

Article

Effects of *Misgurnus anguillicaudatus* and *Cipangopaludina cathayensis* on Pollutant Removal and Microbial Community in Constructed Wetlands

Pengfei Li ¹, Jian Zhang ^{2,*}, Huijun Xie ¹, Zhen Hu ², Haiyan He ² and Wenxing Wang ¹

¹ Environment Research Institute, Shandong University, Jinan 250100, China;

E-Mails: lpflyss@163.com (P.L.); xiehuij@sdu.edu.cn (H.X.); wxwang@sdu.edu.cn (W.W.)

² School of Environmental Science and Engineering, Shandong University, Jinan 250100, China;

E-Mails: huzhen885@sdu.edu.cn (Z.H.); hhy636@126.com (H.H.)

* Author to whom correspondence should be addressed; E-Mail: zhangjian00@sdu.edu.cn;

Tel./Fax: +86-531-88369518.

Academic Editors: Say-Leong Ong and Jiangyong Hu

Received: 17 February 2015 / Accepted: 13 May 2015 / Published: 21 May 2015

Abstract: Aquatic animals play an important role in the energy flow and matter cycling in the wetland ecosystem. However, little is known about their effects on pollutant removal performance and microbial community in constructed wetlands. This work presents an initial attempt to investigate the effects of *Misgurnus anguillicaudatus* (loach) and *Cipangopaludina cathayensis* (snail) on nutrient removal performance and microbial community of constructed wetlands (CWs). Compared with a control group, CW microcosms with aquatic animals exhibited better pollutant removal performance. The removal efficiencies of total phosphorus (TP) in the loach group were 13.1% higher than in the control group, and snails increased the ammonium removal most effectively. Moreover, the concentration of total organic carbon (TOC) and TP in sediment significantly reduced with the addition of loaches and snails ($p < 0.05$), whereas the concentration of total nitrogen (TN) showed an obvious increase with the addition of loaches. High-throughput sequencing showed a microbial community structure change. Loaches and snails in wetlands changed the microbial diversity, especially in the *Proteobacteria* and denitrifying community. Results suggested that benthic aquatic animals might play an important role in CW ecosystems.

Keywords: treatment wetlands; high-throughput sequencing; sediment; benthic animals

1. Introduction

Wetlands are one of the most productive ecosystems, providing unique habitats for birds, plants, fish, and benthic animals. Constructed wetlands (CWs) are artificial wetlands designed to intercept wastewater and remove a wide range of pollutants before being discharged into natural water bodies. Most studies focused on the design of CWs with high pollutant removal efficiency by cultivating different kinds of plants (e.g., *Typha orientalis*, *Phragmites australis*), changing the operating methods of CWs (e.g., subsurface low and free water surface flow systems), and using different kinds of sediment media (e.g., sand, coal gangue) [1–3]. Furthermore, an increasing number of studies have explored benthic animal communities and factors influencing them in CWs [4,5]. Nevertheless, little is known about the effects of aquatic benthic animals on pollutant removal in CWs.

Benthic animals can directly absorb pollutions and transfer organic detritus from sedimentary storage into dissolved nutrients, which are removed by plants and algae absorption or microbial transformation [6]. Through their interaction, such as uptake of food from sediment, burrowing, and defecation, benthic species link energy flow and matter cycling in wetland ecosystems [7]. Furthermore, benthic species increase the sediment–water interface, which facilitates particle exchange between sediment and the water column. As a result, benthic animals can directly and indirectly stimulate pollution removal in sediment by influencing microbial population and activity [8]. However, research about the effects of aquatic benthic animals on microbial community structure and diversity in CWs is scarce.

Information about microbial community structure and diversity is important for understanding the relationship between environmental and ecosystem functions [9]. Microbial communities are also known as major drivers of wetland functions, supporting elemental cycling and biodegradation of pollutants [6,10]. An understanding of the bacterial community composition and structure in wetlands can increase the likelihood of successfully constructing a treatment wetland. The currently available high-throughput sequencing (using 16S rRNA genes) of environmental DNA allows for the rapid analysis of diversity and functionality of microbial communities [11].

As an initial attempt, the effects of loaches and snails on pollutant removal in both water and sediment are evaluated in this study. Microbial community and diversity are evaluated during the process, based on Illumina technology. This work will contribute to a better understanding of pollutant removal and microbial community variation and the involvement of benthic animals in CWs.

2. Materials and Methods

2.1. Benthic Animal Selection

Misgurnus anguillicaudatus (loach) and *Cipangopaludina cathayensis* (snail) are the most common and typical benthic species in wetland systems in China. Those two species can survive on high nutrient levels and low dissolved oxygen conditions [12] and are able to influence the water or sediment condition directly or indirectly [13].

2.2. CWs Microcosm Set-Up

Wetland microcosms were designed in a surface flow constructed wetland (SFCWs) and established under a transparent rain shelter. The experiments lasted from July to November. Twelve experimental wetland units consisting of polyethylene tubs were operated side by side. The treatment units were 65 cm deep and 50 cm in diameter with an outlet at the bottom, filled with washed river sand (particle size <2 mm, 25 kg) as the sediment to a depth of 25 cm. A total of 20 L of water was kept approximately 10 cm above the sand surface. The schematic of wetland microcosms is shown in Figure 1. Samples of *Phragmites australis*, which is common in China and efficient in wastewater treatment, were collected from the natural wetlands of Baiyun Lake in Zhangqiu, Shandong province. *P. australis* stalks approximately 1 m in height were carefully and gently washed with tap water to remove adhering soil and dead plant tissue, then cultivated with 10% Hoagland solution for two weeks. Well-grown plants with similar sizes were chosen and moved into the laboratory-scale CWs systems in May. The planting density of *P. australis* was 65 ± 2.3 plants/m².

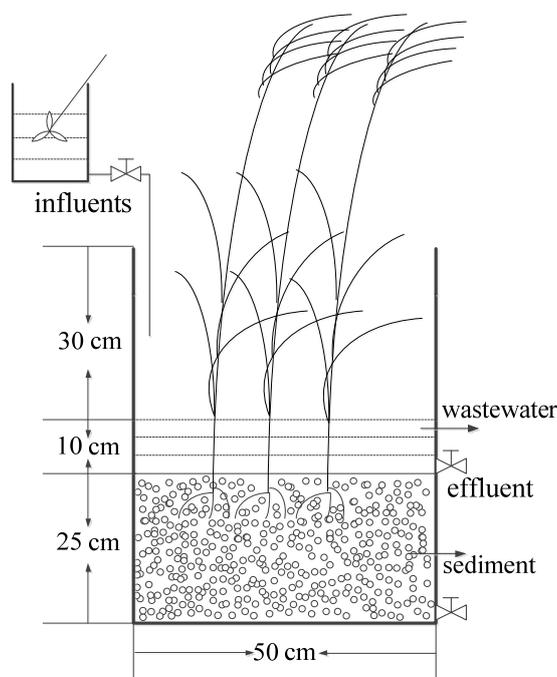


Figure 1. Schematic of the wetland microcosms.

Loaches and snails were bought from the aquarium market. We chose ones with similar size and weight (7.5 ± 0.7 g for loaches and 5.3 ± 0.5 g for snails). All animals were kept in tap water for one week before being put into the CWs systems. Then, animals were put into CWs systems for 30 days to adapt the environment. During this period, dead or unhealthy animals would be excluded. During the whole experimental period of nearly 4 months, CWs were well operated and both plants and animals in CWs were flourishing and healthy.

To minimize experimental variability, a simulation of polluted river water based on Grade II treatment standard of municipal sewage treatment plants in China was used in the experiment. The simulated polluted river water was composed of glucose, $(\text{NH}_4)_2\text{SO}_4$, KH_2PO_4 , and KNO_3 . The primary pollutants of the simulated polluted river water were organic matter, ammonia nitrogen, and phosphorus. The

concentration of chemical oxygen demand (COD), ammonium (NH₄-N), total nitrogen (TN), total phosphorus (TP), and other water parameters are shown in Table 1. Sequencing batch-fed mode was applied in the study. Each wetland unit was drained through the effluent valve of each unit after a cycle. These units were immediately re-filled manually with the simulated polluted river water. Each unit was operated at hydraulic retention time (HRT) of 7 d, with theoretical hydraulic loading rate of 0.015 m³/m²d.

Table 1. Characteristics of influents from the wetland microcosm units ($n = 30$).

Parameter	Mean Concentration	Standard Deviation
COD (mg/L)	61.21	3.24
TN (mg/L)	21.53	1.12
NH ₄ -N (mg/L)	7.94	0.32
TP (mg/L)	1.22	0.14
DO (mg/L)	7.15	1.35
pH	7.56	0.31

2.3. Experimental Design and Sampling

The experiment was divided into four groups; each group had three parallel samples. Every group had a *P. australis* wetland unit with different benthic animals, as described in Table 2.

Table 2. Benthic animal addition in different groups.

Group	Benthic Animals	Density (g/m ²)
Control	-	-
Snail	<i>M. anguillicaudatus</i>	149.43 ± 3.34
Loach	<i>C. cathayensis</i>	150.62 ± 5.42
Mixture	<i>M. anguillicaudatus</i>	75.21 ± 2.31
	<i>C. cathayensis</i>	76.13 ± 3.53

Note: - no animals added.

Effluents at SFCWs were sampled to evaluate the transformations of organics, nitrogen, and phosphorus. Water samples were collected from 8:00 a.m. to 10:00 a.m., using a syringe tube at 5 cm depth below the water surface of each unit every 2 d. Each group comprised three parallel samples. Laboratory analysis was performed on the water samples for COD, NH₄-N, nitrate (NO₃-N), nitrite (NO₂-N), TN, and TP. All the parameters mentioned above were determined based on standard methods [14]. COD was measured by a HACH DR 2800TM Spectrophotometer, USA.

To evaluate TN, TP, and total organic carbon (TOC) storage in the sediment media during the experiment, for each barrel, sediments were collected at five individual locations (four corners and the center of the microcosms) in each parallel and then mixed into a composite sample. Sediment samples from the three parallel samples were subsequently combined with a composite sample before DNA extraction and then air-dried and ground before the TN, TP, and TOC measurement [15,16]. Analyses in media samples were conducted with a 3-AA3 Auto Analyzer (Bran-Luebbe, Hamburg, Germany).

2.4. DNA Extraction and Pyrosequencing

DNA was extracted using the Power Soil DNA isolation kit (MoBio Laboratories, Inc., Carlsbad, CA, USA), following the manufacturer protocols. The DNA concentrations were determined using a Nanodrops ND-1000 UV-vis spectrophotometer (NanoDrop Technologies, Wilmington, DE, USA). 16S rDNA gene amplification was generated using the universal bacterial primers 11f and 1428r. The Miseq pyrosequencing was performed at the Major Biocompany (Shanghai, China).

Sequences that were shorter than 250 bp in length and of a lower quality score than 30 were removed from the pyrosequencing-derived data sets. Clustering of the sequencing into operational taxonomic units (OTUs) was performed upon UCLUST software at 97% sequence identity [17]. Rarefaction analysis, Simpson diversity, Chao1 richness estimations, and Good's coverage were calculated by Mothur analysis (<http://www.mothur.org>) at 3% distance level.

2.5. Statistical Analysis

All statistical analyses were performed with the statistical program SPSS 17.0 (SPSS Inc., Chicago, IL, USA), including analysis of variance (ANOVA). In all tests, differences were considered statistically significant when $p < 0.05$.

3. Results and Discussion

3.1. Pollutant Removal in Water

The variation of pollutants removal efficiency is shown in Figure 2. The removal efficiencies of TP and NH₄-N were relatively high compared to those of COD and TN. COD and TN removal efficiency in all samples represented similar trends, with gradual decrease (Figure 2a,c). In general, TN removal increased early and then gradually decreased over time from the beginning to the end, with the maximum removal efficiency (80%) occurring in July. No significant difference between COD and TN removal efficiency among treated and control samples ($p > 0.05$) was observed.

NH₄-N removal efficiencies of treated units indicated obvious differences from the control group ($p < 0.05$), highest at 86.85% in snail groups, followed by loach and mixture groups, and lowest at 70.09% in control samples. In the entire experimental time, the removal efficiency in the snail group showed significant difference from the control group ($p < 0.05$). In traditional aquaculture, aquaculture wastewater always has high concentrations of ammonia [18,19] because of high stocking density and ammonia excretion of aquatic animals. In the present research, the density of animals was lower than that in aquaculture, and the addition of animals into constructed wetland did not increase the concentrations of ammonia. The density of animals is one of the important influences on water quality. In the wetland microcosms of the present study, the fauna population only slightly increased in the entire experimental time because of the limited food supplement. Removal efficiency of NH₄-N and TN indicated certain differences, whereas TN removal indicated no obvious difference from other groups. This result indicated a possibility of differences in NO₃-N or NO₂-N removal.

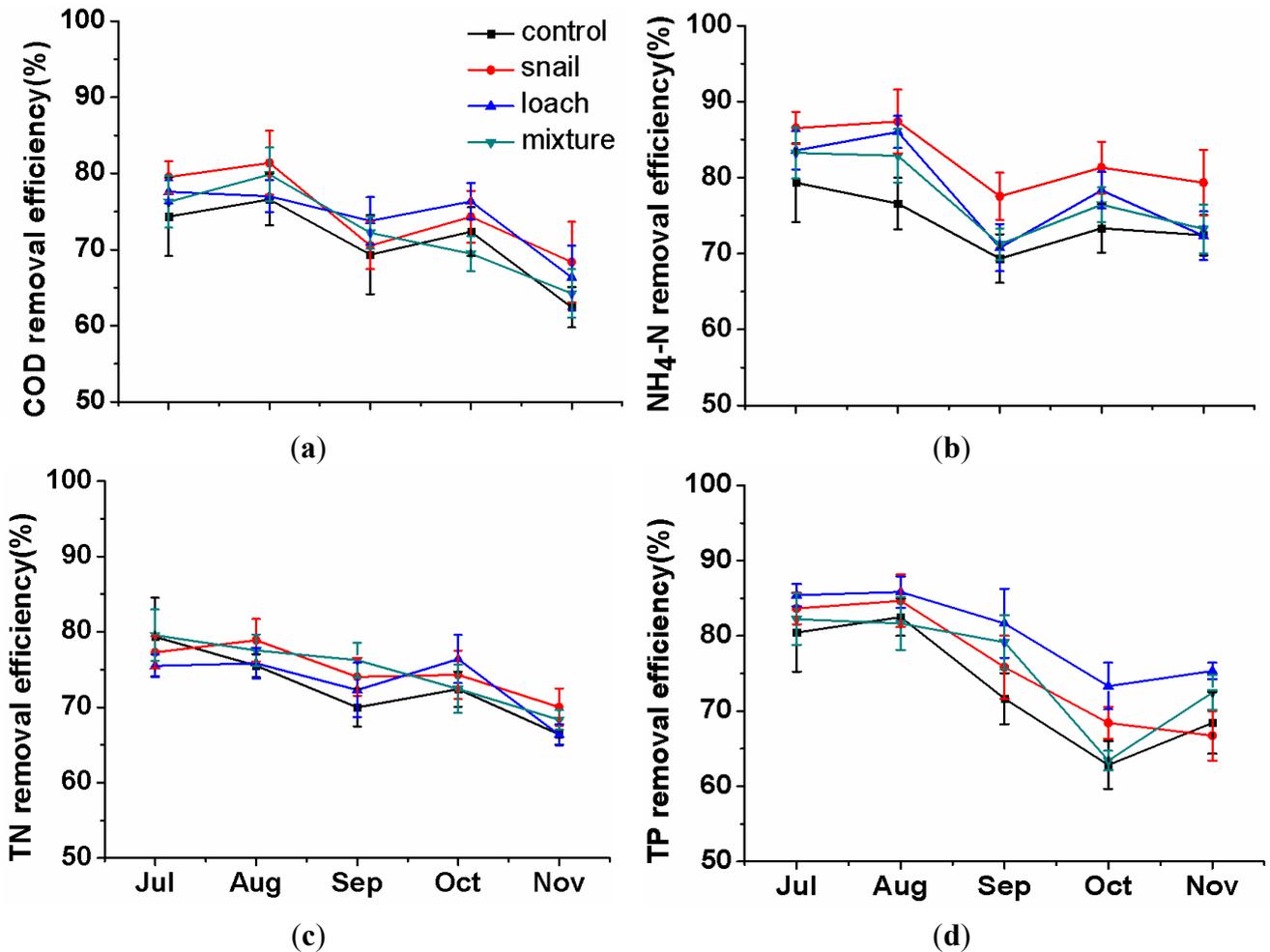


Figure 2. Removal efficiency of COD (a); NH₄-N (b); TN (c); TP (d).

Loach units showed highest removal performance of TP. The average TP removal efficiency ranged from 76.32% to 86.42%, which was much higher than the lowest level of 62% in the control unit ($p < 0.05$). The removal performance of TP in the loach group was also higher than that in the snail group. However, in July and August the difference was not significant ($p > 0.05$). Phosphorus was removed in CWs through substrate adsorption, chemical precipitation, bacterial action, plant and algal uptake, and incorporation into organic matter. Benthic animals in CWs may increase P uptake owing to their ingestion, bacterial action, and plant and algal uptake. Sediment adsorption also plays an important role [20].

The nutrient removal of all units in summer was higher than that in the winter. Consistent with other reports [21,22], we think temperature was one of the most important effects on the removal of nutrients. Temperature in summer are much higher than in winter, and in general, high temperature usually increase the activity of the microorganisms, oxygen availability, and plant uptake.

The difference between NH₄-N and TN removal in each group showed certain differences in NO₃-N or NO₂-N removal. Thus, to further understand the effects of benthic animals on N removal, concentrations of NO₃-N and NO₂-N in effluent were analyzed (Figure 3). Different wetland animals play different roles in NO₃-N and NO₂-N concentration. The concentration of NO₂-N was highest in the snail group, followed by the mixture group and the control group. The loach group had the highest concentration of NO₃-N and the lowest concentration in NO₃-N among the four groups. During the study

period, the average NO₃-N concentrations in the loach group were reduced from 3.21 ± 1.04 mg/L to a minimum of 2.64 ± 0.81 mg/L. The average NO₂-N concentrations in the loach group were reduced from 0.27 ± 0.07 mg/L to a minimum of 0.18 ± 0.03 mg/L. Blair *et al.* [23] found that the addition of earthworms increased the NO₃-N concentration in agroecosystems over a two-year period, corroborating the results of the present study.

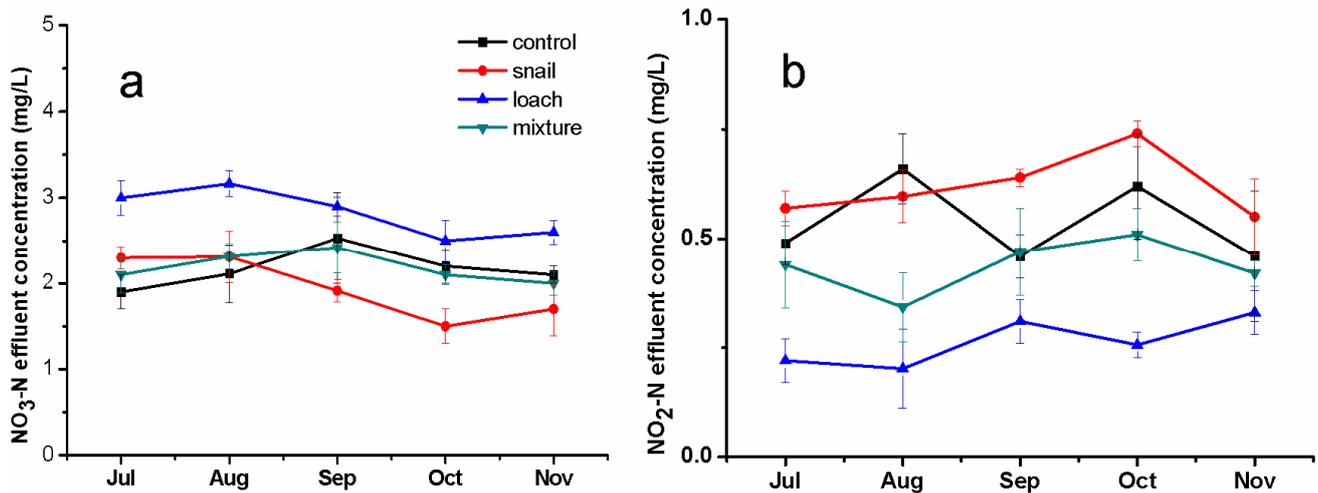


Figure 3. NO₃-N (a) and NO₂-N (b) concentrations of effluent.

3.2. Nutrient Changes in the Sediment

Notably, bioturbation can significantly change sediment characteristics. Results of nutrient changes in the sediment are shown in Figure 4.

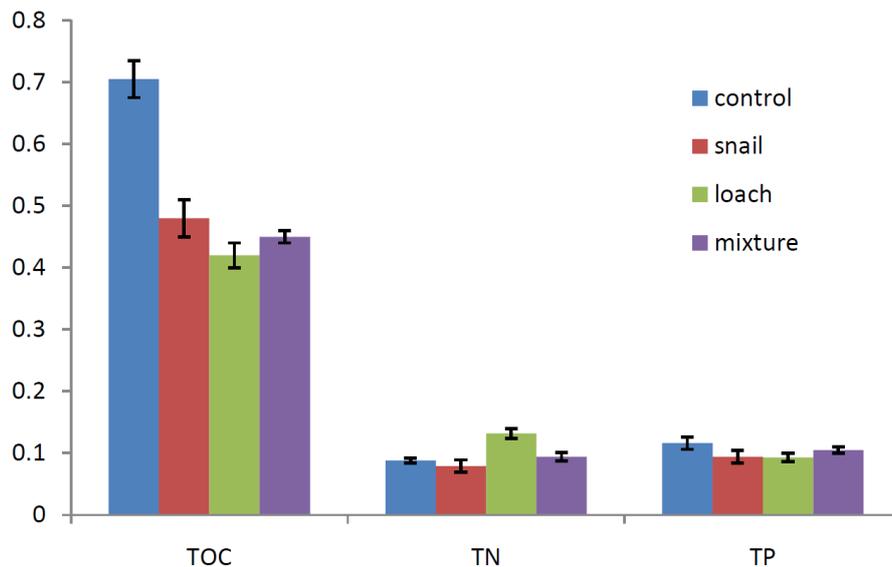


Figure 4. Nutrient and organic matter content of the sediment (average ± standard error) in the treatment and the control at the end of the experiment.

Our results showed that the four units have similar TP concentration. The average TP concentrations in all units throughout the experimental period ranged from 0.11 ± 0.09 mg/g to 0.16 ± 0.05 mg/g. The

concentration of TP in sediment in treated groups was not significantly different from the control group ($p > 0.05$), but the removal efficiency of TP in water (Figure 2d) in the loach group was much higher than in the control group ($p < 0.05$). Therefore, the addition of loach in CWs may increase the removal efficiency of TP in water, which could contribute to higher P uptake owing to bacterial action and plant and algal uptake.

The concentration of TN in the loach group was much higher than in the three other groups, up to 0.149 mg/g ($p < 0.05$), followed by the mixture group, the control group, and the snail group. The high concentration of TN in the loach group may have been caused by the excretion of the loaches and the significant high concentration of $\text{NO}_3\text{-N}$ in the water, which may cause adsorption in sediment.

The TOC concentration in the control group was much lower than in the other three groups ($p < 0.05$). The concentration of TOC was 0.71 ± 0.02 , 0.48 ± 0.03 , 0.42 ± 0.02 , and 0.45 ± 0.01 mg/g in the control, snail, loach, and mixed animal units, respectively. TOC plays an important role in many biogeochemical processes in wetland systems. TOC can strongly affect the transport of contaminants from soils to surface water because of its complexation capacity [24]. The decrease of TOC in the experimental group may have been caused by the ingestion of benthic animals; different animals possess different ingestion abilities. The presence of benthic animals in CWs decreases the concentration of TOC in sediment, which could also decrease the release of organic carbon from sediments at the same time.

3.3. Microbial Communities

The key processes for nutrient removal in CWs include microbial conversion, decomposition, plant uptake, sedimentation, and adsorption-fixation reactions [25]. Microbe activity is the main factor influencing the ammonia removal. Thus, more research must be conducted about the microorganism.

A total of 146,859 reads were obtained from the four samples through pyrosequencing analysis. The average read length is 401–418 base pairs (bp). Each library contained 32,378–40,422 reads. The good coverage index revealed that 93.60%–95.02% of the animals were obtained, with OTUs ranging from 3501 to 5191 (Table 3).

Chao1 and ACE were employed as community richness estimators at 3% dissimilarity. The Chao1 richness estimator showed that the richness ranged from 6852.16 to 9955.64. Compared with the control group, a decreasing trend was observed in the loach group. The mixture and snail groups, however, showed crosscurrent tendency. Therefore, the benthic species in the present study changed the community richness of this CWs. CWs with snails had the highest amount of bacteria among the four groups, which probably occurred because of the ingestion and movement of the snails [26].

Table 3. Comparison of phylotype coverage, diversity, and richness estimators at a phylogenetic distance of 3%.

Group	Cutoff	OTUs	ACE	Chao 1	Shannon	Simpson ($\times 10^{-3}$)	Coverage (%)
control	0.03	4,216	9,417.33	7,367.66	6.03	41.62	94.73
Snail	0.03	5,191	13,097.03	9,955.64	6.43	18.50	93.43
Loach	0.03	3,501	9,009.30	6,852.16	5.66	31.29	95.03
mixture	0.03	4,233	10,029.95	7,917.18	6.85	3.645	93.62

The community diversity was analyzed by calculating the Shannon and Simpson diversity index. For the four groups, an order of mixture > snail > control > loach was obtained in terms of community diversity. Based on these results, the snails had positive effects on the bacterial community. The loach group had less density of microbes, as it ingests microbes from sediment as food.

Figure 5 shows the relative abundance of the bacterial phylogenetic groups for each wetland unit. Proteobacteria represented the majority of the community composition of all sediments. Nevertheless, the relative abundance of this phylum was particularly high in the environments of the CWs, ranging from 30% to 42% in four groups. The Proteobacteria phylum includes a very high level of bacterial metabolic diversity related to global carbon, nitrogen, and sulfur cycling [27]. The wide distribution of this phylum in nature [28,29] and in CWs [30,31] has been documented.

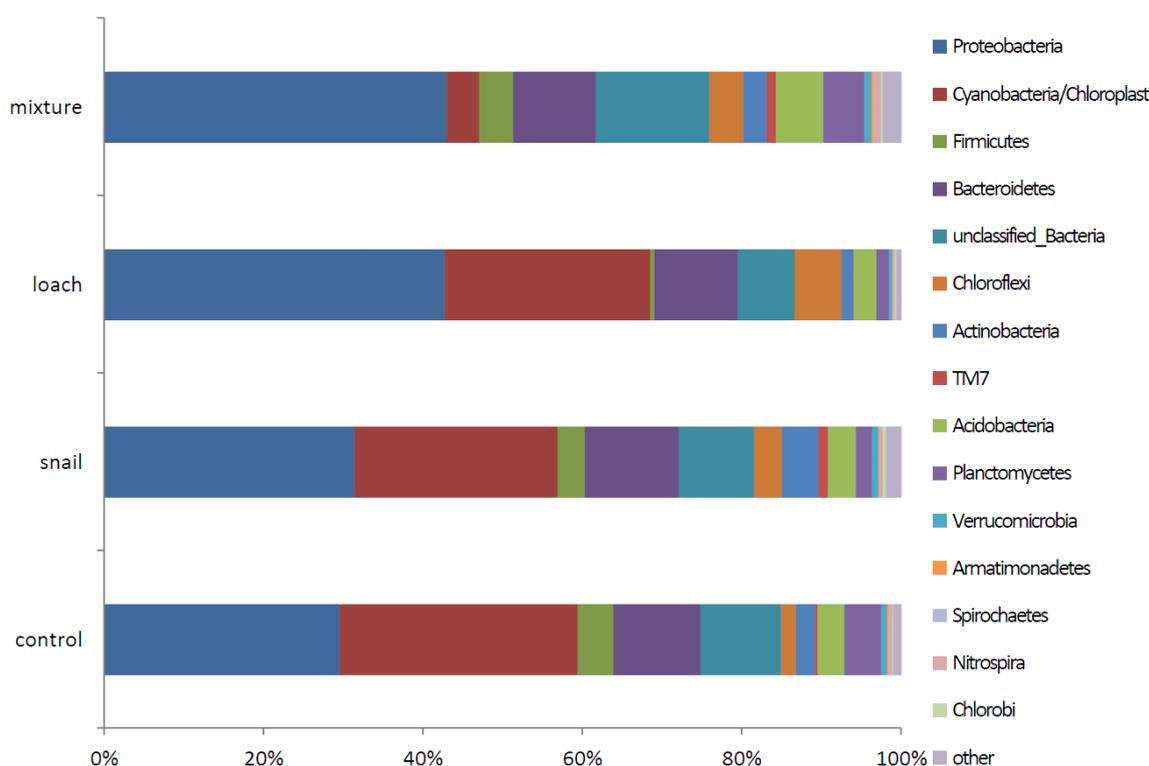


Figure 5. Abundances of the bacterial phylogenetic groups.

Within the phylum Deltaproteobacteria, particularly the Syntrophorhabdus, Desulfonema, Smithellagenera, and Gammaproteobacteria, mainly Pseudomonas were enriched in the soil environments of the CWs (Figure 6). Bacteria belonging to the phylum Verrucomicrobia are nearly ubiquitous in soil [32]. The evidence available indicates that members of this phylum are globally distributed, abundant, and active groups of soil bacteria [33]. Apparently, the activities of loach lead to the increase of Proteobacteria phylum. The cyanobacteria in snail and loach groups had a significant decrease compared with the three other groups. The snails thrived and had much larger numbers than did the other groups. Snails ingested the cyanobacteria as food.

As known, the process of nitrate reduction is dependent on the denitrifier communities. *Bacillus*, *Micrococcus*, and *Pseudomonas* are the main denitrifier communities in wetland sediment [34]. The consortiums of these three microbes were found to be efficient in nitrate reduction. Figure 7 shows the relative abundance of the denitrifier communities. *Bacillus*, *Micrococcus*, and *Pseudomonas* in the loach

group were lower than those in the three other groups. *Bacillus* in the control group was much higher than in the three other groups. The snail group had the most *Micrococcus* species, and *Pseudomonas* was the highest in the mixture group.

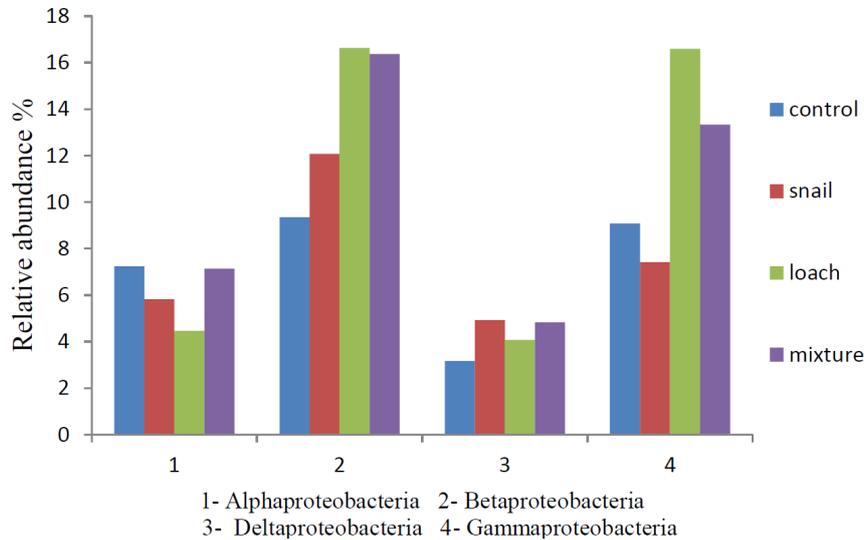


Figure 6. Relative bundances of the Proteobacteria groups.

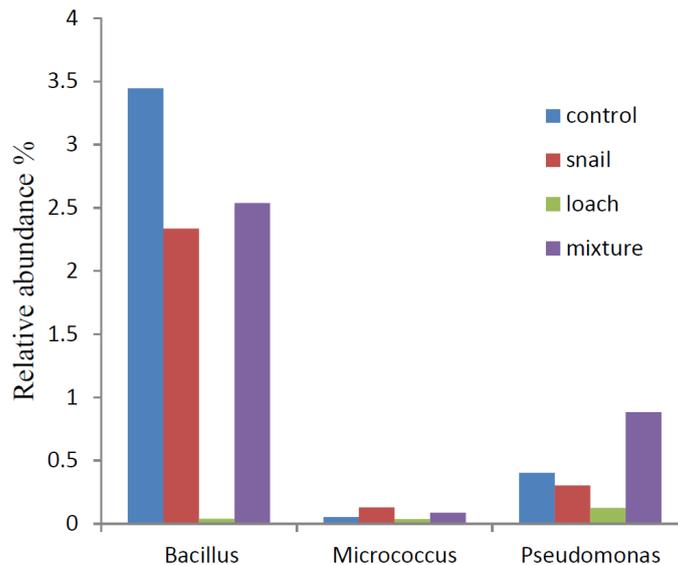


Figure 7. Relative abundances of the denitrifier communities.

Figure 2 shows that the loach group has high NO₃-N and low NO₂-N content, and has significant difference from the three other groups ($p < 0.05$). Investigation of the aerobic metabolism of *Bacillus*, *Micrococcus*, and *Pseudomonas* revealed many interesting features of assimilatory nitrate reduction to ammonia. *Bacillus* species could carry out anaerobic dissimilatory reduction of nitrate to ammonia via nitrite [35]. *Pseudomonas* in the wetland system attained assimilatory denitrification [36]. The genera of *Bacillus*, *Micrococcus*, and *Pseudomonas* were found to be nitrate reducers. Among the three, *Pseudomonas* and *Bacillus* were found to be the most efficient in terms of nitrate reduction [37]. These two kinds of genera were just numerically superior. When the denitrifier communities were low, nitrate was reduced, and the concentration of NO₃-N was high.

4. Conclusions

Benthic animals in wetland systems can improve the removal efficiencies of TP, TN, and NH₄-N in effluent. Loach units showed the highest removal performance of TP and reduction of the concentration of TOC and TP in sediment. The loach activities led to the increase of the Proteobacteria phylum. The mixture group increased the community diversity of microorganisms. The presence of benthic animals in CWs changed the microbial structure and increased the biodiversity of the microcosms. The results suggest that benthic aquatic animals can reduce the concentration of pollutants and improve the quality of CWs ecosystems. The high diversity of the communities sampled in the natural ecosystem could be due to a higher number of ecosystem functions.

Acknowledgments

This work was supported by the Independent Innovation Foundation of Shandong University (2014JC023), the National Natural Science Foundation of China (No. 21307076), the National Water Special Project (2012ZX07203-004), and the Fundamental Research Funds of Shandong University (No. 2014TB003).

Author Contributions

The experimental work was conducted by Jian Zhang, Wenxing Wang, Huijun Xie and Pengfei Li. The manuscript was written by Pengfei Li, Huijun Xie and Zhen Hu. Data analysis was performed by Pengfei Li and Haiyan He. Microbial analysis was performed by Huijun Xie and Pengfei Li. All authors approved the final version of the article, including the authorship list.

Conflicts of Interest

The authors declare no conflict of interest.

References

1. Thullen, J.S.; Sartoris, J.J.; Nelson, S.M. Managing vegetation in surface-flow wastewater-treatment wetlands for optimal treatment performance. *Ecol. Eng.* **2005**, *25*, 583–593.
2. Nahlik, A.M.; Mitsch, W.J. Tropical treatment wetlands dominated by free-floating macrophytes for water quality improvement in Costa Rica. *Ecol. Eng.* **2006**, *28*, 246–257.
3. Ávila, C.; Reyes, C.; Bayona, J.M.; García, J. Emerging organic contaminant removal depending on primary treatment and operational strategy in horizontal subsurface flow constructed wetlands: Influence of redox. *Water Res.* **2013**, *47*, 315–325.
4. Spieles, D.J.; Mitsch, W.J. Macroinvertebrate community structure in high-and low-nutrient constructed wetlands. *Wetlands* **2000**, *20*, 716–729.
5. Jurado, G.B.; Johnson, J.; Feeley, H.; Harrington, R.; Kelly-Quinn, M. The potential of integrated constructed wetlands (ICWs) to enhance macroinvertebrate diversity in agricultural landscapes. *Wetlands* **2010**, *30*, 393–404.
6. Zak, D.R.; Holmes, W.E.; White, D.C.; Peacock, A.D.; Tilman, D. Plant diversity, soil microbial communities, and ecosystem function: Are there any links? *Ecology* **2003**, *84*, 2042–2050.

7. Fanjul, E.; Escapa, M.; Montemayor, D.; Addino, M.; Alvarez, M.F.; Grela, M.A.; Iribarne, O. Effect of crab bioturbation on organic matter processing in South West Atlantic intertidal sediments. *J. Sea Res.* **2014**, *95*, 206–216.
8. Covich, A.P.; Palmer, M.A.; Crowl, T.A. The role of benthic invertebrate species in freshwater ecosystems: Zoobenthic species influence energy flows and nutrient cycling. *Bioscience* **1999**, *49*, 119–127.
9. Sims, A.; Zhang, Y.; Gajaraj, S.; Brown, P.B.; Hu, Z. Toward the development of microbial indicators for wetland assessment. *Water Res.* **2013**, *47*, 1711–1725.
10. Adrados, B.; Sánchez, O.; Arias, C.A.; Becares, E.; Garrido, L.; Mas, J.; Brix, H.; Morató, J. Microbial communities from different types of natural wastewater treatment systems: Vertical and horizontal flow constructed wetlands and biofilters. *Water Res.* **2014**, *55*, 304–312.
11. İnceoğlu, Ö.; Al-Soud, W.A.; Salles, J.F.; Semenov, A.V.; van Elsas, J.D. Comparative analysis of bacterial communities in a potato field as determined by pyrosequencing. *PLoS ONE* **2011**, *6*, doi:10.1371/journal.pone.0023321.
12. Alonso, Á.; Camargo, J.A. Nitrate causes deleterious effects on the behaviour and reproduction of the aquatic snail *Potamopyrgus antipodarum* (Hydrobiidae, Mollusca). *Environ. Sci. Pollut. Res.* **2013**, *20*, 5388–5396.
13. Gonçalves, A.F.; Castro, L.F.C.; Pereira-Wilson, C.; Coimbra, J.; Wilson, J.M. Is there a compromise between nutrient uptake and gas exchange in the gut of *Misgurnus anguillicaudatus*, an intestinal air-breathing fish? *Comp. Biochem. Physiol. Part D Genomics Proteomics* **2007**, *2*, 345–355.
14. American Public Health Association (APHA). *Standard Methods for the Examination of Water and Wastewater*, 20th ed.; American Public Health Association/American Water Works Association/Water Environment Federation: Washington, DC, USA, 1998.
15. Wu, H.; Zhang, J.; Li, P.; Zhang, J.; Xie, H.; Zhang, B. Nutrient removal in constructed microcosm wetlands for treating polluted river water in northern China. *Ecol. Eng.* **2011**, *37*, 560–568.
16. Wu, S.Q.; Chang, J.J.; Dai, Y.; Wu, Z.B.; Liang, W. Treatment performance and microorganism community structure of integrated vertical-flow constructed wetland plots for domestic wastewater. *Environ. Sci. Pollut. Res.* **2013**, *206*, 3789–3798.
17. Edgar, R.C. Search and clustering orders of magnitude faster than BLAST. *Bioinformatics* **2010**, *26*, 2460–2461.
18. Lin, Y.; Jing, S.; Lee, D.; Wang, T. Nutrient removal from aquaculture wastewater using a constructed wetlands system. *Aquaculture* **2002**, *209*, 169–184.
19. Gong, L.J.; Yang, X.F.; Xiong, B.X.; Li, G.P.; Chen, X.L. Study on Nitrogen, Phosphor and Chemical Oxygen Demand of Different Categories of Aquaculture Lakes by Means of Principal Component Analysis, Factor Analysis and Cluster Analysis. *Adv. Mater. Res.* **2012**, *340*, 369–377.
20. Xu, D.; Xu, J.; Wu, J.; Muhammad, A. Studies on the phosphorus sorption capacity of substrates used in constructed wetland systems. *Chemosphere* **2006**, *63*, 344–352.
21. Bachand, P.A.; Horne, A.J. Denitrification in constructed free-water surface wetlands: II. Effects of vegetation and temperature. *Ecol. Eng.* **1999**, *14*, 17–32.
22. Poach, M.E.; Hunt, P.G.; Reddy, G.B.; Stone, K.C.; Johnson, M.H.; Grubbs, A. Swine wastewater treatment by marsh–pond–marsh constructed wetlands under varying nitrogen loads. *Ecol. Eng.* **2004**, *23*, 165–175.

23. Blair, J.M.; Parmelee, R.W.; Allen, M.F.; McCartney, D.A.; Stinner, B.R. Changes in soil N pools in response to earthworm population manipulations in agroecosystems with different N sources. *Soil Biol. Biochem.* **1997**, *29*, 361–367.
24. Tipping, E. Modeling the competition between alkaline earth cations and trace metal species for binding by humic substances. *Environ. Sci. Technol.* **1993**, *27*, 520–529.
25. Tchobanoglous, G. Constructed wetlands and aquatic plant systems: Research, design, operational, and monitoring issues. In *Constructed Wetlands for Water Quality Improvement*; Lewis Publishers: Boca Raton, FL, USA, 1993; pp. 23–34.
26. Xu, D.; Li, Y.; Howard, A. Influence of earthworm Eiseniafetida on removal efficiency of N and P in vertical flow constructed wetland. *Environ. Sci. Pollut. Res.* **2013**, *20*, 5922–5929.
27. Kersters, K.; de Vos, P.; Gillis, M.; Swings, J.; Vandamme, P.; Stackebrandt, E. Introduction to the Proteobacteria. In *The Prokaryotes: A Handbook on the Biology of Bacteria*; Springer: New York, NY, USA, 2006; Volume 5, pp. 3–37.
28. Wang, Y.; Sheng, H.; He, Y.; Wu, J.; Jiang, Y.; Tam, N.F.; Zhou, H. Comparison of the levels of bacterial diversity in freshwater, intertidal wetland, and marine sediments by using millions of illumina tags. *Appl. Environ. Microbiol.* **2012**, *78*, 8264–8271.
29. Peralta, R.M.; Ahn, C.; Gillevet, P.M. Characterization of soil bacterial community structure and physicochemical properties in created and natural wetlands. *Sci. Total Environ.* **2013**, *443*, 725–732.
30. Gorra, R.; Coci, M.; Ambrosoli, R.; Laanbroek, H.J. Effects of substratum on the diversity and stability of ammonia-oxidizing communities in a constructed wetland used for wastewater treatment. *J. Appl. Microbiol.* **2007**, *103*, 1442–1452.
31. Ahn, C.; Gillevet, P.M.; Sikaroodi, M. Molecular characterization of microbial communities in treatment microcosm wetlands as influenced by macrophytes and phosphorus loading. *Ecol. Indic.* **2007**, *7*, 852–863.
32. Zhang, L.; Xu, Z. Assessing bacterial diversity in soil. *J. Soils Sediments* **2008**, *8*, 379–388.
33. Sangwan, P.; Kovac, S.; Davis, K.E.; Sait, M.; Janssen, P.H. Detection and cultivation of soil Verrucomicrobia. *Appl. Environ. Microbiol.* **2005**, *71*, 8402–8410.
34. Chitra, A.V.; Lakshmanaperumalsamy, P. Biodegradation of nitrate in wastestreams from explosives manufacturing plants. *Res. J. Microbiol.* **2006**, *1*, 142–151.
35. Tiedje, J.M. Ecology of denitrification and dissimilatory nitrate reduction to ammonium. *Biol. Anaerob. Microorg.* **1988**, *717*, 179–244.
36. Ayyasamy, P.M.; Shanthi, K.; Lakshmanaperumalsamy, P.; Lee, S.; Choi, N.; Kim, D. Two-stage removal of nitrate from groundwater using biological and chemical treatments. *J. Biosci. Bioeng.* **2007**, *104*, 129–134.
37. Rajakumar, S.; Ayyasamy, P.M.; Shanthi, K.; Thavamani, P.; Velmurugan, P.; Song, Y.C.; Lakshmanaperumalsamy, P. Nitrate removal efficiency of bacterial consortium (*Pseudomonas* sp. KW1 and *Bacillus* sp. YW4) in synthetic nitrate-rich water. *J. Hazard. Mater.* **2008**, *157*, 553–563.