson, The behaviour of heavy metals in sewage sludge-amended soil. Science of the Total Environment, 1991, 100: 151-176.

[54] W.G. Zumft, Cell biology and molecular basis of denitrification. Microbiology and Molecular Biology Reviews, 1997, 61: 533-616

[55] R.J. Ellis, B. Neish, M.W. Trett, J.G. Best, A.J. Weightman, P. Morgan, J.C. Fry, Comparison of microbial and meiofaunal community analysis for determining impact of heavy metal contamination. Journal of Microbiological Methods, 2001, 45: 171-185.

[56] T. Roane and S. Kellogg, Characterization of bacterial communities in heavy metal contaminated soils. Canadian Journal of Microbiology, 1996, 42: 593-603.

[57] C.M. Magalhães, A. Machado, P. Matos, A.A .Bordalo, Impact of copper on the diversity, abundance and transcription of nitrite and nitrous oxide reductase genes in an urban European estuary. FEMS Microbiology Ecology, 2011, 11: 1-11.

[58] C, Magalhães, J, Costa, C, Teixeira, A.A. Bordalo, Impact of trace metals on denitrification in estuarine sediments of the Douro River estuary, Portugal. Marine Chemistry, 2007, 107: 332-341.