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The responding and ecological contribution of biofilm-leaves of submerged macrophytes on phenanthrene dissipation in sediments

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Abstract

The bacterial communities and ecological contribution of biofilm-leaves of the *Vallisneria natans* (VN), *Hydrilla verticillata* (HV) and artificial plant (AP) settled in sediments with different polluted levels of phenanthrene were investigated by high-throughput sequencing in different growth periods. There was no significant difference among the detected Alpha diversity indices based on three classification, attached surface, spiking concentration and incubation time. While Beta diversity analysis assessed by PCoA on operational taxonomic units (OTU) indicated that bacterial community structures were significantly influenced in order of attached surface > incubation time > spiking concentration of phenanthrene in sediment. Moreover, the results of hierarchical dendrograms and heat maps at genus level were consistent with PCoA analysis. We speculated that the weak influence of phenanthrene spiking concentration in sediment might be related to lower concentration and smaller concentration gradient of phenanthrene in leaves. Meanwhile, difference analysis suggested that attached surface was inclined to influence the rare genera up to significant level than incubation time. In general, the results proved that phenanthrene concentrations, submerged macrophytes categories and incubation time did influence the bacterial community of biofilm-leaves. In turn, results also showed a non-negligible ecological contribution of biofilm-leaves in dissipating the phenanthrene in sediments (>13.2%-17.1%) in contrast with rhizosphere remediation (2.5%-3.2% for HV and 9.9%-10.6% for VN).

Capsule: The bacterial community structures were influenced in order of attached surface > incubation time > spiking concentration of phenanthrene in sediment.

Key words: phenanthrene bioremediation; submerged macrophyte; biofilm-leaf; bacterial community; high-throughput sequencing

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are widely distributed environmental pollutants (Chen et al., 2015; Li et al., 2014), which result from incomplete combustion of fossil fuels and organic materials, and are often associated with industrial and human activities (Bamforth and Singleton, 2010). PAHs are highly carcinogenic, teratogenic and mutagenic compounds and can pose a great threat to human health through food-chain bioaccumulation (Moscoso et al., 2012). As demonstrated by studies on occurring, transporting, adsorbing and biodegrading, the concentrations of PAHs in natural environments can be detected in a wide scope from 1 ng/g to 300 mg/kg (Lu et al., 2012). PAHs typically precipitate and accumulate in sediments due to their hydrophobic properties in aquatic environment (Liang et al., 2007; Wang et al., 2003), which result in the sediment being the major sink of PAHs. Meanwhile, several studies indicate that sediments may be the second-most concentrated source of PAHs (Jérôme Cachot et al., 2007; Yang et al., 2008). PAHs observed in sediments mainly consist of 3-rings PAHs (e.g. phenanthrene) and 4-rings PAHs (e.g. pyrene) (Hassan et al., 2018; Lin et al., 2016). Dredging contaminated sediments is a common but disruptive practice which can lead to the re-suspension of pollutants (Agarwal et al., 2007). Therefore, exploring cost-effective, in situ approaches for sediment remediation has become increasingly important (Perelo, 2010).

Phytoremediation is a prospective alternative means because of its affordability, effectiveness and low environmental impact (Cheema et al., 2010; Gomes et al., 2013). A considerable amount of work has been done investigating the rhizosphere, which could

improve microbial population and diversity of soil surrounding plant roots (Ma et al., 2010). Such studies mainly focus on the rhizosphere of terrestrial plants, while submerged plants are the key and widespread species in coastal shallow water. Diverse microbiomes were often associated with submerged rhizoplanes, due to the unique environment that persists compared to their terrestrial counterparts (Srivastava et al., 2016; Zhao et al., 2017). This plant-microbes ecosystem could be manipulated to alleviate the condition of polluted sediments naturally. The high root oxygen loss (ROL) capacity of submerged macrophytes could contribute to nitrogen cycling in sediments: higher abundance of ammonia oxidizers increased denitrification, while nitrifies and denitrifying communities may associate more closely in these environments (Vila‐Costa et al., 2016).

Recently, the remediation strategy of using submerged macrophytes to deal with sediments polluted by PAHs has attracted widespread attention. Meng et al. (2015) showed that microbial degradation, rather than plant uptake, played a major role in the plant-promoted the dissipation of PAHs in sediments, and microbial degradation was not controlled by the amount of bioavailable PAHs - even if these were readily bioavailable. Dissipation ratio of PAHs was correlated with PAH-degrading bacteria population which in turn, linked positively with sediment redox potential, and low plant density of *Vallisneria spiralis* should be a better selection for phytoremediation (Liu et al., 2014). In addition, *Potamogeton crispus* greatly improved the bioavailability (73.9%) and biodegradation activity (277%) of pyrene in aged sediments in contrast with unaged sediments (13.1% and 150%, respectively) (Meng and Chi, 2016).

Diverse substances such as organic matter, silt, zoogloea, algae and other microorganisms often accumulate on the leaf surface of submerged plants. A micro-interface is therefore established which varies in its composition, structure and thickness depending on the actual environmental properties. This impacts the gas exchange between water and plants and induces specific heterogeneous oxidation – reduction environments. Numerous investigations have focused on the micro-interface of submerged macrophytes. Sand-Jensen et al. (1985) reported that dissolved oxygen (DO) on the micro-interfaces of leaves of *Potamogeton crispus*, *Littorella uniflora*，*Zostera marina* and *Scytosiphon lomentaria* increased with shorter distance from the leaf surfaces, and with increasing illumination intensity. In the daytime, the leaf surfaces of submerged macrophytes produced an oxygen-enriched environment, while a highly anaerobic environment appeared in night. Meanwhile, the pH in the micro-interfaces was greater than surrounding water (Jones et al., 2000; Sandjensen et al., 1985), and the difference value increased with the thickness of biofilm (Jones et al., 2000). Recently, high throughput sequencing, and next generation sequencing technology has provided gene sequence outputs that could be further analyzed to provide in-depth bacterial taxonomic assignments (Kim and Isaacson, 2015). Thus, some researchers studied the response and role of functional bacteria on biofilm-leaves for eutrophication, especially for nitrogen in aquatic environments through high throughput sequencing technology (Pang et al., 2016; Yan et al., 2017; Zhang et al., 2016). Little is known about the cumulative capacity of PAHs in the leaves of submerged macrophytes and their impact on the biofilm-leaves

when they release from sediments to water due to the characterization of hydrophobicity normally. However, Lipotropy of leaves of submerged plants makes them enrich PAHs from water (Diepens et al., 2014). PAHs may be mediated by microorganisms in micro-interfaces, especially bacteria. Thus, we hypothesized that the bacterial community in biofilm attached on leaves of submerged macrophytes suffering from PAHs might be altered and biofilm-leaves might contribute to the dissipation of PAHs in turn.

In our present study, phenanthrene was chosen as one of representative low molecular weight (LMW) PAHs because it is commonly found in sediments. The bacterial community and ecological contribution of biofilm-leaves were identified by high throughput sequencing, compared between an artificial plant and two submerged macrophytes in different growth periods, settled in sediments spiked with different phenanthrene concentrations. The objective of this study is to explore and identify the key driving factor influencing the bacterial community of biofilm-leaves and the ecological contribution of different dissipation mechanism (e.g. biofilm, submerged macrophytes, and background degradation) on phenanthrene dissipation in water and sediment.

2. Materials and methods

2.1 Materials

Vallisneria natans (VN) and *Hydrilla verticillata* (HV) (Nanjing Sam Creek aquatic breeding research base) were selected as the tested aquatic plants. A control group (CG) and artificial plants group (bio-racks with organic glass, AP) with similar surface area to submerged macrophytes were also tested. Sediments (pH=7.32, organic matter=2.14%,

130 background phenanthrene level=0.056 mg/kg) were collected from a suburb river of
131 Nanjing (not in the main industrial area), air-dried, manually crushed, and then sieved
132 with 2-mm mesh to remove plant residues and stone. Organic glass containers (40 × 50
133 cm) were chosen to cultivate submerged plants, to avoid loss of phenanthrene via
134 adsorption. The experiment was carried out in the ecological greenhouse with three
135 replicates for 35 d.

136 *2.2 Experimental setup*

137 The 0.6 g phenanthrene dissolved in acetone (1600 mL) was spiked into 6 kg sediment.
138 After acetone evaporating, the polluted sediment was mixed with unpolluted sediment
139 with their respective proportion, and laid in each container smoothly. The final contents
140 of phenanthrene in sediment (dry weight) were 20 mg/kg and 10 mg/kg, respectively.

141 0.01 g norfloxacin and 0.015 g roxithromycin / L water) were added into water to
142 domesticate for 3 – 5 d, which could remove or destroy the original biofilm-leaves. After
143 the completion of plant domestication, the robust and uniform plants were transplanted
144 into the containers. 50 L water was added to the container and the water-line was marked
145 clearly to replenish water to a uniform level throughout cultivation.

146 Water, leaf and sediment samples were collected for phenanthrene analysis at 14, 28
147 and 35 d. The samples were stored at -20℃ for PAHs analysis. The biofilm-leaves of
148 submerged plants and biofilm-surface of AP were extracted in 14 and 28 d, respectively.

149 *2.3 The separation of biofilm attached on the plants leaves*

150 The method of biofilm separation from leaf surfaces was modified from He et al. (2012).

With precool ethanol-PBS buffer as eluent, appropriate amount of leaves were put in the polyethylene test tube, then Triton solution and several 3 mm glass beads were added. All the sample tubes were placed in reciprocating oscillator with constant temperature and were shaken for 10 min (225 r/min), then underwent ultrasounds (150 W, 40 kHz) for 1 min. After filtrating elution liquor, the filtrate was centrifuged for 10 min (10000 rpm), and the centrifugal precipitate was collected. Artificial plant biofilms could be scraped directly with a sterile scalpel.

2.4 Analysis methods

2.4.1 Extracting and purifying of samples (Hussain et al., 2016; Zhao et al., 2014)

Sediment samples: 5.0 g of lyophilization sediment samples and 2.0 g anhydrous sodium sulfates were mixed and soaked in 15 mL extractant agent (hexane / acetone = 2/1, *v/v*) for 1 h in 50 mL glass centrifuge tube, and processed 10 min ultrasonic extraction. The above-mentioned process was repeated twice, and the supernatants were moved to the pear-shaped bottle and concentrated to 1 mL with vacuum rotary evaporator for purifying. After the Solid Phase Purification Column (SPPC, 0.5 g copper powder + 1 g anhydrous sodium sulfate + 1g silica gel + 1 g alumina pretreated) was rinsed and activated with 5 mL *n*-hexane, the concentrated extracting liquor was transferred to the SPPC and eluted by 15 mL *n*-hexane and 10 mL dichloromethane. The obtained eluent was concentrated and adjusted to 1 mL with *n*-hexane.

Water samples (Zhao et al., 2018): After each water sample (1 L) was filtrated through glass fiber membrane of 0.45 μm and extracted with C18 SPE column, the column was

172 washed by HPLC water, and pumped for 30 minutes to remove redundant moisture. The
173 PAHs in the SPE column was eluted with 10 mL of acetone/*n*-hexane (*v/v*=1/2) and 12
174 mL of dichloromethane/*n*-hexane (*v/v*=3/2). The eluent was concentrated and adjusted to
175 1 mL with *n*-hexane.

176 Plant samples (Tao et al., 2006; Zhao et al., 2018): 5.0 g plant samples and 5.0 g
177 anhydrous sodium sulfates were packed in a filter paper parcel and extracted in Soxhlet
178 Extractor for 10 h with 100 mL 10% of acetone/*n*-hexane (*v/v*) mixed solvent, and the
179 extracted liquid was washed twice in separating funnel by 5% sodium sulfate solution for
180 removing acetone. The extract of *n*-hexane was concentrated to about 1 mL and dealt with
181 20 mL concentrated sulfuric acid to get rid of the fat in plant samples twice. Finally, the
182 extract was put into SPPC, the purification and elution steps were same as sediments.

183 *2.4.2 Determination of phenanthrene* (Jiao et al., 2007).

184 The phenanthrene was analyzed by Agilent1100 HPLC with fluorescence and UV -
185 adsorption detector. A 250 mm × 4.6 mm × 5 μm reversed phase C18 column (Agilent
186 ZORBAX Eclipse XDB-C18) was used as the stationary phase. A solution of acetonitrile
187 and ultrapure water was delivered as the mobile phase in a gradient programme at 1
188 mL/min. The volume ratio of acetonitrile and water was 75: 25. Phenanthrene was
189 quantified by using external standard solutions sourced from Ehrenstorfer (Augsburg,
190 Germany). Detective wavelength with FLD signals: Exλ=257 nm, Emλ=380 nm.

191 *2.4.3 DNA extraction, PCR amplification, sequencing and data analysis*

192 Weighing 0.5 g biofilm-leaves/-surface samples in 2 mL centrifuge tube, the bacterial

DNA 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 contained 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 Plantium Taq (5 U/μL), 10 ng DNA template, with sterile water added to make the final volume to 50 μL. The following PCR cycle was performed: 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 using Illumina MiSeq platform (Illumina, San Diego, CA, USA).

Sequencing analysis was executed by using QIIME (Yu et al., 2017). The Uclust method was used to pick the representative sequences for each operational taxonomic units (OTUs). Then the taxonomic information was annotated with the SILVA database after subsampling based on the lowest number of reads. The number of reads ranged from 35410 to 46153. Alpha diversity refers to the diversity of microorganism in a special region or ecosystem. A number of Alpha diversity measures were evaluated using the

Mothur software including the abundance based coverage estimator (ACE), terminal richness estimation (Chao1), the Shannon index, Simpson index and the Good's coverage estimation. Statistical analyses were performed using R software, including rank-abundance curves, microbial community composition at phylum and genus level, PCoA and hierarchical cluster analysis using Bray-Curtis algorithm. Among them, PCoA and hierarchical cluster analysis were usually used to research the similarity or difference of microbial community composition of different samples group. Heat maps generated by HemI can reflect visually the difference in abundance distribution of species between different groups. The difference of microbial communities at genus level was analyzed by Kruskal-wallis H test and Wilcoxon rank-sum test using STAMP software, which is used to assess the difference of Alpha diversity indices based on three classifications. In addition, one-way ANOVA was applied to check the difference of ECI between four systems.

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

3. Results and discussion

3.1 Phenanthrene dissipation and ecological contribution of submerged macrophytes and their biofilm-leaves

3.1.1 Dissipation of phenanthrene in water, sediment and leaves

During the experiment, phenanthrene concentration in sediments declined with the time in all the treatments (Fig. 1a). The dissipated efficiencies were in the order of VN

(62.7% for 20 mg/kg and 64.1% for 10 mg/kg) > HV (51.1% and 58.4%) ≧ AP (51.6% and 53.5%) > CG (35.8% and 42.4%). The results indicated that submerged macrophytes settled in sediments could contribute to the removal of phenanthrene, with dissipation rates dependent on the type of submerged macrophytes. This may relate to the capabilities of oxygenation and microbiological degradation of root tissue (He and Chi, 2016) and the biofilm-leaves.

The phenanthrene concentrations in water were rather low because of its hydrophobicity and low solubility (Fig. 1b); most phenanthrene was adsorbed in sediments (Fig. 1a) or on the surface of submerged macrophytes (Fig. 1c). The phenanthrene concentrations in water showed an initial increase followed by a gradual decrease slowly (Fig. 1b), while the phenanthrene concentrations in leaves always kept a relatively stable level in two kinds of hydrophyte leaves and were approximately 200 times greater than in water (Fig. 1b, Fig. 1c). The concentrations of phenanthrene in leaves were lower by an order of magnitude than in sediments (Fig. 1a, Fig. 1c), due to the mutual exchange balance of PAHs among overlying water, leaves and sediment (Diepens et al., 2014) and low level of phenanthrene in water naturally.

3.1.2 Ecological contribution of submerged macrophytes and their biofilm-leaves

Actually, the dissipation mechanisms of phenanthrene in different treatments system are very complicated and include many dissipated processes, which may contain synergies and antagonisms and be difficult to identify the sole dissipated contribution. In order to understand the combined dissipation ecological contribution of different

256 treatments (e.g. background dissipation, volatilization, photolysis) well, we regarded the
257 dissipation of phenanthrene as the sum of dissipated contribution of different dissipation
258 mechanism and ignored the synergies and antagonisms among them probably.
259 Considering the giant area of leaves and its ability of releasing oxygen of submerged
260 macrophytes at the same time, we should give weight to the contribution of biofilm-leaves
261 in remediating PAHs polluted sediments. So, we set an ecological contribution index (ECI)
262 to evaluate the contribution of each system in remediating Phe-polluted sediments (Fig.
263 1d).

264 ECI= (spiked conc.-residual conc.) ∗100/ spiked conc. % (1)

265 ECI was in order of VN (36.8%-62.7%) > HV (30.2%-51.10%) > AP (27.6%-51.6%) >
266 CG (7.1%-35.8%) in 20 mg/kg phenanthrene system, and VN (40.3%-64.1%) > HV
267 (33.2%-58.4%) > AP (30.3%-53.5%) > CG (14.6%-42.4%) in 10 mg/kg phenanthrene
268 system from 14 d to 35 d, respectively. One-way ANOVA followed by DUNCAN post
269 hoc test was performed to check the difference of ECI among these four systems. And
270 results showed that there were no significant difference appeared regarding the PAHs
271 concentration (p=0.078 for 20 mg/kg; p=0.233 for 10 mg/kg); however, the ECI of
272 VN/HV system, especially for VN system was higher than AP/CG system spiked by 20
273 mg/kg phenanthrene rather than 10 mg/kg phenanthrene. Actually, the dissipation of
274 phenanthrene in CG system comes from the background dissipating ability, which
275 includes the volatilization, photolysis, release from sediment to water, microbiological
276 degradation in water and sediment. The dissipation of phenanthrene in AP system comes

from the background dissipating ability and the adsorption of artificial plant and the degradation by the biofilm attached on the artificial plant surface. In the submerged macrophytes system, phenanthrene dissipation can be explained by the action of rhizosphere microbes, in addition to the factors acting in the AP. The difference of dissipation between the AP and CG systems can represent the ecological contribution of artificial plant biofilm (15.0%-20.5% for 20 mg/kg phenanthrene and 11.1%-15.6% for 10 mg/kg phenanthrene, and the averages were 17.1% and 13.2%, respectively), and the ecological contribution of rhizosphere microbes can be represented by the difference of dissipation between submerged macrophytes system and AP system (9.2%-11.7% in 20 mg/kg phenanthrene and 9.1%-10.6% in 10 mg/kg phenanthrene system for VN, the average was 9.9%-10.6%; and 0%-5.4% in 20 mg/kg phenanthrene system and 1.6%-4.9% in 10 mg/kg phenanthrene system for HV, the average was 2.5%-3.2%).

Our research confirmed phenanthrene released from sediments to water then accumulated on the leaves surface of submerged macrophytes. We also noticed the dissipation of phenanthrene in water and sediments during the cultivation process (Fig. 1a and Fig. 1b). It may be related to the dissipation induced by physical and chemical process (e.g. photodecomposition), the enrichment and dissipation of hydrophyte (Li et al., 2009), and the degradation of microorganisms. In addition, the added organic matters could improve the adsorption capacity of sediment on phenanthrene because the dead leaves sank into sediments later (Ying-Heng et al., 2014). However, the steady phenanthrene concentration in the leaves of submerged macrophytes might be related to

298 the length of incubation time (Liu et al., 2014).

299

300 **Figure 1**

301

302 *3.2 Bacterial diversity analysis based on attached surface, spiking concentration and*
303 *incubation time*

304 *3.2.1 Alpha diversity*

305 Alpha diversity can show the diversity of microorganism in a special region or
306 ecosystem. In this study, the alpha diversities of biofilm microbial indices for leaf surface
307 were shown in Table 1. ACE and Chao 1 are designed to estimate the community richness
308 of biofilm samples based on the OTU numbers. The values of ACE and Chao 1 showed
309 that the community richness of microbes in artificial plant samples (1C and 2C) were far
310 higher than those submerged plant samples (1A, 2A, 3A, 4A and 1B, 2B, 3B, 4B), and
311 the community richness of VN were slightly higher than those of HV. The Shannon and
312 Simpson indices reflect community diversity. In our present study, the community
313 diversity was consistent with community richness. The incubation time significantly
314 affected the community diversity of biofilm samples, and those of 14 d samples were
315 higher than those of 28 d samples. However, the effect of phenanthrene concentration was
316 indistinctive. The coverage index represents the sequencing depth. In the study, the
317 coverage was approximately equal to 0.99 except for 1C (0.97) and 2C (0.97) after
318 subsampling based on the lowest number of reads, and indicating all the samples reflected

the actual situation. Meanwhile, the difference was analyzed among Alpha indices based on three classifications (attached surface, spiking concentration of phenanthrene and incubation time) using Wilcoxon rank-sum test (Table S1). However, there was not statistically significant difference regretfully ($p < 0.05$, see the Table S1).

Table 1

3.2.2 Taxon richness and distribution evenness analysis: Rank-abundance distribution curves, Shannon-Wiener curves and Rarefaction curves of the OTUs

Applying the rank-abundance curve to analyze species distribution was an imperative manner. On account of computing the sequencing numbers which each OTU contained, researchers sorted by OTUs in descending order and depicted the relevant relations in accordance with abundance. The curves can reflect both species abundance and distribution evenness (Cheng et al., 2016). Fig. 2 showed that the distribution ranges of AP were wider than SM, which demonstrated that the species in the biofilm-surface of AP were more abundant. The curve graph of SM was smaller than that of AP, which meant that the species distribution was more even in AP. The rank-abundance distribution corresponded to the analysis of community diversity using Alpha diversity indices.

The Shannon-Wiener curves takes richness and evenness of samples into account. Sample 10B had the highest diversity (6.64) and followed by sample 9B (6.53), while the sample 3B had the lowest diversity (4.00) (Fig S1a). The reads of each sample were large

enough (>25,000 tags per sample) to reflect huge diversity of microbial community because they reached the plateau since less than 5,000 tags for each sample. Rarefaction analysis was applied to standardize and compare taxon richness observed among samples (Fig. S1b). The rarefaction curves based on OTUs (97% similarity) presented a generally consistent tendency for SM rather than AP. These results showed that recovered sequences reflected the diversity of microbial community well, and further sampling could reveal the diversity of microbial community in AP to some extent.

3.2.3 Beta diversity

The Beta diversity is good at comparing the microbial community composition among different sample groups. Here, it was assessed by PCoA based on OTU level, and the results were illustrated in Fig. 3a. Principal components 1 (PC1) and PC2 explained 42.5% and 18.79% of variation of microbial community composition, respectively. Generally, the samples were divided into three groups (group A, group B and group C). The different attached surface (AP: group C *vs.* SM: group A and group B) had a dominant influence on the microbial community structure, but the difference among submerged macrophytes was small. The separation of group A and group B indicated that incubation time also influenced the bacterial community composition. However, the spiking concentration of phenanthrene just presented marked effect on the community structure in VN only.

Figure 2

3.3 Comparison and difference analysis based on attached surface, spiking concentration

and incubation time at genus level

The bacterial community structures of AP and SM samples at phylum level are shown in Fig. S3. The results reflected that the diversity and evenness of bacterial community on the surface of artificial plant were higher than those on the leaves of submerged macrophytes again. In order to identify and understand the key impact factors (e.g. submerged plants, spiking concentration of and incubation time) on the microorganism composition of biofilm-leaves, Hierarchical clustering analysis, heat map, Kruskal-wallis H test and Wilcoxon rank-sum test were used to analysis and visually show the difference significant of different impact factors.

3.3.1 Hierarchical clustering analysis

Hierarchical clustering dendrograms were usually used to research the similarity or difference of microbial community composition of different samples. And in our study, they were generated at genus level based on attached surface, spiking concentration and incubation time, respectively. From Fig. 3b, all the samples were separated into two distinct groups: AP and SM. And compared to HV, VN had a stronger influence on bacterial community composition at genus level except for sample 1A. While different spiking concentration of phenanthrene had a weak impact on the bacterial community structures at genus level (Fig. 3c). Moreover, bacterial community structures changed along with incubation time at genus level except for 3A (Fig. 3d). These results were consistent with PCoA.

3.3.2 Visual display of difference: Heat maps of bacterial communities

Attached surface, spiking concentration of phenanthrene and incubation time affected bacterial community in one way or another. Heat maps of bacterial communities in top 50 genera based on attached surface, spiking concentration and incubation time were drawn to highlight distinctly different specific genera (Fig. 5). Fig. 5a showed that 8 genera were abundant in the bacterial community of samples AP, but were rarely identified and appeared in samples SM, including *Pseudomonas*, *norank_c__Acidobacteria*, *norank_f__Anaerolineaceae*, RB1, *Geobacter*, *Nitrospira*, *unclassified_f__Micrococcaceae* and *Sphingomonas*. Previous studies showed that *Pseudomonas* and *Sphingomonas* were PAH-degrading bacteria, and *nitrospira sp.* was associated with nitrogen cycling. *Norank_c__Cyanobacteria*, *unclassified_f__Comamonadaceae* and *Pirellua* were abundant in samples SM. A few genera were abundant in samples VN, such as *unclassified_c__Cyanobacteria*, *Limnothrix* and *unclassified_f__Family*I_*o__ Subsection*I.

Fig. 5b presented *unclassified_f__Family*I_*o__Subsection*I, *unclassified_f__ Comamonadaceae*, *unclassified_c__Cyanobacteria*, *Limnothrix* and *Pseudorhodoferax* were observed to be abundant in the lower concentration groups (10 mg/kg), while only one genus, *norank_c__Cyanobacteria*, was found in abundance among the higher concentration groups (20 mg/kg).

Only two genera, *Limnothrix* and *Ideonella*, were detected to be abundant in 14 d (Fig. 5c), while *unclassified_c__Cyanobacteria*, *Pesudorhodoferax*, *norank_f__Family*I, *unclassified_f__Family*I_*o__Subsection*I and *norank_o__Caenarcaniphilales* were

403 abundant in 28 d.

404 **Figure 3**

405

406 *3.3.3 Difference significance level analysis: Kruskal-wallis H test and Wilcoxon rank-*
407 *sum test*

408 In order to assess the significance level of abundance difference of species and obtain
409 the significantly different species, Kruskal-wallis H test and Wilcoxon rank-sum test were
410 used to identify the difference between the top 60 genera based on attached surface (Fig.
411 6a), and top 15 genera based on spiking concentration of phenanthrene (Fig. 6b) and
412 incubation time (Fig. 6c), respectively. Only five genera including
413 *norank_c__Cyanobacteria* (*p*=0.04227), *Limnothrix* (*p*=0.04973), *norank_f__MNG7*
414 (*p*=0.0197), *norank_f__Gemmatimonadaceae* (*p*=0.0379) and
415 *unclassified_f__Family*I_*o__Subsection*III (*p*=0.01927) presented a significant
416 difference from top 40 genera in Fig. 4a. We observed that *norank_c__Cyanobacteria*,
417 *Limnothrix* and *unclassified_f__Family*I_*o__Subsection*III belonged to the phylum of
418 Cyanobacteria, *norank_f__MNG7* belonged to the phylum of Proteobacteria, and
419 *norank_f__Gemmatimonadaceae* belonged to the phylum of Gemmatimonadetes.
420 However, there were 9 genera presented significant difference from the last 15 rare genera
421 in top 60 genera. There was no significant difference between the top 15 genera (Fig 4b).
422 Seen in Fig. 4c, four genera in top 15 genera presented significant difference consisting
423 of *Ideonella* (*p*=0.03038), *Bryobacter* (*p*=0.03038), *Gemmata* (*p*=0.03038) and

Planctomyces (p=0.03038). *Ideonella* belonged to the phylum of Proteobacteria, *Gemmata* and *Planctomyces* belonged to the phylum of Planctomycetes. These results indicated that attached surface dominating in bacterial community tended to influence the genera with lower relative abundance more than incubation time, while spiking concentration of phenanthrene did not tend to affect the genera significantly.

Figure 4

3.4 The influence mechanism of attached surface, spiking concentration and incubation time on microbial community

In our present study, SM had a more important role in biofilm establishment than AP. This was most likely related to allelochemicals secreted by submerged macrophytes (e.g. phenolic acids, fatty acids, alkaloids, terpenes and flavonoids) (Weston and Mathesius, 2013; Zi et al., 2014), which could restrict the growth of algae, cyanobacteria and heterotrophic bacteria, and affected biofilm composition. In addition, submerged macrophytes not only secreted organic substances to provide carbon source for the microorganisms of the biofilm, but also released oxygen by photosynthesis, which leaded to the change of micro-interfaces environment (e.g. DO, ORP and pH) day and night. These activities also influenced the composition of bacterial communities attached on leaves of submerged plants (Hempel et al., 2009). Of course, the morphological and physiological complexity of submerged macrophytes could provide a diversity of

445 microhabitats for organisms to live in (Goldsborough et al., 2005). Several researchers
446 had insisted that periphyton communities correlated with macrophytes were highly host-
447 specific (Kahlert and Pettersson, 2002; Wetzel, 1983).

448 The bacterial community was not obviously affected by the phenanthrene spiked in
449 surficial sediments in different concentrations. This might be related to the lower
450 concentration and smaller concentration gradient of phenanthrene on the leaves surface
451 by different migration routine (Fig. 1c). Phenanthrene can transfer in the macrophytes
452 tissues from root to leaves after it is absorbed from sediment; however, this may not be a
453 reliable pathway as phenanthrene translocation from roots to shoots can be limited (Liu
454 et al., 2014), let alone to leaves. An alternative explanation is that phenanthrene is released
455 from sediments into water and then to leaves. Recent studies suggested that phenanthrene
456 could be dissipated by rhizospheric microorganisms or fixed by sediments (He and Chi,
457 2016; He et al., 2016; Liu et al., 2014), and small amounts of phenanthrene were released
458 from sediments due to low solubility in water (1.18 mg/L). In addition, leaves of
459 submerged macrophytes could absorb from or release to overlying water for phenanthrene
460 (Diepens et al., 2014). Thus the contents of phenanthrene accumulated in leaves of
461 submerged macrophytes were rather limited (Fig. 1c and Fig. 1d), making it difficult to
462 change the composition of biofilm. If phenanthrene was directly accumulated by leaves
463 from water, results would be other cases which needed our future study.

464 Incubation time had a marked effect on the bacterial community of biofilm attached on
465 leaves of submerged macrophytes. We attributed this phenomenon to the growth status of

biofilm possibly (Cai et al., 2013). Several researches suggested that epiphytic microbes were difficult to attach on the healthy leaves (Jennings and Steinberg, 1997; Peterson et al., 2007). And the self-destruction of aged leaves produced plenty of dissolved substances to facilitate the development of epiphytic microbes. Meanwhile, the phenanthrene accumulated in leaves of submerged macrophytes was stable from 14 d to 35 d. We did not find out the PAH-degrading bacteria presented significant difference (Fig. 6c), which might be related to the reality that these bacteria did not occupy the dominating positions in nature. In our future study, we will put more emphasis on the ascertaining response of the functional bacteria in the biofilm attached on leaves of submerged macrophytes to organic and high-toxic pollutants like PAHs.

4. Conclusions

Our study suggested that biofilm-leaves could contribute to remediation of sediment polluted by phenanthrene. Attached surface had an important effect on bacterial community composition of biofilm-leaves/-surface due to the active interface influence of submerged macrophytes. Incubation time changed the bacterial community, which might explain differences in growth and mature state of the biofilm. Phenanthrene spiking concentration in sediment did not appear to markedly influence bacterial community – which may be due to the lower concentration and smaller concentration gradient of phenanthrene on the leaf surfaces. On the whole, phenanthrene concentration spiked in sediment, aquatic plant categories and incubation time affected the bacterial community on biofilm-leaves with varying degrees.

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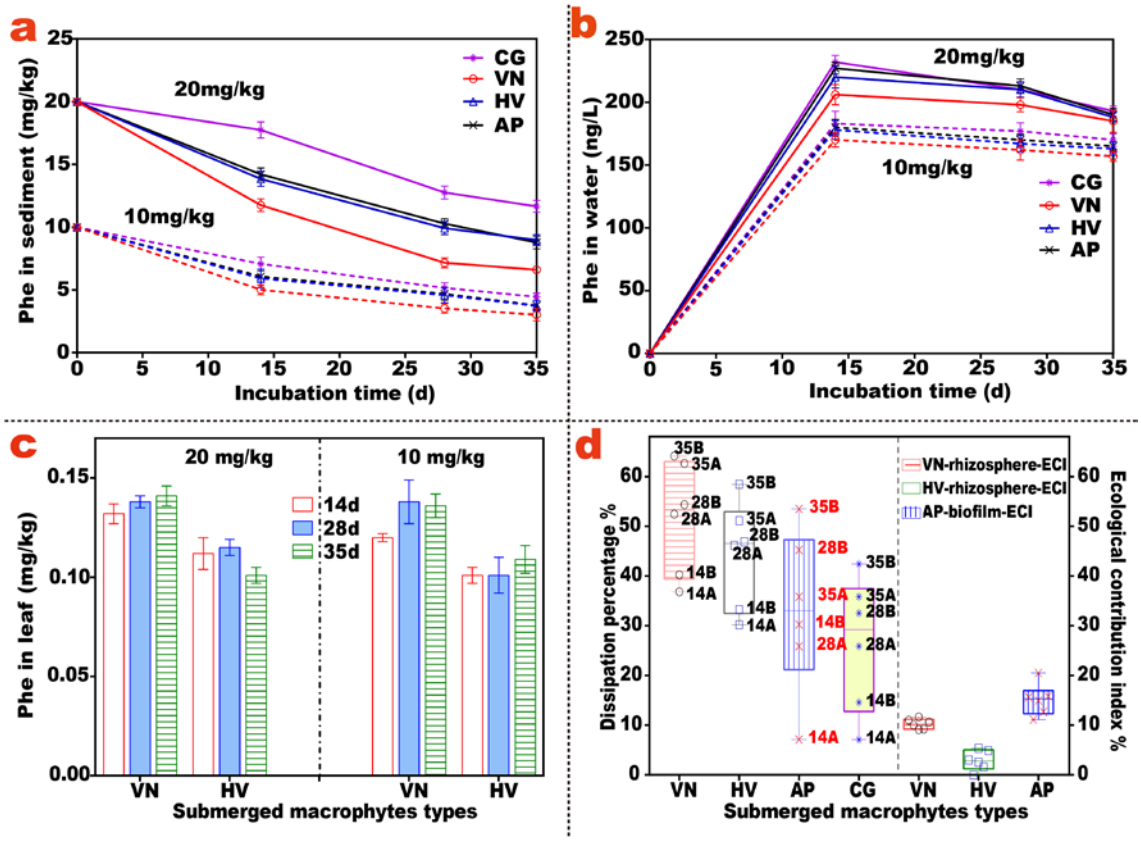
646
647

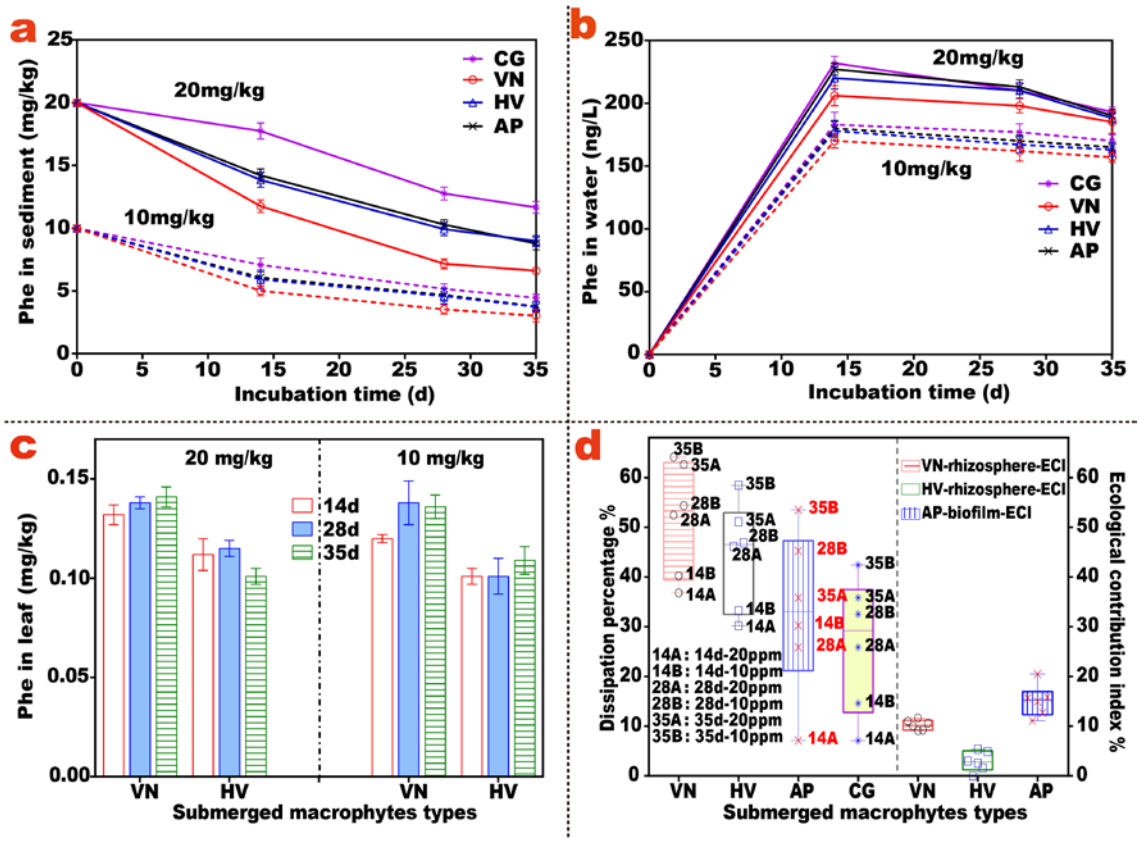
Table 1. Microbial diversity indices in all ten samples

Samples NO	Phe Conc. mg/kg	Sampling Time	Submerged plants	ACE	Chao 1	Shannon	Simpson	Coverage (%)
1A	20	14d	VN	1535	1526	5.24	0.0156	98.8
2A	20		HV	1561	1593	5.08	0.0254	98.8
3A	10		VN	1551	1617	5.11	0.0240	98.7
4A	10		HV	1591	1621	5.39	0.0129	98.8
1B	20	28d	VN	1568	1590	5.11	0.0236	98.8
2B	20		HV	1451	1550	4.90	0.0407	98.9
3B	10		VN	1894	1694	4.00	0.0761	98.5
4B	10		HV	1262	1265	4.94	0.0213	99.0
1C	20	28d	AP	3916	3823	6.53	0.0053	96.7
2C	10		AP	4070	4036	6.63	0.0061	96.6

Note: VN: *Vallisneria natans;* HV: *Hydrilla verticillata;* AP: artificial plants

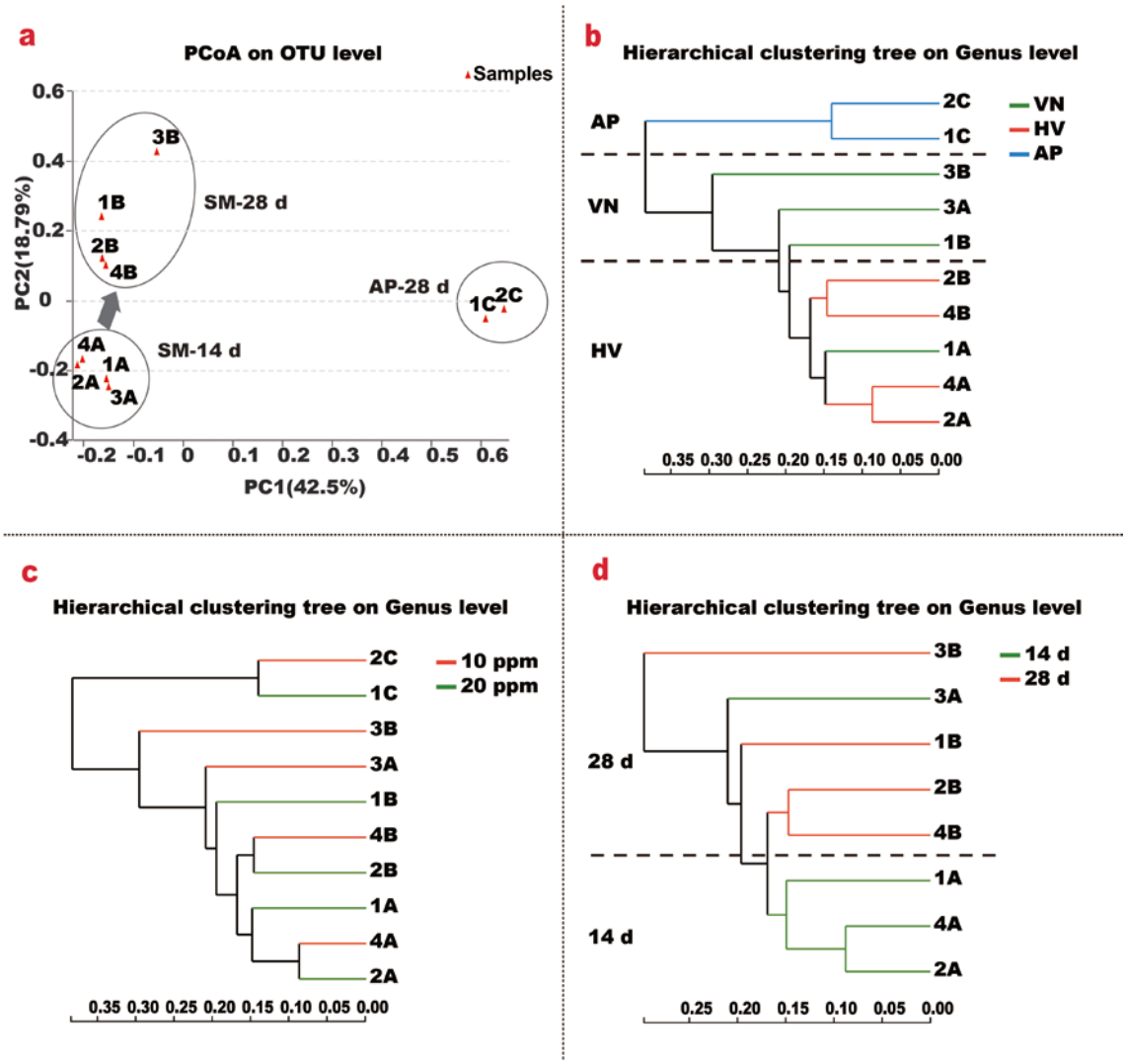

a
Phe in sediment (mg/kg)
20mg/kg
10mg/kg
CG
VN
HV
AP
Incubation time (d)
b
Phe in water (ng/L)
20mg/kg
10mg/kg
CG
VN
HV
AP
Incubation time (d)
c
Phe in leaf (mg/kg)
20 mg/kg
10 mg/kg
14d
28d
35d
VN
HV
VN
HV
Submerged macrophytes types
d
Dissipation percentage %
Ecological contribution index %
VN-rhizosphere-ECI
HV-rhizosphere-ECI
AP-biofilm-ECI
35B
35A
28B
28A
14B
14A
VN
HV
AP
CG
VN
HV
AP
Submerged macrophytes types

651


a
Phe in sediment (mg/kg)
20mg/kg
10mg/kg
CG
VN
HV
AP
Incubation time (d)
b
Phe in water (ng/L)
20mg/kg
10mg/kg
CG
VN
HV
AP
Incubation time (d)
c
Phe in leaf (mg/kg)
20 mg/kg
10 mg/kg
14d
28d
35d
VN
HV
VN
HV
Submerged macrophytes types
d
Dissipation percentage %
Ecological contribution index %
VN-rhizosphere-ECI
HV-rhizosphere-ECI
AP-biofilm-ECI
35B
35A
28B
28A
14B
14A
14A: 14d-20ppm
14B: 14d-10ppm
28A: 28d-20ppm
28B: 28d-10ppm
35A: 35d-20ppm
35B: 35d-10ppm
VN
HV
AP
CG
VN
HV
AP
Submerged macrophytes types

652

Fig. 1. The residual characteristics of phenanthrene in sediment (**a**), in water (**b**), in leaves of VN and HV (**c**). The dissipation percentage in sediment of VN, HV, AP and CG system, ecological contribution index (ECI) of VN, HV-rhizosphere and AP-biofilm (**d**). The 20 mg/kg and 10 mg/kg of phenanthrene concentrations were spiked in sediment initially, respectively. (CG: control group; AP: artificial plant; HV: *Hydrilla verticillata*; VN: *Vallisneria natans*. 14A: 14 d-20 ppm; 14B: 14 d-10 ppm; 28A: 28 d-20 ppm; 28 B: 28 d-10 ppm; 35A: 35 d-20 ppm; 35B: 35 d-10 ppm.)


a
PCoA on OTU level
Samples
PC2(18.79%)
PC1(42.5%)
SM-28 d
SM-14 d
AP-28 d
3B
1B
2B
4B
4A
1A
2A
3A
1C
2C
b
Hierarchical clustering tree on Genus level
AP
VN
HV
VN
HV
AP
0.35 0.30 0.25 0.20 0.15 0.10 0.05 0.00
c
Hierarchical clustering tree on Genus level
10 ppm
20 ppm
0.35 0.30 0.25 0.20 0.15 0.10 0.05 0.00
d
Hierarchical clustering tree on Genus level
14 d
28 d
28 d
14 d
0.25 0.20 0.15 0.10 0.05 0.00

662

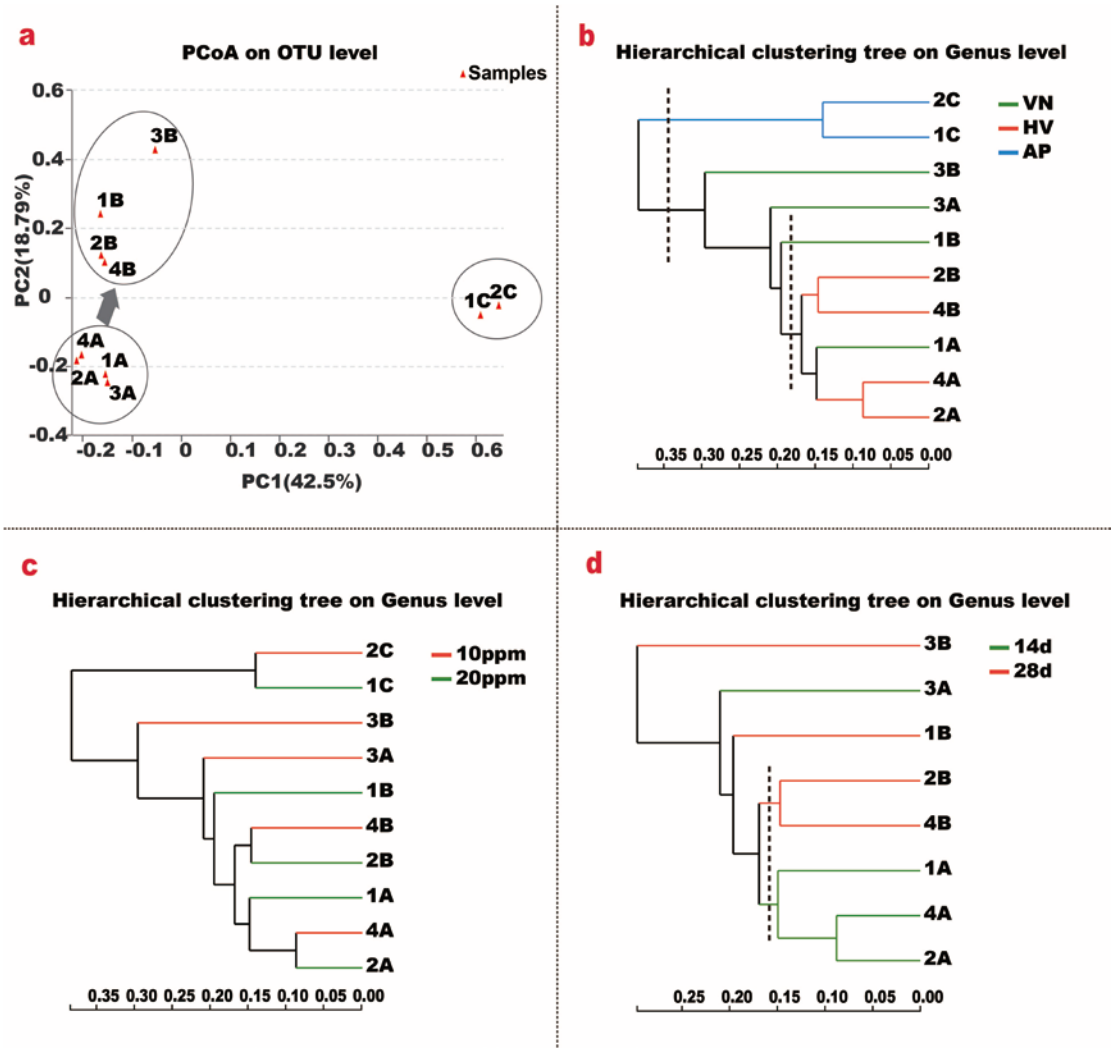
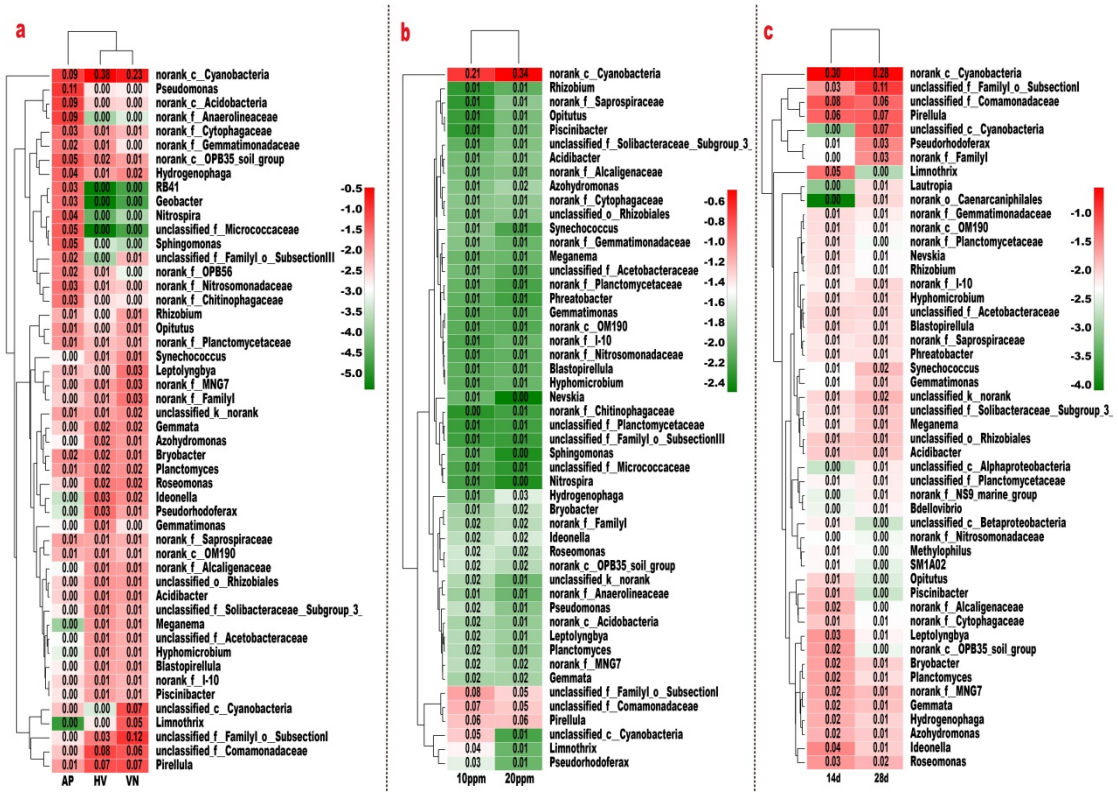


Fig. 2. a: Principal coordinates analysis (PCoA) using Bray-Curtis distances for all the samples on OTU level; **b**, **c** and **d**: Hierarchical clustering tree using average linkage between samples at genus level based on attached surface (**b**), spiking concentration of phenanthrene in sediment (**c**, 10 mg/kg and 20 mg/kg) and incubation time (**d**, 14 d and 28 d: the time of collected samples), respectively. (VN for *Vallisneria natans*; HV for *Hydrilla verticillata*; SM means submerged macrophytes for VN+ HV; AP for artificial plants)



673

674

675 **Fig. 3.** Heat maps of the bacterial community in top 50 genera based on attached surface

676 (**a**, AP for artificial plant; HV for *Hydrilla verticillata*; VN for *Vallisneria natans*), spiking

677 concentration (**b**, the sediments were spiked by 10 mg/kg and 20 mg/kg phenanthrene)

678 and incubation time (**c**, 14 d and 28 d: the time of collected samples). The red corresponds

679 to higher relative abundance and the green to lower relative abundance.

680

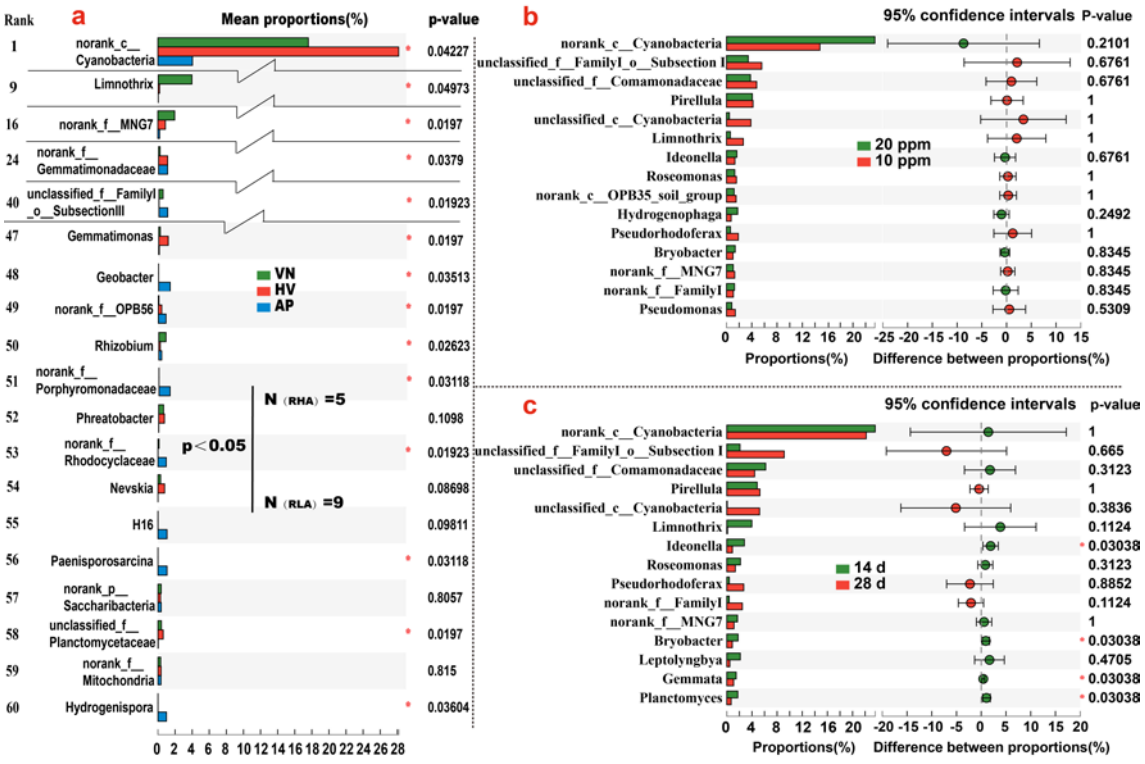


Fig. 4. Difference test between genera based on attached surface (**a**, AP for artificial plant; HV for *Hydrilla verticillata*; VN for *Vallisneria natans*; $N_{(RHA)}$ means the number of species with relative high abundance; $N_{(RLA)}$ means the number of species with relative low abundance), spiking concentration (**b**, the sediments were spiked by 10 mg/kg and 20 mg/kg phenanthrene) and incubation time (**c**, 14 d and 28 d: the time of collected samples) .

Notes: "*" denotes differences were statistically significant ($p < 0.05$)