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Effects of Phenanthrene on the key processes and functional genes of denitrification in sediments of Jiaozhou Bay

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Abstract. Polycyclic Aromatic Hydrocarbons (PAHs) will combine with the particulate matter and settle in the sediments, which will influence the denitrification process. The study chooses the Dagu River estuary and the bay area of the northwest of the Jiaozhou Bay as sampling sites, and select Phenanthrene (Phe), the model compound of PAHs as a pollutant. The concentrations of Phe are 0mg/kg, 50mg/kg and 500mg/kg. The impact of Phe on nitrate reduction ability, nitrite reduction ability, related enzyme activity, and relative abundance of functional genes are explored. The results showed that in Jiaozhou Bay and Dagu River estuary Phe had a markedly effect on nitrate reduction, nitrite accumulation, nitrate reductase activity and nitrite reductase activity. The relative abundance of *narG* and *nirS* genes were markedly affected by Phe, and the inhibitory effect was enhanced with the increase of concentration. Compared with Jiaozhou Bay, the reduction of nitrate and nitrite in the Dagu River estuary are more sensitive to the pollution of Phe.

1. Introduction

Denitrification refers to the process in which the N elements in nitrate (NO_3^-) or nitrite (NO_2^-) are reduced to N_2 or N_2O by microorganisms. It is one of the important steps in the nitrogen cycle that generates energy [1]. Because of its unique anoxic environment, the sediment in the gulf is the aggregation site of anaerobic denitrification by denitrifying bacteria. Among the process, the reduction of nitrate under the control of nar gene is particularly important as the first step of the whole reaction [2]. The catalytic process of nitrite reductase under the control of nir gene is a signature reaction of nitrification which is different from other nitrate metabolism. It is the most important rate-limiting step in this process and often used as the molecular marker of denitrifying bacteria to study its community structure and diversity [3]. Denitrification can remove nitrogen and play an important role in maintaining the balance of nitrogen in nature and regulating eutrophication of water [4]. Denitrification reduces NO_2^- in rivers, oceans and other water by converting NO_2^- into NO, and effectively prevents the accumulation of NO_2^- in aquatic animals and plants [5]. Therefore, it is of great significance to study the influence of various environmental factors on denitrification.

Polycyclic aromatic hydrocarbons (PAHs) are a class of persistent organic pollutants widely existing in the environment [6]. And PAHs have the functions of "carcinogenic, teratogenic and gene mutagenesis" [7]. PAHs in the environment are mainly generated through incomplete combustion of

fossil fuels, forest fires, volcanic eruptions and endogenous biosynthesis [8]. PAHs are less soluble in water, and most PAHs which have entered into water will combine with particulate matter and settle in sediments [6]. Then the PAHs may be eventually removed by microbial degradation [9]. Therefore, sediments are important sites for PAHs migration and transformation. At present, there have been many studies on PAHs pollution in surface sediments of the gulf in China, indicating that PAHs pollution in China should be paid attention to [10-12].

Jiaozhou Bay and Dagu River estuary area were selected as the study areas. After surface sediments were collected, simulated culture was carried out in the laboratory. The effects of different concentrations of phenanthrene on the reduction capacity of NO_3^-, NO_2^-, denitrification enzyme activity and the abundance of *narG* and *nirS* functional genes were studied.This study provides a scientific basis for the in-depth study of the ecological impact mechanism and environmental effects of the Phe on the offshore sedimentary environment.

2. Materials and methods

2.1 Sediment collection and analysis

The sediment sample was collected from the surface (0-5 cm in depth) of two typical sea areas located in Jiaozhou Bay (36.1269°N, 120.1838°E) and Dagu River estuary (36.1688°N, 120.1365°E) in July 2017 (Fig.1). After passing through a 1mm sieve, the sediment sample was stored at 4℃ until further experiments were conducted. Sediment particle size was determined by laser particle analyser [13]. Phenanthrene (Phe) in air-dried sediment samples was extracted by ultrasonic extraction method and quantitatively analyzed by GC-MS. Total organic matter was determined by LOI [14]. The moisture content was determined by gravimetric method. The main environmental parameters of sediments are shown in Table 1.
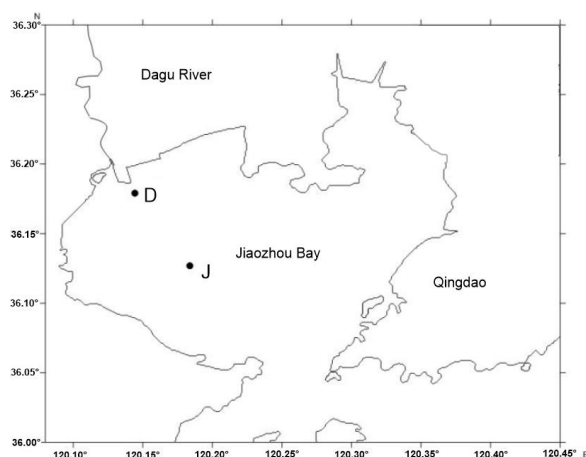

Fig.1. The sampling stations in Dagu River Estuary and Jiaozhou Bay

Table.1. Physical and chemical characteristics of sediments from the two stations

	D	J
Phe(mg·kg^{-1})	0.035 ±0.003	0.041±0.004
Eh(mv)	125.83 ± 3.23	48.56 ± 2.16
OM (%)	1.14 ± 0.14	1.28 ± 0.20
H$_2$O(%)	33.1 ±1.2	34.7 ± 0.9
TN (%)	0.105 ± 0.011	0.086 ± 0.012
Particle size	silty clay	sandy silt

2.2 Experiment setup

Phe used in this study was purchased from Sigma-alorich. Phe was dissolved in acetone and added to the air-dried sediment samples with Phe levels of 0 (Phe-free, with acetone only), 50 and 500 mg kg^{-1}

dry weight sediment. After completely mixing, each sediment subsample was left overnight in darkness to allow the solvent to evaporate. After adding 0.5g sample with Phe, 60g wet sediment and 60mL filtered (0.22 µm, membrane filters) seawater was mixed in serum bottle. The incubation water was amended with 50 mM KNO_3 and 1% glucose. The serum bottle was hermetically sealed with a butyl stopper and aluminium crimp, then purged with N_2 for 15 min to remove O_2. Each experiment group was performed in the dark for 24 h at a constant temperature (25 ℃) and stirring rate (80 rpm.).

2.3 Determination of NO_3^- , NO_2^- concentration
The sample was centrifuged for 10min at 5000 r·min^{-1}. Then the supernatant was filtered by 0.22 m microporous filter membrane and placed in the centrifuge tube. The concentration of f NO_3^- and NO_2^- was determined by
QuAAtro nutrient automatic analyzer.

2.4 Assays for nitrate reductase and nitrite redu-ctase
Sediment nitrate reductase (NAR) and nitrite reductase (NIR) were measured via incubation tests following the description by Kristjansson et al. (1980) [15]. After centrifugation at 8000r·min^{-1} for 10min, the supernatant was discarded. The sample was washed with 100mmol·L^{-1} phosphate buffer (pH=7.4). The cell structure was broken by ultrasound and removed by centrifugation at 12000r·min^{-1} for 10min. The supernatant was used for the determination of enzyme activity. Adding 2ml extract to anaerobic oxygen bottle with 5mmol·L^{-1} $Na_2S_2O_4$, 10mmol·L^{-1} PBS (pH=7.4), 10mmol·L^{-1} Methylviologen, 1mmol·L^{-1} $NaNO_3$ or 1mmol·L^{-1} $NaNO_2$, makes the final volume to 2.1ml. Then the bottle was performed in the incubator for 30min at a constant temperature (30℃). The concentration of f NO_3^- and NO_2^- was determined by QuAAtro nutrient automatic analyzer. Unit (U) of NAR/NIR activity was defined as the amount of NO_3^- /NO_2^- loss after 30min incubation with each gram sediment.

2.5 DNA extraction and Real-time quantitative PCR
The genomic DNA was extracted from 1 g wet weight of sediment (stored at -80℃) using a Fast DNA SPIN kit for soil (SK8233, Sangon Biotech Co., Ltd) according to the manufacturer's instructions. The DNA solution was stored at -80℃ for real-time quantitative polymerase chain reaction (RT-qPCR).
Quantification of *narG* and *nirS* genes was performed on a Roche LightCycler480 using the previously described primer sets (Table.2). The 25µL reaction mixtures contained 0.4µL of each 20mM primer, 10µL of SYBR Green Fast, qPCR Master Mix (Roche, Switzerland), 7.2µL ddH$_2$0, and 2µLof template DNA. Thermal cycling conditions for *narK*, *nirS* genes were as follows: pre-incubation at 95℃ for 3min, 45cycles consisting of denaturation at 95℃ for 7s, annealing at 57℃ for 10s, extension at 72℃ for 15s, followed by melting curve analysis at 60-95℃ with a heating rate of 0.1℃ s^{-1} and a continuous fluorescence measurement) and finally a cooling step to 40℃. The DNA extraction and measurement of gene expression by RT-qPCR were performed in triplicate, and the mean of all these values was used for final analysis. The relative abundance of gene was analyzed by $2^{-\triangle\triangle CT}$ method [16]. All quantitative PCR reactions including unknown samples were performed in triplicate and no template control (NTC) treatments were included in all runs.

<div align="center">Table 2 Primers used in real-time PCR</div>

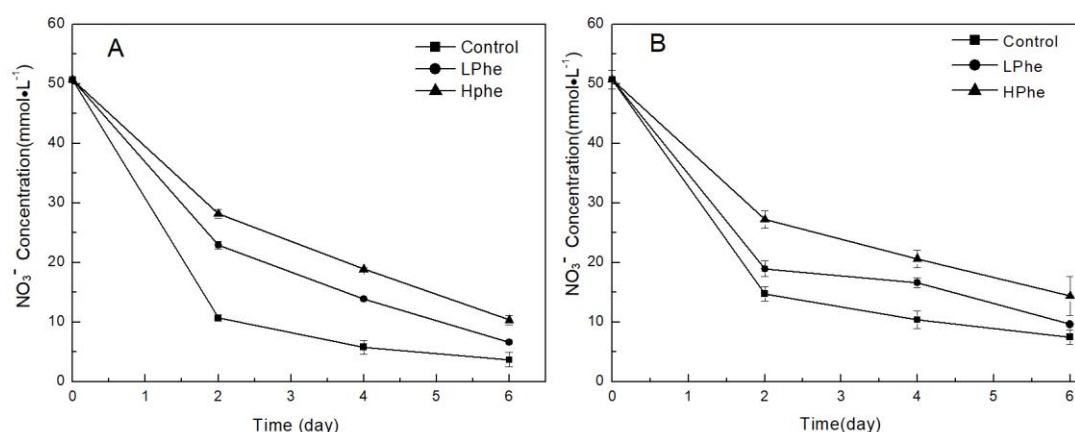| Primers	Bacteral target genes	Sequence (5'–3')	References
1055f 1392r	16srDNA	ATGGCTGTCGTCAGCT ACGGGCGGTGTGTAC	[17]
narG1960m2F narG2050m2R-GC	*narG*	TAYGTSGGGCAGGARAAACTG CGTAGAAGAAGCTGGTGCTGT	[18]
Cd3aF R3cdR-GC	*nirS*	TACCACCCSGARCCGCGCGT GCCGCCGTCRTGVAGGAA	[19]

Fig. 2. Variation of NO_3^- concentration of sediments among different concentrations of Phe in Jiaozhou Bay(B) and Dagu River estuary area(A)

2.6 Data analyses
Origin was used for the preliminary processing and calculation of data. One-way ANOVA was performed to determine significant variations at the level of $P < 0.05$ by using SPSS (11.0).

3. Results

3.1 Nitrate reduction
The change of NO_3^- concentration under the treatment of Phe is shown in Fig. 2. During the incubation of Dagu river estuary (station: D) and Jiaozhou Bay (station: J), the NO_3^- concentrations in control group was significantly ($p<0.05$) different from the NO_3^- concentrations in treatment groups (LPhe, HPhe), which indicates that the addition of Phe has a significant inhibitory effect on NO_3^- concentration. And the NO_3^- concentrations in HPhe group was significantly higher than that in LPhe group ($p<0.05$), which suggests that the inhibitory intensity is related to the concentration of Phe. After 2 days of incubation, the amount of NO_3^- reduction in LPhe and HPhe group at station D was 69.6% and 56.2% of the control group. And at station J, the amount of NO_3^- reduction in LPhe and HPhe group was 88.4% and 65.3% of the control group, which suggests that the inhibitory effect of Phe on the reduction of NO_3^- at station D is stronger.

3.2 Nitrate reductase activity
During the incubation of station D and station J, the nitrate reductase (NAR) activity in control group was significantly ($p<0.05$) higher than that in treatment groups(Fig.3). And NAR activity in HPhe group was significantly lower than that in LPhe group ($p<0.05$), which indicates that the addition of Phe has a significant inhibitory effect on NAR activity and the inhibitory intensity is related to the concentration of Phe. By calculating the inhibitory rate of NAR activity, it was found that the inhibitory rates of NAR in LPhe group at two sattions were the highest at day 2, which were 50.4%(station D) and 39.4%(station J). And the inhibitory rate continued to decrease in later incubation. The inhibition rates of the two HPhe have the same trend. Therefore, when NAR activity is taken as the ecotoxicological index, it is particularly important to observe the changes in day 2 after incubation. And Phe has a stronger inhibitory effect on NAR activity at station D.

3.3 narG relative abundance
In LPhe group, the relative abundance of *narG* at station D ranged from 1.04 to 0.63 during two-day incubation (Fig.4A). The relative abundance of *narG* at station D ranged from 1.04 to 0.39 during two-day   incubation   in   HPhe   group   (Fig.4A).   In   the   control   group   the   relative
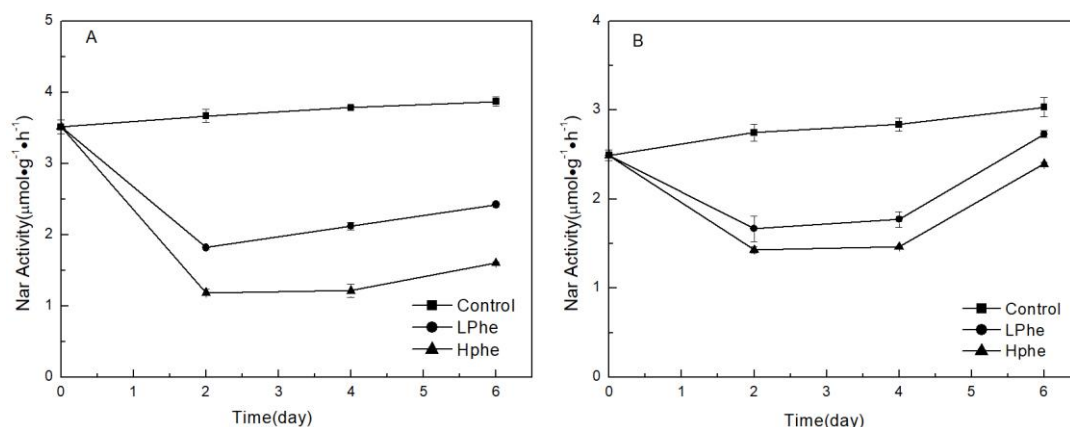
Fig. 3. Variation of NAR activity among different concentrations of Phe in Jiaozhou Bay (B)and Dagu River estuary(A)
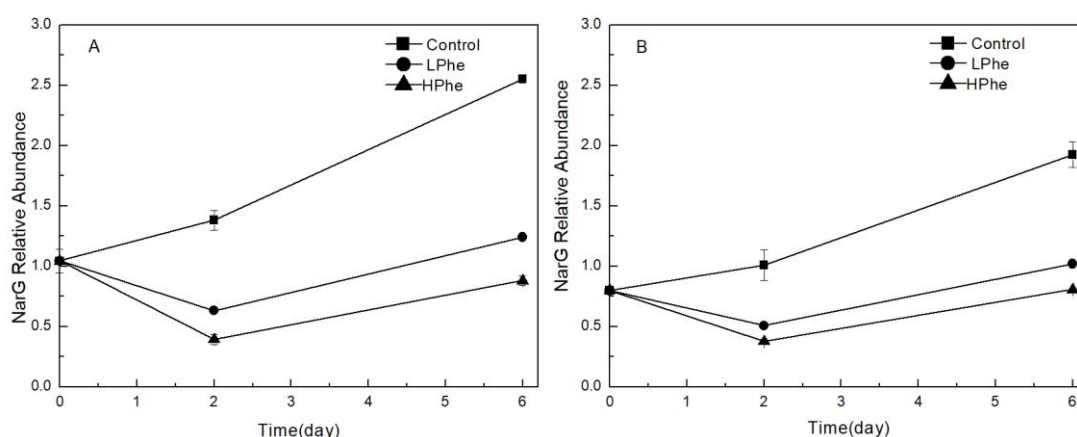

Fig. 4. Variation of the relative abundance of *narG* among different concentrations of Phe in Jiaozhou Bay(B) and Dagu River estuary(A)

abundance of *narG* keep growing during the incubation. The relative abundance of *narG* in the treatment groups was significantly lower than that in the control group ($p<0.05$). In LPhe group, the relative abundance of *narG* at station J ranged from 0.80 to 0.51 during two-day incubation (Fig.4B). The relative abundance of *narG* at station J ranged from 0.80 to 0.38 during two-day incubation in HPhe group (Fig.4B). Therefore, Phe has a stronger inhibitory effect on the relative abundance of *narG* at station D. Meanwhile the relative abundance of *narG* in the treatment groups decreased significantly at day 2 and recovered at day 6 at both D and J stations, which was tha same as the results of NAR.

3.4 Nitrite reduction

According to the concentration of NO_3^- and NO_2^- in the sediment sample，the reduction amount of NO_2^- can be calculated(Fig. 5). The reduction of NO_2^- in both control group and treatment groups presents an upward trend during the incubation. The reduction of NO_2^- in treatment groups were significantly lower than that in control group ($p<0.05$). And HPhe group was markedly lower than LPhe group ($p<0.05$). The amount of NO_2^-reduction in LPhe and HPhe group is 78.16% and 37.33% of the control group at station D in the second day. While they were 43.59% and 18.83% at station J. With the extension of time, the inhibition of NO_2^-reduction kept reducing. The inhibitory effect of Phe

on NO_2^- reduction was related to the concentration of Phe.

3.5 Nitrite reductase activity

During the incubation of station D and station J, the nitrite reductase (NIR) activity in treatment groups were significantly ($p<0.05$) lower than that in control groups (Fig.6). And NIR activity in HPhe group was significantly lower than that in LPhe group ($p<0.05$) at station D. which indicates that the inhibitory intensity is related to the concentration of Phe. But there was no significant difference at station J. The inhibitory rates of NIR activity in LPhe and HPhe group were the highest at day 2, which were 37% and 59.6% at station D. The inhibitory rates of NIR activity in LPhe and HPhe group were the highest at day 4, which were 49.7% and 50.9% at station J. The effect of Phe on NIR activity was delayed. And Phe has a stronger inhibitory effect on NIR activity at station D.
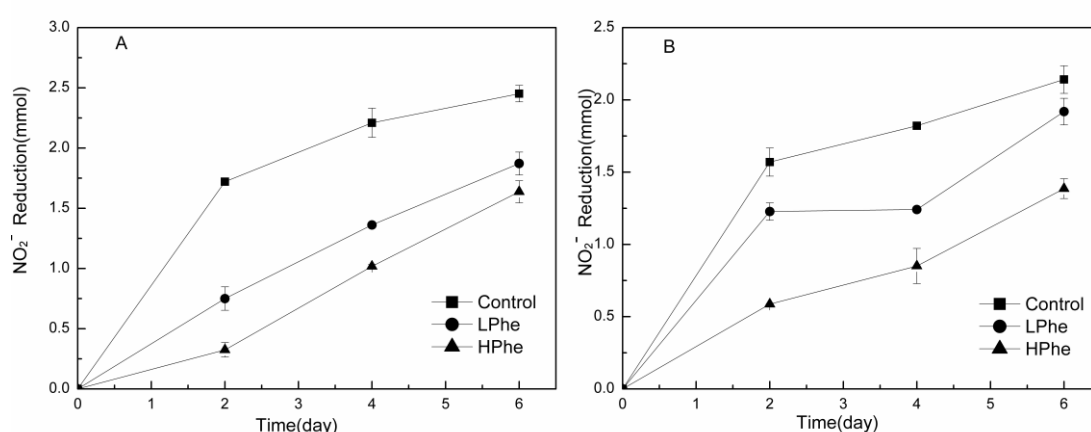

Fig. 5. Variation of NO_2^- reduction of sediments among different concentrations of Phe in Jiaozhou Bay(B) and Dagu River estuary area(A)
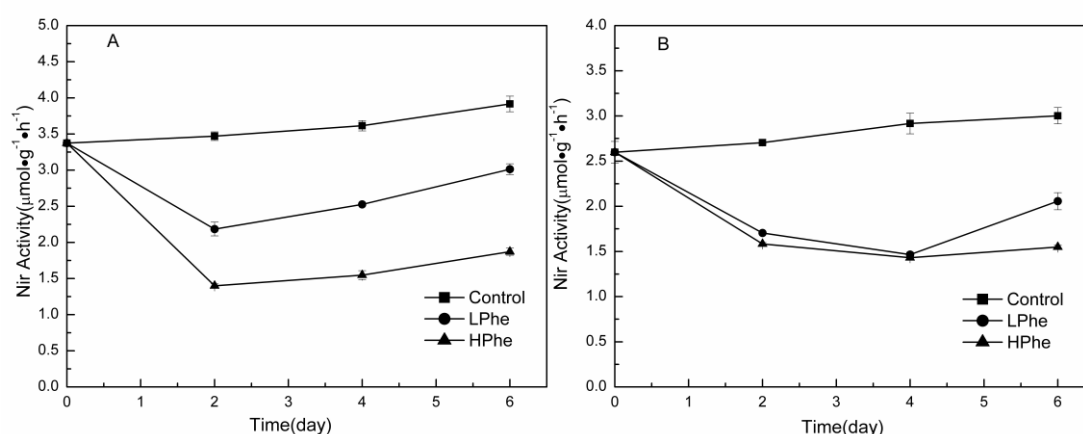

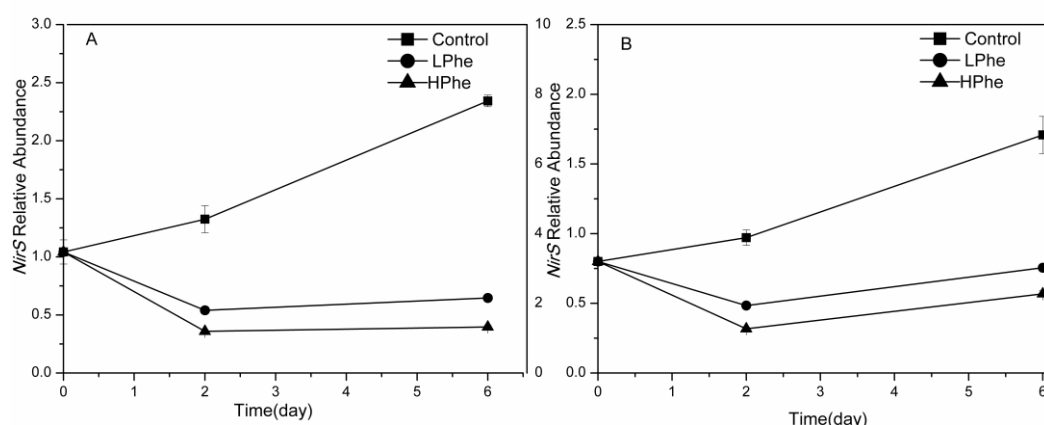Fig. 6. Variation of NIR activity among different concentrations of Phe in Jiaozhou Bay (B)and Dagu River estuary(A)

Fig. 7. Variation of the relative abundance of *nirS* among different concentrations of Phe in Jiaozhou Bay(B) and Dagu River estuary(A)

3.6 nirS relative abundance

In the control group the relative abundance of *nirS* showed an upward trend during the incubation. In LPhe group, the relative abundance of *nirS* at station D ranged from 1.04 to 0.55 during two-day incubation (Fig.7A). The relative abundance of *nirS* at station D ranged from 1.04 to 0.36 during two-day incubation in HPhe group (Fig.7A). In LPhe group, the relative abundance of *nirS* at station J ranged from 0.80 to 0.48 during two-day incubation (Fig.7B). The relative abundance of *nirS* at station J ranged from 0.80 to 0.32 during two-day incubation in HPhe group (Fig.7B). The relative abundance of *nirS* in the treatment groups was significantly lower than that in the control group ($p<0.05$). Phe has a stronger inhibitory effect on the relative abundance of *nirS* at station D.

4. Discussion

The functional genes of denitrification control the expression of enzyme activity, and the changes of NO_3^- and NO_2^- concentration can reflect the enzyme activity. The results showed that Phe significantly affect NO_3^- concentration, NO_2^- accumulation, functional gene abundance and enzyme activity. Guo et al. [20] studied the influence of Pyrene on the denitrification process in agricultural soil, and found that the nitrification rate and the abundance of denitrification functional genes were negatively correlated with the concentration of Pyrene. Jiang et al. [21] found that PAHs significantly inhibited the activity of denitrification reductase and the abundance of functional genes in mangrove sediments. By comparing the inhibition rates of enzyme activity and the relative abundance of genes, it can be found that genes are more sensitive to the addition of Phe than enzyme activity. Guo et al. [22] also showed that the effect on *nirS*, *nirK* and *nosZ* genes are stronger than denitrification potential after adding PAHs. The results may suggest that Phe has a direct effect on genes, while the effect on enzyme activity is caused by the dual effects of the process of enzyme action and the expression of enzyme.

Nitrate reduction process and nitrite reduction process are considered as the key steps in the denitrification process, which have important influence on the denitrification rate. *NarG* and *nirS*, which control these two processes, are also the genes that have been studied most in functional genes of denitrifying bacteria, and are used as molecular markers of denitrifying bacteria to study their population structure and diversity [23]. According to the inhibitory rate of key functional genes, the inhibitory effect of pheniramine on *nirS* is stronger than that on *narG*. Research shows that PAHs can form electrophile with high activity such as epoxy, diketone in the process of decomposition. Molecules in the environment react with excited PAHs molecules to produce singlet oxygen, superoxide, free radicals and other active intermediates, which can cause the peroxidation of lipid, the destruction of cell membrane structure and the damage of RNA and DNA [24].

Compared with Jiaozhou Bay, the NO_3^-, NO_2^- reduction in Dagu River estuary are more sensitive to the pollution of Phe. The difference in physical and chemical properties between the gulf region and the estuary region may lead to such a result. The sediment organic matter content in Jiaozhou Bay is high. In a general way the higher the organic matter is, the higher the denitrification rate of sediments is [25]. The particle size of sediments in the estuary area is lower. Study has shown that the denitrification capacity may increase with the increase of particle size [26]. Under the same concentration of Phe, the regions with stronger denitrification capacity is inhibited less. Wang et al. [27] found that the stations with high PAHs in the offshore sediments of Qingdao were concentrated in Jiaozhou Bay and the nearshore area.

5. Conclusions

(1) Phe markedly inhibited NO_3^- reduction, NO_2^- accumulation, nitrate reductase activity and nitrite reductase activity in Jiaozhou Bay and Dagu River estuary. The degree of influence was related to the concentration of Phe. On the second day after the addition of Phe the difference between each treatment and control group was the most significant. When studying the process of NO_3^- reduction and NO_2^- reduction by Phe, the changes of indicators before and after the second day could be focused on.

(2) Compared with Jiaozhou Bay, the reduction of nitrate and nitrite in the Dagu River estuary are more sensitive to the pollution of Phe.

(3) The relative abundance of *narG* and *nirS* genes were markedly affected by Phe, and the inhibitory effect was enhanced with the increase of concentration. And the inhibitory effect on the relative abundance of *nirS* was stronger than *narG*.

References

[1] L. Philippot, BBA-Gene Struct. Expr. **1577**, 355 (2002)
[2] W. G. Zumft, Microbiol Mol Biol Rev. **61**, 533(1997)
[3] G. Braker, A. Fesefeldt, K. P. Witzel, Appl. Environ. Microbiol. **64**, 769(1998)
[4] J. N. Galloway , A. R. Townsend , J. W. Erisman , et al. Science. **320**, 889(2008)
[5] H. Y. Yildiz, A. C. Benli. Ecotoxicol. Environ. Saf. **59**, 370(2004)
[6] G. Witt. Mar. Pollut. Bull. **31**, 237(1995)
[7] J. B. Sutherland. Biodegradation. **9**, 53(1992)
[8] H. H. Soclo, P. Garrigues, M. Ewald. Mar. Pollut. Bull. **40**, 387(2000)
[9] H Budzinski, I. Jones, J. Bellocq, et al. Mar. Chem. **58**, 85(1997)
[10] X. He , X. Song, Y. Pang, et al. Environ. Monit. Assess. **186**, 4001(2014)
[11] W. Jiao, T. Wang , J. S. Khim, et al. Environ. Geochem. Health, **34**, 445(2012)
[12] B. Li, C. Feng, X. Li, et al. Mar. Pollut. Bull. **64**, 636(2012)
[13] C. C. Huang, J. Pang , X. Zha, et al. Quat. Sci. Rev. **30**, 460(2011)
[14] O. Heiri, A. F. Lotter, G. Lemck. J. Paleontol. **25**, 101(2001)
[15] J. K. Kristjansson, T. C. Hollocher. J. Biol. Chem.,**255**, 704(1980)
[16] K. J. Livak, T. D. Schmittgen, Methods. **25**, 402(2001)
[17] G. Harms, A. C. Layton, H. M. Dionisi, et al. Environ. Sci. Technol. **37**, 343(2003)
[18] J. C. Lopez-Gutierrez, S. Henry, S. Hallet. J. Microbiol. Methods. **57**, 399(2004)
[19] G. Braker, A. Fesefeldt, K. P. Witzel. Appl. Environ. Microbiol. **64**, 3769(1998)
[20] G. X. Guo, H. Deng , M. Qiao, et al. Environ. Pollut. **159**, 1886(2011)
[21] S. Jiang, Y. Su, H. Lu, et al. Mar. Pollut. Bull. **122**, 366(2017)
[22] G. X. Guo, H. Deng , M. Qiao, et al. Environ. Sci. Technol. **47**, 3015(2013)
[23] S. A. Wakelin, P. N. Nelson, J. D. Armour , et al. Soil Res. **49**, 65(2011)
[24] M. Certík, K. Dercová, Z. Sejáková, et al. Biologia. **58**, 1111(2003)
[25] E. A. Strauss, G. A. Lamberti. Limnol. Oceanogr., **45**, 1854(2000)
[26] A. M. Laverman, C Meile, P. Van Cappellen, et al. Appl. Environ. Microbiol. **73**, 40(2007)
[27] J. T. Wang, L. J. Tan, W. H. Zhang, et al. Environ. Sci. **31**, 2014(2010)