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The dissipation and risk alleviation mechanism of PAHs and nitrogen in constructed wetlands: The role of submerged macrophytes and their biofilms-leaves

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ABSTRACT

The role of submerged macrophytes (*Vallisneria natans, Hydrilla verticillata* and artificial plant) and their biofilms-leaves for the dissipation and risk alleviation mechanism of PAHs (phenanthrene and pyrene) and nitrogen in constructed wetland systems with PAH-polluted sediments were investigated. Biofilms-leaves/surface might contribute to PAHs degradation, which was positively correlated with PAHs degrading bacteria. Nitrogen-fixing bacteria in biofilms on surface might cause total nitrogen in sediment (TNs) increasing by 4% from 14th d to 28th d indirectly when suffering PAHs pollution. The relative abundance of nitrogen-fixing bacteria significantly increased with the increase of PAHs concentrations in early period ($p < 0.01$), which might lead to risk of nitrogen accumulation further. Heat maps showed that the relative abundance of functional bacteria were influenced in order of attached surface > incubation time > spiking concentration of PAHs. Interestingly, differences of deduced bacterial functions were affected in order of incubation time > attached surface > spiking concentration. Thus, submerged macrophytes and their biofilms on leaves not only played an important role in PAHs degradation, but also regulated the nitrogen cycling in constructed wetland systems, which could reduce these pollutants risk for natural environment, organisms and human health.

1. Introduction

Submerged macrophytes are the imperative component in aquatic environment and play an important role in improving the quality of water environment (Barko and James, 1998). In numerous studies, submerged macrophytes were investigated to absorb and accumulate various pollutants in natural environment, which were therefore applied to remove these contaminants (Cardwell et al., 2002; Mazej and Germ, 2009; Shivers et al., 2018). Nitrogen accumulation in water body causes various environmental problems including microcystin production with algae explosion, high concentration ammonium and N_2O release, which threaten several aquatic organisms, ecological environment and human health (Liu et al., 2018; Ma et al., 2018; Yang et al., 2017). Researchers applied submerged macrophytes to deal with nitrogen pollution. Meanwhile, they observed that nitrogen removal was associated with environment conditions (e.g. DO and pH) (Choudhury et al., 2018; Yan et al., 2017), seasons (Soana and Bartoli, 2014; Ye et al., 2018), submerged macrophytes categories (Choudhury et al., 2018) and relative microorganisms (Yan et al., 2017).

In natural ecosystems, a micro-interface system (biofilms-leaves) is established on the leaves surface of submerged macrophytes, which is composed of organic matter, algae and microorganism. The concentrations of microbes and algae on biofilms on submerged macrophytes' leaves were higher than those of floating macrophytes (Pang et al., 2016). Microbes are important and imperative parts of wetland systems and have great effects on nitrogen removal through nitrification and denitrification processes (Hou et al., 2017; Li et al., 2014). The role of biofilms on leaves in nitrogen cycling had been focused on and studied gradually. Submerged macrophytes played a major role in determining the bacterial community structure (Zhang et al., 2016), and

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the relative abundance of nitrifying bacteria were higher in sediment than in biofilms on leaves but denitrifying bacteria had the opposite result (Pang et al., 2016). Nitrogen loading increased the abundance of nitrifiers and denitrifiers, as reflected by the nitrogen cycling genes (Yan et al., 2017; Zhang et al., 2016). In addition, ammonium stress altered the chemical states of leaf surface, which might reduce the absorption ability of leaves for nutrients from water body and then the growth status of submerged macrophytes might be affected (Gong et al., 2017).

PAHs are regarded as one of Persistent Organic Pollutants (POPs), which have potential threat on flora, fauna and even human health due to their carcinogenic, teratogenic and mutagenic capacity. Recently, submerged macrophytes had been applied to deal with the PAH-polluted sediments, which were regarded as one of phytoremediation methods. PAHs stimulated the growth of submerged plants, in turn the presence of submerged plants enhanced the dissipation of PAHs (Meng and Chi, 2015). Compared with remediated ability of different submerged macrophytes, He et al. (2016) deduced that dissipation of PAHs might be attributed to the differences of oxygen production capabilities in root. Further, the removal of PAHs were related to the changes in microbial community structure possibly in sediments planted by different submerged macrophytes (He et al., 2016). Especially, PAH-degrading bacteria were positively correlative with the dissipation of PAHs (Liu et al., 2014). The transfer chain of sediment-water-leaf had been confirmed to be important for PAHs translocation (Diepens et al., 2014). The ecological contribution of submerged macrophytes and their biofilms-leaves had been calculated and the performance of PAH-degrading bacteria on biofilms-leaves was consistent with rhizosphere (Zhao et al., 2018).

There were numerous researches referring to the effect of trace substances for nitrogen cycling and related microorganisms (Ahmad et al., 2012; Kapoor et al., 2015; Liang and Tabatabai, 1978; Lim et al., 2003; Yadav et al., 2016). PAHs were also certainly studied for the effects of nitrogen cycling and nitrogen transforming microorganisms in sediment. Nitrate addition could stimulate biodegradation of PAHs, which might be owing to enrichment of functional genes involved in N-, carbon (C)-, sulfur (*S*)- and phosphorus (P)- cycling processes, especially those microorganisms with diverse metabolic abilities leading to PAHs reduction/degradation (Xu et al., 2015; Xu et al., 2014). Meanwhile, the presence of PAHs reduced the diversity of nitrogen-fixing bacteria, and prevented the growth of many nitrogen fixing bacteria, such as proteobacteria and cyanobacteria (Sun et al., 2012).

Although recent studies presented that submerged macrophytes and their biofilms-leaves played an important role in nitrogen cycling and PAHs degradation, little is known about nitrogen cycling involved into submerged macrophytes planted in PAH-polluted sediment. Thus in our present study, we hoped to figure out PAHs and nitrogen dissipation and interaction between them in constructed wetland systems through researching the response of submerged macrophytes and functional bacteria on biofilms on leaves, and combining with habitat indicators. The aim of this study, following hypotheses had been put forward: (1) the presence of PAHs concentration gradients in constructed wetland system might influence nitrogen cycling; and (2) the effect of PAHs on nitrogen cycling might be alleviated due to the application of submerged macrophytes in PAHs-polluted sediment in constructed wetland systems.

2. Materials and methods

2.1. Experiment design

Vallisneria natans (VN) and *Hydrilla verticillata* (HV) (Nanjing Sam Creek aquatic breeding research base) were selected as the tested submerged macrophytes. Meanwhile, we also set up artificial plants group (AP, bio-racks made by polymethyl methacrylate) with similar surface area with submerged macrophytes. Sediments (pH = 7.32, organic matter = 2.14%, the phenanthrene and pyrene of background value were 0.056 mg/kg and 0.042 mg/kg, respectively, TN = 892 mg/kg, NO_3^--*N* = 5.20 mg/kg, NH_4^+-*N* = 0.15 mg/kg) were collected from a suburb river of Nanjing, air-dried, crushed, and then sieved with 2-mm mesh to remove plant residues and stone. Organic glass containers (diameter × height = 40 cm × 50 cm) were chosen to cultivate submerged macrophytes, which could avoid the adsorption loss of phenanthrene and pyrene. The experiment was carried out in the ecological greenhouse with three replicates for 35 d.

The 0.6 g phenanthrene and 0.06 g pyrene dissolved in acetone (1600 mL) was spiked into 6 kg sediments, respectively. The spiked sediments were mixed with the unpolluted with their respective proportion equably after acetone evaporating and then laid in each container smoothly. The final contents in sediments (dry weight) were 20 mg/kg, 10 mg/kg (phenanthrene) and 2 mg/kg, 1 mg/kg (pyrene), respectively.

Norfloxacin (0.01 g/L water) and roxithromycin (0.015 g/L water) were added into water to domesticate for 3–5 d, which could remove or destroy the biofilms on leaves originally. In domesticated process, there was no sediment put in container in order to avoid affecting later experiments. Robust plants (grew uniformly) were chosen randomly to transplant into the containers after domestication. 50 L tap water (TN = 1.24 mg/L, NO_3^--*N* = 0.14 mg/L, NH_4^+-*N* = 0.09 mg/L) was added to the container and the water-line was marked clearly to replenish water to a uniform level throughout cultivation. The specific arrangement of experiment groups was showed in Table 1.

2.2. Sample collection

Water, leaf and sediment samples were collected for detecting the phenanthrene and pyrene contents in 14th, 28th and 35th d. The samples were stored at −20 °C for PAHs analysis and nitrogen determination. Besides, the biofilms on leaves of submerged macrophytes were extracted in 14th and 28th d, respectively. The method of separating biofilm modified from He et al. (2012). With precooling ethanol-PBS buffer as eluent, proper amount of leaves were put in the polyethylene pipe, then triton solution and several 3 mm diameter glass beads were added. All the sample bottles were placed in constant temperature (25 °C) shaking bed for 10 min (225 rpm), and then underwent ultrasounds (150 W, 40 kHz) for 1 min. After filtrating elution liquor, the filtrate was centrifuged for 10 min (10,000 rpm) and the precipitate was collected. Biofilms on surface of artificial plant could use a sterile scalpel to scrape directly.

2.3. Sample preparation and instrumental analysis

The method of PAHs extraction and purification from sediment, water and plant samples were referred from our previous studies (Zhao et al., 2014; Zhao et al., 2018). The phenanthrene and pyrene were analyzed by Agilent1100 HPLC with fluorescence and UV - adsorption detector, respectively (SI, S1).

Table 1
The experimental protocols.

Samples NO.	Phe Conc. (mg/kg)	Pyr Conc. (mg/kg)	Sampling time	Submerged plants
A1	20	2	14th d	VN
A2	10	1		HV
A3	20	2		VN
A4	10	1		HV
B1	20	2	28th d	VN
B2	10	1		HV
B3	20	2		VN
B4	10	1		HV
C1	20	2		AP
C2	10	1		AP

Dissolved oxygen (DO), pH, oxidation-reduction potential (ORP) and conductivity (COND) were measured in situ by using SX 751 series portable electrochemical meters (SanXin Instrumentation Inc., Shanghai, China). Total nitrogen, nitrate nitrogen and ammonia nitrogen in water (TNw, NO_3^--Nw, NH_4^+-Nw) were measured in the laboratory with 24 h after fixing water samples with concentrated sulfuric acid; TNw, NO_3^--Nw and NH_4^+-Nw were measured by potassium persulfate oxidation-ultraviolet spectrophotometry method, ultraviolet spectrophotometric screening method and Nessler's reagent spectrophotometry, respectively. Sediment and leaf samples were frozen and dried for one week and sieved by 2-mm mesh. Total nitrogen, nitrate nitrogen and ammonia nitrogen of sediment (TNs, NO_3^--Ns, NH_4^+-Ns) were analyzed by hypobromate oxidation method, ultraviolet spectrophotometry and indiphen blue colorimetric method, respectively. TN of plants was measured by micro kjeldahl method (Mohee et al., 2008).

2.4. DNA extraction, PCR amplification and high through-put sequencing

Weighing 0.5 g biofilms on leaves/surface samples in 2 mL centrifuge tube, The DNA in bacteria were extracted by Soil DNA Kit (Omega E.Z.N.A.™, Omega Bio-Tech) according to manufacturer's protocol. The PCR primers were V3-V4 universal primers 341F/805R (341F: CCTACGGGNGGCWGCAG; 805R: GACTACHVGGGTATCTAATCC) provided by Sangon Biotech Co., Ltd., Shanghai, China. The PCR reaction mixture composed of 5 μL 10 × PCR buffer, 0.5 μL dNTPs (10 mM each), 0.5 μL Bar-PCR primer F (50 μM), 0.5 μL Primer R (50 μM), 0.5 μL Platinum Taq (5 U/μL), 10 ng DNA template and added sterile water to make the final volume to 50 μL. The PCR was performed under the conditions pertaining to: initial denaturation at 94 °C for 3 min, denaturation at 94 °C for 30 s, renaturation at 94 °C for 30 s, annealing at 45 °C for 20 s, extension at 65 °C for 30 s with a total of 30 cycles, and the final extension at 72 °C for 5 min. Amplification products were detected by 1% agarose gel electrophoresis, and then recycled using DNA Recycle Kit, SK8131 (Sangon Biotech Co., Ltd., Shanghai, China), and finally quantified using Qubit 2.0 DNA Assay Kit (Sangon Biotech Co., Ltd., Shanghai, China). Paired-end sequencing was performed by using Illumina MiSeq platform.

Raw fastq files were transformed into effective tags through splicing, filtering and removing chimeric. UCLUST was used to cluster sequences into Operational Taxonomic Units (OTUs) at ≥97% 16 s rRNA gene sequence similarities. The taxonomic information was annotated with the RDP database.

The raw DNA sequence data was uploaded to NCBI Short Read Archive (SRA) with the accession number of SRP125077 and SRP137091.

2.5. Statistical analysis

Before statistical analysis was performed, collected data were checked for the assumption of normal distributions using evaluating IBM SPSS 23 software (evaluation version). The statistically differences of ORP, DO, pH, COND, functional bacteria were analyzed by one-way ANOVA and Tukey's post hoc comparison test in SPSS 23 software for windows at a significant level of $p < 0.05$. Difference analysis of deduced bacterial functions was evaluated by Kruskal-Wallis test and Mann-Whitney U test. Data are presented in mean ± standard deviation. Redundancy analysis (RDA) was performed using Canoco 5.0 software (evaluation version).

3. Results and discussion

3.1. The dissipation of PAHs in constructed wetlands

During the experiment, the concentrations of phenanthrene and pyrene in sediments declined in all the treatments (Fig. 1a). The dissipated efficiencies of phenanthrene were in order of *Vallisneria natans* (VN) (62.7% for 20 mg/kg and 64.1% for 10 mg/kg) > *Hydrilla verticillata* (HV) (51.1% and 58.4%) ≥ artificial plant (AP) (51.6% and 53.5%). Besides, that spiked by 2 mg/kg of pyrene were in order of VN (33.5%) > HV (32.3%) > AP (29.3%), while spiked by 1 mg/kg were in order of VN (43.0%) > AP (37.5%) > HV (33.5%). The results indicated that submerged macrophytes settled in sediments could contribute to the removal of phenanthrene and pyrene, the dissipated ability depended on the type of submerged macrophytes, which were related to the oxygenation capabilities and microbiological degradation of root (He and Chi, 2016) and the biofilms on leaves. Obviously, we observed that the dissipation capacity was in order of VN > HV, which was consistent with the study of He et al. (2016). In addition, we also discovered that the dissipation capacity of AP was similar to HV.

The concentrations of PAHs showed a gradual decrease slowly in water (Fig. 1b), while always kept a relatively stable level in leaves of two kinds of submerged macrophytes (Fig. 1c). The concentrations of PAHs in leaves were far too lower than sediments due to the mutual exchange balance of PAHs among overlying water, sediment and leaves and low level of PAHs in water naturally (Diepens et al., 2014). Leaves possess giant area and also release oxygen, so we should give importance to the contribution of biofilms-leaves system in remediating PAHs.

3.2. Analysis of environmental parameters and nitrogen in constructed wetland systems

In our study, we detected and analyzed the water quality parameters including oxidation-reduction potential (ORP) (mV), dissolved oxygen (DO) (mg/L), pH and conductivity (COND) (μS/cm) in different sediment systems spiked with different concentrations of phenanthene and pyrene during incubation process (S2, Fig. S1).

In our present study, we divided the whole incubation time into two time spans, 14 d and 28 d. The values of ORP ($F = 21.726$, $p = 0.000$), DO ($F = 5.731$, $p = 0.017$), pH ($F = 115.185$, $p = 0.000$) and COND ($F = 93.377$, $p = 0.000$) presented significant differences among these two periods. Detected ORP ($F = 6.854$, $p = 0.009$), DO ($F = 7.107$, $p = 0.008$) and pH ($F = 4.544$, $p = 0.034$) values were different between low and high PAHs concentration except for COND ($F = 0.618$, $p = 0.433$). ORP in AP systems (146.76 ± 24.43 mV) were higher than VN systems (140.07 ± 29.62 mV) and HV systems (133.90 ± 27.91 mV). But there were no differences in ORP between VN and HV systems ($p > 0.05$). The values of DO showed differences and were in order of HV (6.50 ± 1.60 mg/L) > VN (5.98 ± 1.78 mg/L) > AP (5.28 ± 1.51 mg/L). Compared with the leaves of submerged macrophytes, the surface of artificial plant could not produce O_2 (Yan et al., 2017). The values of pH in AP systems (8.81 ± 0.43) were lower in contrast with VN (9.45 ± 0.62) and HV (9.56 ± 0.47) systems. This might be associated with the absorption of dissolved carbon dioxide (Liu et al., 2016; Schumacher et al., 2003). Similar to ORP, no significant differences showed among VN and HV systems ($p > 0.05$). COND in AP systems (268.94 ± 32.19 μS/cm) were higher compared to VN systems (250.07 ± 22.01 μS/cm), and then followed by HV systems (226.66 ± 29.81 μS/cm). This might be related with the absorption of VN or HV systems on the ions and N, which was consistent with Choudhury et al. (2018). In his study, he found that N removal in water was negatively affected by COND.

The measured physicochemical indicators included TNw, NO_3^--Nw, NH_4^+-Nw, TN in submerged plants, and TNs, NO_3^--Ns, NH_4^+-Ns of cultivated systems on 14th d and 28th d, respectively (Fig. 2). We divided these samples into two groups including submerged plants (VN and HV), and artificial plant (AP). The concentrations of TNw increased in VN and HV systems while decreased in AP systems from 14th d to 28th d. This might be attributed to the releasing of organic nitrogen from sediment mostly along with the incubation time (Zhang et al., 2018). The concentrations of NH_4^+-Nw in all samples increased, which might be associated with high abundance of nitrogen-fixing bacteria

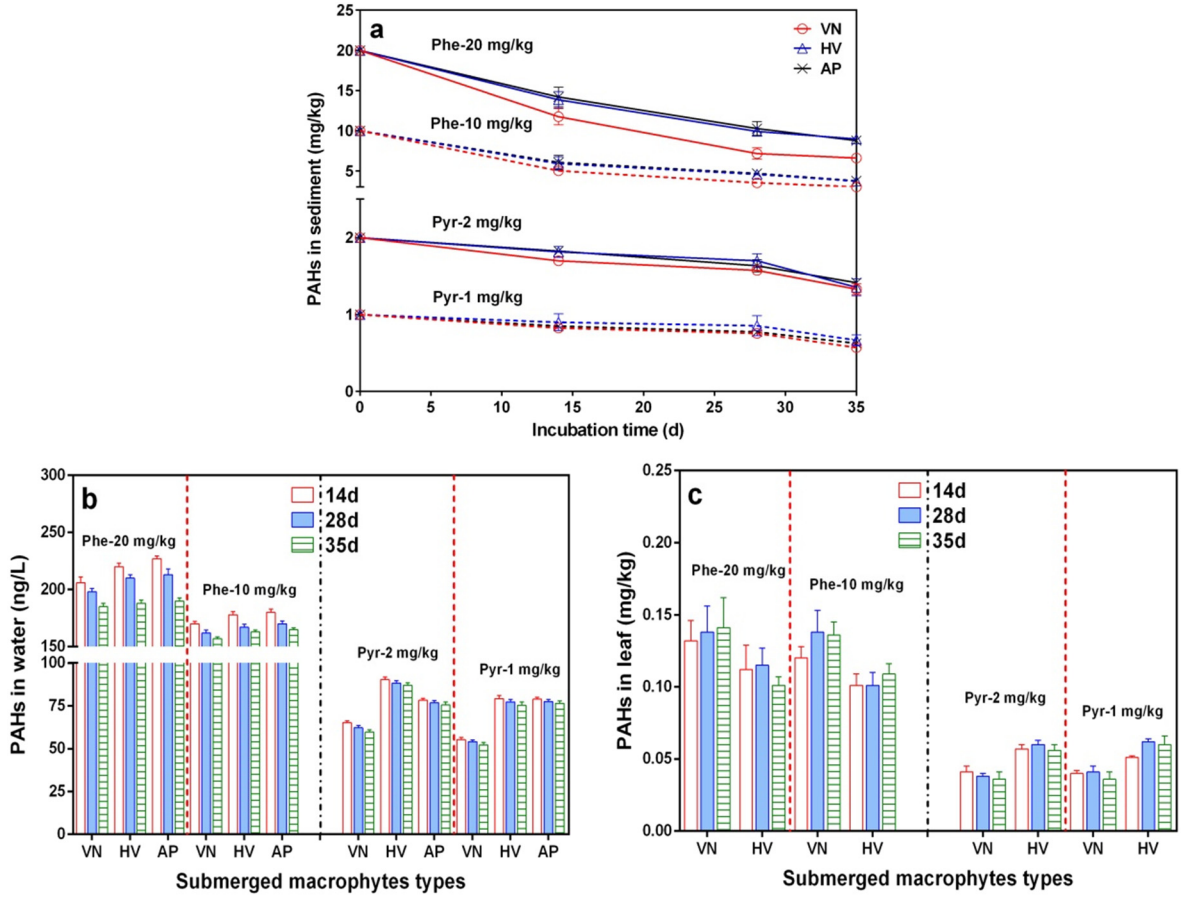

Fig. 1. Residual characteristics of PAHs (phenanthrene and pyrene) in sediments, water and leaf in different treatments. (AP: artificial plant; HV: *Hydrilla verticillata*; VN: *Vallisneria natans.*)

detected in biofilms on leaves/surface. The concentrations of NO_3^--Nw decreased in AP systems, while kept stable in VN and HV systems. This might be related with higher abundance of denitrifying bacteria observed on biofilms on surface of AP in contrast with biofilms on leaves of VN and HV.

The concentrations of TNs in 28th d were 4% higher than in 14th d for AP systems but always kept a stable level in VN and HV systems, which might be related to organic nitrogen sedimentation from overlying water (Small et al., 2014). Although this process also happened in VN and HV systems, higher sediment microbial biomass and activity led to decomposition of nutrients (Kai et al., 2010; Silva et al., 2012) and the roots of submerged macrophytes could absorb the inorganic nitrogen for growth. The contents of NO_3^--Ns in AP systems decreased along with incubation time rather than in VN and HV systems, which might result from the coupling-digestion of NO_3^--Ns and PAHs (Dou et al., 2009), and the NH_4^+-Ns was prone to transform into NO_3^--Ns in aerobic environment due to O_2 releasing from roots of submerged macrophytes. Meanwhile, anaerobic environment in AP systems stimulated the denitrifying process leading to the decrease of NO_3^--Ns. The concentrations of NH_4^+-N decreased in sediments of AP systems, while rose in VN and HV systems. This might be related to the microorganisms in rhizosphere of VN and HV systems were more abundant and diverse than in AP systems. They played an important role in large part of NH_4^+-N releasing from organic nitrogen (Zhao et al., 2015). In addition, the concentrations of TN of submerged macrophytes decreased from 14th d to 28th d, which were attributed to biological dilution. But no doubt, the total contents of TN increased along with the growth of plants (Jarrell and Beverly, 1981).

3.3. Comparison of functional bacteria on biofilms on leaves/surface

In our study, we managed to pick out the functional bacteria including PAH-degrading bacteria and nitrogen transforming bacteria at genus level on biofilms on leaves of submerged macrophytes in 14th d and 28th d and biofilms on surface of artificial plant in 28th d which were spiked by phenanthrene (Fig. 3a) and pyrene (Fig. 3b) to analyze their community structure. As far as biofilms on leaves of submerged macrophytes were concerned, we could easily figure out that the relative abundance of these four kinds of functional bacteria were in order of nitrogen-fixing bacteria > denitrifying bacteria > PAH-degrading bacteria > nitrifying bacteria in 14th d generally. The results suggested that N_2 fixer might be the main source of new N accumulation in the systems (Karl et al., 2002). However, the relative abundance of PAH-degrading bacteria and nitrogen-fixing bacteria decreased significantly in 28th d. The relative abundance of denitrifying bacteria decreased on biofilms on leaves of VN, but increased on biofilms on leaves of HV in 28th d, while the relative abundance of nitrifying bacteria still kept at a low level. The results were consistent with the study by Pang et al. (2016). For biofilms on surface of AP, the relative abundance of these four kinds of bacteria were in order of PAH-degrading bacteria > denitrifying bacteria > nitrogen-fixing bacteria > nitrifying bacteria generally. The relative abundance of each of them was higher compared with the biofilms on leaves of submerged macrophytes, which might be associated with host-specificity (Kahlert and Pettersson, 2002; Wetzel, 1983).

In order to display the abundance of specific functional bacteria visually, heat map was chosen to draw (Fig. S2) and the differential species were pointed out (SI, S3) based on attached surface, spiking concentrations of PAHs and incubation time.

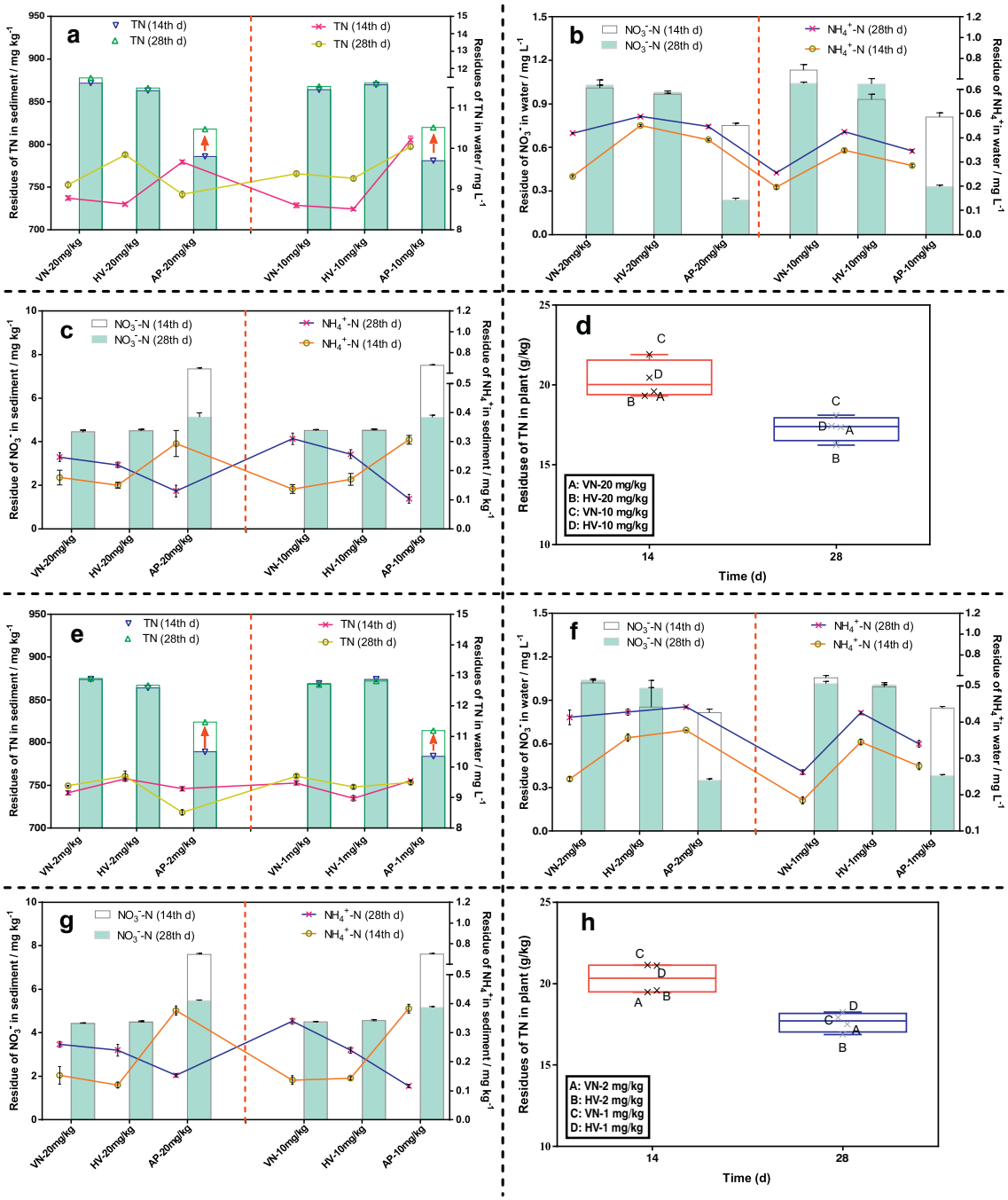

Fig. 2. Measured indicators including TN (**a, e**), NO_3^--N (**b, f**), NH_4^+-N (**b, f**) in water, TN (**d**, **h**) of submerged plants and TN (**a, e**), NO_3^--N (**c, g**), NH_4^+-N (**c, g**) in sediment which spiked by phenanthrene and pyrene in constructed wetland systems, respectively.

On the whole, PAH-degrading bacteria (*Comamonas* and *Arthrobacter*) and denitrifying bacteria (*Comamonas*, *Meganema*, *Rhodobacter*, *Hyphomicrobium*, *Flexibacter* and *Azospira*) were more abundant in VN and HV than AP generally, but nitrogen-fixing bacteria (*Desulfuiomonas*, *Anaeromyxobacter*, *Desulfovibrio*, *Geobacter* and *Bradyrhizobium*) were lower. There were much functional bacteria affected in 28th d than in 14th d, especially for denitrifying bacteria (*Pseudomonas*, *Azospira*, *Meganema*, *Acidovorax*, *Hydrogenophaga* and *Rhozoplanes*) and PAH-degrading bacteria (*Pseudomonas*, *Arthrobacter*, *Sphingomonas*, *Acidovorax* and *Aeromonas*). This might be associated with growth status of biofilms (Zhao et al., 2018). In addition, the higher abundance of PAH-degrading bacteria (*Pseudomonas*, *Sphingomonas*, *Brevundimonas* and *Aeromonas*) appeared in the sediment spiked by 10 mg/kg phenanthrene or 1 mg/kg pyrene, which presented that PAHs concentration and types affected the abundance and species of functional bacteria.

3.4. Responses of TNs to PAHs degradation, environmental conditions and functional bacteria

Total nitrogen in sediments (TNs) could reflect N content of whole system. So, the impacts of PAHs, environment factors and functional bacteria in biofilms on leaves/surface on residual TNs were analyzed by using RDA (Fig. 4). The eigenvalues of the first two species axes were 0.3859 and 0.3589, respectively, the total eigenvalue was 1.0000, and the first two ordination axes could account for 74.48% of the total

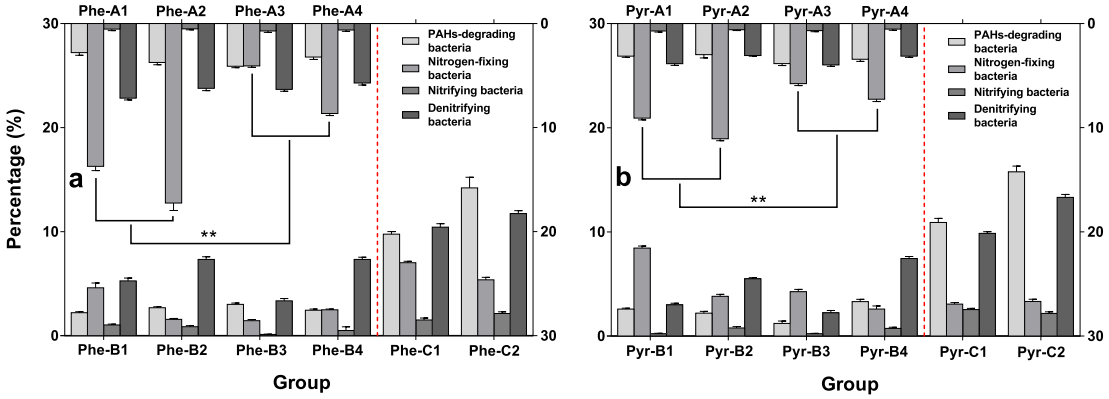

Fig. 3. The community structure of functional bacteria including PAHs-degrading bacteria, nitrogen-fixing bacteria, nitrifying bacteria and denitrifying bacteria on biofilms on leaves/surface settled in sediments spiked by phenanthrene and pyrene.
Note: "*" means $p < 0.05$; "**" means $p < 0.01$.

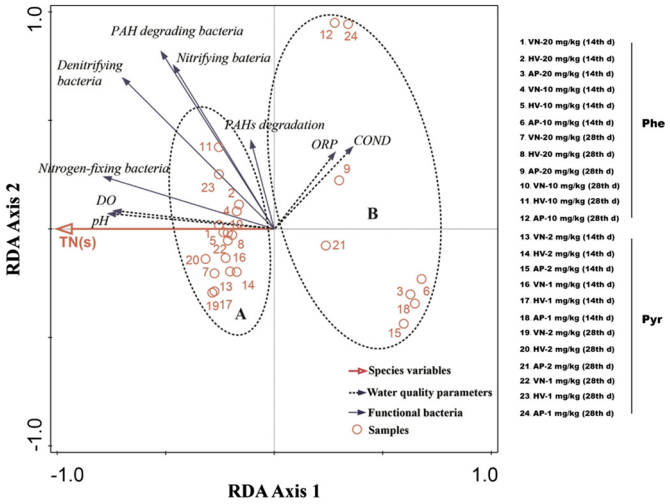

Fig. 4. RDA (redundancy analysis) depicted the relationships between total nitrogen in sediment (TNs) and the dissipation of PAHs, environmental factors and functional bacteria in biofilms on leaves/surface of all samples. (Group A: submerged macropytes samples; Group B: artificial plant samples). Note: PAHs degradation shows as percent reduction rate; functional bacteria shows as relative abundance. ORP (mV), COND (μS/cm) and DO (mg/L) show as number.

amount of information. The results also showed that the correlation coefficient between the first species axes and the first environmental axes was 0.7640, indicating that these axes were well correlated. The correlation coefficient between the two environmental axes was 0.000, indicating they were perpendicular. This demonstrated that the ordination results could reflect the relationships among TNs, functional bacteria and environmental parameters. We observed that TNs were positively correlated with pH and DO, but negatively correlated with ORP and COND. This result revealed that aquatic environmental conditions created by submerged macrophytes tended to cause TNs increasing, which were consistent with the study by Yan et al. (2017). While due to absorption of submerged macrophytes through root for growth, the concentration of TNs kept steady level generally (Fig. 2). PAHs degradation was positively correlated with PAHs degrading bacteria in biofilms on leaves/surface, which was consistent with similar studies in sediments (Guo et al., 2011). In addition, we observed that most of PAH-degrading bacteria pertained to denitrifying bacteria in our study. So, we speculated that sediment spiked by PAHs with higher concentration maybe cause releasing risk of greenhouse gas, N_2O (Battaglia and Joos, 2018). In addition, PAHs degradation percentage was positively correlated with nitrifying bacteria and denitrifying bacteria. In other words, the presence of PAHs might affect the nitrification and denitrification processes, which resulted in N accumulation in systems. Interestingly, nitrogen-fixing bacteria in biofilms on leaves/surface were positively correlated with TNs of sediment. Meanwhile, we observed that the relative abundance of nitrogen-fixing bacteria in biofilms-leaves of submerged macrophytes spiked by a high concentration of PAHs in sediments was significantly higher than those spiked by a low concentration of PAHs in sediments in 14th d ($p < 0.01$) (Fig. 3). Thus, we surmised that high concentration PAHs spiked in sediments might lead to TNs accumulation further, especially in AP systems in our study. However, there was no TNs accumulation in VN/HV systems generally, which might attribute to the role of submerged macrophtes, absorption on nitrogen and regulation on functional bacteria in biofilms-leaves. This contributes to avoiding the risk of threating aquatic organisms, ecological environment and human health, for example, microcystin production with algae explosion, high concentration ammonium and N_2O release (Liu et al., 2018; Ma et al., 2018; Yang et al., 2017).

3.5. Analysis of deduced bacterial functions based on COGs database

Microbial genome sequencing projects produce numerous sequences of deduced proteins. In order to annotate these proteins and assign to their functions, the Clusters of Orthologous Groups of proteins (COGs) database (http://www.ncbi.nlm.nih.gov/COG/) had been created and became a popular tool. Through matching with COG databases, there were 25 major functional COG categories found in biofilms-leaves/surface in our study (Fig. S3). We observed that several functions were associated with PAHs degradation and nitrogen transformation including amino acid transport and metabolism, nucleotide transport and metabolism, carbohydrate transport and metabolism, coenzyme transport and metabolism, translation, ribosomal structure and biogenesis, transcription, inorganic ion transport and metabolism, signal transduction mechanisms and defense mechanisms. The relative abundance of carbohydrate transport and metabolism in AP samples presented a little higher than in VN and HV samples. And coenzyme transport and metabolism and translation, ribosomal structure and biogenesis were more abundant in VN samples than in HV samples in 28th d. However, transcription and signal transduction metabolisms were found to be more abundant in HV samples than in VN samples in 28th d.

For understanding further, we figured out their difference among different groups divided by attached surface, spiking concentration and incubation time (Fig. 5). When sediment spiked by phenanthrene, cell mobility presented significant difference ($p < 0.05$) based on attached surface. Moreover, signal transduction mechanism, amino acid transport and metabolism and function-unknown presented significant difference ($p < 0.05$); and energy production and conversion, replication

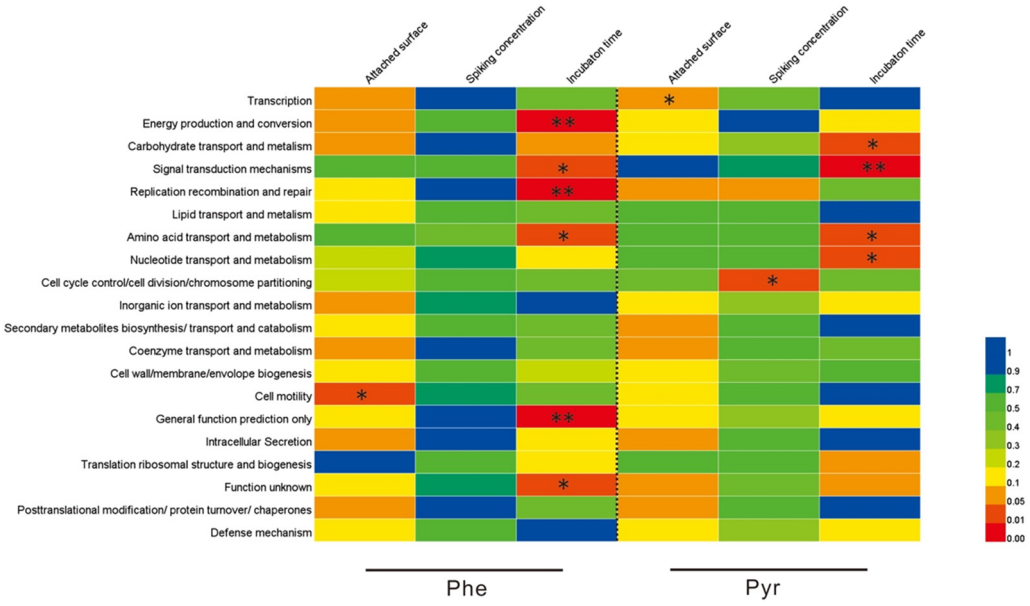

Fig. 5. Difference test among deduced functions depicted based on attached surface, spiking concentration of PAHs and incubation time. Note: "*" means $p < 0.05$; "**" means $p < 0.01$.

recombination and repair and general function prediction only presented extremely significant difference ($p < 0.01$) based on incubation time.

For sediment spiked by pyrene, transcription based on attached surface and cell cycle control/cell division/chromosome partitioning based on spiking concentration presented significant difference ($p < 0.05$). In addition, carbohydrate transport and metabolism, amino acid transport and metabolism and nucleotide transport and metabolism presented significant difference ($p < 0.05$); and signal transduction mechanism was up to extremely significant level ($p < 0.01$). We could easily figure out that the differences of deduced function were affected in order of incubation time > attached surface > spiking concentration. Meanwhile, we observed that six kinds of functions, energy production and conversion, carbohydrate transport and metabolism, signal transduction mechanism, amino acid transport and metabolism, nucleotide transport and metabolism and general function prediction, were all associated with nitrogen transformation and PAHs degradation. In terms of function-unknown, it needed our future research to explore.

4. Conclusion

PAHs degradation in sediment was positively correlated with PAH-degrading bacteria in biofilms on leaves/surface. Nitrogen (N) accumulation that appeared in the presence of PAH-polluted sediment might be associated with nitrogen-fixing bacteria increasing. Nitrogen-fixing bacteria tended to rise in early period as the increase of PAHs concentrations. PAHs residuals were negatively correlated with nitrifying and denitrifying bacteria, which might prevent the N releasing from aquatic systems. Besides the absorption ability of submerged macrophytes for nitrogen, their host-specificity suppressed the fluctuation of nitrogen transforming bacteria, which might alleviate the N accumulation of PAH-polluted sediments in constructed wetland systems. The relative abundances of functional bacteria were influenced by three factors, attached surface, incubation time and spiking concentration, which was not consistent with differences of deduced function. This study had shown that functional bacteria in biofilms on leaves were important for the phytoremediation of contaminated sediments and the regulation of nitrogen cycle. Therefore it is necessary to apply submerged macrophytes in remediating water pollution.

Declaration of Competing Interest

We declare that we have no financial and personal relationships with other people or organizations that can inappropriately influence our work, there is no professional or other personal interest of any nature or kind in any product, service and/or company that could be construed as influencing the position presented in, or the review of, the manuscript entitled, "The dissipation and risk alleviation mechanism of PAHs and nitrogen in constructed wetlands: the role of submerged macrophytes and their biofilms-leaves".

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.envint.2019.104940.

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