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Enhanced removal of organic matter and nitrogen in a vertical-flow constructed wetland with *Eisenia foetida*

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ABSTRACT

Constructed wetlands (CWs) have been widely applied for water quality amelioration. However, soil macrofauna usually considered as ecosystem engineers are scarce in wetlands. We hypothesize that introduction of an epigeic earthworm *Eisenia foetida*, a common ecosystem engineer, into wetlands would improve removal of organics and nitrogen in vertical-flow CWs. This paper quantitatively analyzed the effects of addition of *E. foetida* on removal of organic matter and nitrogen in vertical-flow wetlands. Results demonstrated that average removal efficiencies of COD, NH_4^+–N, and TN in the earthworm-amended wetlands were 15.7, 21.3, and 20.6%, higher than those observed in control wetlands with no addition of *E. foetida*, respectively. Moreover, the improvement was mainly achieved in the upper layer (0–20 cm) matrix, where *E. foetida* inhabited. Meanwhile, the total bacterial count, the numbers of ammonifier, ammonia-oxidizing bacteria and nitrite-oxidizing bacteria, as well as urease and protease activities in the upper layer of the earthworm-amended wetlands were higher than those in control wetlands. Therefore, it is suggested that *E. foetida* improved the removal of nitrogen and organics by stimulating the proliferation of bacteria and enzyme activities.

Keywords: Constructed wetland; Earthworm; Microorganism; Nitrogen; Organic matter

1. Introduction

Constructed wetlands (CWs) are effective and suitable systems for treatment of urban wastewater from small to medium communities with considerable benefits, such as low construction and operation costs, easy to handle, CO_2 consumption, and O_2 production [1,2]. CWs gain purification capacity from ecological functions of the wetland ecosystem itself. According to the fundamental theory of environmental eco-engineering, the longer the food chain, the greater the energy consumption is, thus the better its pollutant removal efficiency is. In CWs, however, important parts of the food chain, naturally formed soil animals, are not only poor in biomass but rare in numbers.

Earthworms, as one of the animal groups with the greatest biomass, play an irreplaceable role in maintaining the function of soil ecosystem. Numerous studies suggest that earthworms can improve the

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physical structure, aeration, and water permeability of soil [3]. They can also accelerate decomposition of organic carbon [4] and mineralization and recycling of nutrients such as nitrogen and phosphorus [5], as well as increase microbial activity in soil and promote plant growth [6]. Recently, an earthworm (*Pheretima peguana*) was introduced into two-stage pilot-scale subsurface-flow CWs for swine wastewater treatment and successfully alleviated the clogging of CWs [7].

Motivated by these previous studies, we sought to strengthen the purifying function of CW ecosystem by introducing *Eisenia foetida* into a vertical flow CW (VF-CW) to supplement the animal part of food chain. The aims of the present study were first to test whether addition of *E. foetida* is able to improve the performance of VF-CW with regard to the removal of nitrogen and chemical oxygen demand (COD) and second to study whether addition of *E. foetida* altered the spatial distribution of bacteria and microbial activities.

2. Materials and methods

2.1. Description of the wetlands

Four VF-CWs with identical dimensions of 1.50 m × 0.75 m × 0.50 m ($L \times W \times D$) were divided into two groups, earthworm-added treatment and non-earthworm treatment (served as control wetlands). Each treatment was composed of duplicate reactors. In 2009, the VF-CWs were implemented at the Taihu Lake field research station for water environment research (31°18′87″N, 119°56′73″E). The wetland tanks were made of concrete and the inner wall and bottom were lined with glass fiber reinforced epoxy resin with a thickness of 1 mm. A slope of 1% was created at the bottom of the wetlands to allow easier drainage. A drainage pipe of perforated PVC was placed at the bottom of the VF-CWs. Each tank had three sampling points at 10, 20, and 30 below the matrix surface. The tanks were filled with a support matrix of coal cinders to 40 cm in height (Fig. 1). Thirty seedlings of reed (*Phragmites communis* Trin.) collected from the shore of Taihu Lake were distributed evenly across the surface area of each wetland at the end of April 2009.

After planting, the wetlands were flooded for one month with tap water before they were loaded with raw sewage. The water quality of the actual rural sewage used for the experiments is shown in Table 1. The raw sewage was pumped from a septic tank to a perforated PVC pipe 10 cm above the surface of wetlands and gravity fed into the pilot-scale VF-CWs. All systems were loaded intermittently 6 times a day with a batch volume of 56.5 L, resulting in a hydraulic loading rate of 0.3 $m^3/(m^2\,d)$. It takes about 20 min to drain the outflow from wetlands, after each injection of wastewater. As a result, the minimum exposure time of the earthworms to the air was about 220 min during each cycle. In total, the exposure time of the earthworms to the air was 22 hours/day. All the wetlands were run in parallel under identical conditions.

For the two earthworm-amended wetlands, a total of 1,700 individuals of earthworm (*E. foetida*) were put into each wetland when the wetlands started to receive raw sewage. *E. foetida* was selected for these experiments due to its tolerance of a wide range of temperature and humidity, and its high proliferation rate relative to other species. *E. foetida* (Fig. 2) is cylindrical in shape with a length of 30–130 mm (shorter than 70 mm in most cases), a diameter of 3–5 mm, and

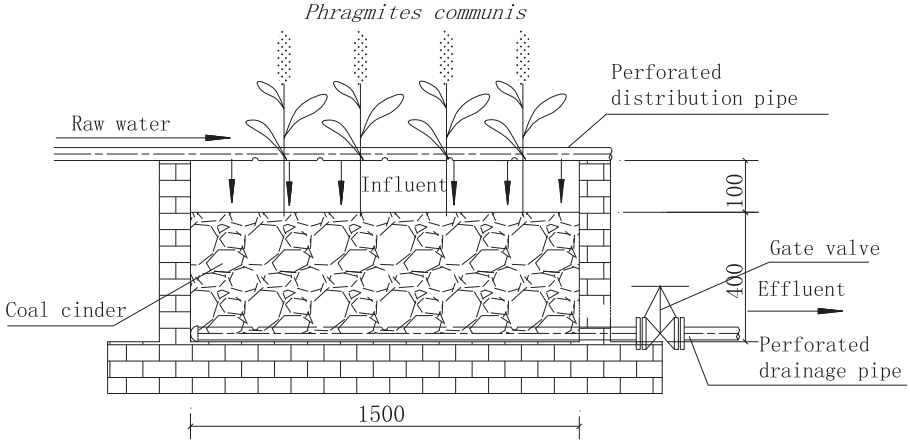

Fig. 1. Schematic diagram of the vertical-flow constructed wetland (Dimension Units = cm).

Table 1
Water quality of the rural sewage (mg/L)

Parameter	COD	NH_4^+–N	TN	SS
Range	95.42–509.38	7.37–55.51	13.57–72.13	40.7–58.2
Mean	324.35	27.39	63.49	44.7

Fig. 2. Image of the *E. foetida* used in the experiments.

80–110 somites per organism. *E. foetida* prefers a humid and quiet habitat. The suitable temperature for *E. foetida* is 5–30 °C. A temperature below 0 °C will lead to retardation while greater than 39 °C will lead to death. The life span of *E. foetida* is 2–10 years under natural conditions and extends to 15 years under a condition of artificial feeding [8].

2.2. Water sample analysis

After a stabilization period of two months, monitoring of the wetland for water quality began and continued for 3 months (from July 28 to Oct 30, 2009). The samples were taken every 2–3 days at five points in the system: at the input of the system and at the output of each wetland. In addition, water samples were collected across a transect of matrix (i.e. 10, 20, 30 cm below the matrix surface, respectively) through the pipes pre-installed along the depth of matrix. Water samples were analyzed for COD, ammonia nitrogen (NH_4^+–N), total nitrogen (TN), and suspended solids (SS) according to the protocols described in Chinese Standard Methods [9]. Once a sample was collected, dissolved oxygen (DO) was measured immediately by a WTWOxi330 DO analyzer (Germany).

2.3. Bacteria enumeration

Samples of coal cinder matrix for microbial community analyses were collected three times from different depths (5–10 and 30–35 cm below the wetland surface, respectively) of the wetlands. After roots and macro-fauna were removed by hand, the field-moist samples were sieved (around 2 mm) and divided into three subsamples. Two subsamples were stored at 4 °C for analysis of enzyme activities and for MPN (most probable number) enumeration. The third subsample was immediately fixed with paraformaldehyde and dehydrated with ethanol series and kept at −20 °C until subject to Fluorescence in Situ Hybridization (FISH) analysis. All analytical results were calculated on the basis of the oven-dry (105 °C) weight of coal cinder matrix.

2.3.1. Total bacteria and nitrifier

Total bacteria and nitrifiers were enumerated with a FISH protocol [10,11] that was slightly modified for this study. The cells were washed with phosphate-buffered saline (PBS, 130 mM NaCl and 10 mM NaH_2PO_4/NaH_2PO_4, pH 7.4) and stored in a 1:1 mixture of PBS and ethanol at −20 °C. Before hybridization, the samples were dispersed into individual cells by ultrasonicator and placed in wells on the gelatin-coated microscopic glass slides, then dehydrated by successive ethanol solution of 50%, 80% and 96% each for 3 min, dried in the air. All hybridizations were performed by placing the samples within vials containing the hybridization buffer and the probe at 46 °C for 3 h. The hybridization buffers were composed of 0.9 M NaCl, 20 mM trishydroxymethylaminomethane–hydrochloric acid (Tris–HCl), 0.01% sodium dodecyl sulfate (SDS), and a probe-specific formamide percentage as indicated in Table 2. The probe concentration within the hybridization buffer, for each of the three probes used in this study, was 5 mg L^{-1}. Hybridization was followed by rinsing the samples with a washing buffer at 48 °C for 20 min. The washing buffers consisted of 20 mM Tris–HCl, 5 mM ethylenediamine tetraacetic acid (EDTA), 0.01% SDS and a probe specific NaCl concentration as shown in Table 2. After being rinsed with sterile distilled water, and being air-dried, the slides were analyzed with fluorescence microscope (OLYMPUS-BX42, Japan) immediately. At least 20 different visual fields (each containing more than 1,000 cells) were examined for each sample.

2.3.2. Ammonifiers and denitrifiers

Ammonifiers and denitrifiers were enumerated with an MPN technique. Matrix samples were diluted in 1:10 serial dilutions in 8.5 g/L NaCl distilled water. Five replicates were prepared for each decimal dilution tube. The tubes were incubated at 22 ± 2 °C for at least 4 weeks. The composition of 1 L basal medium for the MPN method was: 5.30 g K_2HPO_4, 2.65 g

Table 2
The probe sequence of total bacteria, nitrite-oxidizing bacteria and ammonia-oxidizing bacteria and the hybridization conditions

Target bacteria	Probe name	Sequence(5′-3′)	5′-probe labeling		
				Formamide concentration (%)	NaCl concentration (mM)
Total bacteria	EUB338	GCT GCC TCC CGT AGG AGT	HEM	20	0.225
Nitrite-oxidizing bacteria	NIT3	CCT GTG CTC CAT GCT CCG	FITC	40	0.056
	CNIT3*	CCT GTG CTC CAG GCT CCG			
Ammonia-oxidizing bacteria	NSO190	CGA TCC CCT GCT TTT CTCC	HEX	55	0.020

Note: CNIT3* is the competitive probe of the NIT3.

KH_2PO_4, 5.0 mg Na_2S, 1.00 g NH_4Cl, 0.50 g yeast extract, 5.0 mL trace mineral solution [12], and 2.0 mg resazurin. After sterilization, 0.08 g $CaCl_2$ and 0.10 g $MgCl_2$ were added from sterile stock solutions. Denitrifiers were enumerated by MPN in the basal medium supplemented with 1.84 g/L potassium acetate and 0.72 g/L potassium nitrate. Acetylene (10% v/v) was added to the headspace and tubes were considered positive for denitrification, when accumulation of N_2O occurred in the head-space. Ammonifiers were enumerated by MPN in the basal medium supplemented with 0.50 g/L tryptone, 5 g/L glucose and 0.72 g/L potassium nitrate. Nitrate, nitrite and ammonium were measured after growth had ceased. Tubes with an increase in ammonium concentration of at least 30% of the added nitrate were considered positive for ammonification.

2.4. Determination of protease and urease activity

Urease and protease activities were determined in 0.1 M phosphate buffer at pH 7 [13]. 1 M urea and 0.03 M *N*-α-benzoyl-argininamide (BAA) were used as substrates, respectively. 2 ml of buffer and 0.5 ml of substrate were added to 0.5 g of the soil sample, which was incubated at 30°C (urease) or 39°C (protease) for 90 min. Both activities were determined by the NH_4^+ released and expressed as mg $NH_3-N/100$ g matrix/24 h.

2.5. Statistical analysis

The removal efficiency was calculated as percentage removal (R) for each parameter, calculated by $R = [1-(Q_eC_e)/(Q_iC_i)] \times 100$, where C_i and C_e are, respectively, the influent and effluent concentrations, while Q_i and Q_e are, respectively, the influent and effluent flow rates. All statistical analyses were done with SPSS 15.0 (SPSS, Chicago, USA). Two-sample *t*-tests were used to evaluate the significance of differences between the two types of CWs and between the replicates.

3. Results

3.1. Performance of the VF-CWs

There were no significant differences between the two replicates of each wetland type for all measured parameters throughout the experiments. As shown in Fig. 3, significant decreases in COD, NH_4^+–N, TN and SS concentrations of outflow water were observed for all wetlands, compared to inflow water. The average removal efficiency ($n = 34$) for the replicated control wetlands was 48.5% for COD, 42.3% for NH_4^+–N, 49.4% for TN, and 58.8% for SS, respectively. As for the earthworm-added wetlands, the average removal efficiency increased by 15.7% for COD, 21.3% for NH_4^+–N, 20.6% for TN, and 11.2% for SS, respectively. Further statistical analysis indicated that the increases in removal efficiencies were significant ($p < 0.05$) for each of the water quality parameters. Obviously, the purification performances of earthworm-amended wetlands were significantly higher than the performances of control wetlands, which was very likely attributable to the introduction of earthworms.

3.2. Vertical distribution of E. foetida

At the end of the experiments, earthworm samples were collected at different depths. Table 3 shows the distribution of *E. foetida* at different depths. The number

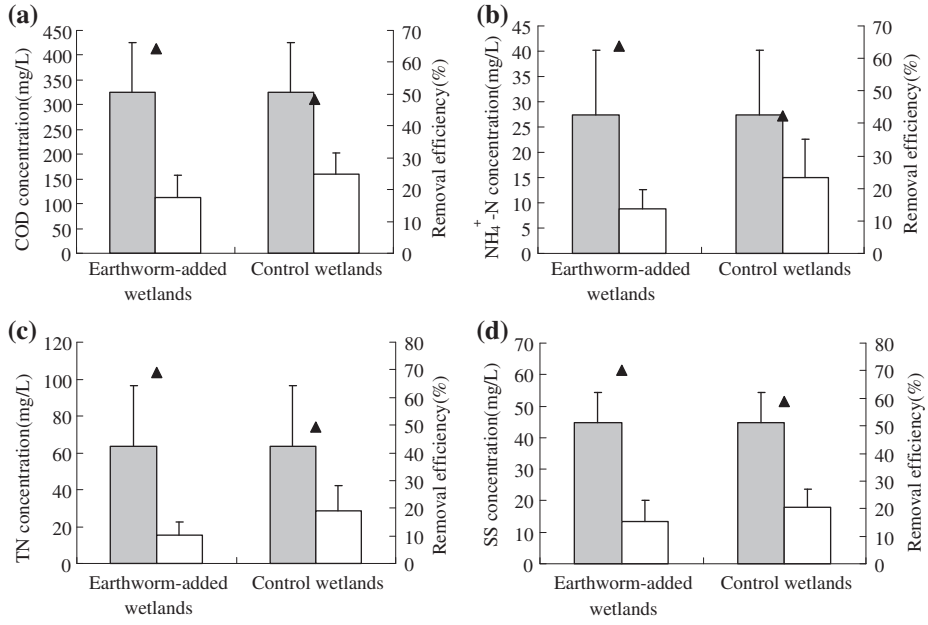

Fig. 3. Removal of (a) COD, (b) NH_4^+–N, (c) TN and (d) SS in earthworm-added wetlands and control wetlands with no addition of earthworm. (▲: Removal efficiency; ■: earthworm-added wetlands; □: control wetlands).

of *E. foetida* was highest in the top layer and decreased gradually with the depth. In addition, no *E. foetida* could be found in lower layer of 20–40 cm. Almost all the *E. foetida* inhabited in the upper layer of cinder, especially in the layer of 0–5 cm. The distribution pattern of *E. foetida* in the wetlands could be related to their physiological characteristics, since *E. foetida* is mainly litter-dwelling and lives on the soil surface or in the upper reaches of the mineral soil due to the oxygen requirement of earthworms for respiration [14].

3.3. Depth profiles of organic matter and nitrogen

In order to examine the variation of water quality along the vertical profile of wetlands, water samples were collected across a transect at 10, 20, and 30 below the matrix surface besides inflow and outflow. For the convenience of data plotting and analysis, the inflow and outflow samples are considered as water samples at depth of 0 cm and 40 cm, respectively. Fig. 4 shows the vertical profiles of the average concentrations of COD, NH_4^+–N, and TN. One can see that the average concentrations of all three water quality variables gradually decreased downward along the profile. Additionally, the decline of concentration was more rapid in earthworm-added wetlands than that in control wetlands, irrespective of the water quality variables, especially for the 0–20 cm layer. For example, at the same sampling point of 20 cm, the average outflow concentration of earthworm-added wetlands was lower than that of control wetlands by 54.8 mg/L for COD, 6.2 mg/L for NH_4^+–N, and 9.4 mg/L for TN, respectively. Moreover, about 73.5% of COD, 68.6% of NH_4^+–N, and 58.2% of TN in the raw water were removed from the 0–20 cm layer of earthworm-added wetlands. In contrast, from the 0–20 cm layer of control wetlands, only about 59.5% of COD, 47.8% of NH_4^+–N, and 45.2% of TN were removed. Therefore, the removal ability of the upper half of earthworm-added wetlands was much greater than that of the upper half of control wetlands with regard to COD, NH_4^+–N, and TN, which resulted in the superior performance of earthworm-added wetlands.

Taking into account that all *E. foetida* inhabited in the upper half of the earthworm-added wetlands

Table 3
The vertical distribution of *E. foetida* in the matrix

	Depth of the matrix (cm)	Density of the *E. foetida* (individuals/dm^3)
Upper layer	0–5	10–20
	5–10	6–12
	10–15	2–7
	15–20	1–3
Lower layer	20–40	None

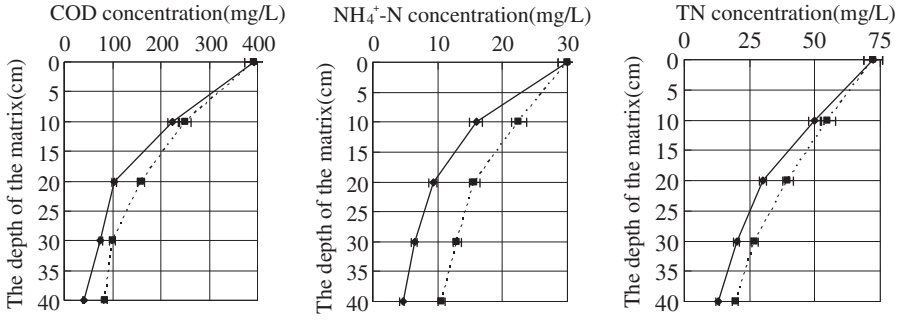

Fig. 4. Vertical profiles of COD, NH_4^+–N and TN in earthworm-added wetlands and control wetlands with no addition of earthworms. —◆—: Earthworm-added wetlands; —■—: Control wetlands.

(Table 3), the reason that the upper half of earthworm-added wetlands had the higher organic matter and nitrogen removal in comparison to the control wetlands was more probably related to the vertical distribution of earthworm.

3.4. Distribution of bacteria in wetlands

In present study, the total number of bacteria, distribution of nitrifying bacteria, and denitrifying bacteria in the upper (0–20 cm) and lower layer (20–40 cm) of the matrix were determined for both the earthworm-added wetlands and the control wetlands to investigate the effect of *E. foetida* addition on bacteria densities in wetlands.

3.4.1. The distribution of bacteria density

With regard to the upper layer of matrix, the average total bacterial density was 2.63×10^9cells/g·matrix (dry weight) in the earthworm-added wetlands, which was approximately 1.9 times higher than that in control wetlands (Fig. 5). Similarly, the average total bacterial density in the lower layer was 2.35×10^8cells/g·matrix (dry weight) in the earthworm-added wetlands, while it was only 1.18×10^8cells/g·matrix in the lower layer of control wetlands. The total bacterial density in the lower layer was one order of magnitude lower than that in the upper layer irrespective of the addition of *E. foetida*. However, the total bacterial density was remarkably higher in the earthworm-added wetlands than that in control wetlands for upper layer and lower layer, respectively. A similar pattern was found for ammonifiers, ammonia-oxidizing bacteria, and nitrite-oxidizing bacteria (Fig. 5). With respect to the upper layer of matrix, the densities of ammonifier, ammonia-oxidizing bacteria and trite-oxidizing bacteria in the earthworm-added wetlands were higher than those in the corresponding layer of control wetlands by 2.5, 1.47, and 1.63 times, respectively. As for the lower layer of matrix, although the densities of these microbial communities were slightly higher in the earthworm-added wetlands than in control wetlands, these differences were not significant.

However, a completely converse distribution pattern was observed for the denitrifier density (Fig. 5). The denitrifier densities were nearly the same in the upper layers of earthworm-added wetlands and control wetlands, which were one order of magnitude lower than the densities in the lower layer. Moreover, for the lower layer, the denitrifier density in the earthworm-added wetlands was still 1.3 times higher than that in control wetlands.

Table 4
The spatial distribution of urease activity and protease activity in the matrix of wetlands

	Urease activity mg NH_3–N/(100 g matrix 24 h)		Protease activity mg NH_3–N/(1 g matrix 24 h)	
	Earthworm-added wetlands	Control wetlands	Earthworm-added wetlands	Control wetlands
Upper layer of matrix	56.92 (8.918)	47.33 (9.209)	1.25 (0.100)	1.15 (0.005)
Lower layer of matrix	16.35 (5.046)	13.14 (3.131)	0.86 (0.149)	0.79 (0.044)

Note: Standard deviation values are presented in parentheses.

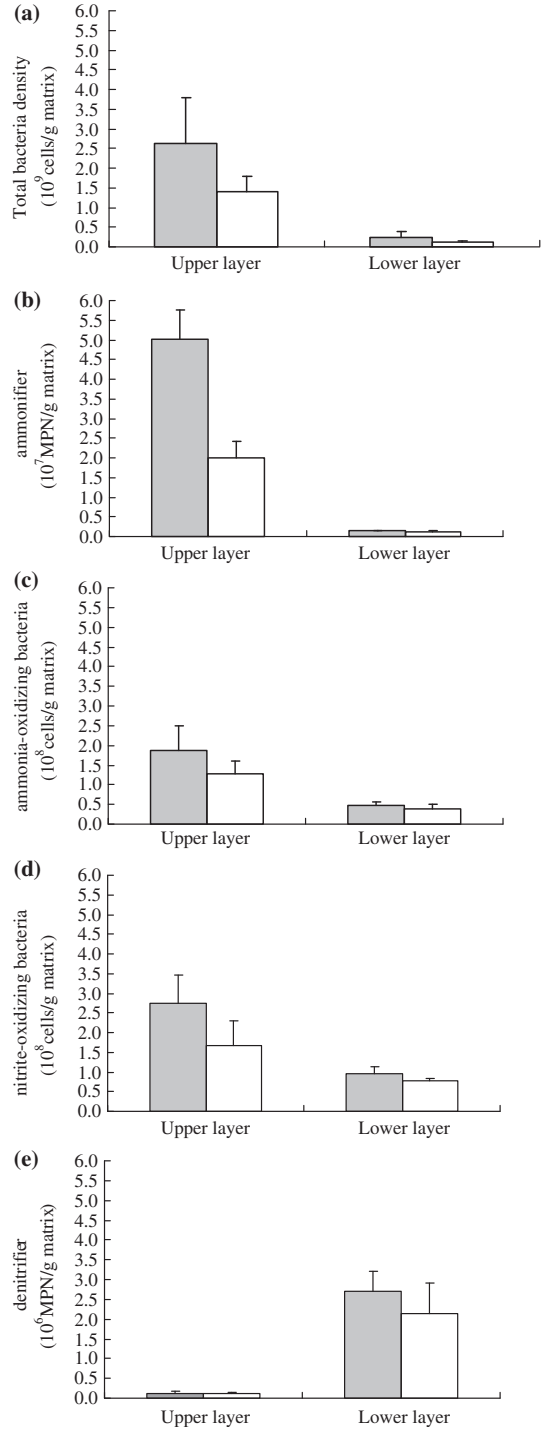

Fig. 5. The spatial distribution of bacteria in the matrix of earthworm-added wetlands and control wetlands with no addition of earthworms. (a) Total bacterial count; (b) ammonifiers; (c) ammonia-oxidizing bacteria; (d) nitrite-oxidizing bacteria; (e) denitrifiers; ■: earthworm-added wetlands; □: control wetlands.

3.4.2. Spatial distribution of microbial activities

Urease and protease are important hydrolytic enzymes directly related to transformation of organic carbon and nitrogen. It can be seen from Table 4 that the average urease and protease activities in the earthworm-added wetlands were higher than in control wetlands for the upper layer and lower layer, respectively.

4. Discussion

We hypothesized that introduction of earthworms into vertical-flow CWs would improve removal of COD and nitrogen, which was confirmed by the results of experiments. In addition, higher densities of bacteria, such as total bacteria, ammonifiers, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and denitrifiers, were found in earthworm-added wetlands as compared to control wetlands. Furthermore, increased protease and urease activity were also observed in the earthworm-added wetlands. Therefore, enhanced treatment of COD and nitrogen could be attributed to the augmentation of microbial biomass and enzyme activity which resulted from the addition of earthworms.

The enhancement in microbial biomass in the earthworm-added wetlands may be explained partly by production of mucus by the earthworms, because mucus is known to have a stimulating effect on microorganisms [15]. In addition, it is also known that microbial biomass is stimulated in earthworm burrows [16] and by breakdown of organic matter, a process that would be increased by high earthworm densities. Moreover, earthworms can modify the structure of soil and matrix, thereby increasing the surface available for microorganisms, and released new nutrient pools as a result of its feeding and casting activities, which stimulates microbial metabolism [17,18]. These processes would increase with the high earthworm densities in the upper layers of earthworm-added wetlands (Table 3). The vertical distribution of earthworms was closely related to the surface-dwelling nature of *E. foetida*. *E. foetida* is an epigeic earthworm species which lives on or near the soil surface, typically in the litter layers of forest soils and does not burrow, and also requires high moisture content and adequate amounts of suitable organic material [8,19]. In present study, the experimental wetland systems had a vertical down-flow design, thus the organic matter was largely trapped by the upper layer matrix, which provided abundant food for *E. foetida*. Captured particulate organics could be transformed to dissolved and more degradable forms

by the feeding and casting activities of earthworm, and become more readily utilized by bacteria. Field and laboratory studies have indicated that interactions between earthworms and microorganisms increase soil carbon turnover, soil nutrient availability, and microbial activity [20]. For instance, CO_2 emission from farmland soil with organic fertilizer inoculated with *E. foetida* increased by about 40% in comparison to the control soil with no earthworms inoculated [21]. As a result, the higher COD removal achieved by earthworm-added wetlands in comparison to control wetlands could be explained by the accelerated degradation of organic matter by earthworms, which was consistent with the higher density of total bacteria and enzyme activities in the upper layer matrix.

Ammonifiers, ammonia-oxidizing bacteria, nitrite-oxidizing bacteria, and denitrifiers, which take part in the processes of nitrogen cycle, play an important role in nitrogen transformation. The increases in the populations of these microorganisms were mainly responsible for the enhanced nitrogen removal in earthworm-added wetlands. Ammonification and denitrification can convert organic nitrogen and nitrate into NH_3, N_2O, and N_2, while nitrosification and nitrification can transform NH_3 and NO_2^- into NO_2^- and NO_3^-, respectively. Parkin and Berry [22] found that the density of ammonifiers, nitrifying bacteria and denitrifying bacteria in the soil of *E. foetida* hole was higher than that of the control soil, which is consistent with the results of present study. The high density of ammonifiers in the upper layer of earthworm-added wetlands promoted transformation of organic nitrogen into ammonia, which laid the foundation for the succeeding coupled nitrification and denitrification in wetlands.

Nitrification is essentially an autotrophic process and usually, considered as the limiting step for biological nitrogen removal. The ammonia-oxidizing bacteria and nitrite-oxidizing bacteria are specialized chemolitotrophic bacteria, and they are the key agents of autotrophic nitrification. Earthworms excrete ammoniacal compounds including NH_4^+, urea, allantoin, and uric acid [18,22], which would be favorable to the development of nitrifying bacterial populations in the earthworm-added wetlands in present study. In addition, earthworms have the ability to elevate populations of nitrifying bacteria [22]. Effective nitrification would provide abundant electron acceptor for the subsequent denitrification.

Anoxic circumstances and organic matter as electron donors are the main factors affecting denitrification. As the organic matter was firstly degraded in the upper layer of wetlands, the DO was mostly consumed in this layer. Therefore, the lower layer became anoxic which favors the denitrification process, which was consistent with the higher density of denitrifiers. Furthermore, the feeding and intestinal digestion of *E. foetida* in the upper layer could increase the soluble organic matter content, supplying adequate electron donors for the denitrification process in the lower layer of vertical-flow wetlands. In present study, the density of nitrifying bacteria in the upper layer matrix was high, while the density of denitrifying bacteria in the lower layer matrix was high, which corresponded with the sequence of nitrification and denitrification, and was conducive to biological nitrogen removal in earthworm-added vertical-flow wetlands.

Soil enzymes play a pivotal role in organic matter decomposition by converting macromolecules into smaller molecular moieties. Urease and protease are involved in hydrolyzing organic nitrogen compounds to inorganic nitrogen. The enhancement of transformation of organic to inorganic nitrogen will be favored by biological nitrification and denitrification, and therefore improve removal of TN and NH_4−N. As a result, the higher urease and protease activities measured in the earthworm-added wetlands were closely related to enhanced removal of nitrogen. The presence of earthworms might account partly for the increase in urease and protease activities in the earthworm-added wetlands. Urease and protease activities in three soils of varying texture were enhanced by addition of *E. foetida* [23]. The results of enzyme analysis were in accordance with the bacteria distributions, and further confirmed the function of earthworm improving the removal of nitrogen and organics.

5. Conclusions

(1) With the introduction of *E. foetida* into vertical-flow CWs, the average removal efficiencies of COD, NH_4^+–N, and TN were improved by 15.7, 21.3 and 20.6%, respectively.
(2) The average removal efficiencies of COD, NH_4^+–N, and TN in the upper half layer of earthworm-added wetlands were 73.5, 68.6 and 58.2%, respectively, while they were only 59.5, 47.8 and 45.2%, respectively, in control wetlands with no *E. foetida* addition. *E. foetida* was found inhabited mainly in the upper half layer of earthworm-added wetlands, which could be related to the higher performance of the upper half layer.
(3) The total bacterial count, as well as the populations of ammonifiers, ammonia-oxidizing bacteria and nitrite-oxidizing bacteria in the upper half layer of

the earthworm-added wetlands were 1.9, 2.5, 1.5, and 1.6 times as much as that in control wetlands, respectively. Similarly, the urease activity and protease activities were improved by 1.2 and 1.1 times, respectively. The denitrifying bacteria density in the lower layer of the earthworm-added wetlands was 1.3-fold greater than that in control wetlands. It is suggested that the addition of *E. foetida* improved the microbial biomass and enzyme activities, thus enhancing the removal of nitrogen and organics.

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References

[1] H. Sellami, S. Benabdallah, A. Charef, Performance of a vertical flow constructed wetland treating domestic wastewater for a small community in rural Tunisia, Desalin. Water Treat. 12 (2009) 262–269.
[2] Y.-C. Chen, S.-L. Lo, Y.-C. Lee, Distribution and fate of perfluorinated compounds (PFCs) in a pilot constructed wetland, Desalin. Water Treat. 37 (2012) 178–184.
[3] Q.M. Ketterings, J.M. Blair, J.C.Y. Marinissen, Effect of earthworms on soil aggregate stability and carbon and nitrogen storage in a legume cover crop agroecosystem, Soil Biol. Biochem. 29 (1997) 401–408.
[4] H. Bossuyt, J. Six, P.F. Hendrix, Interactive effects of functionally different earthworm species on aggregation and incorporation and decomposition of newly added residue carbon, Geoderma 130 (2006) 14–25.
[5] Y. Araujo, F.J. Luizao, E. Barros, Effects of earthworm addition on soil nitrogen availability, microbial biomass and litter decomposition in mesocosms, Biol. Fertil. Soils 39 (2004) 146–152.
[6] S.S. Pati, S.K. Sahu, CO_2 evolution and enzyme activities (dehydrogenase, protease and amylase) of fly ash amended soil in the presence and absence of earthworms (*Drawida willsi Michaelsen*) under laboratory conditions, Geoderma 118 (2004) 289–301.
[7] C. Nuengjamnong, N. Chiarawatchai, C. Polprasert, R. Otterpohl, Treating swine wastewater by integrating earthworms into constructed wetlands, J. Environ. Sci. Health Part A Toxic/Hazard. Subst. Environ. Eng. 46 (2011) 800–804.
[8] G. Tripathi, P. Bhardwaj, Comparative studies on biomass production, life cycles and composting efficiency of *Eisenia fetida* (Savigny) and *Lampito mauritii* (Kinberg), Bioresour. Technol. 92 (2004) 275–283.
[9] Editorial Board of Monitoring and Analytical Method of Water and Wastewater, State Environmental Protection Administration of China, Monitoring and Analytical Method of Water and Wastewater, fourth ed, China Environmental Science Press, Beijing, (in Chinese) 2002.
[10] A. Alfreider, J. Pernthaler, R. Amann, B. Sattler, F. Glöckner, A. Wille, R. Psenner, Community analysis of the bacterial assemblages in the winter cover and pelagic layers of a high mountain lake by in situ hybridization, Appl. Environ. Microbiol. 62 (1996) 2138–2144.
[11] R.I. Amann, W. Ludwig, K.H. Schleifer, Phylogenetic identification and in situ detection of individual microbial cells without cultivation, Microbiol. Rev. 59 (1995) 143–169.
[12] J.P. Touzel, G. Albagnac, Isolation and characterization of *Methanoeoecus* mazei strain MC3, FEMS Microbiol. Lett. 16 (1983) 241–245.
[13] P. Nannipieri, B. Ceccanti, S. Cervelli, E. Matarese, Extraction of phosphatase, urease, protease, organic carbon and nitrogen from soil, Soil Sci. Soc. Am. J. 44 (1980) 1011–1016.
[14] C.A. Edwards, P.J. Bohlen, Biology and Ecology of Earthworms, Chapman and Hall, London, p. 426, 1996.
[15] B.M. Doube, G.G. Brown, Life in a Complex COMMUNITY: Functional Interactions between Earthworms, Organic Matter, Microorganisms, and Plant Growth, In: C.A. Edwards (Ed), Earthworm Ecology, St. Lucie Press, Boca Raton, 1998, pp. 179–211.
[16] A.V. Tiunov, S. Scheu, Microbial respiration, biomass, biovolume and nutrient status in burrow walls of *Lumbricus terrestris* L. (Lumbricidae), Soil Biol. Biochem. 31 (1999) 2039–2048.
[17] W. Devliegher, W. Verstraete, Lumbricus terrestris in a soil core experiment—nutrient-enrichment processes (NEP) and gut-associated processes (GAP) and their effect on microbial biomass and microbial activity, Soil Biol. Biochem. 27 (1995) 1573–1580.
[18] E.K. Tillinghast, R.O. Donnell, D. Eves, E. Calvert, J. Taylor, Water-soluble luminal contents of the gut of the earthworm *Lumbricus terrestris* L. and their physiological significance, Comp. Biochem. Physiol. A: Mol. Integr. Physiol. 129 (2001) 345–353.
[19] L. Zirbes, J.-L. Deneubourg, Y. Brostaux, E. Haubruge, A new case of consensual decision: Collective movement in earthworms, Ethology 116 (2010) 546–553.
[20] M.L. Schindler-Wessells, P.J. Bohlen, D.A. McCartney, S. Subler, C.A. Edwards, Earthworm effects on soil respiration in corn agroecosystems receiving different nutrient inputs, Soil Biol. Biochem. 29 (1997) 409–412.
[21] F. Caravaca, A. Pera, G. Maseiandaro, B. Ceccanti, A. Rolda, A microcosm approach to assessing the effects of earthworm inoculation and oat cover cropping on CO_2 fluxes and biologieal properties in an amended semiarid soil, Chemosphere 59 (2005) 1625–1631.
[22] T.B. Parkin, E.C. Berry, Microbial nitrogen transformations in earthworm burrows, Soil Biol. Biochem. 31 (1999) 1765–1771.
[23] F. Caravaca, A. Roldán, Effect of *Eisenia foetida* earthworms on mineralization kinetics, microbial biomass, enzyme activities, respiration and labile C fractions of three soils treated with a composted organic residue, Biol. Fertil. Soils 38 (2003) 45–51.