Title: Performance of constructed wetlands and associated mechanisms of PAHs removal with mussels

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Abstract

The role of mussels in laboratory-scale constructed wetlands (CWs) where wastewater was contaminated with five typical polycyclic aromatic hydrocarbons (PAHs) (three 3-ring PAHs and two 4-ring PAHs) was investigated in this study. The CWs performance and PAHs removal mechanisms were well studied. Results indicated that these five PAHs improved removal efficiencies of NO_3-N but in the case of NH_4-N accumulation occurred. Of the five added PAHs, the 4-ring PAHs were more refractory with higher concentrations in effluent than 3-ring PAHs. By monitoring the five PAHs concentration in water, mussels had excellent removal efficiency of the five PAHs (97%). According to the mass balance calculation, mussels promoted plant uptake of five PAHs, contributing 15.2% of five PAHs removal in CWs. The PAHs could also accumulate in mussels through ingested substrate. Thus, mussels presented a positive correlation with five PAHs purification at a depth of 0-10 cm in substrate, which was 34.7 μg/kg lower than the control group. Due to the purification and enhanced aerated degradation, mussels performed better in removing 3-ring PAHs in substrate, decreasing 8.3% for 3-ring PAHs compared to the control. A positive correlation between five PAHs addition and *nirS*, *nrfA* genes was observed. However, PAHs showed negative impact on nitrifying bacteria (*amoA*). The significant correlations between mussels and five PAHs made it possible to improve CWs for PAHs treatment with aquatic faunas.

Keywords: Constructed wetlands; Mussels; PAHs; Removal mechanism.

1. Introduction

Polycyclic aromatic hydrocarbons (PAHs) are important organic contaminants and they are widespread with hyper toxicity, and carcinogenic properties [1]. Over the past 30 years, the PAHs mainly originated from anthropogenic processes in urbanized or industrialized regions, such as vehicle exhausts and coal combustion [2]. PAHs accumulate in the aquatic environment and are difficult to be biodegraded. Due to their low solubility and high hydrophobicity, PAHs contaminants in the aquatic ecosystem are adsorbed onto suspended particles and finally accumulate in sediments instead of dissolving in water [3]. They have been shown to constitute a major threat to a wide variety of fishes, invertebrates, and even natural ecosystems [4, 5].

Constructed wetland (CW), as an economic technology, has been used to remove common pollutants such as chemical oxygen demand (COD), nitrogen and phosphorus. However, not much research has focused on specific organic pollutants such as PAHs in CWs [6, 7]. In the presented study, settling and sedimentation onto a substrate were considered to be the main removal processes for PAHs in examined systems, but they could not remove the contaminants permanently. PAHs-organic matter bonding to a substrate reduces the mobility of contaminants and encourages PAHs to be more resistant to degradation in the anoxic environment [8]. Therefore, the contamination caused by the PAHs was much more harmful in the substrate phase than in the water phase. To mitigate the risks to ecological health induced by PAHs contamination, several current research projects have attempted to increase the bioavailability of PAHs through surfactants and co-substrates addition. However, this increases the costs and causes secondary pollution [9]. Although limited by the generally low bioavailability of PAHs, transformation by microorganisms is one of the main contributors to decreased PAH-contaminated sites in deep substrate [10]. Compared to physical and chemical remediation technologies,

bioremediation is widely recognized as a 'green' or environmentally friendly technique for remediating PAH-contaminated soils. The bioremediation technologies consist of bioaugmentation and biostimulation [11]. It occurs through microbial degradation of PAHs in aerobic or anaerobic conditions [12, 13]. It is more effective, exerts a minimal environmental impact and releases no other pollutants.

The benthic fauna is an essential element for PAHs degradation, especially inhabiting the substrate phase in CWs. Firstly, the bio-accumulation of pollutants by benthic fauna through the aquatic food web is an important pathway of PAHs [14]. Secondly, the PAHs, having low molecular weight, could be degraded by soil microorganisms in aerobic conditions [15], but the deep substrate always lacks oxygen. Plants could also degrade PAHs through plant uptake and enhanced aerobic degradation through radial oxygen loss [16]. Benthic fauna are of great significance for changing the oxygen transfer through bioturbation and enhancing plant growth [17]. Thirdly, microbial degradation of PAHs depends on various environmental conditions, such as nutrients, chemical properties of PAHs, denitrifying conditions, oxygen levels and microbial composition [15]. Under anaerobic conditions, the efficiency of microbial biodegradation highly depends on electron receptors such as nitrate (NO_3-N), sulfate or ferric iron [18]. As one of the more favorable electron acceptors, NO_3-N can be reduced by microorganisms associated with the consumption of a variety of organic carbons, including PAHs. According to our previous research, benthic fauna can change the nitrogen cultures in substrate [19, 20]. Thus, benthic fauna are considered to significantly influence PAHs removal in an ecological and sustainable way. Mussels are the typical benthic fauna in CWs. The mussels in an aquatic environment mainly inhabit the deep substrate, with excellent filtration capacity for organic pollutants, and change the nutrient level in substrate [21]. Consequently, it is considered that mussels may have an effect on

PAHs removal during metabolism.

The majority of previous research focused on the effect of plants and microbes addition on PAHs degradation in the aquatic environment. This is the first study, to the best of our knowledge, that revealed the importance of mussels involved in determining degradation behavior of PAHs in CWs. Here, we analyzed typical 4-ring and 3-ring PAHs contained in the 16 priority PAHs. The typical 3-ring PAHs of acenaphthene (ACE), acenaphthylene (ACY), fluorene (FLU) and 4-ring PAHs of fluoranthene (FLT) and pyrene (PYR) were selected as the target PAHs. All of the five PAHs have high water quality guidelines that were developed by the U.S. Environmental Protection Agency (EPA) [22]. The specific objectives of this study are to: 1) evaluate the influence of mussels on the purification of five PAHs in CWs; 2) evaluate the influence of these five PAHs on standard water quality variables (COD, NO_3-N, ammonium (NH_4-N), total nitrogen (TN)); and 3) determine the contributions of CWs components (plants, substrate, and others) and their relationship to the removal of the five PAHs under the influence of mussels. This research study was crucial for developing CWs treatment technologies utilizing benthic fauna whilst also providing useful information on the design of CWs for PAHs removal.

2. Material and methods

2.1 Laboratory-scale CW systems and chemicals

The CW systems under this study were located in the Environmental Research Institute of Shandong University, Jinan, northern China. Four units were constructed outdoors under a transparent shelter, with a length of 60 cm, width of 40 cm and depth of 50 cm. Gravel (2-3 cm in diameter) and river sand (1-2 mm in diameter, mainly Si_2O_3, Al_2O_3,

and Fe_2O_3) were washed in advance. They were used as substrate with a depth of 5 cm and 20 cm from bottom to top. The water depth was 10 cm during experimental period. An outlet was set at the bottom of each unit. The CWs were mono-cultured with water celery (*Oenanthe javanica* (Blume) DC). The *O. javanica* is confirmed to survive in winter. Sprouted *O. javanica* were collected from Taian, Shandong Province, China. The *O. javanica* were planted in September. The four experimental units were divided in two parts (CW-C and CW-M), which were constructed without and with mussels. The freshwater mussels used in experiments were *Lamprotula leai*, with a density of 15 individuals per unit [23]. Processes for the domesticated of mussels were detailed discussed in our previous research [21]. The CW-C and CW-M were further divided into two sections. Two microcosms were spiked with a mix of five PAHs in the influent (CW-CP and CW-MP). Four units in total were operated as: CW-C (without benthic fauna); CW-M (with mussels); CW-CP (contained Σ_5 PAHs and without benthic fauna); and CW-MP (contained Σ_5 PAHs and with mussels).

The ACE (>99.5% purity), ACY (90% purity), FLT (98% purity), FLU (>99% purity), and PYR (99% purity) used in this experiment were purchased from Aladdin Reagent (Shanghai, China). The standard substitute (p-Terphenyl-d14 and 2-fluorobiphenyl) used for pretreatment recovery rate calculation of PAHs extraction was obtained from the ANPEL Laboratory Technologies (Shanghai) Inc. The internal standards injected into the sample extracts for the purposes of quantitation were also obtained from ANPEL Laboratory Technologies (Shanghai) Inc.

2.2 Experimental procedure

A laboratory-scale sequencing batch-operated procedure was implemented in this experiment. The total experiment period lasted from September 2017 to February 2018,

with the air temperature ranging from 20 °C to 4 °C. The hydraulic retention time (HRT) of each system was 5 days, corresponding to the hydraulic loading rate (HLR) of 2 cm/d. The experimental microcosms were stable for six cycles. The data was steady from December to February with the temperature ranging from 6 °C to 4 °C. The influent was at Class I (B) level according to the Wastewater Discharge Standard (GB 18918-2002) [24]. Synthetic wastewater was used in this study by mixing the following components in tap water (mg per liter): sucrose 51.33, $(NH_4)_2SO_4$ 37.60, KH_2PO_4 10.33, KNO_3 76.60, $CaCl_2$ 10.00, $MgSO_4$ 10.00. After each experimental cycle the wastewater was drained from the outlet at the bottom. Then immediately filled with the synthetic wastewater.

The concentrations of each PAH were as follows (μg/L): ACE and ACY at 70, FLU, FLT and PYR at 20 according to the U.S. EPA. Due to the high hydrophobicity of PAHs, the five PAH compounds were increased at a 10-fold working concentration by dissolving the PAHs in 35 mL of acetonitrile (chromatographically purity). The PAHs solution was stored in amber bottles with Teflon-lined lids at 4 °C. Before use, 3.5 mL concentrated acetonitrile was added to the influent water. The concentrated solutions were subsequently diluted to create the final concentrations in the synthetic water and divided into the two units (CW-CP and CW-MP), which modelled the five synthetic EPA PAHs mixture. To ensure the conditions remained consistent, the CW-C and CW-M units both contained the same amount of acetonitrile.

2.3 Sample processing and analysis

Water samples were taken from influent and overlying water at the beginning and end of each cycle (every 5 days) using a Teflon polyethylene bottle. After collection, the water samples were filtered through a 0.45 μm cellulose acetate membrane by syringe. Specific parameters, i.e. TN, NH_4-N, NO_3-N and COD were determined in the laboratory

according to the standard methods [25].

For each unit the substrate samples of 25 g were taken every two cycle. The substrate samples at different depths (0-5 cm and 5-10 cm) below the water-sediment interface were collected using a tapered column. Samples were homogenized from five individuals (same height, equal amount). Then they were powdered to pass through a 2 mm sieve and stored at -20 °C prior to analysis. The NO_3-N content in the substrate was determined by reacting the sand with 2 M KCl (800 g/L) and this entailed 2 h shaking and 20 min centrifuge [26].

The plant heights were tested monthly. The plant samples were randomly collected from each unit every fourth cycle. Plant samples were carefully rinsed with distilled water several times to remove any adhering sand particles, and separated into stem and leaf tissues. Then the leaf was used for chlorophyll detection according to the methods described by Lan et al. [27].

To understand the effect of five PAHs on mussels' activity, the respiration rate was tested by analyzing the decrease in dissolved oxygen (DO) per unit time per mussel individual in a closed system [28]. Once the testing was completed, the mussels were replaced into the units. Two mussels were picked up for analyzing the element and five PAHs contents every fourth cycle. At the same time the mussels with the same weight were added into the systems. After collection the mussels were dissected and the bivalve tissue was removed from the shell. Then the tissues were rinsed with distilled water and dried at 45 ^{o}C for 96 h. The C and N contents in animal samples were determined using an elemental analyzer located in the Qilu University of Technology, Jinan, Shandong Province.

2.4 PAHs extraction and determination

2.4.1 Wastewater

Extraction of PAHs in wastewater was subsequently done using a solid-phase extraction method. Before extraction the water was filtered through a quantitative filter paper. 1000 mL water samples with surrogates were passed through a C18 extraction membrane using a solid phase extractor (Extrapid). The organic compounds in the membrane were eluted with dichloromethane (20 mL) for 20 min. The organic phase containing PAHs was then collected, and the water phase remaining in the extracts was absorbed with Na_2SO_4. The solvent was further concentrated to 1 mL by a vacuum concentrator (Vortex 600). The sample components were identified and measured by injecting the concentrated extract into a gas chromatography/mass spectrometry (GC/MS) system. The PAHs GC/MS analytical procedure was conducted according to EPA 525.2 on a Shimadzu (Japan) QP2020 GC/MS with a Rxi® 5Sil MS column, working in SIM mode [29]. The PAHs concentrations were calculated by the internal standard method (target ion peak areas were used for the calculation).

2.4.2 Substrate

The PAHs in the substrate samples were extracted using an accelerated solvent extractor (Thermo Fisher ASE 350) based at Shandong Academy of Environmental Science with acetone-hexane mixture (1:1). The instruments used for substrate sample testing were similar as described in section 2.4.1. The PAH GC/MS analysis method for substrate was performed according to EPA 8270C [30]. Furthermore, the PAHs contents in substrate were calculated by the internal standard method.

2.4.3 Plants and aquatic animals

The aboveground plant parts, i.e. the stems were measured. The stem parts of plants were naturally dried by air before extraction. For extraction, 5 g of the dried plant sample was

ground and placed into a 50 mL Teflon tube with 25 mL dichloromethane. Then they were extracted using the ultrasound extraction technique for 1 h, shaken for 1 h, and centrifuged at 1600 rpm for 10 min. Following extraction the water phase was removed, and the dichloromethane layer was purified by the silica gel column which contained neutral chlorine dioxide, silica gel, and Na_2SO_4. Then the residue was reduced to the final volume of 1 mL using the vacuum concentrator. Internal standard was added prior to GC-MS analysis. The extraction and detection method was implemented according to the description by He et al. [31].

For PAHs determination, the mussels' tissue was cut into small pieces. 5 g samples were placed into a 50 mL Teflon tube with 30 mL mixture of n-hexane: dichloromethane (1:1). They were then extracted by ultrasound extraction technique for 1 h, shaken for 1 h, and centrifuged at 1600 rpm for 10 min. The extraction of mussel samples were subjected to Na_2SO_4 to remove water. The remaining extraction solvent was cleaned up with an alumina purification column containing silica, alumina and Na_2SO_4. Then the residue was reduced to 1 mL for detection. The PAHs analysis was done according to the methods documented by Wang et al. [32].

2.5 Microbial study

For microbial analysis, DNA and RNA in substrate were extracted using the MOBIO PowerSand™ DNA and RNA Isolation Kits according to the manufacturer's instructions. Complementary DNA (cDNA) was generated by RNA using the superscript reverse transcriptase (TaKaRa, Japan). The reverse transcription PCR program was implemented according to the method described by Wang et al. [33]. The DNA and cDNA products were stored at -80 °C before qPCR analysis.

A Roche LC-480 real-time PCR system was used to quantify the 16S rRNA and

functional genes such as *nir* (*nirS* and *nirK*), *amoA*, *NSR*, and *nrfA*. The abundance of the targeted genes in each sample was determined by Abs Quant/2nd Derivative Max from Roche LC-480 Install. The reaction mixture and qPCR cycling conditions were established as reported in our previous research [19].

2.6 Data calculation and analysis

The mass balance of five PAHs was calculated by using the following equation,

$$Total\ mass = substrate\ accumulation + plants\ absorption + effluent + animal\ assimilation + others$$

The total mass of PAHs in influent and the mass of PAHs in effluent were calculated according to the PAHs concentrations and water volume. The total PAHs contents in plant, mussels and substrate were determined according to their weight.

2.7 Quality control

Method limit of quantification (LOQ) ranged from 0.04 μg/L (ACY, ACE and FLU) to 0.05 μg/L (FLT and PYR) for water samples. The method for substrate analysis was characterized by LOQs of 0.30 μg/kg (PYR), 0.37 μg/kg (FLT, ACE), 0.39 μg/kg (FLU) and 0.40 μg/kg (ACY). The pretreatment recovery rate for water analyses ranged from 62%-95%, 74%-100% for the substrate, and 42%-96% for the plant and mussel analyses. The recovery efficiencies ranged from 62%-85%.

2.8 Statistical significance

Data detected and their concentrations were analyzed using Mean ± standard deviation (SD). The statistical significance of difference was determined with a one-way analysis of variance (ANOVA) using SPSS 13.0. Correlations at $p<0.05$ were considered statistically significant. The sum of all the five PAHs detected in samples represented the total PAHs (expressed as Σ_5 PAH).

3. Results and discussion

3.1 Pollutants removal performance in different CW units

The concentrations of priority contaminants in wastewater (NH_4-N, NO_3-N, and COD) are shown in Fig. 1. During the experiment the concentrations of these three common pollutants reached a steady state in the effluent. PAHs addition could significantly enhance NO_3-N removal, but greatly inhibit NH_4-N removal in CWs. Specifically, the average concentrations of NO_3-N in CW-C and CW-M effluents were 11.8±1.04 mg/L and 10.4±1.58 mg/L, respectively, which was almost double that recorded for CW-CP and CW-MP with 5.02±1.85 mg/L and 4.08±1.49 mg/L, respectively (Fig. 1A). However, PAHs addition increased NH_4-N concentration and reached as high as 13.6±1.60 mg/L and 10.5±2.54 mg/L in CW-CP and CW-MP, respectively, while the data in CW-C and CW-M were only at 2.82±1.43 mg/L and 2.18±1.91 mg/L, respectively (Fig. 1B). A decline in the DO concentration with PAHs addition was also detected (Table S1). Similar results were also reported by Xu et al. [34]. Two possibilities can account for these: firstly, the organic matter stimulated soil microbiological activity due to the additional source of carbon, which was highly beneficial for denitrification and NO_3-N degradation [10]; and secondly, the dissimilatory N reduction to ammonium (DNRA) process was the dominant pathway of NO_3-N removal and NH_4-N accumulation with rich electron donors, such as organic carbon [34].

The enhanced DNRA process and NH_4-N accumulation with PAHs were verified by the N isotope experiment with $K^{15}NO_3$. Results for NH_4-N production confirmed the improved DNRA process with PAHs addition (Table S2). The CWs containing mussels performed the best at removing NH_4-N. The mussels enhanced NH_4-N removal through

their filtration, which attributed to lower NH_4-N concentration in CW-MP than that in CW-CP. But the NH_4-N concentration in CW-MP was still higher than that without PAHs. Moreover, the Σ_5 PAHs addition reduced the final COD concentrations than without PAHs contaminants (Fig. 1C), which was responsible for higher NO_3-N removal efficiency. Organic carbon abundance emerged as an important factor in the heterotrophic denitrification process, meaning that more COD was utilized for denitrification.

The mean concentration values for total PAHs in the influent and effluent of different CW units are shown in Fig. 1D. Apparently, both CW units could efficiently remove Σ_5 PAHs and revealed no difference with the $\sum_5$ PAHs effluent concentrations of 3.76 μg/L and 3.49 μg/L for CW-CP and CW-MP, respectively ($p>0.05$). The CW-MP has a higher removal rate of $\sum_5$ PAHs than that of CW-CP (Fig. S1). More than 96% of the $\sum_5$ PAHs were removed from the CW-MP, as the mussels demonstrated excellent capacity for organic removal due to their filtration [35]. The 3- and 4-ring PAHs had varying compositions in the different CW units. The average removal efficiencies of 3-ring and 4-ring PAHs in two microcosms were 99.8 % and 88.0%, respectively. The removal efficiency of 4-ring PAHs was 10% poorer than that of 3-ring PAHs. A predominance of 4-ring PAHs was observed in wastewater after treatment. The 4-ring PAHs in our experiment (FLT and PYR) accounted for 95.7% and 97.0% among the final $\sum_5$ PAHs in CW-CP and CW-MP, respectively. The other three compounds accounted for only 4.32% and 2.97% in two groups of the total PAHs. This is the likely result of PAHs being used as a carbon source and providing energy for denitrification, through co-metabolization with other carbohydrates via the catalysis of dioxygenase [36]. The 4-ring PAHs had low utilization rates and proved to be more difficult to biodegrade than the 3-ring PAHs [10]. For this reason the 3-ring PAHs were preferred for the removal process.

3.2 Σ_5 PAHs distribution and removal mechanism in CW units

3.2.1 Σ_5 PAHs accumulated in substrate along the height of CWs

Substrate plays a very important role in the removal of various pollutants in CWs, especially at the 0-10 cm depth [37]. Settling and adsorption onto substrates are considered to be the most likely removal processes for PAHs, which leads to less bioavailability towards biodegradation. The organics and nutrients settled on the superficial substrate, stimulated microbial activity at the sediment-water interface, reduced DO levels and restrained NH_4-N removal (Fig. 1B). In the present study, the Σ_5 PAHs contents in the 0-5 cm and 5-10 cm depths of substrate were detected (Fig. 2). The amount of Σ_5 PAHs deposited in substrate ranged from 41.8-62.8 μg/kg substrate. The CW-MP had lower accumulation of Σ_5 PAHs than the CW-CP, especially in the 5-10 cm depth substrate. Diverse micro-environments along the height of the substrate provided various habitats for benthic fauna and microbes, and this affected pollutants removal. The mussels mainly inhabited the 5-10 cm depth. The mussels could generally take up the organic matter from these depths within the substrate by either direct ingestion or by bacteria within their filtration capacity [14]. Consequently, they were able to purify the Σ_5 PAHs in substrate. The mean Σ_5 PAHs concentrations highly correlated with NO_3-N concentration in the substrate. At the 0-5 cm depth, the concentrations of NO_3-N were 0.673±0.420 mg/kg and 0.999±0.086 mg/kg in CW-CP and CW-MP, respectively. At the 5-10 cm depth, the NO_3-N concentration showed the same order of CW-MP>CW-CP. Anaerobic biodegradation of PAHs pollutants is an important pathway in deep substrate due to the lack of oxygen and photo degradation. Its efficiency is highly dependent on terminal electron receptors such as NO_3-N. Thus, higher NO_3-N contents with mussels may enhance PAHs removal by anaerobic degradation.

Amongst the five PAHs, the percentages of the 3-ring PAHs in 0-10 cm depth substrate amounted to 25.0% and 17.7% in the CW-CP and CW-MP, respectively. This

was probably due to the low molecular weight of PAHs (2 to 3-ring PAHs) and their preference for being volatilized into the gas phase, whilst the 4-ring PAHs were predominant in the particulate phase and could generally be deposited in increased proportions [38]. The mussels had 8.3% less proportions of 3-ring PAHs accumulated in the substrate. The 3-ring PAHs were most likely degraded via aerobic microbial degradation. The 4-ring PAHs, being "more difficult" components were mostly degraded after the 3-ring PAHs were degraded [39]. It is generally known that the relatively high concentrations of PAHs in substrate mainly existed due to the limited water exchange [40]. Mussel feeding activity and bioturbation may have mixed and aerated the sediments, thus promoting the biodegradation of light PAHs with 3-rings, and contributed to the removal of PAHs in aquatic environments [41].

3.2.2 Plant uptake of Σ_5 PAHs

The role of plants has been extensively studied for PAHs removal in CWs [42, 43]. For our study, the plants were analyzed to investigate their absorption of Σ_5 PAHs amounts over the experimental period (Fig. 3A). Prominent PAHs found in the plants were in the decreasing order of FLT, PYR, FLU, ACE, and ACY. Amongst the five PAHs, both plants in two CWs had a higher uptake of FLT and PYR. The PAHs in each group were dominated by the 4-ring PAHs, which represented approximately 65% of the total PAHs in plants. This agrees with the amounts of these five PAHs distributed in the substrate.

The overall plant uptake of Σ_5 PAHs had significantly greater accumulation, which was reported by Wang et al. [44]. In the late experimental period, PAHs in plants were slightly removed and this was attributed to plant growth with transpiration and metabolism, as well as utilization of carbon sources (Fig. S2) [36]. The CW plants were able to accumulate PAHs and this meant that harvesting of plants contributed to mitigation of organic pollutants in the CWs. The five PAHs were detected in plants at

high concentrations (51.3-248 μg/g dry weight (DW)) and varied with the average concentrations of 144±28.9 μg/g DW and 206±36.2 μg/g DW in CW-CP and CW-MP, respectively. The Σ_5 PAHs accumulation was higher in CW-MP, which confirmed that the mussels promoted the plants' uptake of PAHs. Plants may enhance the degradation of PAHs in the rhizosphere by: firstly, increasing the activity and density of root-associated microorganisms; and secondly, facilitating the PAHs transport to the roots by releasing the root-associated enzymes [45]. The enhanced plant growth by mussels was reflected in the higher chlorophyll content and plant height in CW-MP (Fig. S3), and facilitated the plants' uptake of Σ_5 PAHs. The presence of roots increased the aeration in substrate, thereby promoting PAHs aerobic degradation in the deep layer and transforming PAHs into more bio-accessible compounds [46]. The mussels promoted plant growth and increased the oxygen level due to root function and further improved the 3-ring PAHs removal in substrate.

3.2.3 Σ_5 PAHs in mussel samples

The factors that affected the accumulation of PAHs in aquatic animals include the magnitude of contamination in their habitats, the trophic status, feeding habits, and metabolic capacities [14]. The Σ_5 PAHs residues accumulated in mussels during the experiment period shown in Fig. 3B. The concentrations of Σ_5 PAHs increased from 0.861 to 17.0 μg/g fresh weight tissue. The mussels accumulated hydrophobic pollutants through ingestion. The lipids of mussels are also an important factor for the bioaccumulation of PAHs [32]. Higher percentages of the 4-ring PAHs were found in the mussels. The proportions of two 4-ring PAHs decreased at the end of the experiment, probably due to enhanced biotransformation as well as reduced gut assimilation in mussels. Therefore, the extra uptake via ingested substrate by mussels was also a pathway for PAHs removal in the substrate, resulting in the relatively flat trend of contaminant

concentrations in the CWs. However, the PAHs pollution in the sampling area caused a decrease in filtration rates of the mussels (Fig. S4).

3.3 Relationships between microbes and nutrient removal

Few studies have focused on the effect of PAHs on the substrate microbial communities in contaminated aquatic ecosystems [47]. The microbial functional genes involved in key N-cycling processes were studied. Bacterial activity was indicated by determining the RNA copy numbers. The DNA and RNA values were obtained and their Log values are shown in Tables S4 and S5, respectively, which represent the order of microbial magnitude in Log values. In the present study, both the microbial abundance and activity were influenced by the mussels and PAHs contaminants. According to the qPCR results, the relative abundance of nitrifying bacteria (*amoA* and *NSR*) decreased with Σ_5 PAHs addition, especially the *amoA* genes. The activity of nitrifying bacteria was clearly restrained with Σ_5 PAHs pollution, the abundance and activity of *nirS* did increase in CW-MP, and it was higher than that in CW-M. The CWs with mussels still had higher nitrifying and denitrifying bacteria than the control group. The qPCR data showed that the *nrfA* genes abundance was enriched by the addition of Σ_5 PAHs. Coupled with NH_4-N accumulation and NO_3-N removal (section 3.1), we postulate that the abundant PAHs settled on the substrate and restrained the nitrification process but promoted DNRA and the denitrification processes.

3.4 Proposed mechanism of Σ_5 PAHs removal in CWs with the addition of mussels

Except for substrate adsorption, aquatic animal bioaccumulation and plant absorption, the PAHs can also be removed by other mechanisms. These other processes cannot be measured directly. Thus, a mass balance calculation was adopted to estimate the removal contribution by CWs components. According to Fig. 4, total loss due to other mechanisms

contributed much to Σ_5 PAHs removal. Microbial degradation processes, as well as photo-biodegradation and volatilization, are regarded as the primary pathways for the removal of most PAHs [48]. The estimated percentages of Σ_5 PAHs accumulated by substrate in CW-CP and CW-MP were 15.4% and 10.9%, respectively. Adsorption on substrates made only a minor contribution to the accumulation of Σ_5 PAHs compared to other loss processes, but contributed more to the entire degradation component of the accumulation of PAHs in each CW. Apart from substrate accumulation, the rhizosphere of plants also contributed to the removal of organic pollutants. Considering the Σ_5 PAHs concentrations absorbed by plants, the percentage of plant uptake in the CW units followed the order of: CW-MP>CW-CP. To summarize, the addition of mussels could promote the removal of Σ_5 PAHs by enhancing plant absorption and uptake of contaminants in the substrate. The amount of Σ_5 PAHs effluent from wastewater took into account no more than 4.3% of the total PAHs removal, with no significant difference ($p>0.05$) being observed.

Based on the results presented here, there were significant correlations between functional microbial abundance, environmental factors and PAHs concentration in the CWs with mussel addition (Fig. 5). The mussels in CWs enhanced plant growth and further promoted plant uptake of Σ_5 PAHs. Meanwhile, mussels in CWs had a significant positive correlation with Σ_5 PAHs removal in substrate, especially in the 5-10 cm depth that the mussels inhabited. The purification capacity and ingestion of mussels contributed to the enhanced PAHs removal. Furthermore their bioturbation activity and enhanced plant growth were attributed to an oxygenated substrate, therefore the 3-ring PAHs removal was improved by mussels. The changes in microbial abundance and activity with PAHs were responsible for the different performances in removing NO_3-N and NH_4-N [49]. Denitrification, coupled with a carbon source provided by PAHs, was speculated to have occurred resulting in NO_3-N removal. The inhibited NH_4-N removal was attributed

to the enhanced DNRA and restrained nitrification processes. Our findings confirmed that the use of mussels to remove PAHs in CWs is feasible.

4. Conclusion

The potential of mussels to influence the removal of five typical PAHs in CWs and the effect of PAHs on CWs performance were studied. The results indicated that the Σ_5 PAHs promoted NO_3-N removal but increased NH_4-N concentrations in wastewater in CWs. Mussels had excellent purification capacity with the Σ_5 PAHs removal efficiency improving up to 97% in wastewater. The 4-ring PAHs had higher concentrations than the 3-ring PAHs for each CW in the substrate, probably because of settlement and recalcitrance. Through mass balance calculation, it was found that the mussels decreased the Σ_5 PAHs accumulation at 0-10 cm depth substrate, especially for 3-ring PAHs. This was mainly due to the ingestion, purification, and enhanced oxygenated environment in the deep substrate where the mussels are located. Mussels highly promoted plant uptake of Σ_5 PAHs. The Σ_5 PAHs were assimilated in the mussels. Regarding the microbial results, Σ_5 PAHs in CWs inhibited the abundance and activity of nitrifying bacteria (*amoA* and *NSR*), and increased the DNRA process (*nrfA*). This contributed to the differences in NH_4-N and NO_3-N removing in wastewater. Finally, our reported results suggest that mussels can promote Σ_5 PAHs removal in the CWs.

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