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Improving nutrient removal performance of surface flow constructed wetlands in winter using hardy submerged plant-benthic fauna systems†

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Constructed wetlands (CWs) have been widely used as an ecological technology for removing nutrients from aquatic ecosystems. However, the treatment efficiency of surface flow constructed wetlands (SFCWs) in winter is generally low. To enhance the nutrient removal performance of SFCWs in winter, we developed a novel hardy submerged plant-benthic fauna system by adding *Chironomus riparius* (*C. riparius*) larvae and planting *Potamogeton crispus* L. in SFCWs. Compared to a system without *C. riparius*, the paired system greatly enhanced TN and TP removal with the average removal efficiencies of 54.73% and 94.76%, respectively. Furthermore, the paired system improved NO_3^-–N removal efficiency by 29.51% and reached NH_4^+–N removal efficiency as high as 86.20% simultaneously. The mass balance analysis indicated that *C. riparius* larvae enhanced substrate absorption and plant uptake in the CWs. The results of microbial analysis agreed with the nutrient removal performance, showing that *C. riparius* larvae influence the abundance and community structure of microbes related to N removal. As a whole, this study provides a promising ecological strategy for performance intensification of SFCWs in winter.

1. Introduction

Nutrient loads in aquatic systems have increased notably, leading to eutrophication and harm to ecosystems.[1] Constructed wetlands (CWs), an effective ecological technology for wastewater treatment, simulate the structure and function of natural wetlands to remove superfluous nutrients. They have been widely used due to their low cost, low operation and maintenance requirements, and landscaping function.[2,3] CWs could remove nutrients by utilizing substrate adsorption and precipitation, plant uptake, and microbial degradation.[4,5] However, due to withered plants and inhibited microorganism activity at low temperatures, the treatment performance of CWs decline in winter.[6,7]

Several studies have been conducted to enhance the treatment efficiency of CWs at low temperatures. Some researchers concentrated on design innovation of CWs to achieve performance intensification, and various types of CWs were developed such as vertical subsurface flow wetlands and two-stage subsurface flow wetlands, but these CWs were expensive and prone to clogging risk.[8,9] Using artificial aeration in winter can also benefit pollutants removal in CWs,[10] but this approach has high costs and wastes energy. The need for a low-cost and environmentally friendly method has prompted some researchers to use ecological engineering methods, such as plant selection.[11,12] A few kinds of plants can survive at low temperatures and benefit CWs in terms of removing pollutants in winter.[11] Fan *et al.* compared some hardy plants and found that the submerged plant *Potamogeton crispus* L. (*P. crispus*) could improve the nutrient removal performance of CWs in winter especially ammonium (NH_4^+–N) removal.[12] Nevertheless, the research they conducted showed limited nitrate (NO_3^-–N) removal efficiency, which was due to high NH_4–N conversion efficiency to NO_3^-–N and insufficient denitrification.[12] Thus, a more effective ecological engineering method is required.

Benthic fauna is a crucial segment of natural wetland,[13,14] but it is not valued in CWs. Recently, the function of some kinds of benthic fauna attracts interest for their special performance in NO_3^-–N consumption with the bioturbation and the stimulation on related microbes.[15,16] In a previous study, benthic fauna were added in CWs planted with emerged plant in winter and achieved the enhancement of NO_3^- removal.[17] However, the NH_4^+–N removal efficiency was only 56.93% in the previous system due to the excretions of benthic fauna and insufficient

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nitrification.[17] In the present study, we tried to add benthic fauna to submerged plant CWs and hope to achieve synergic removal of NO_3^-–N and NH_4^+–N at low temperature. On the one hand, the bioturbation of benthic fauna can create burrows in the substrate.[18,19] Then more NO_3^-–N and carbon would move to the anaerobic zone *via* the burrows, and denitrification can be facilitated.[15,20] On the other hand, with the prominent NH_4^+–N removal ability of submerged plant,[21,22] the NH_4^+–N released by benthic fauna would be removed.

Additionally, benthic fauna may stimulate microbial metabolism,[23,24] which may influence N circulation and conversion further. Besides nitrogen (N), previous studies indicated that benthic fauna also contributed to the phosphorus (P) flux between sediments and the overlying water.[19,25] Based on these, microbial abundance and P removal performance were also involved in this study.

For submerged plant, hardy plant *P. crispus* was selected in the present study. For the benthic fauna, *Chironomus riparius* (*C. riparius*) larvae, the larvae of one of the common chironomid specie, were chosen due to its cold resistance and distinct behavioural modality. In natural wetland and other aquatic ecosystem, *C. riparius* larvae molt into their adult form when temperature rise and the adults will oviposit in the water when the following autumn coming.[16,26,27] There are a great number of *C. riparius* larvae distributed in natural wetland and other aquatic ecosystem every winter.[28] They roam at the sediment surface and burrow downwards, which would influence both the superficial and deeper substrate and probably affect plant thereby. Then the N and P removal could also be impacted by these behavioural modality.

So, a novel hardy submerged plant-benthic fauna system, specifically a *P. crispus*–*C. riparius* larva CW, was developed creatively to enhance the nutrient removal performance of surface flow constructed wetlands (SFCWs) in winter. Dynamic measurements were performed on the N and P concentrations in the overlying water. The fate of nutrients in this system was evaluated based on calculations of mass balance. The parameters of the microorganisms were also determined by quantitative real-time polymerase chain reaction (qPCR) and Illumina high-throughput sequencing.

2. Materials and methods

2.1 *P. crispus* and *C. riparius* larvae preparation

P. crispus were transferred from Nansi Lake, Shandong Province, China in October 2016, and were washed to remove any adhering impurities. Then they were transplanted into the laboratory-scale CWs microcosms at a density of 100 rhizomes per square meter.[12] 10% Hoagland solution was used to cultivate sprouted *P. crispus*, which were approximately 10 cm high, for three weeks.

C. riparius larvae were acquired from a commercial breeding facility in Jinan, Shandong Province, China. Then the larvae were bred in the laboratory (10 cm of sediment, the same as experimental sediment; 10 °C; constant aeration; in darkness) for two weeks, feeding with synthetic water that was similar to the microcosms used. Larvae of 7 to 12 mm length were singled out and washed with purified water. After that, they were introduced into the experimental CWs microcosms at a density of 14 000 individuals per m^2, according to the typical density observed in natural environments.[15]

2.2 Experimental setup and operation

The experimental microcosms were established outdoors under a transparent rain shelter in Jinan, northern China (36°40′N, 117°03′E). Six polyethylene barrels of 50 cm depth and 43 cm inner diameter were used to develop laboratory-scale SFCWs. All the systems had an outlet at the bottom to drain water away and had a vertical perforated PVC pipe in the centre of the barrel to measure temperature and dissolved oxygen (DO). Two layers of substrate were added to each CW microcosm: the 5 cm superincumbent substrate layer (SS layer) was sediment which obtained from Baiyun Lake natural wetland (Jinan, Shandong Province) and was sieved through a 2 mm mesh as a suitable habitat for *C. riparius* larvae; the 15 cm underlying substrate layer (US layer) was washed sand (particle size <2 mm; mainly SiO_2, Al_2O_3, and Fe_2O_3). The six CWs were divided into two groups (each group had three parallels): CWs with *P. crispus* larvae and *C. riparius* were named CWs-PC; CWs with only *P. crispus* were named CWs-P and served as controls.

Synthetic wastewater was used for experimental influent to simulate sewage treatment plant effluent (Grade I-B Discharge Standard of Pollutants for Municipal Wastewater Treatment Plant of China (GB 18918-2002))[29] by mixing the following components in tap water: 53.46 mg L^{-1} sucrose, 37.75 mg L^{-1} $(NH_4)_2SO_4$, 4.39 mg L^{-1} KH_2PO_4, 86.66 mg L^{-1} KNO_3, 10 mg L^{-1} $MgSO_4$, 10 mg L^{-1} $CaCl_2$, and a nutrient solution of micronutrients containing Fe, Zn, Cu, Mn, B, and Mo. The CWs were fed the synthetic wastewater in a sequencing fill-and-draw batch mode. They were filled with wastewater for treatment at the beginning of a cycle and drained from the outlet at the last day of a cycle, then refilled immediately for the next cycle. Each CW held 36 L synthetic wastewater when filled, maintained at approximately 25 cm above the substrate. According to technical specification of constructed wetlands for wastewater treatment engineering of China (HJ 2005-2010) and the actual environmental condition, a hydraulic retention time (HRT) of 8 d was adopted. The entire experimental period included eight cycles, lasting from December 2016 to the following February with the average ambient temperature ranging from −4 °C to 7 °C. When the temperature was below zero, the microcosms were wrapped by the thermal insulation materials to avoid freezing. The details about the temperature of air and water during the experimental period were shown in Fig. S1.†

2.3 Sampling and analyses

Water samples were collected from the middle water depth of all units every two days and filtered through a 0.45 mm cellulose acetate membrane. Then the samples were analysed immediately for NO_3^-–N, NH_4^+–N, TP, TN, and chemical oxygen demand (COD), following the standard methods.[30] DO and water temperature were measured at the sediment–water interface *in situ* every two days using a DO meter (HQ40d

53LED™, Hach, USA). Potential of hydrogen (pH) was measured at the middle water depth of all units *in situ* using a portable pH meter (PHS-3D, Rex Instrument, Shanghai, China).

The substrate samples, including SS layer samples and US layer samples, were separately collected at the beginning and the end of the experimental session to estimate N and P storage in the substrate and to analyse microbial characteristics. SS layer samples were taken from the sediment layer at the same height in each unit, and five individual samples of equal amounts were collected. For US layer sampling, the whole sand layer (depth 15 cm) was divided into three increments: 0 to 5 cm, 5 to 10 cm, and 10 to 15 cm. Five individual samples from each depth increment were collected and mixed, and sand samples from all three depth increments in each CW were blended to obtain a representative sample for each system. Then, the SS layer samples and US layer samples were both dried at −60 °C using a freeze dryer (Unicryo MC 2 L freeze dryer, Germany) for 36 h. After desiccation, the SS layer samples and US layer samples were ground separately and passed through 0.2 mm and 1.0 mm sieves, respectively. All the substrate samples were stored at −20 °C for further analysis. The TN contents in the SS layer samples and US layer samples were measured in Xinpu Environmental Technology Company (Shanxi, China) using the semi-micro Macro Kjeldahl method,[31] while the TP contents were determined by molybdenum blue method after digestion with perchloric acid and sulfuric acid.[32]

Plant samples were collected from all parts of the plants in each unit at the beginning and the end of the experiment to estimate N and P storage. The harvested plants were washed to remove impurities and dried in an oven at 65 °C for 72 h before being ground to pass through 0.425 mm sieves. All the *C. riparius* larvae in each larvae-added CW system were sorted at the end of the experiment. The animal samples were then dried at −60 °C using a freeze dryer (Unicryo MC 2 L freeze dryer, Germany) for 36 h and homogenised. Plant samples and animal samples were both stored at −20 °C. The TN and TP contents in the plant samples were measured using the same methods as substrate samples measurement in Xinpu Environmental Technology Company. The TN content in animal samples was determined using an elemental analyser in the School of Chemistry and Chemical Engineering, Shandong University (Jinan, China), while the TP content was measured using an Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES) in the Science Spectrum R & D Center (Qingdao, China).

2.4 Microbial analysis

Microbial analysis was performed on the SS layer samples, US layer samples, and animal samples. The genomic DNA was extracted using MOBIO PowerSand™ DNA Isolation Kits and analysed by qPCR and Illumina high-throughput sequencing.

Quantitative real-time polymerase chain reaction was conducted using a LightCycler® 480 II (Roche, USA) to detect the absolute abundance of bacterial 16S rRNA, and the key functional genes connected with nitrification and denitrification including ammonia monooxygenase (*amoA*), cd1-containing nitrite reductase (*nirS*), copper containing nitrite reductase (*nirK*) using primers: 338F/518R, amo598f/amo718r, nirK583F/nirK909R, nirScd3aF/nirSR3cd, respectively (Table S1†). The reaction mixture of qPCR was 20 μL, composed of 10 μL of SYBR Premix Ex Taq™, 7.2 μL of RNess-free water, 0.8 μL of the forward and reverse primers (0.4 μL of each), and 2 μL of template DNA. Each DNA sample was performed in triplicate and then averaged to get a representative result. A 10-fold dilution series of standard DNA was used to calculate a standard curve. The standard curves for the bacterial and functional genes had R^2 values of 0.991–0.999 and the amplification efficiencies ranged from 80.66% to 98.52%.

In order to detect the microbial communities in the CWs, Illumina high-throughput sequencing was performed on a HiSeq 2500 platform (Illumina, USA) at the Novogene Institute (Beijing, China). Before the Illumina sequencing was performed, the DNA concentration and purity were assessed through agarose gel electrophoresis (1%) and PCR reactions were carried out using Phusion® High-Fidelity PCR Master Mix (New England Biolabs) to amplify the V4–V5 hypervariable regions of the 16S rRNA gene with primers 515F and 907R. The PCR products were assessed by electrophoresis on 2% agarose gel and the ones with the bright strip between 400 to 450 bp were chosen for further experiments. Eligible products were pooled together and purified with Qiagen Gel Extraction Kit (Qiagen, Germany), then 250 bp paired-end sequencing of 16S rRNA gene amplicons were performed using the Illumina HiSeq instrument at the sequencing institutions. After sequencing, FLASH (version 1.2.7) was used to merge paired-end reads and QIIME (version 1.7.0) was used to filter the spliced data. Sequence reads were filtered to exclude low quality reads, chimeric sequence reads and those containing ambiguous base ('N'). Then, high quality 16S rRNA genes were classified into operational taxonomic units (OTUs) under 97% identities. Alpha diversity was calculated using Chao, Shannon, Simpson, ACE, and Good-coverage indices. All these indices were calculated with QIIME (version 1.7.0) and displayed with R software (version 2.15.3).

2.5 Mass balance calculations

The only nutrient source considered in the present system was influent (in), while the nutrients sinks were the effluent (ef), plant uptake (pu), substrate storage involving sediment storage and sand storage (ss), animal assimilation (aa) and other losses (ol) such as microbial metabolism and volatilization.

$$NU_{in} = NU_{ef} + NU_{pu} + NU_{ss} + NU_{aa} + NU_{ol} \quad (1)$$

The mass balance in the CWs was calculated using equations as follows:

$$NU_{in}/NU_{ef} = A^{-1} \times (nT)^{-1} \times \sum_{i=1}^{n} C_i \times V_i \quad (2)$$

$$NU_{pu}/NU_{ss}/NU_{aa} = A^{-1} \times nT^{-1} \times (W_{end} \times C_{end} - W_{initial} \times C_{initial}) \quad (3)$$

$$NU_{ol} = NU_{in} - NU_{ef} - NU_{pu} - NU_{ss} - NU_{aa} \quad (4)$$

NU_{in}/NU_{ef} (mg m^{-2} d^{-1}) is the nutrient load for the influent or effluent, respectively; $NU_{pu}/NU_{ss}/NU_{aa}/NU_{ol}$ (mg m^{-2} d^{-1}) is the nutrient concentration in the different removal pathways (plant uptake, substrate storage, animal assimilation and other losses, respectively) per unit area and days of operation; i is the number of batches in sequence ($i = 1, 2, 3 \ldots 8$); n is the total number of batches; C_i is the nutrient concentration in the influent or effluent of batch i (mg L^{-1}); V_i is the volume of influent or effluent (L; $V = 36$ L); A is the area of the CW (m^2; $A = 0.15$ m^2); and T is the retention time for every batch (d; $T = 8$ d); $W_{initial}/W_{end}$ (g) is the weight of the different removal agents at the beginning or end, respectively; $C_{initial}/C_{end}$ (mg g^{-1}) is the nutrient concentration in the different removal agents at the beginning or end, respectively.

2.6 Statistical analysis

Data analyses were performed by Microsoft® Office Excel 2010 (Microsoft Corporation, USA), with the results expressed as mean ± standard deviation. The mean removal efficiencies of the CWs groups were calculated using the results of four typical cycles, while other mean values were calculated using the results of three replicates. One-way analysis of variance (ANOVA) tests was used to test the significance of results utilizing the statistical program SPSS 19.0 (SPSS, Chicago, USA), and differences were considered to be significant at $P < 0.05$.

3. Results and discussion

3.1 Nitrogen and phosphorus removal performance

Because the performance of the CWs during the first four cycles was unstable (Fig. S2†), the last four experimental cycles were selected as typical cycles. The concentrations of NO_3^-–N, NH_4^+–N, TN, and TP during four typical experimental cycles for the CWs are shown in Fig. 1.

Fig. 1a shows that CWs-PC had much better performance in terms of NO_3^-–N removal than CWs-P ($P < 0.05$, $n = 4$), indicating *C. riparius* larvae addition can enhance NO_3^-–N removal. In the four typical experimental cycles, the final NO_3^-–N removal efficiencies ranged from 29.37 to 49.24% in CWs-PC while they ranged from 7.52 to 20.20% in CWs-P. The similar improvement was also reported by Lu *et al.* with the average NO_3^-–N removal efficiency increased from 10% to 30% by enhancing denitrification in winter.[33] It is generally accepted that denitrification is the main mechanism of removing NO_3^-–N in CWs.[34] In the present study, denitrification was promoted by *C. riparius* larvae in three aspects: O_2 condition, reactants in substrate and the denitrification in *C. riparius* larvae body. On the one hand, the average DO concentration during the typical operating cycle was 1.87 ± 0.53 mg L^{-1} in CWs-PC ($n = 4$), while it was as high as 4.71 ± 1.07 mg L^{-1} in CWs-P ($n = 4$) (Fig. S3†), demonstrating that *C. riparius* larvae consumed O_2 and then provided more microscopic anoxic zone in the substrate,[15] which could serve as suitable habitat for denitrification microbes.[35,36] On the other hand, many previous studies also illuminated that *C. riparius* larvae led to higher NO_3^-–N penetration and more particulate organic matter sedimentation into substrate, facilitating denitrification.[15,16,20] Furthermore, *C. riparius* larvae body were distinct microsites with low O_2 concentrations, high NO_3^-–N concentrations and plenty of denitrifiers, where denitrification could happen.[20] Interestingly, no significant difference ($P > 0.05$, $n = 4$) was observed in COD removal between CWs-PC and CWs-P (Fig. S3†), indicating that carbon sources in the water might were not used for denitrification by *C. riparius* larvae, which should be further studied. Fig. 1a also shows differences between experimental cycles. The final NO_3^-–N concentrations in CWs-PC in the last two cycles were higher than those in the preceding cycles, which were related with the eclosion of *C. riparius* larvae.

The performance of NH_4^+–N removal was consistent with our expectation and no excessive accumulation of NH_4^+–N was observed with the addition of *C. riparius* larvae ($P > 0.05$, $n = 4$). As Fig. 1b shows, the treatment efficiency of NH_4^+-N was 86.20 ± 4.49% in CWs-PC and 89.31 ± 7.01% in CWs-P, and there was no significant difference observed between them ($P > 0.05$, $n = 4$). Compared with previous similar studies, the NH_4^+–N removal efficiency in the present study was much higher. In a previous study, Gao *et al.* achieved NH_4^+–N removal efficiency at 62.1 ± 8.8% in CWs by planting *Iris sibirica* in winter.[37] In another study, Zhi *et al.* reported that the NH_4^+–N removal efficiency was 76 ± 3.9% in winter using tidal flow CWs.[38] The better performance in the present study is mostly due to the effect of *P. crispus*. Firstly, submerged in the water, *P. crispus* could release oxygen produced by photosynthesis directly into the water and supply more DO to the CW system,[39] which was beneficial to aerobic nitrification. Although *C. riparius* larvae consumed DO in CWs-PC, they could periodically ventilate the burrows created by them to supply DO to substrate,[15] so the nitrification was not inhibited in CWs-PC. Secondly, *P. crispus* absorbed NH_4^+–N from the substrate and water through its roots and leaves.[12] A previous study showed prominent NH_4^+–N removal rates by utilizing *P. crispus*, with NH_4^+–N absorption rates of 1.59 mg N min^{-1} g DW^{-1} in winter.[39] Furthermore, the treatment efficiency of NH_4^+–N in CWs-PC and CWs-P improved in the later experimental cycles as shown in Fig. 1b, which was related to the growth of *P. crispus*.

Better NO_3^-–N removal performance and comparable NH_4^+–N removal performance resulted in better TN removal performance in CWs-PC than CWs-P ($P < 0.05$, $n = 4$). Fig. 1c showed that the average removal efficiency of TN reached 54.73 ± 3.19% in CWs-PC, while it was 39.57 ± 3.55% in CWs-P. In previous studies, Zhang *et al.* improved the TN removal efficiency of SFCWs to 44.7% in winter by plant collocation.[39] Wang *et al.* achieved 47.5% TN removal efficiency by the subsurface flow constructed wetlands planted with hardy plant.[40] Compared with these CW systems treating similar wastewater, CWs-PC had comparable TN removal efficiency and even higher by combing with *C. riparius* larvae and *P. crispus*.

As for TP removal, the performance of the two purification systems during four typical experimental cycles is shown in Fig. 1d. The TP removal efficiency was 94.76 ± 4.10% in CWs-PC, which was much higher than CWs-P ($P < 0.05$, $n = 4$). In fact, the TP removal efficiency in CWs-PC was even higher than some subsurface flow CWs. Yan and Xu mentioned the TP

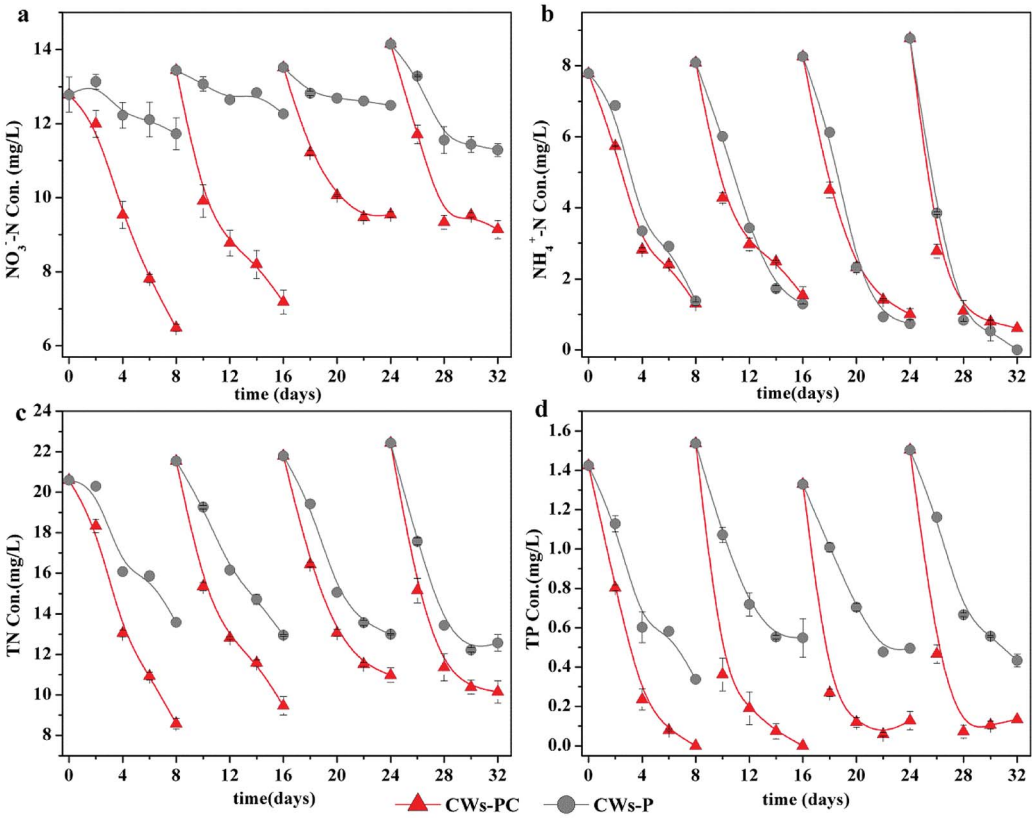

Fig. 1 Treatment performance on NH_4^+–N, NO_3^-–N, TN and TP for each group during four typical operating cycles ($n = 3$).

removal efficiency of subsurface flow CWs in north area of China ranged from 19% to 70% in winter.[11] The TP removal mechanisms in CWs-PC are mainly related to substrate adsorption and plant assimilation,[4] which would be discussed in Section 3.2.

3.2 Quantification and evaluation of nitrogen and phosphorus balance

In the present study, the contributions of different removal pathways to removing N and P from different wetland microcosms were quantified using mass balance. Based on areal loads of the influent (NU_{in}), effluent (NU_{ef}), and removal rates of plant uptake (NU_{pu}), substrate storage (NU_{ss}), animal assimilation (NU_{aa}), and other losses (NU_{ol}), the results of the mass balance are displayed in Fig. 2. The TP content of larvae body changed from 4.54 mg g^{-1} to 4.72 ± 0.24 mg g^{-1} ($P > 0.05$, $n = 3$) during the experimental period, which was statistically insignificant, so the animal assimilation was not taken in into account in Fig. 2b.

For N removal, other losses and substrate absorption were the dominant pathway in both CWs-PC and CWs-P (Fig. 2a). Other losses include volatilization and gas emission *via* microbial reactions such as nitrification–denitrification, accounting for 18.89–24.24% of the TN input in all the CWs. In the present study, the volatilization of ammonia is negligible because both CWs-PC and CW-P were slightly alkaline.[41] Therefore, microbial degradation might play an important role in N removal in the two systems. The results were consistent with some previous studies which suggested the significance of microbial reactions for N removal and showed a high proportion of nitrification–denitrification for N removal.[42,43] It is noteworthy that the proportion of other losses in the CWs-PC accounted in TN input was 5.35% higher than in the CWs-P ($P < 0.05$, $n = 3$), which suggest that adding *C. riparius* larvae might influence microbial N removal and we would discuss this part in Section 3.3. Substrate absorption accounted for 15.45–22.39% of the TN input. The proportions of substrate absorption were comparable to the proportions of 20.5–34.4% found in the previous study.[44] Furthermore, the proportion of plant uptake accounted in TN input varied from 0.44–2.24% in all the CWs. These values are lower than those seen in previous studies that also assess the contribution of submerged plant uptake on N removal.[45,46] The minor contribution of plant uptake in the present study was mainly because of the lower biomass production of *P. crispus*. Besides, a previous study compared four aquatic plant treatment systems and found the *P. crispus* system had lowest NH_4^+–N uptake rate but highest NH_4^+–N removal efficiency,[47] suggesting *P. crispus* mostly removed N by nitrification rather than absorption by itself. In this study, *C. riparius* larvae also assimilated N in CWs-PC, and the TN content of larvae body increased from 406.4 mg g^{-1} to 433.05 ± 2.65 mg g^{-1} ($P < 0.05$, $n = 3$). Due to the eclosion of *C. riparius* larvae and the limit of manual sorting from substrate at the end

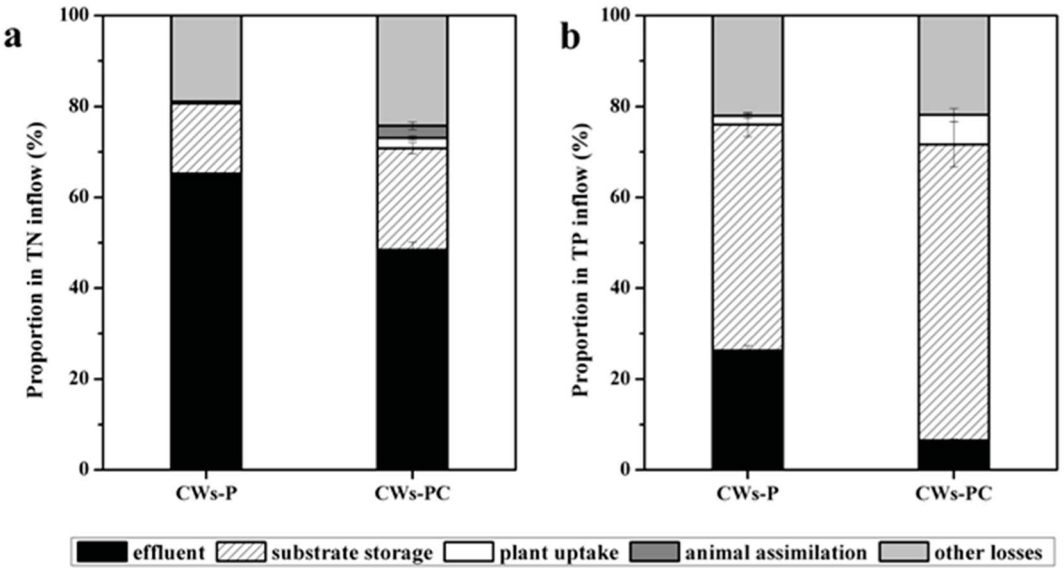

Fig. 2 Proportion of N and P removed by different pathways among different wetlands during the experimental period ($n = 3$).

of the experiment, the larvae mass (shown in Table S2†) did not increase over the course of the experiment ($P > 0.05$, $n = 3$). However, although some larvae mass was not taken into account, animal assimilation in CWs-PC accounted for 2.71% of the TN input due to the increased TN content of larvae body, indicating direct assimilation by *C. riparius* larvae also contribute to N removal.

On the other hand, the proportion of TN input stored in the substrate was 6.92% higher in the CWs-PC than in the CWs-P ($P < 0.05$, $n = 3$), indicating that *C. riparius* larvae enhanced substrate absorption, which also contributes to TN removal. In the present study, *C. riparius* larvae dug downwards and irrigated the burrows they produced.[15] A mass of *C. riparius* larvae were found in the substrate at a depth of 15 cm (Fig. S4†). Fluxes of N between the deep substrate and water could be enhanced using the burrows in the deeper substrate so that fresh inorganic N could bind to the deeper substrate and be adsorbed.[48] Moreover, the burrows were rich in NH_4^+–N excreted by *C. riparius* larvae and this portion of the NH_4^+–N would precipitate in the deep substrate following excretion.[49] As can be seen in Table 1, the TN content of the US layer was higher in the CWs-PC than in the CWs-P ($P < 0.05$), although there was no obvious difference in the SS layer. Thus, *C. riparius* larvae could improve the TN storage of US layers and increase TN removal. Besides, plant uptake in the CWs-PC removed 1.80% of the TN input higher than in the CWs-P ($P < 0.05$), suggesting that the contributions of plant uptake to N removal were strengthened by adding *C. riparius* larvae. This is because *C. riparius* larvae increase the biomass and N content of *P. crispus*. Table S2† shows the total dry weight of *P. crispus* in CWs-PC was 2.15 fold higher than in the CWs-P at the end of the experiment ($P < 0.05$), suggesting that *P. crispus* in CWs-PC grew better. The better growth condition was related to the higher TN storage in the substrate stimulated by *C. riparius*. Table 1 shows *P. crispus* in CWs-PC has higher TN content than CWs-P ($P < 0.05$), which might be related to the regulation of nitric oxide produced by larvae-based denitrification on plant assimilation.[50] As a result, the plant uptake in CWs-PC was promoted by *C. riparius* and TN removal was promoted thereby.

Table 1 TN and TP storage in the substrate and *P. crispus* of different CWs

		Parameters	
CWs	Items	TN (mg g^{-1})	TP (mg g^{-1})
CWs-P	SS layer	1.033 ± 0.035	0.065 ± 0.001
	US layer	0.028 ± 0.002	0.015 ± 0.002
	Plant	23.520 ± 1.470	5.944 ± 0.427
CWs-PC	SS layer	0.915 ± 0.056	0.062 ± 0.001[a]
	US layer	0.128 ± 0.010[a]	0.026 ± 0.001[a]
	Plant	32.620 ± 0.770[a]	6.321 ± 0.050

[a] Indicate significant differences ($P < 0.05$).

The contributions of different pathways in different wetland microcosms to TP input were shown in Fig. 2b. The results showed that substrate storage removed 49.75–65.11% of the TP input in CWs-PC and CWs-P, while P removal through plant uptake was quantified as 1.96–6.50% and other losses accounted for 21.86–22.03%. The substrate storage of P was much higher in the CWs-PC than in the CWs-P ($P < 0.05$), indicating that *C. riparius* larvae had a great influence on TP storage. By adding *C. riparius* larvae, more soluble and labile P was pumped into the deeper substrate layer through bioturbation and the burrows created.[25] Metal ions such as aluminium (Al), iron (Fe), and calcium (Ca), and organic matter in the substrate were then able to take up soluble and labile P, binding them in the substrate. As can be seen in Table 1, the TP contents of US layer were significantly higher with the addition of *C. riparius* larvae. Table 1 also showed the TP content of the SS layer was lower, which suggested that the P in the overlying wastewater and released from the SS layer would be absorbed by the US layer in

CWs-PC. Furthermore, the pH value was 8.70 ± 0.10 in CWs-PC while the pH value was 8.41 ± 0.08 in CWs-P ($P < 0.05$, $n = 3$), and the higher pH value could increase P sorption capacity of alkaline CW.[51] Therefore, the effect of substrate adsorption on TP removal can be enhanced in CWs-PC. Similar to the effect of *C. riparius* on plant uptake in terms of removing TN, *C. riparius* improved the performance of *P. crispus* on TP removal and a higher proportion of plant uptake can be obtained in CWs-PC ($P < 0.05$, $n = 3$). As to other losses, they mainly included microbe assimilation and algal component removal and no differences were observed between the CWs-PC and CWs-P ($P > 0.05$, $n = 3$). In previous studies, researchers found that plant uptake removed 36% of TP input and the P load due to adsorption, desorption, precipitation, and exchange with groundwater was estimated to be approximately 26%.[52] Another study used SFCWs to treat the eutrophicated waters of a lake and showed that the TP in plants was 19.2% of TP removed.[53] It seems that the contributions of different pathways to TP removal vary between different plant species and substrates.

3.3 Microbial abundance and structure

There were obvious differences in the percentage of other losses accounted in TN input observed between the CWs-PC and CW-P (as described in Section 3.2), which may be related to microbial degradation. Based on the well-founded impact of *C. riparius* larvae on microbial N removal,[23,24,54] the abundance and structure of N-related microbes were detected in this study.

It is accepted that nitrite reductase genes (*nirK* and *nirS*) and ammonia monooxygenase gene (*amoA*) can be used to detect denitrifying bacteria and nitrifying bacteria in environmental samples, respectively.[55,56] Table 2 shows the copy numbers of these functional genes and bacterial 16S rRNA in the substrate and *C. riparius* larvae samples of wetland microcosms, reflecting the absolute abundance of related bacteria in the two systems. From Table 2, it can be seen that the copy number of *nirS* in the SS layer of the CWs-PC was higher than in the CWs-P ($P < 0.05$, $n = 3$) and the mean copy number of *nirK* and *nirS* in the US layer of CWs-P and CWs-PC were at different magnitudes, indicating that *C. riparius* larvae increased the abundance of denitrifying bacteria in substrate. The copy number of *nirK* gene and *nirS* gene in larval bodies were $(7.90 \pm 2.90) \times 10^6$ copies per g and $(5.62 \pm 1.73) \times 10^6$ copies per g, respectively, suggesting that denitrification could occur in the larval body. These results were consistent with the NO_3^-–N removal performance of CWs-PC. Moreover, the copy number of *amoA* in the SS layer of the CWs-PC was higher than in the CWs-P ($P < 0.05$), which was due to the stimulation of *C. riparius* larvae on nitrifying bacteria. The results agree with a previous study which showed *C. riparius* larvae could facilitate nitrification by concomitant ventilation and ammonium excretion in the burrows.[15] The probable effect of *C. riparius* larvae on nitrification may be a reason for NH_4^+–N removal in CWs-PC. As for the absolute abundance of bacterial 16S rRNA, no obvious difference was detected between the two layers in either CWs-PC or CWs-P ($P > 0.05$, $n = 3$), indicating that *C. riparius* larvae did not alter the total bacterial abundance in CWs, probably changed the bacterial abundance of associated pollutants only.

Also, the microbial communities were investigated in the present study using high-throughput sequencing. The diversity index and species richness of samples from CWs-PC and CWs-P (Table S3†) elucidated that the species diversity and richness increased imperceptibly in response to the addition of *C. riparius* larvae. Fig. 3a shows the bacterial composition of different samples at the phylum level. In the *C. riparius* larvae bodies, *Proteobacteria* and *Bacteroidetes* were the two predominant phyla, which include many types of denitrifiers.[57] In the substrates of both CWs-PC and CWs-P, *Proteobacteria*, *Actinobacteria*, and *Chloroflexi* dominated, comprising 70.20–88.80% of all detected OTUs, and they are related to N and C cycling.[58] Kang *et al.* also indicated *Proteobacteria* and *Actinobacteria* predominated in their benthic fauna added CWs.[2]

Previous studies have reported that the three typical phyla of *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* strains play a crucial role in N-transformation.[59,60] To detect the effect of *C. riparius* larvae on microbial communities that relate to N removal, subdivisions of these three phyla were all further analysed at class level (Fig. 3). From Fig. 3b, it can be seen that the relative abundance of gamma-*Proteobacteria* in the SS layer of the CWs-PC was 4.86% higher than that of the CWs-P, while in the US layer the relative abundance was 10.96% higher in the CWs-PC than in the CWs-P. Fig. 3c shows that *Flavobacteriia*, within the phylum *Bacteroidetes*, in the SS layer of the CWs-PC

Table 2 The quantities of functional genes and bacterial 16S rRNA in the substrate and *C. riparius* larvae samples of wetland microcosms

Genes (copies per g)	CWs	SS layer	US layer	Larvae body
nirK	CWs-P	$(1.23 \pm 0.33) \times 10^8$	$(3.43 \pm 2.56) \times 10^5$	—
	CWs-PC	$(1.36 \pm 0.16) \times 10^8$	$(4.62 \pm 3.42) \times 10^6$	$(7.90 \pm 2.90) \times 10^6$
nirS	CWs-P	$(3.47 \pm 0.33) \times 10^7$	$(5.66 \pm 0.00) \times 10^4$	—
	CWs-PC	$(6.01 \pm 0.53) \times 10^{7a}$	$(4.79 \pm 3.75) \times 10^5$	$(5.62 \pm 1.73) \times 10^6$
amoA	CWs-P	$(2.29 \pm 0.32) \times 10^5$	$(9.85 \pm 0.03) \times 10^3$	—
	CWs-PC	$(3.53 \pm 0.17) \times 10^{5a}$	$(1.64 \pm 0.16) \times 10^4$	$(3.27 \pm 0.62) \times 10^4$
16S rRNA	CWs-P	$(7.67 \pm 0.74) \times 10^9$	$(4.76 \pm 0.01) \times 10^7$	—
	CWs-PC	$(9.16 \pm 0.99) \times 10^9$	$(4.87 \pm 3.66) \times 10^8$	$(1.36 \pm 0.16) \times 10^9$

[a] Indicate significant differences ($P < 0.05$); – means no data.

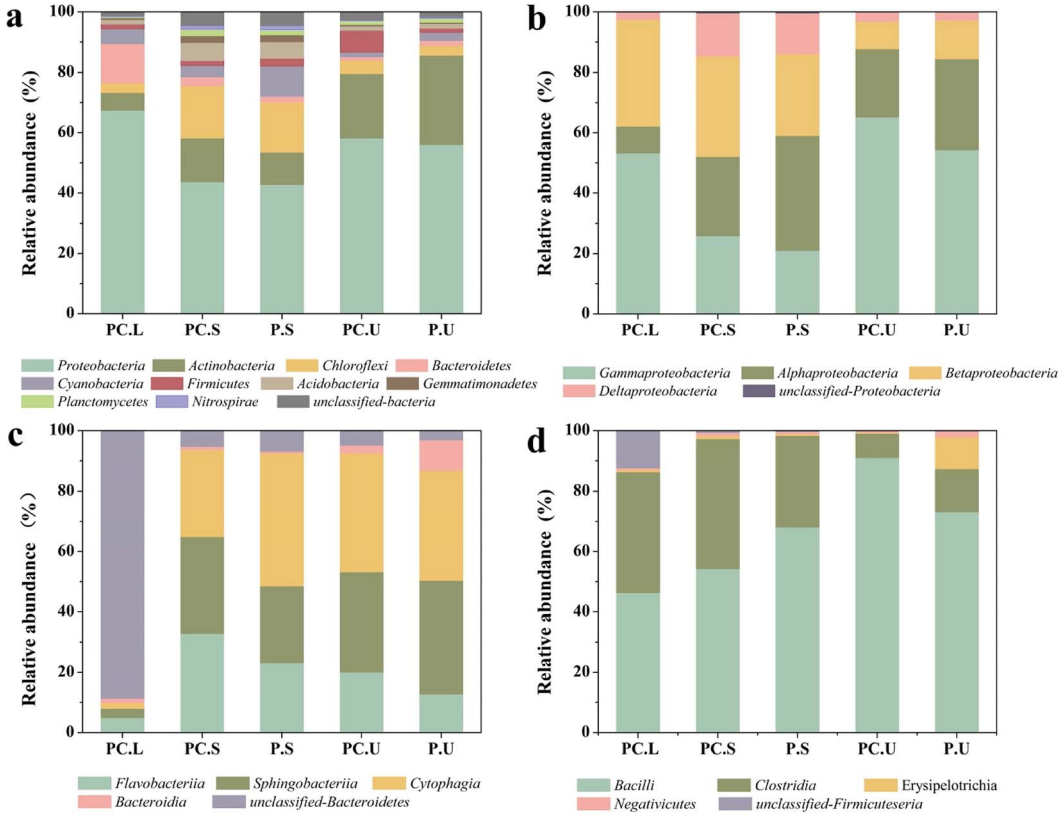

Fig. 3 Bacterial community composition as revealed by high-throughput sequencing analyses in different samples of CWs. Sequences that could not be classified into any known group were assigned as unclassified bacteria (a) bacterial community composition at phylum level, (b) relative abundance of *Proteobacteria* subdivisions in CWs at class level, (c) relative abundance of *Bacteroidetes* subdivisions in CWs at class level, (d) relative abundance of *Firmicutes* subdivisions in CWs at class level. PC.L: *C. riparius* larvae samples of CWs-PC; PC.S: SS layer samples of CWs-PC; P.S: SS layer samples of CWs-P; PC.U: US layer samples of CWs-PC; P.U: US layer samples of CWs-P.

was 9.71% higher than that of the CWs-P, and US layer was 7.26% higher. For *Firmicutes* phyla (Fig. 3d), the relative abundance of *Bacillus* in the SS layer was 13.78% lower in the CWs-PC than in the CWs-P, but in the US layer the relative abundance of *Bacillus* was 17.89% higher in the CWs-PC. The relative abundance of *Clostridia* in the SS layer of the CWs-PC was 12.74% higher than in the same layer of the CWs-P, and there was no obvious difference observed between relative abundance in the US layer of the CWs-PC and CWs-P. Among these classes, gamma-*Proteobacteria*, *Flavobacteriia* and *Clostridia* are all related to denitrifying processes.[61,62] *Bacillus* is reported to be an aerobic bacterium and involved in heterotrophic nitrification.[63] The results of the relative abundance of *Bacteroidetes*, *Proteobacteria*, and *Firmicutes* subdivisions in the CWs at the class level indicated that *C. riparius* larvae could improve the relative abundance of denitrifiers in both the SS layer and US layer. Specifically, the results from the *Bacillus* class also suggested that the *P. crispus–C. riparius* CWs could supply oxygen to deeper substrates and could stimulate nitrifiers.

To further detect the influences of *C. riparius* larvae on denitrifiers and nitrifiers, the main potential functional bacteria responsible for denitrification and nitrification were identified by genus-level analysis in Fig. 4. The results showed that abundant denitrifiers and nitrifiers were observed in *C. riparius* larvae bodies, which provide evidence for microbial denitrification and nitrification prevailed. As for the substrate samples, no obvious difference was detected between the CWs-PC and CWs-P in SS layer. In US layer, the relative abundance of denitrifiers was higher in the CWs-PC than in the CWs-P, indicating that adding *C. riparius* larvae enhance denitrification in US layer, which would benefit NO_3^-–N removal. Besides, the relative abundance of nitrifiers was slightly higher in CWs-PC, which was related to the ventilation of *C. riparius* larvae burrows.

3.4 Implications for practical application of hardy submerged plant-benthic fauna systems

In the present study, we tested the performance of the *P. crispus–C. riparius* system on performance intensification of SFCWs in winter. Our results, based on laboratory-scale microcosms, clearly showed that the *P. crispus–C. riparius* system intensified the nutrient removal in winter. Because *C. riparius* can acclimate and maintain populations in CWs every winter with its high reproductive ability and the tolerance in varieties of environment,[27] we are confident on the long-term nutrients removal performance of such ecological system. Now, further experiments are needed to validate the long-term viability of large scale *P. crispus–C. riparius* CWs.

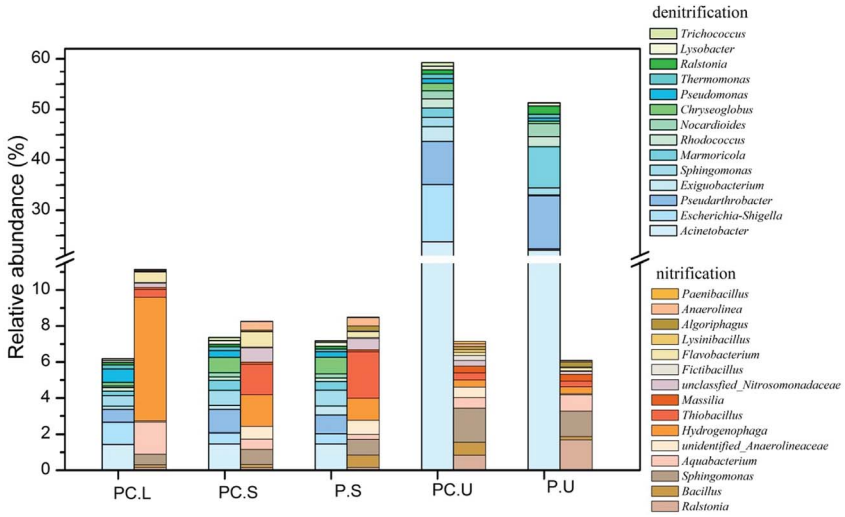

Fig. 4 Relative abundance variations of main potential functional genus in different samples of CWs. Sequences that could not be classified into any known group were labelled as "unclassified" PC.L: *C. riparius* larvae samples of CWs-PC; PC.S: SS layer samples of CWs-PC; P.S: SS layer samples of CWs-P; PC.U: US layer samples of CWs-PC; P.U: US layer samples of CWs-P.

4. Conclusions

The *P. crispus*–*C. riparius* system could enhance the nutrient removal performance of CWs in winter. In this system, the NH_4^+–N removal efficiency was high with the prominent oxygen supply ability of *P. crispus* and the ventilation of burrows created by *C. riparius* larvae. The bioturbation and burrows of *C. riparius* larvae facilitated fluxes of nutrient between US layer and water. As a result, N transformation was enhanced and NO_3^-–N removal efficiency was improved with *C. riparius* addition. Besides, the substrate storage and plant uptake towards nutrient were enhanced, contributing to the enhanced TN and TP removal. In addition, the microbial analysis reflected that *C. riparius* larvae influenced the abundance and structure of N related microbes, which also beneficial to N removal. Thus, hardy submerged plant-benthic fauna systems could be a promising ecological technology to improve the performance of SFCWs in winter.

Conflicts of interest

There are no conflicts to declare.

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