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Performance and mechanisms of a microbial-earthworm ecofilter for removing organic matter and nitrogen from synthetic domestic wastewater

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ABSTRACT

The performance of a microbial-earthworm ecofilter for the treatment of synthetic domestic wastewater is evaluated, and the mechanisms of organic matter and nitrogen transformation investigated. Vermifiltration efficiently reduced chemical oxygen demand (COD) and ammonia nitrogen (NH_3-N) from the influent. A combination of soil with sawdust possessed higher porosity and specific surface area than other media, and this microporous structure together with wormcast surface greatly facilitated COD reduction at depths from 5 to 35 cm. Nitrogen variations in wastewater were influenced by soil properties, earthworm activities, and wormcast characteristics. Their interaction with added nitrogen determined soil nitrogen distribution. In addition, denaturing gradient gel electrophoresis (DGGE) profiles revealed a highly diverse community of ammonia-oxidizing bacteria (AOB) and *Nitrospira* in soil layers. There was a positive correlation between the Shannon biodiversity index for AOB and decreasing NH_3-N concentration, indicating that dominant soil microbes played a major role in removing NH_3-N and nitrogen conversion. In contrast to previous reports, identification of retrieved sequences of AOB species showed that most belonged to an uncertain AOB genus. This biofiltration system is a low cost, efficient alternative for decontaminating local domestic wastewater.

1. Introduction

In China, watershed pollution is now a prominent form of water contamination. In contrast to the United States and other developed countries, rural people account for most of the Chinese population, and more than 96% of domestic wastewater in rural areas is discharged directly into aquatic environments without any treatment [1]. It is reported that rural wastewater has seriously affected surface water and groundwater quality, and is the main source of serious river and lake pollution [2,3]. Nitrogen (mainly in the form of ammonia) in wastewater causes eutrophication and potential toxicity to aquatic species [4]. Therefore, the key to watershed pollution is decontamination of wastewater (especially of nitrogen compounds) emitted in rural China.

The characteristics of rural wastewater change greatly in quality, quantity, and spatial distribution; therefore, the conventional fixed centralized wastewater treatment technologies used for municipal wastewater treatment are often not suitable [5]. In order for resource-scarce, economically developing rural areas to use wastewater treatment, the treatment system should be low-cost, easy to maintain, and highly efficient. To date, many onsite independent and synthetic systems for decentralized sewage treatment have been applied in rural areas, including constructed wetlands (CWs), soil trenches, high-rate algal ponds, vegetation-based wastewater treatment, septic tanks, compound media filter bed combined systems, and three-stage step-feed wastewater treatment systems combined with drop-aeration biofilms [6–11]. All of these methods have their own unique advantages, with different treatment efficacies for different pollutants. Currently, vermifiltration (VF) is a promising method that combines biological technologies and ecological methods, and has been applied in small pilot-scale tests.

VF is a process that adapts traditional vermicomposting to a passive wastewater treatment. A typical system will separate the wastewater solids by allowing wastewater to be gravity-fed over filtration material such as fine mesh [12]. Sinha [13] reported that earthworms work as biofilters; they can reduce the 5-day BOD (Biochemical Oxygen Demand) level (BOD_5) by over 90% and chemical oxygen demand (COD) by 80–90%. The worms ingest and biodegrade organic wastes by absorption through their body walls. Li [14,15] found that a VF system could be a stage in the reuse of swine wastewater by demonstrating that it could successfully treat the sewage produced each day by more than 100

Abbreviations: CWs, constructed wetlands; OM, organic matter; DO, dissolved oxygen; S1, topsoil; S2, midlevel soil; S3, subsoil; SD, sand; DE, detritus; VF, vermifiltration.

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Table 1
Physical characteristics of initial fillings.[a]

Items	Unit	Soil + sawdust	Sand	Detritus
Bulk density	$g\,cm^{-3}$	0.86	1.96	2.23
Porosity	%	67	23	16
Saturated hydraulic conductivity	$cm\,s^{-1}$	0.19	0.27	0.45
pH		7.14	8.02	8.33
Specific surface area	$m^2\,g^{-1}$	40.59	24.35	1.01

[a] All data represent average of triplicates.

swine. COD, ammonia nitrogen (NH_3-N), and BOD_5 were efficiently reduced in these studies. Furthermore, some innovative technologies combined with traditional methods have been described at both laboratory and pilot scales. These include extensive systems to treat diluted manure consisting of a screen, vermifilter, and settling tank, macrophytes ponds and constructed wetlands. To recycle the water, laboratory-scale ceramsite-vermifilters and VF enhancement with a slag–coal cinder converter filter have been used for domestic wastewater treatment [16–18].

Although a considerable reduction in the level of COD and NH_3-N in end effluents has been reported using VF, some of the key processes remain to be understood or optimized. If this technology is to be widely applied, then for example, the composition and properties of the different layers, characteristics of wormcast in soil, nitrifying bacterial community structure, and how these interact to effect COD, NH_3-N removal and nitrogen transformation in the process need to be better understood.

To obtain a more detailed understanding of organic matter (OM) degradation, NH_3-N removal, and N distribution processes in VF, this study focused on the following four areas: (1) determining the concentration of COD, dissolved oxygen (DO), NH_3-N, nitrate nitrogen (NO_3-N), and nitrite nitrogen (NO_2-N) before and after VF of wastewater at different substratum layers in a laboratory experiment; (2) assessing the effects of the physical characteristics of each layer, the nutrient constitution of soil and wormcast, and the structural features of the wormcast surface on the removal of organic matter, NH_3-N, and nitrogen conversion in sewage; (3) identifying and evaluating the ammonia oxidizing bacterium (AOB) and *Nitrospira* in filters by polymerase chain reaction-denaturing gradient gel electrophoresis (PCR-DGGE); and (4) investigating microbial community diversity and composition and determining how the microbial population influences nitrogen distribution.

2. Experimental

2.1. Experimental design

A cuboid shaped plexiglass VF system (40 cm in length, 40 cm in width, and 115 cm in depth) was equipped with a plexiglass wastewater tank. Wastewater was introduced to the apparatus via a peristaltic pump (Fig. 1). The reactor was packed with a 30 cm soil-earthworm bed (6–9 mm diameter), 30 cm sand (100–800 μm diameter), 30 cm detritus (3–10 mm diameter) and a 20 cm supporting layer of cobblestone (10–50 mm diameter) layered from top to bottom. Soil and sawdust were mixed at a volume ratio of 3:1. Sawdust was added as a bulking agent because it has been shown to improve soil permeability and enhance earthworm growth and survival [19]. Some physical characteristics of the fillings are presented in Table 1. The artificial soil was inoculated with *Eisenia foetida* (Savigny) at an initial earthworm density of $12.5\,g\,L^{-1}$. This species has been widely used in VF [18].

During operation the surface loading of the wastewater was adjusted to $1\,m^3\,m^{-2}\,d^{-1}$, and the wet to dry time ratio was 1:3. These approaches prevented the blockage of the soil layers and sustained the penetrability of the ecofilter. The entire test volume of the synthetic sewage was applied in a single batch through a rotating glass pipe (2 cm in diameter and 36 cm in length) with 1.5 mm holes to ensure uniform distribution of the influent. The perspex pipe was placed on the upside of the VF surface, 15 cm from the top layer of VF. Synthetic wastewater based on that of Fang [20] was used, see Table 2. From June, 2010 to August, 2010 at Nanjing University, Nanjing (32°03′N, 118°47′E), the system was fed daily and one operation cycle was performed each day, controlled by a digital timer (Kerde TW-K11, Zhejiang, China). Each cycle included wastewater flow for 1 h, retention for 3 h, and finally emptying of the water. The operation and monitoring of the VF were conducted between operating periods. All the samples including controls were performed in triplicate.

2.2. Water sampling and chemical analysis

Influent and effluent water from the laboratory-scale VF were sampled every week to evaluate treatment performance. Effluent outlets 1–3 allowed separate analysis of direct soil-earthworm layer outflow, sand layer outflow, and detritus layer outflow (Fig. 1) at depths of 35, 65, and 95 cm below the surface, respectively. The total effluent outlet was at the bottom left. Water samples were analyzed for COD, TN, total phosphorus (TP), NH_3-N, NO_3-N, and NO_2-N according to the methods described in Examination of Water and Wastewater [21]. The DO, pH, and temperature were measured *in situ* (using a YSI Model no. 550A DO meter, USA, and a pH meter from Shanghai Kangyi Instrument Co. Ltd., PHS-2C, China).

2.3. Sampling and analysis of soil layers and wormcast

When emptying the sewage packing, samples of topsoil (S1, 5–15 cm), midlevel soil (S2, 15–25 cm), and subsoil (S3, 25–35 cm) were collected from sampling points at intervals of 15 days. In total, 35–65 cm sand (SD) and 65–95 cm detritus (DE) were collected at the end of each period. Samples from the same depth were mixed to give one composite sample. Subsequently, plant roots, earthworms, and other waste were removed from the substrates. Earthworm cast was gathered before and after the operation as described by Zhao [22], and the system was replenished with an equivalent weight of earthworms. Finally, both the filter materials and the casts were freeze-dried, sieved (<2 mm) except DE, and stored at −20 °C for analysis.

The specific surface area of various filters was measured at room temperature and atmospheric pressure by a BET method with nitrogen using a NOVA 3000e surface area and pore size analyzer (Quantachrome Instruments, USA). Porosity and bulk density were determined using standard soil science methods [23]. The saturated hydraulic conductivity was measured using the penetration tube method. The pH of the filter material was measured in a 10% (w/v) aqueous solution using a digital pH meter. The measurements of OM, total organic carbon (TOC), TN, NH_3-N, and NO_3-N were carried out in soil before the operation of VF (at day 0) and every 15 days thereafter, and in wormcast before and after the final running period. Organic matter was determined by the loss on ignition at 550 °C for 2 h [24]. Total organic carbon and TN were determined using an elemental analyzer (Elementar Vario EL III, Germany), while NH_3-N and NO_3-N were measured using the KCl extraction–distillation method and the Cu–Cd reduction-colorimetry method, respectively [23]. Organic nitrogen (N_{org}) was calculated by subtracting NH_3-N and NO_3-N from TN. Scanning electron microscopy (SEM) of wormcast collected at the end of the operation period was conducted using an S-3400N II microscope (Hitachi, Japan).

Table 2
Physical and chemical characteristics of the synthetic wastewater used as the influent.[a]

Parameters	pH	Temperature (°C)	DO (mg L^{-1})	COD (mg L^{-1})	TN (mg L^{-1})	NH_3-N (mg L^{-1})	TP (mg L^{-1})
Values[a]	7.62 ± 0.25	27.82 ± 1.36	5.76 ± 0.51	243.76 ± 13.97	41.00 ± 1.17	39.30 ± 0.77	3.12 ± 0.11

[a] Values (mean ± standard deviation) are averages of three replicates.

Table 3
Primers used in this study.

Prime set	Target group	Primer sequence 5′–3′	Reference
amoA-1f-GC	ammonia oxidizing bacteria	**CGCCCGCCGCGCGCGGCGGGCGGGGCGGGG GCACGGGGGG**[a] GGG GTT TCTA CTG GTG GT	[25,26]
amoA-2r	ammonia oxidizing bacteria	CCC CTC KGS AAA GCC TTC TTC	[25]
NSR1113f-GC	*Nitrospira*	**CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G**[a] CCT GCT TTC AGT TGC TAC CG	[26,27]
NSR1264r	*Nitrospira*	GTT TGC AGC GCT TTG TAC CG	[27]

[a] The GC-clamp sequences used for PCR-DGGE are written in bold.

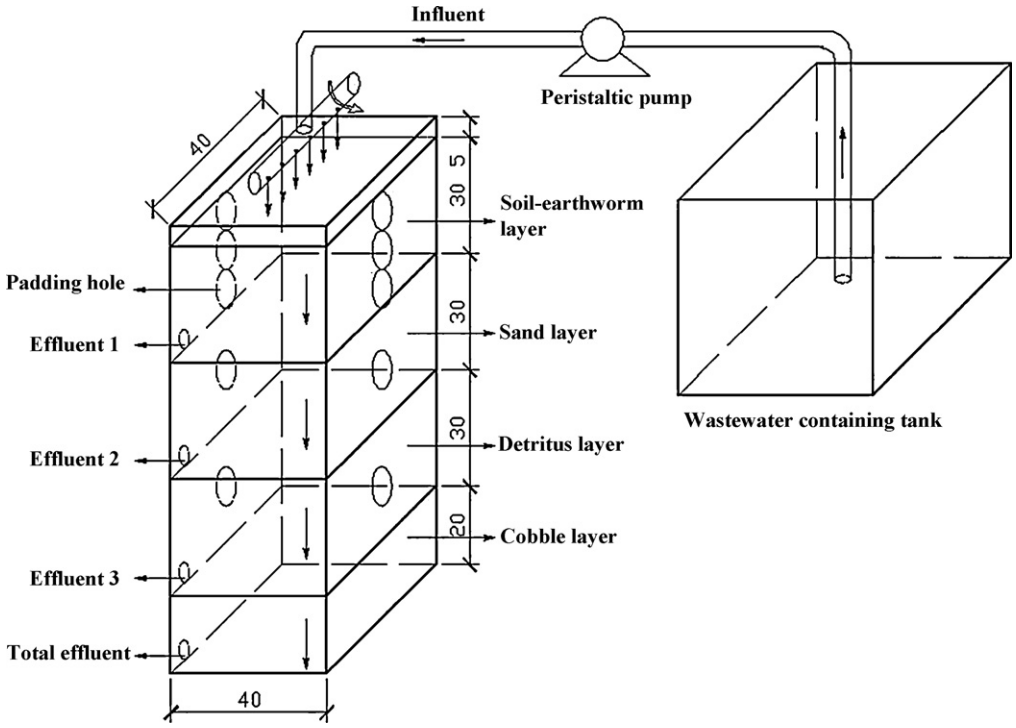

Fig. 1. Diagram of the laboratory-scale VF system used in this study (unit: cm).

2.4. DNA extraction and PCR-DGGE

Samples of DNA were extracted from soil, sand, and detritus samples taken after 90 days of wastewater treatment using the Ultra Clean Soil DNA Isolation Kit (MO BIO Laboratories, Loker Ave West, Carlsbad, CA, USA), according to the manufacturer's instructions. These DNA preparations were used as template DNAs for PCR. To amplify specific 16S rDNA from samples, we used a nested PCR approach. Each PCR reaction was conducted in a volume of 50 μL using EDC-810 Thermal Cyclers (Eastwin, Beijing, China). The PCR mixture contained 0.25 μL DNA polymerase (5 U μL^{-1}) (TaKaRa, Ex Taq, Japan), 5 μL 10× Ex Taq Buffer, 4 μL $MgCl_2$ (25 mM), 4 μL dNTP mixture (2.5 mM each), and 1 μL primer (20 μM each). One μL of template DNA (0.6–1.0 ng) was added to each reaction with the specific primer pair amoA-1F and amoA-2R for ammonia oxidizing bacteria or NSR1113f/NSR1264r for *Nitrospira*, see Table 3. The latter genus was selected because it is the most abundant nitrite oxidizer in wastewater treatment systems [28]. As PCR was performed to generate products for subsequent DGGE analysis, we used primers containing GC-clamp at the amoA-1F and NSR1113f 5′-end. The cycling program for the PCR was 94 °C for 3 min, followed by 35 cycles of 1 min at 94 °C, 90 s at 55 °C, and 90 s at 72 °C, and a final extension of 10 min at 72 °C. The program used for amplification of *Nitrospira* 16S rDNA sequences was 30 cycles of 1 min at 95 °C, 1 min at 55 °C, and 2 min at 72 °C. For verification, aliquots of the PCR products were separated on an agarose gel (1.2%, 100 V, 25 min) and DNA bands were visualized by ethidium bromide.

Denaturing gradient gel electrophoresis (DGGE) was performed using the Bio-Rad D gene system (Bio-Rad, USA). Aliquots of the PCR fragments were loaded onto 8% (wt/vol) polyacrylamide gels in 1 × TAE buffer. The gel was run on denaturing gradients of 35–60% (100% denaturant is 7 M urea plus 40% formamide) for AOB, or 40–60% for *Nitrospira* for about 6 h at 60 °C and 150 V. After electrophoresis, the gels were soaked for 15 min in ethidium bromide (250 mL Milli-Q water, 25 μL ethidium bromide stock of 5 mg mL^{-1}) and subsequently rinsed for 20 min with Milli-Q water. Gel images using UV translumination were stored by using the Gel Doc 2000 System from Bio-Rad (Bio-Rad, USA).

Table 4
Organic matter, total organic carbon, and nitrogen distribution in the layers of soil and wormcast.

Parameters	Initial soil + sawdust[a]	5–15 cm Soil[b]	15–25 cm Soil[b]	25–35 cm Soil[b]	Initial wormcast[a]	Wormcast[a]
OM (%)	8.59 ± 0.21	12.64 ± 0.85	12.78 ± 1.03	12.90 ± 0.46	15.56 ± 1.34	17.31 ± 0.13
TOC ($g\,kg^{-1}$)	29.4 ± 0.5	39.7 ± 1.7	35.4 ± 2.1	46.9 ± 2.4	66.1 ± 4.7	64.3 ± 3.6
TN ($g\,kg^{-1}$)	4.6 ± 0.1	5.2 ± 0.3	4.8 ± 0.6	6.1 ± 1.04	6.2 ± 0.01	6.3 ± 0.01
NH_3-N ($g\,kg^{-1}$)	1.7 ± 0.01	3.1 ± 0.1	2.7 ± 0.04	3.4 ± 0.5	0.042 ± 0.012	0.036 ± 0.003
NO_3-N ($mg\,kg^{-1}$)	24.8 ± 0.2	37.6 ± 2.7	38.5 ± 3.9	40.2 ± 3.2	75.2 ± 5.4	86.3 ± 4.8
N_{org} ($g\,kg^{-1}$)	2.9 ± 0.03	2.1 ± 0.03	2.1 ± 0.2	2.7 ± 0.06	6.16 ± 0.051	6.18 ± 0.062

[a] Values (mean ± standard deviation) are averages of three replicates.
[b] Arithmetic averages of six samplings (mean ± standard deviation) during operation period.

2.5. Cloning, sequencing, and phylogenetic analysis

Gel slices from the DGGE were put into 1.5 mL micro-centrifuge tubes containing 25 μL TE buffer and incubated for 24 h at 4 °C. The DNA eluted from a small gel chip was used as a direct template for PCR to recover the DNA fragments. The PCR conditions were the same as for the original PCR, except that the primer pairs amoA-1F/amoA-2R and NSR1113f/NSR1264r were without GC-clamps. The PCR products amplified from DGGE gel were subjected to agarose gel electrophoresis. Subsequently, DNA fragments were repurified with an Agarose Gel DNA Purification Kit Ver.2.0 (TaKaRa, Japan). Purified DNA from AOB was used as a template for direct sequencing at GenScript Inc. (Nanjing, China). Fragments less 200 bp were not suitable for direct sequencing [29], so purified PCR-amplified DNA from *Nitrospira* (151 bp) DGGE gel were further cloned using the pTG19-T PCR Product Cloning Kit (Generay, Shanghai, China) prior to sequencing, following the same method described above.

The Shannon index was calculated to assess the structural diversity (richness and evenness) of the microbial community:

$$H = -\sum_{i=1}^{s} p_i \log p_i = -\sum_{i=1}^{s} \left(\frac{n_i}{N}\right) \log \left(\frac{n_i}{N}\right)$$

where H is the Shannon Index, n_i is the height of the peak, and N is the sum of all peak heights in the curves [22]. The nucleotide sequences were compared with those deposited in the GenBank (NCBI) using Basic Local Alignment Search Tool (BLAST), then the sequences determined in this study and obtained from the DNA database were aligned using ClustalW. Distance matrix analyses using the p-distance, and neighbor-joining trees were constructed by pair-wise deletion using MEGA version 4.0 (Molecular Evolutionary Genetics Analysis). Tree topology was evaluated by bootstrap analysis using 1000 replicates. The sequences generated in this study have been deposited in the National Center for Biotechnology Information under accession numbers JF742542 to JF742558.

3. Results and discussion

3.1. The performance and mechanisms involved in the OM removal

The effluent COD declined sharply from 5 to 35 cm, whereas it was more or less constant between 35 and 115 cm (Fig. 2a). The total effluent COD was maintained below 50 mg L^{-1} at all times in the VF unit during synthetic wastewater treatment. The physical, chemical, and biological processes and synergistic effects of earthworms and microorganisms, including the adsorption of small particle organisms, colloid organisms, and organic molecules, as well as the oxidation-reduction of organic matter and activity of earthworms, made VF effective for COD removal [30]. Among the VF layers, the soil-earthworm layer with added sawdust played a major role because of its higher porosity and surface area (Table 1), which

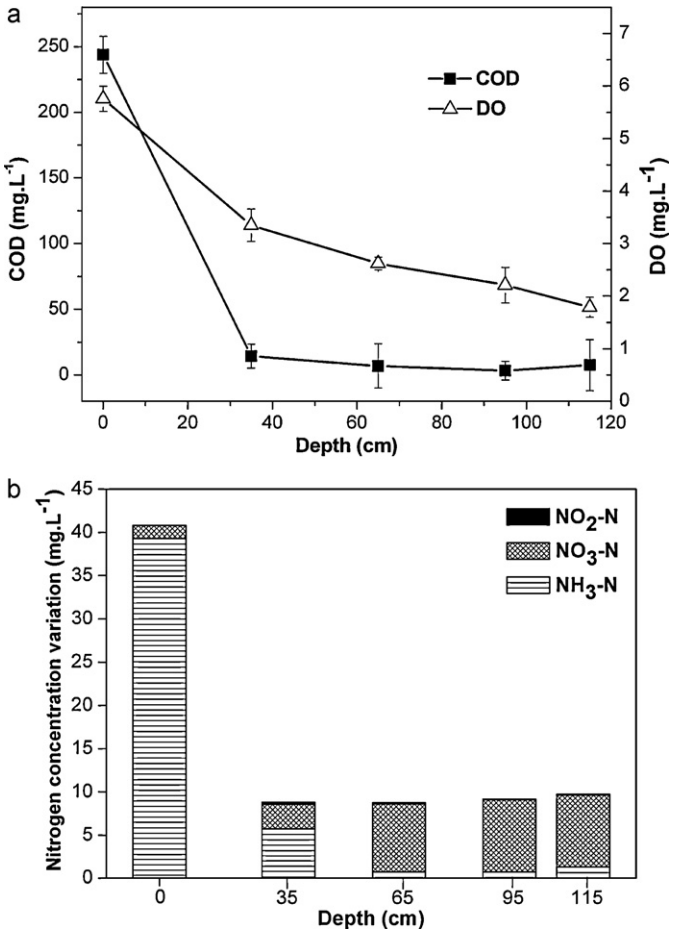

Fig. 2. Performance of laboratory-scale microbial-earthworm ecofilters for wastewater treatment a: Removal of chemical oxygen demand (COD) and dissolved oxygen (DO) at different depths; b: ammonia nitrogen (NH_3-N), nitrate nitrogen (NO_3-N) and nitrite nitrogen (NO_2-N) variations in inflow and different effluents by analysis of distribution in the filters.

proved beneficial for removing most of the organic contaminants by precipitation and adsorption in the voids of the soil. Meanwhile, the micrographs of wormcast (Fig. 3) revealed micro-pores on the surface and abundant cylindrical organic matter within, suggesting that wormcast acted as a kind of wastewater filter medium for removing OM. Since the main decrease in COD content was found over the first 5–35 cm depth, and the porosity and specific surface area were lower in the sand and detritus layers than those of the soil, the organic substrates had poor availability. Thus, COD content did not change dramatically over these lower layers.

Because the COD was removed efficiently from wastewater mainly by means of precipitation and adsorption, the soil OM and TOC contents were observed to increase (Table 4). A large number of wormcasts in the VF produced by earthworms were rich in

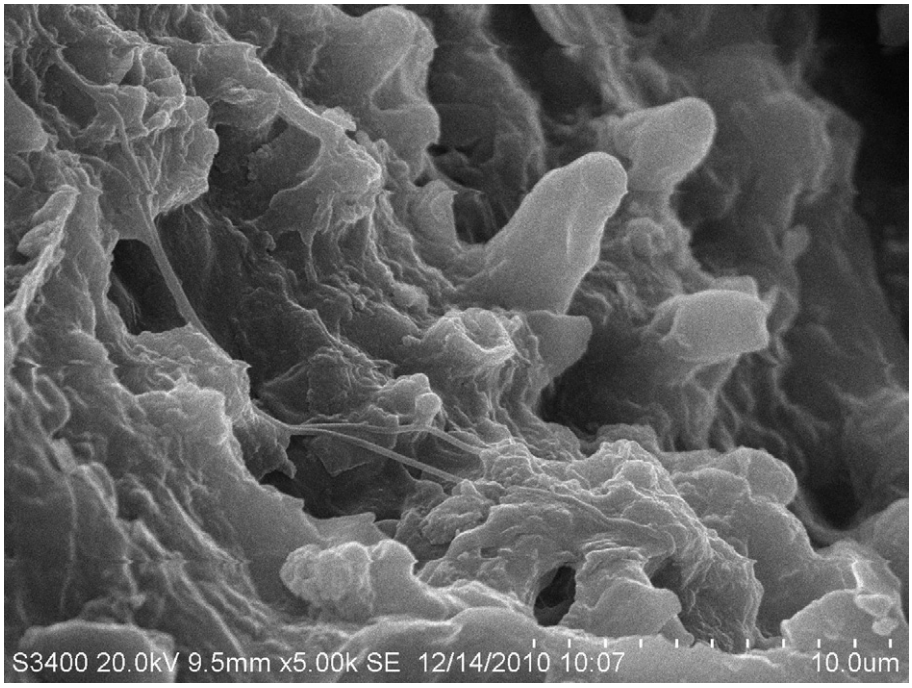

Fig. 3. A SEM image of a sample of wormcast (5000×).

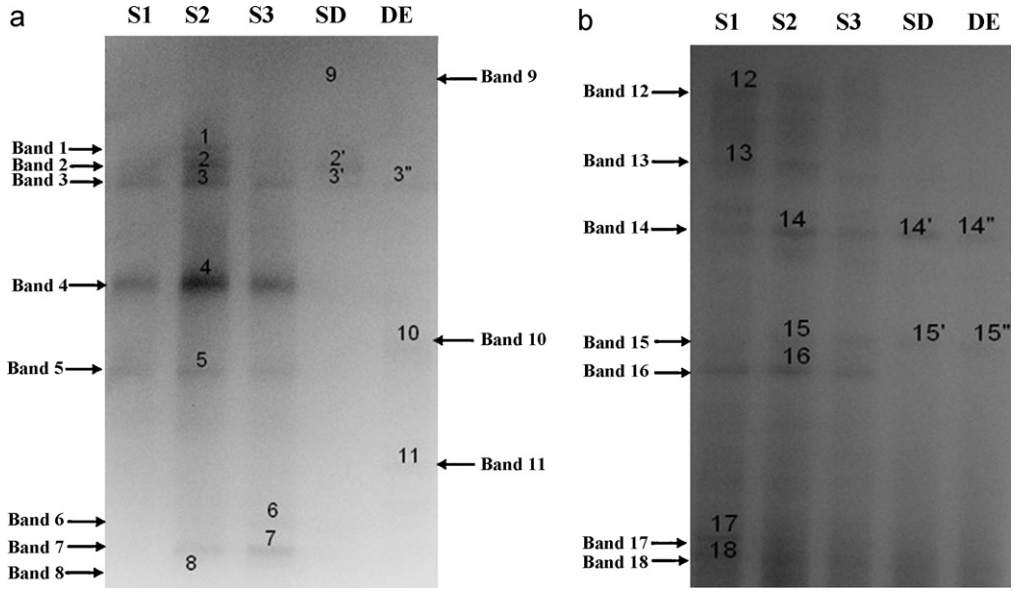

Fig. 4. Denaturing gradient gel electrophoresis (DGGE) analysis of ammonia-oxidizing bacteria (AOB) (a) and *Nitrospira* (b) fragments retrieved from layers of the VF system. The excised bands 1–11 and bands 12–18 denote AOB and *Nitrospira*, respectively. S1–S3, SD, and DE represent topsoil, midlevel soil, subsoil, sand, and detritus, respectively. The same mobilities in the DGGE gel are represented by ′ and ″.

organic matter and TOC (Table 4). It has been estimated that after several years all the surface soil in an earthworm habitat may be completely transformed into casts [31]. Therefore, the content of wormcast brings about sustained growth of the nutrient content of soil.

3.2. NH_3-N removal and nitrogen transformations

3.2.1. The performance of nitrogen variation in wastewater

Fig. 2b shows the effect of depth on N species variation in sewage. The NH_3-N in inflow and outflow at 35 cm depth comprised the largest component of the N-species. NH_3-N content underwent a dramatic decline between depths of 5 and 35 cm, followed by a further decline from 35 cm to 65 cm. No further significant change occurred below 65 cm. There was both a low concentration and low variation of NO_2-N between the influent and different effluents. It was also shown that effluent NO_3-N concentration greatly increased from 35 to 65 cm, while it was dominant in the various forms of N between 65 cm and 115 cm. The majority of nitrogen present as NH_3-N from the synthetic wastewater was removed mainly in the soil and sand parts of the reactor through rapid adsorption by the biomass and filters,

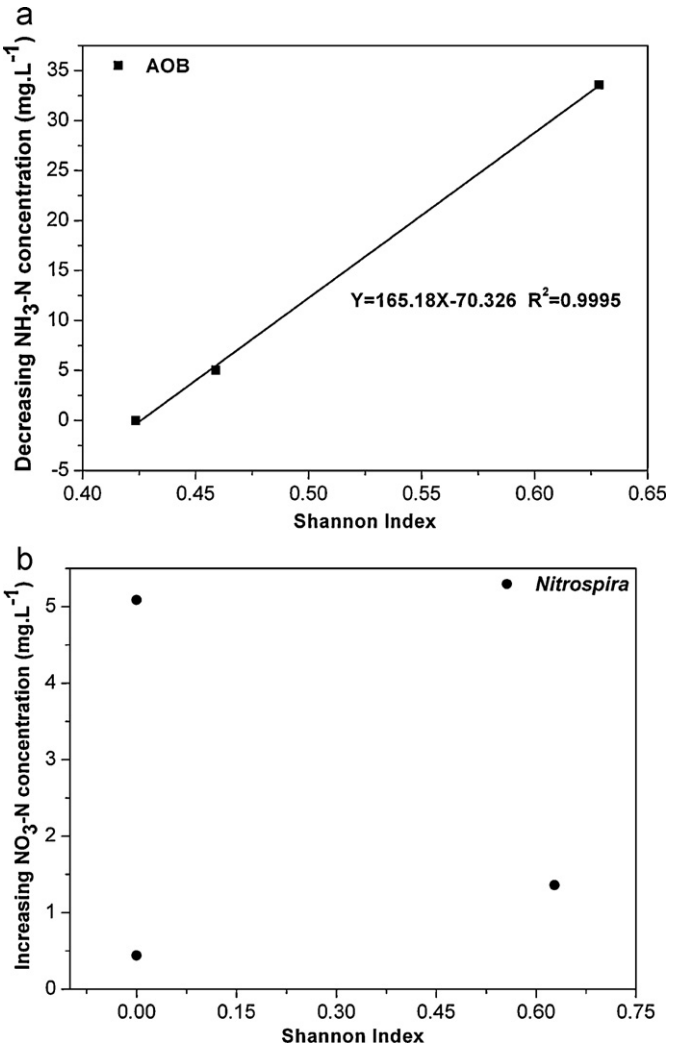

Fig. 5. The relationship between decreasing NH_3-N and Shannon index for AOB (a), and increasing NO_3-N and Shannon index for *Nitrospira* (b) in different padding. The soil Shannon index is the average value of topsoil, midlevel soil, and subsoil.

and the adsorbed NH_3-N was subsequently converted to NO_3-N via biological nitrification, which was carried out by aerobic, autotrophic bacteria using molecular oxygen as an electron acceptor [32]. Meanwhile the high surface DO concentration (Fig. 2a) was beneficial for aerobic microbial survival, which in turn was advantageous for nitrification. Thus, the majority of the NH_3-N removal occurred at depths between 5 and 65 cm. In addition, NO_2-N concentrations remained low in all effluent samples. This is thought to be due to the role of nitrites as intermediates in nitrification. Furthermore, the nitrification step for NH_3-N removal led to a substantial increase in NO_3-N and high DO consumption, the accumulated NO_3-N could not be removed effectively between 65 cm and 115 cm through denitrification because of the large quantities of organic carbon which served as a carbon source for denitrification in upper layers but not in deeper layers [33].

3.2.2. Effect of the physicochemical characteristics of soil and wormcast

The nitrogen distribution across the soil strata and wormcast, which play dominant roles in the N cycle, was studied to better understand nitrogen transformation mechanisms in VF. In the upper soil, TN increased, mainly due to the NH_3-N profile that increased from 1.7 g kg^{-1} initially, to 3.1 g kg^{-1} at 5–15 cm, 2.7 g kg^{-1} at 15–25 cm and 3.4 g kg^{-1} at 25–35 cm (Table 4). Conversely, the ratio of N_{org}/TN decreased slightly with soil depth, although the majority of N in the form of N_{org} was detected in wormcast. Consistent with previous work [34], earthworm activity was found to be a major determinant of N_{org} in soil, as these animals promote nitrogen mineralization. The increased NH_3-N in the top soil might be from the adsorption or absorption of NH_4^+ ions from influent onto the mineral or organic fraction of the soil, as ammonium ions are relatively inert to cation exchange [35]. The increased VF NH_3-N was concomitant with removal from the wastewater. Meanwhile, earthworm activity and the production of cast was found to be critical for NH_3-N reduction in the wastewater, as it oxygenates the influent and this oxygenation facilitates the nitrification of ammonia by microbes [36]. The fiber shown in Fig. 3 might be derived from sawdust. The inclusion of sawdust was also useful to decrease NH_3-N in sewage because it acted as a biosorbent through complexation with NH_4^+ ions [37].

The concentration of NO_3-N was low in all soils and wormcast, but increased with soil depth (Table 4). This is likely caused by surface earthworm activity, nitrification, and soil properties. It is well known that earthworms mediate the conversion of organic nitrogen to inorganic nitrogen and that nitrification promotes the formation of nitrate [33,34]. Furthermore, soil particles have the capacity to retain pollutants and capture NO_3-N from wastewater. Similar findings were obtained by Wong et al. [38]. Though nitrate nitrogen in soil increased with depth, the majority represented downward transport with sewage outflow.

3.2.3. The effect of AOB and Nitrospira community structure and diversity

The AOB and *Nitrospira* communities in soil, sand, and detritus from the lab-scale VF are shown in Fig. 4. The DGGE profiles revealed that the dominant communities were in S1–S3, with no significant differences between them. However, soil nitrifying bacterial structure greatly differed between sand and detritus. Similarly, evaluation of the bacterial diversity (Table 5) based on the Shannon index showed that microbial diversity was higher in soil than in sand or detritus. These results indicate that soil is the most suitable substrate for nitrifying bacterial communities in this reactor, and that earthworm activities had no significant effect on the community structure of nitrobacteria at different soil depths. Filter body effects in combination with the loss of organic substrates most likely contributed to this observation. Microorganisms easily attach to the surfaces and micro-pores of soil particles. Some groups like indigenous ammonium oxidizers attach more strongly to clay particles than do most heterotrophic bacteria [39,40]. In addition, lower substrate and nutrient availability in the sand and detritus, due to removal of organic pollutants in the upper layer of the VF, resulted in lower growth of microbes in these media [41]. It is probable that the earthworms maintain the balance of nitrifiers with the help of aerobic and anaerobic microflora in their guts, which could explain why adding earthworms had no obvious influence on the diversity of nitrobacterium. The distinct features of these bands are also shown in Fig. 4. The majority (e.g., bands 2, 3, 4, and 14) exhibited higher intensities in S1–S3 (especially S2), indicating that particular bacterial species existed in soil at relatively high abundance within the nitrification groups. These findings may also be due to the higher density of earthworms in the 10–25 cm strata. Here, sufficient oxygen was available, and the burrowing action of the earthworms resulted in improved aerobic conditions in the soil body, thereby creating a favored microenvironment for aerobic nitrobacteria.

By examining the relationship between concentration changes of different N forms and the Shannon index for AOB and *Nitrospira*, the effect of bacterial diversity on NH_3-N removal and NO_3-N distribution for wastewater treatment was determined. It was found that there was a significant positive correlation between

Table 5
Contrast of Shannon-Wiener diversity index in different paddings of the VF.

	Targets	S1	S2	S3	SD	DE
Number of bands	AOB	4	6	6	3	3
	Nitrospira	7	6	6	1	1
Shannon index (H)	AOB	0.58476	0.65495	0.64583	0.45888	0.39854
	Nitrospira	0.76296	0.69021	0.63185	0	0

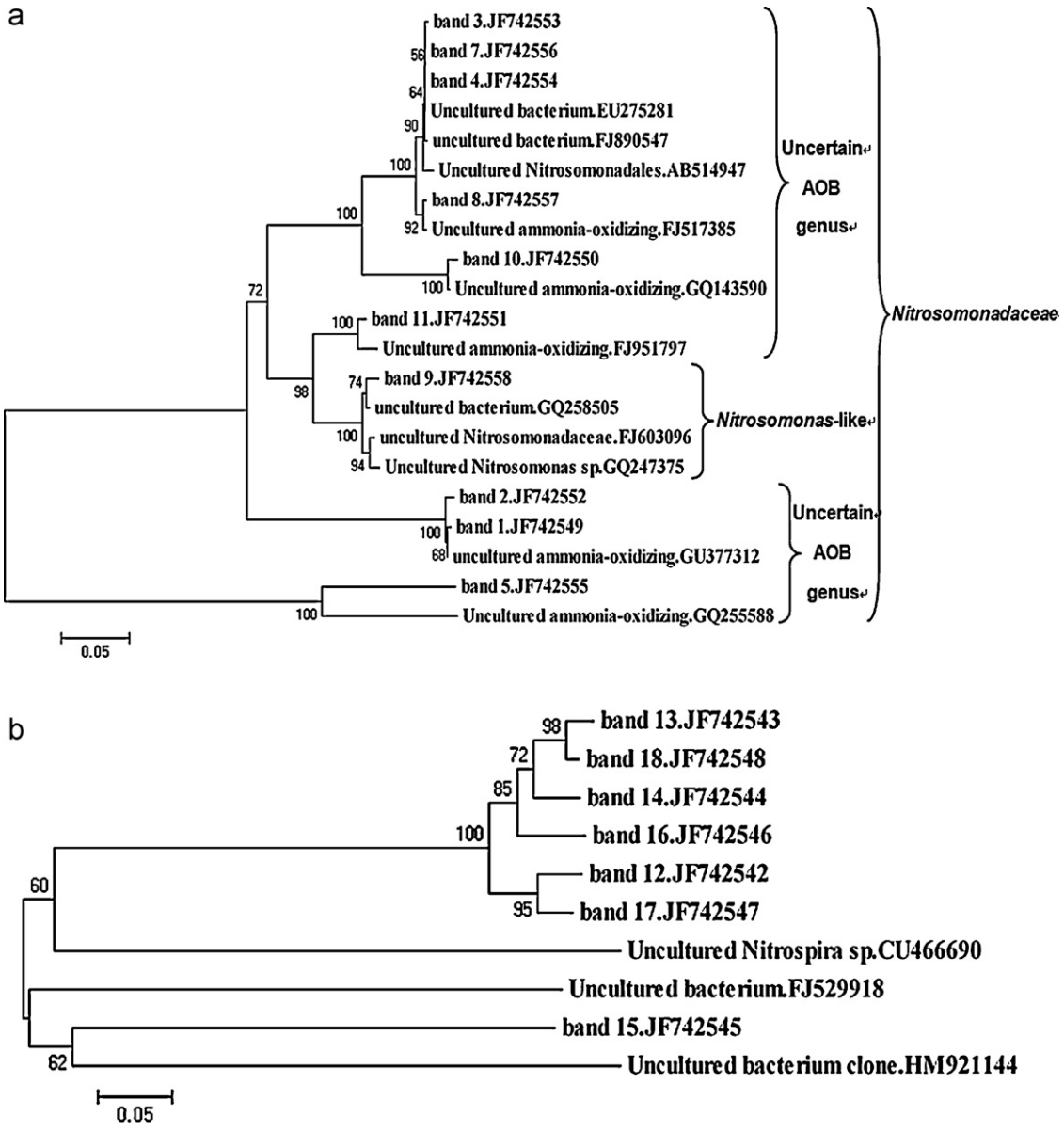

Fig. 6. Phylogenetic tree based on neighbor-joining analysis of gene sequences from ammonia oxidizers with a length of 491 bp (a) and *Nitrospira* with a length of 151 bp (b). Bands 1–18 represent sample sequences. Numbers at the branches represent bootstrap values, and the scale bar indicates 5 changes per 100 amino acid positions.

decreasing NH_3-N and Shannon index for AOB ($R^2 = 0.9995$), while there was no apparent relation between increasing NO_3-N and Shannon index for *Nitrospira* (Fig. 5). It is obvious that AOB diversity increased with decreasing ammonia nitrogen concentration (or as a function of improved ammonia nitrogen removal), which is consistent with the relatively large decline in the NH_3-N concentration in the soil-earthworm layer (Fig. 2). The correlation between nitrate nitrogen concentration and Shannon index suggests that there are other factors affecting NO_3-N transformation besides the diversity of *Nitrospira.* Because of nitrate transformation into nitrogen via denitrification [33], a minor increase in NO_3-N concentration with increasing Shannon index for *Nitrospira* in TOC-enriched soil was observed.

3.2.4. Identification of AOB and Nitrospira in the VF

The numbered bands in the DGGE gel were excised for sequencing. Bands with the same mobility in the DGGE gel (e.g., bands 3, 3′, and 3″) had the same nucleotide sequences. Thus, the same band name (e.g., band 3) was used to describe the sequences used for phylogenetic analysis. Their phylogenetic positions are illustrated by the neighbor-joining tree in Fig. 6. The closest matches of amoA for all sequences amplified from these bands

(except band 6 which was not related to others) were either uncultured ammonia oxidizers or environmental clone sequences falling within the beta *proteobacterial* class. The obtained sequences showed between 97 and 100% similarity with previously identified gene sequences. Results indicated that the AOB community consisted mainly of unidentified ammonia oxidizers and a unique *Nitrosomonas*-like genus grouped in the *Nitrosomonadaceae* family. The unidentified AOB contributed four bands, six bands, five bands, two bands, and three bands to the ammonia oxidizer community in S1, S2, S3, SD, and DE, respectively (Fig. 4a and 6a). Sequences similar to these unidentified fragments have been observed in other soils (EU275281, GQ143590, FJ890547, AB514947, FJ517385, GU377312), and in a sediment (FJ951797) and constructed wetland (GQ255588), where they had a significant influence on nitrification and the AOB community composition. Furthermore, the only sequence retrieved from band 9 in SD was placed within the *Nitrosomonas*-like genus. Obtained sequences from AOB in VF were chemolithoautotrophs, having in common the ability to utilize ammonia as a sole source of energy and mediate transformation of NH_3-N to NO_2-N [42]. In contrast, within the population of AOB, a higher percentage of *Nitrosospira* and *Nitrosomonas* sequences have been found in constructed wetlands and conventional wastewater treatment plants [43–45]. This apparently distinct microbial distribution in different biofilters could arise for two types of reason; (1) if there was an insufficient quantity of bacterial template DNA for PCR amplification for the rarer groups in the AOB community, then the single more abundant group could appear to be the only one present, or, (2), it may also be that the system is relatively immature and thus the different populations have not had time to reach a long-term equilibrium.

As shown in Fig. 5b, all the strains shared 95–97% of the sequences identical with those retrieved from the database. Sequences analysis revealed that the whole 16S DNA sequences retrieved from bands 12 to 18, except for 15, were related to *Nitrospira*, while band 15, which was present in all filters, was grouped with the unidentified bacterium in biofilm or filters. Since *Nitrospira* thrive under conditions of relative nitrite scarcity and neutral pH [46,47], they were able to survive in this system (see Table 2 and Fig. 2) and were responsible for an important step in the N cycle, the oxidation of NO_2-N to NO_3-N [42]. This outcome is consistent with results showing that *Nitrospira* members of nitrite-oxidizing bacteria, usually dominant in biofilms from wastewater treatment plants and soils [47,48], were present here.

4. Conclusion

Because the microbial-earthworm ecofilter was able to significantly reduce the COD and NH_3-N from artificial wastewater, it may be an effective technology for domestic wastewater treatment. A stable removal of COD was achieved and the majority was trapped by the upper 5–35 cm depths. This COD reduction was greatly facilitated by the addition of sawdust to the soil, which enhanced porosity and specific surface area, and by the structure of the wormcast surface. The soil layers were responsible for the major fraction of nitrogen variations, in addition to determining wormcast characteristics, earthworm activity, and nitrifying bacteria diversity. According to sequences analysis, the dominant AOB members were unidentified ammonia oxidizers and the majority of sequences retrieved from bands 12 to 18 were related to *Nitrospira*. In conclusion, these results could yield a deeper understanding of the mechanism of organic matter and nitrogen removal in VF systems. These laboratory-scale studies may allow extension of this methodology to applications like controlling water pollution in rural areas that cannot be served by conventional sewage systems.

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