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Comparison of nutrient removal and bacterial communities between natural zeolite-based and volcanic rock-based vertical flow constructed wetlands treating piggery wastewater

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ABSTRACT

This study designed two vertical flow constructed wetlands (VFCW) as a secondary treatment for piggery wastewater. The effect of effluent recirculation and substrate type on treatment efficiency of constructed wetlands on piggery waster was firstly investigated. Recirculation considerably improved the removal of NH_4-N, total nitrogen (TN), and total phosphorus (TP), but not that of chemical oxygen demand (COD). The natural zeolite-based VFCW performed better than volcanic rock-based VFCW for all four parameters. At the highest recirculation ratio, the average treatment efficiencies of natural zeolite-based VFCW reached 50.7, 91.6, 48.3, and 80.7% for COD, NH_4-N, TN, and TP, respectively. The diversity and composition of total bacteria and ammonia-oxidizing bacteria (AOB) in the bioflim of natural zeolite and volcanic rock were then determined. Both total bacteria and AOB in biofilm of natural zeolite were more diverse than those in biofilm of volcanic rock. Phylogenetic analyses further revealed two points: first, genera *Alcaligenes* and *Comamonas* dominated in the β-*proteobacteria* of natural zeolite and volcanic rock, respectively, and second, genera *Nitrosospira* and *Nitrosomonas* dominated in AOB of natural zeolite and volcanic rock, respectively. Our results suggest that natural zeolite-based VFCW with effluent recirculation is a good choice for piggery wastewater treatment. In addition, substrate type not only influences nutrients removal, but also plays a part on shaping microbial communities in constructed wetlands.

Keywords: Constructed wetlands; Microbial community; Natural zeolite; Volcanic rock; Piggery wastewater

1. Introduction

Intensive livestock farming produces a large amount of wastewater that contains high concentrations of suspended solids (SS), organic compounds, and nutrients. Since traditional anaerobic digestion takes a longer time than aerobic degradation to reach satisfactory treatment efficiency, the piggery wastewater is often partially treated and directly sprayed onto the agricultural lands afterwards. However, the nutrients loading usually exceed the requirement of plants and deteriorate the

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quality of ground water and aquatic environments nearby. Further treatment is required after anaerobic digestion to reduce the environmental impact. Constructed wetlands are feasible facilities to fulfill this demand [1,2], with their advantages of low cost and easy maintenance [3,4]. Vertical flow constructed wetlands (VFCW) possess greater oxygen transfer ability than horizontal flow ones, thus they are more effective to remove NH_4-N and organic matter [5].

In order to achieve approving treatment efficiency for constructed wetlands, it is very necessary to ameliorate the processes and choose suitable substrates. Additional effluent recirculation has the potential to facilitate the interaction of contaminants with substrates or with micro-organisms [6]. This process is probably useful to enhance the treatment of piggery waste, which is characterized by high-concentration ammonium and organic matter. It has been observed that various materials, including furnace-slag [7], gravel [8], natural or synthetic zeolite [9], lime, ceramsite [10], and brick, have the ability to remove nitrogen and phosphorus from wastewater. Natural zeolites were gradually employed as filter material of constructed wetlands because of their high affinity for ammonium ion and for many toxic cations and their availability and inexpensiveness [5,11]. But the treatment efficiency of natural zeolite-based constructed wetlands on complex piggery wastewater remains unknown. Volcanic rocks are readily available in countries and regions that had volcanic activities in the past [12,13]. These dark particles are very porous and have a good hydraulic conductivity. However, the capacity of volcanic rocks in removing nitrogen and phosphorous from wastewater is not well understood. Their performance in the constructed wetlands treating piggery wastewater needs further exploration.

Micro-organisms play a major role in the degradation of organic pollutants and cycling of nutrients within constructed wetlands [14]. Thus, it is important to obtain knowledge about the microbial assemblages for understanding the bioremediation processes. Recently, researches on constructed wetlands are not limited to achieve good treatment efficiency, the diversity and composition of microbial community in constructed wetlands has also been investigated [2]. Besides total bacterial community, several studies paid attentions to functional groups like AOB [15–18] and denitrifying bacteria [18,19]. Some factors, including plant species [20], substrate type [21], operational condition, and spatial distribution [22], were thought of having potential influences on the microbial community of constructed wetlands. However, systematical comparison of microbial community between the biofilms of different substrates has not yet been done previously. Among various molecular tools, denaturing gradient gel electrophoresis (DGGE) was frequently applied to observe community changes and dynamics in constructed wetlands [2,15,20,22]. Compared to DGGE, clone library and sequencing can provide more comprehensive and reliable data of community composition [23].

In this study, natural zeolite and volcanic rock were used as main substrates to construct two pilot VFCWs, respectively. The effect of substrate type and effluent recirculation on the removal efficiency of constructed wetlands treating piggery wastewater was investigated. Further, clone library and sequencing were introduced to compare the diversity and composition of total bacteria and AOB in the biofilm of natural zeolite and volcanic rock.

2. Materials and methods

2.1. Description of wetland systems and operation

In a greenhouse, two pilot VFCWs were constructed based on the equipment described by Sklarz et al. [24] with some modification, consisting of two containers placed one above the other (Fig. 1). The upper container (1.0 m length, 1.0 m width, and 0.7 m height) is designed as a vertical flow wetland bed filled with a 10 cm high bottom layer of gravel, a 30 cm high medium layer of natural zeolite (or volcanic rock), and a 20 cm high top layer of red loam. The diameter of natural zeolite and volcanic rock ranges from 1 to 2 cm. It was perforated at the bottom of upper container. *Pennisetum hybrid* was planted on the top of both wetland systems. The lower container (1.0 m length, 1.0 m width, and 0.4 m height) is used as a reservoir. A filtration column (0.16 m diameter and 0.8 m height) filled with oyster shells is linked to the effluent pipe of lower container for further purification before the effluent is released. The wetland systems are named as natural zeolie-based VFCW and volcanic rock-based VFCW according to their different medium substrates. The textural and physicochemical characteristics of natural zeolite and volcanic rock are given in Table 1.

The progress of system operation is described as follows briefly. The piggery wastewater was delivered to the upper wetland using a peristaltic pump and trickled down to the lower container. The hydraulic loading from influent tank on both wetlands was maintained at $0.04\,m^3\,d^{-1}$. Then effluent water in the container was partially recirculated back to the upper wetland by another pump. Recirculation ratios (recirculation flow rate: feed flow rate) were set at the first 1:1, then 2.5:1, and finally 5:1. The hydraulic retention time (HRT) of our wetlands without recirculation is

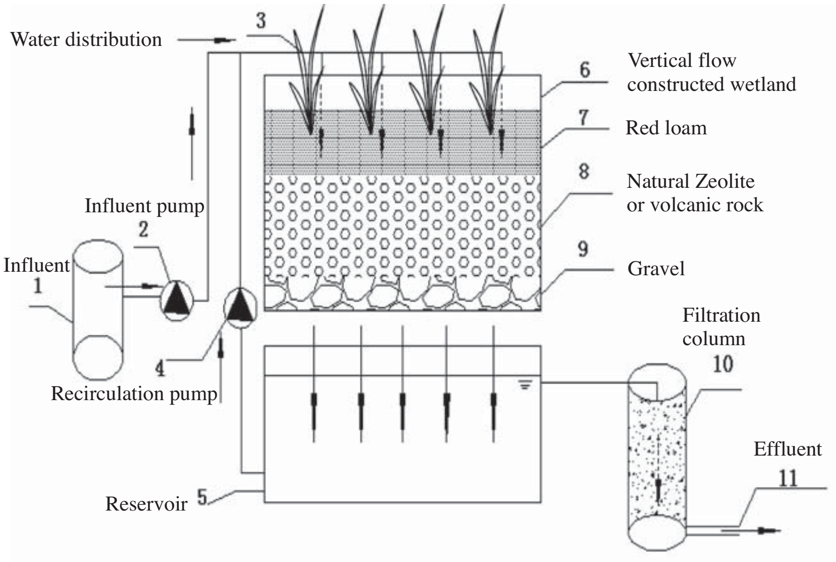

Fig. 1. Schematic description of the VFCW with effluent recirculation.

Table 1
Textural and physicochemical characteristics of natural zeolite and volcanic rock

Property	Natural zeolite	Volcanic rock
pH	5.98	8.69
EC (μs cm^{-1})	70	98
NH_4-N adsorption maxima (mg g^{-1})	11.63	0.211
Particle density (g cm^{-3})	2.07	1.82
Bulk density (g cm^{-3})	0.98	0.62
Porosity (%)	52.5	66.0
Specific surface area ($m^2 g^{-1}$)	2.08	4.72
Micropore volume[a] ($cm^3 g^{-1}$)	4.85×10^{-3}	15.20×10^{-3}
Micropore size[b] (nm)	9.23	6.03
Crystalline species	Montmorillonite $KAlSi_3O_8$ SiO_2	Diopside Olivine Anorthoclase

[a] Single point adsorption total pore volume of pores less than 75.6879 nm diameter at P/Po = 0.973745411.

[b] Adsorption average pore width (4 V/A by BET).

approximately 5 days. The HRT reduces with increasing recirculation ratio theoretically. The wetland systems ran for 30 days continuously within each period of recirculation ratio.

2.2. Sampling strategy and nutrient analysis

The piggery wastewater was taken from the anaerobic lagoon of a pig farm in Xiamen, China. After anaerobic digestion in the lagoon, 67–73% of chemical oxygen demand (COD) in piggery wastewater has been removed. The concentrations of COD, NH_4-N, total nitrogen (TN), PO_4^{3-}, total phosphorus (TP), and SS in influent samples were monitored when every batch of piggery wastewater was sent from the pig farm. Their mean values and ranges (unit: mg L^{-1}) are 859 (613–1,193), 644 (529–1,005), 695 (584–1,095), 37 (20–49), 40 (28–63), and 1,720 (900–2,120) for COD, NH4-N, TN, PO_4^3-P, TP, and SS, respectively. Therefore, the quantified hydraulic loading contents are 34.36, 25.76, 26.36, and 1.624 g $m^{-2}d^{-1}$ for COD, NH_4-N, TN, and TP, respectively.

During each period of recirculation ratio, the effluent samples of both VFCWs were collected at five days interval. The COD, NH_4-N, TN, and TP in the effluent were then determined immediately. Water samples were analyzed based on American standard methods for examination of wastewater [25]. The statistical analyses were performed using SPSS version 16. The model of univariate was employed to test two-way analysis of variance. Significant differences between data-sets were set at $p < 0.05$.

2.3. Structure of bacterial communities and diversity of amoA genes

2.3.1. DNA extraction from biofilm of substrates

Six subsamples of natural zeolites and volcanic rocks were collected from the medium layer of two VFCWs, respectively, when the experiment of nutrient removal had been finished. These subsamples were then combined together and their biofilms were extracted with phosphate buffer (K_2HPO_4 9.3 g L^{-1} and KH_2PO_4 1.8 g L^{-1}). Genomic DNAs were extracted in duplicate from these biofilms using OMEGA soil DNA extraction kit according to the protocol recommended by the manufacturer. The DNA extracts were pooled together and stored in −70°C for further molecular manipulation. The quality and quantity of DNA extracts were examined by gel electrophoresis and nanodrop spectrophotometer (Thermo Fisher Scientific).

2.3.2. PCR, library construction, and sequencing

Bacterial 16S rRNA genes and *amoA* genes were amplified from DNA extracts by polymerase chain reaction (PCR) reaction in a thermal cycler (Eppendorf, Germany) using primer sets 27F/1492R and *amoA*1F/*amoA*2R, respectively [26]. The fragment lengths of the amplification products were about 1,466 bp for 16S rRNA genes and 491 bp for *amoA* genes. The thermal cycling parameters for amplifying 16S rRNA genes were: initial denaturing at 94°C (10 min), followed by 30 cycles of reactions including denaturing at 94°C (30 s), primer annealing at 54°C (30 s) and primer extension at 72°C (45 s), and finally an additional extension step was performed at 72°C for 10 min. The amplification of *amoA* genes differed in the annealing temperature (55°C). All PCR reactions were conducted in triplicate and pooled together to reduce PCR bias.

The libraries of 16S rRNA genes and *amoA* genes from both natural zeolite and volcanic rock were constructed. Briefly, the amplification products were purified using Axygen gel DNA purification kit. They were then ligated into pMD 19T vector (Takara, Japan) and finally transformed into *E.coli* JM109 competent cells according to the manufacturer's instructions. Positive clones were verified by blue/white screen and PCR using universal M13 primer set. Approximately, 70 positive clones were randomly picked out from each clone library. They were then sequenced by an ABI 3730 DNA automated analyzer (Applied Biosystems). The sequenced gene fragments were checked using Pintail program (http://www.bioinformatics-toolkit.org/Web-Pintail/) to get rid of PCR chimera.

2.3.3. Diversity and phylogenetic analyses

All the qualified sequences were analyzed by software DOTUR to identify operational taxonomic units (OTUs). The sequences showing $\geq 97\%$ similarity were considered to be the same phylotype. Five diversity indices, including Richness, Shannon-Weaver index, Simpson index, Evenness and Good's Coverage, were calculated according to the method described in molecular microbial ecology manual [27]. The significance of difference between two libraries was tested by webLIBSHUFF (http://libshuff.mib.uga.edu/). The 16S rRNA genes were compared with reference sequences in Genbank (http://www.ncbi.nlm.nih.gov) using a Blastn search tool. They were then classified into different phyla. One neighbor-joining tree was constructed with representative sequences from β-*proteobacterial* OTUs and relative sequences from Genbank using MEGA 4.0. Another tree was constructed with translated *amoA* OTUs and their relative sequences. Bootstrapping was performed 1,000 times to estimate the reliability of phylogenetic trees. The sequences used in trees construction were deposited in Genbank under accession numbers JF743032-JF743042 for phylum β-*proteobacteria* and JF743010-JF743031 for *amoA*.

3. Results

3.1. Nutrients removal under different recirculation ratios

The average removal efficiencies of COD, NH_4-N, TN, and TP and the accumulation of NO_2^- and NO_3^- in two VFCWs treating piggery wastewater are shown in Fig. 2. Higher recirculation ratio remarkably enhanced the removal of NH_4-N, TN, and TP for both wetland systems. The effect of recirculation on the COD removal was insignificant ($p < 0.05$). At the highest recirculation ratio, the average treatment efficiencies of zeolite-based VFCW reached 50.7, 91.6, 48.3, and 80.7% for COD, NH_4-N, TN, and TP, respectively, while volcanic-based VFCW removed 46.4, 69.6, 34.4, and 70.4% of COD, NH_4-N, TN, and TP, respectively. The higher recirculation ratio also facilitated the accumulation of NO_3^- and $NO_2^- + NO_3^-$ in the influent of both wetlands.

Zeolite-based VFCW performed better than volcanic-based VFCW under the same recirculation ratio especially in removing NH_4-N, TN, and TP. The removal efficiencies of zeolite-based VFCW were 9.2–48.1, 31.5–61.3, 40.5–94.8, and 14.7–99.9% higher than those of volcanic-based VFCW for COD, NH_4-N, TN, and TP, respectively. The accumulations of NO_3^- in the influent of zeolite-based VFCW were totally higher than that in the influent of volcanic-based VFCW. However, more $NO_2^- + NO_3^-$ were accumulated in volcanic-based VFCW than zeolite-based VFCW when the recirculation ratio was raised.

Substrate species and recirculation ratio were considered as two factors for two-way analysis of variance. The results indicated that the substrate

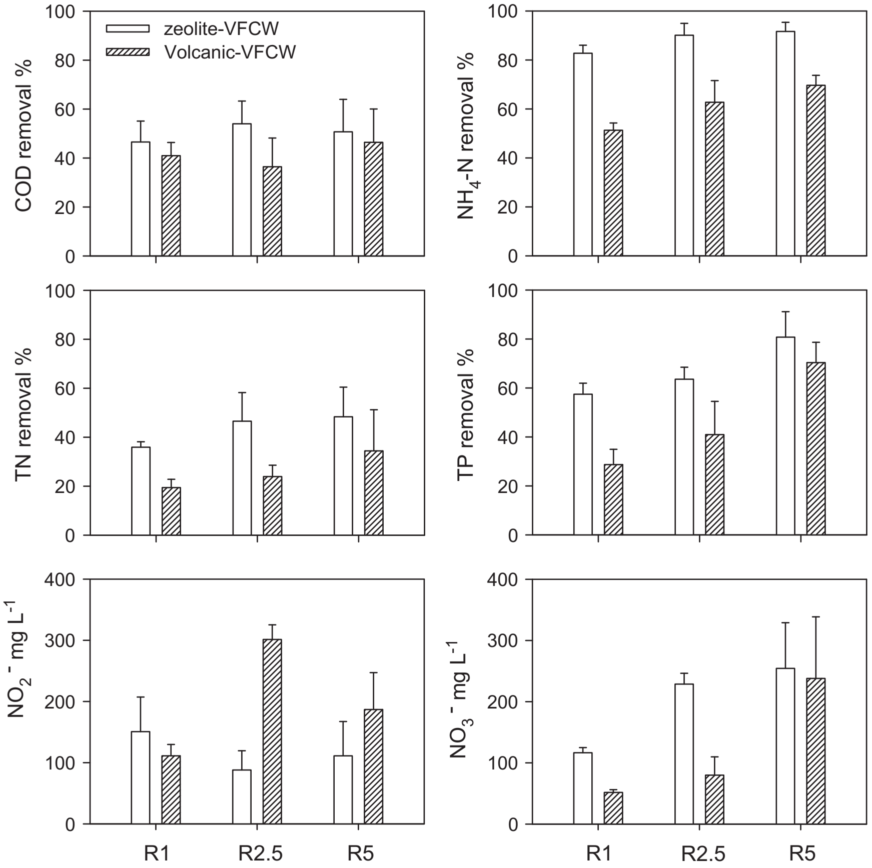

Fig. 2. Average removal efficiencies of COD, NH_4^+-N, TN, and TP, and accumulation of NO_2^- and NO_3^- in natural zeolite-based and volcanic rock-based VFCWs with hydraulic loading at 4 cm/d under different recirculation ratios ($n = 6$). Recirculation ratio 1:1, 2.5:1, and 5:1 was expressed as R1, R2.5, and R5, respectively.

species posed a significant impact on treatment efficiencies for all of the four parameters ($p < 0.05$), whereas recirculation ratio significantly influenced the removal of NH_4-N, TN, and TP ($p < 0.05$), but not that of COD.

3.2. Diversity and composition of bacterial communities

Diversity indices of 16S rRNA gene sequences from the biofilm of two substrates are summarized in the middle column of Table 2. With 97% similarity analyzed by DOTUR, 70 clones of zeolite biofilm were grouped into 42 OTUs, whereas 68 clones of volcanic biofilm were distributed into 35 OTUs. All the Richness, Shannon–Wiener, and Simpson indices of zeolite biofilm were a little higher than that of volcanic biofilm. This suggests that the diversity of bacterial communities of zeolite biofilm is greater than that of volcanic biofilm. In addition, the Evenness of the former was better than that of the latter. The Coverage indices of both l6S rRNA gene libraries were higher than 60%. Comparison made by online software web-LIBSHUFF indicates that there is a significant difference between two l6S rRNA gene libraries ($p < 0.01$).

Table 3 shows the bacterial community composition in the biofilms of natural zeolite and volcanic rock after comparing the retrieved sequences with reference sequences in Genbank database. Sixty (60%) and 54.4% of total clones from zeolite biofilm and volcanic biofilm, respectively, belonged to phylum *Proteobacteria*, which was dominated by β-*proteobacteria* and γ-*proteobacteria*. Of the total clones, 18.6 and 14.7% from natural zeoltie and volcanic rock, respectively, belonged to phylum *Bacteroidetes*, which was dominated by class *Sphingobacteria*. Phylum *Firmicutes* was also an important group occupying 8.6 and 19.1% in library of natural zeolite and volcanic rock, respectively. Fewer clones belonging to phyla *Actinobacteria*, *Fibrobacteres*, and *Planctomycetes* were found in library of natural zeolite and volcanic rock.

It is well-known that a large proportion of bacteria involved in nitrification and denitrification belonged

Table 2
Diversity indices of 16S rRNA gene and *amoA* gene sequences from the biofilms of natual zeolite and volcanic rock

Indices	16S rRNA gene		*amoA*	
	Zeolite	Volcanic	Zeolite	Volcanic
No. of clones	70	68	68	67
No. of OTUs	40	35	12	10
Richness estimator (chao1)	103	96	18	15
Shannon–Wiener	3.40	3.16	1.85	1.03
Simpson	0.954	0.936	0.770	0.429
Evenness	0.922	0.890	0.745	0.452
Good's coverage	60.6%	63.8%	92.6%	90.9%

Table 3
Bacterial community composition in the biofilms extracted from natural zeolite and volcanic rock

Phylum/class	Zeolite biofilm		Volcanic biofilm	
	Clone no.	%	Clone no.	%
Proteobacterium	42	60	37	54.4
α-proteobacterium	9	12.9	3	4.4
β-proteobacterium	15	21.4	15	22.1
γ-proteobacterium	17	24.2	16	23.5
δ-proteobacterium	1	1.4	3	4.4
Bacteroidetes	13	18.6	10	14.7
Sphingobacteria	10	14.3	9	13.2
Firmicutes	6	8.6	13	19.1
Actinobacteria	3	4.3	0	0
Planctomycete	2	2.9	4	5.9
Fibrobacteres	1	1.4	0	0
Unidentified	3	4.3	4	5.9
Total	70	100	68	100

to *β-proteobacteria*. Fig. 3 shows the neighbor-joining tree constructed with representative sequences of *β-proteobacteria* and reference sequences from Genbank. The *β-proteobacterial* composition was strikingly different between libraries of natural zeolite and volcanic rock. The *β-proteobacterial* sequences of natural zeolite fell into six OTUs, whereas those of volcanic rock were classified into five OTUs. However, no OTU contained gene sequences from natural zeolite and volcanic rock simultaneously. In the library of natural zeolite, 53.3% of *β-proteobacterial* clones belonged to family *Alcaligenaceae* and 20% belonged to family *Nitrosmonadaceae*. Three clones of genus *Nitrosospira* and no clone of genus *Nitrosomonas* were found. In the library of volcanic rock, 73.3% of *β-proteobacterial* clones belonged to family *Comamonadaceae*. Only one clone of genus *Nitrosomonas*, but no clone of genus *Nitrosospira* was observed.

3.3. Diversity and composition of AOB

Diversity indices of bacterial *amoA* sequences from biofilm of two substrates are summarized in the right column of Table 2. Sixty-eight clones of zeolite biofilm were grouped into 12 OTUs, whereas 67 clones of volcanic biofilm were distributed into 10 OTUs. The indices of richness, Shannon–Wiener and Simpson of AOB from natural zeolite, were much higher than that from volcanic rock. This suggests that the diversity of AOB from zeolite biofilm is much higher than that from volcanic rock. The evenness of AOB library of natural zeolite was also better than that of volcanic rock. The coverage of both libraries reached up to 90%, which indicated that the sizes of both libraries were large enough. Comparison made by online software web-LIBSHUFF indicates that there is a significant difference between two *amoA* gene libraries ($p < 0.01$).

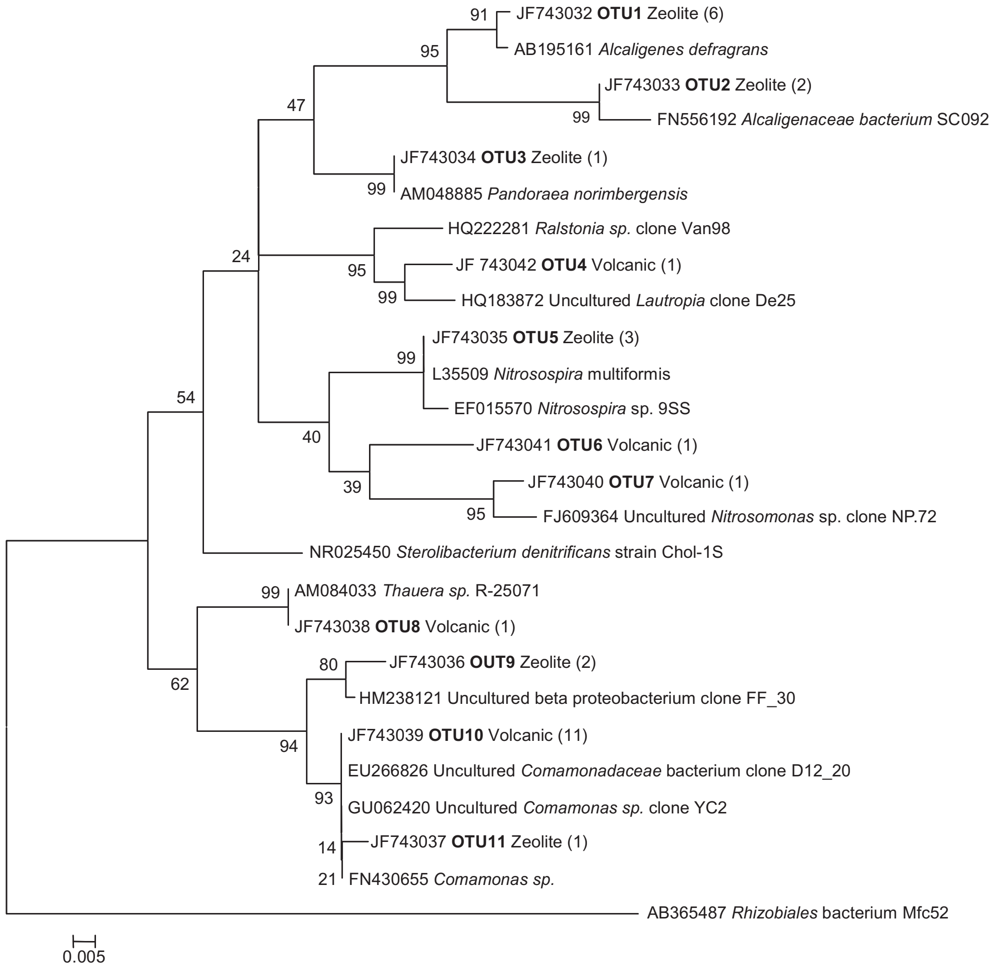

Fig. 3. Neighbor-joining tree constructed using representative sequences (OTUs) belong to phylum β-*proteobacteria* and reference sequences. The number in parentheses is the total number of sequences from biofilms of natural zeolite or volcanic rock that have > 97% similarity with representative sequences. Bootstrapping was performed 1,000 times.

The neighbor-joining tree constructed with amino acid sequences (translated from *amoA*) of OTUs and reference sequences is shown in Fig. 4. The primer set *amoA*1F and *amoA*2R can amplify β-*proteobacterial amoA* from genus *Nitrosomonas* and *Nitrosospira*. The whole phylogenetic tree was composed of three clusters. One of them was made up by *Nitrosospira*-like sequences, whereas two of them were made up by *Nitrosomonas*-like sequences. No sequence from both *amoA* libraries fell into cluster II .The composition and distribution of *amoA* amino acid sequences between two *amoA* libraries was remarkably different. Only OTU9, OTU12, and OTU19 had sequences from both *amoA* libraries. In cluster I, there were nine OTUs and totally 63 sequences from *amoA* library of natural zeolite, whereas eight OTUs and only 16 sequences from *amoA* library of volcanic rock. In cluster III, there were three OTUs and only five sequences from *amoA* library of natural zeolite, where two OTUs and totally 51 sequences from *amoA* library of volcanic rock.

4. Discussion

This study intended to investigate the effect of different substrates and recirculation ratios on the nutrient removal of VFCW. More attentions were then given to compare the diversity and community composition of total bacteria and AOB between the biofilms of natural zeolite and volcanic rock in the constructed wetlands.

In the constructed wetlands, organic compounds are degraded by the heterotrophic micro-organisms aerobically and anaerobically depending on the oxygen concentration in the substrate. In this study, COD

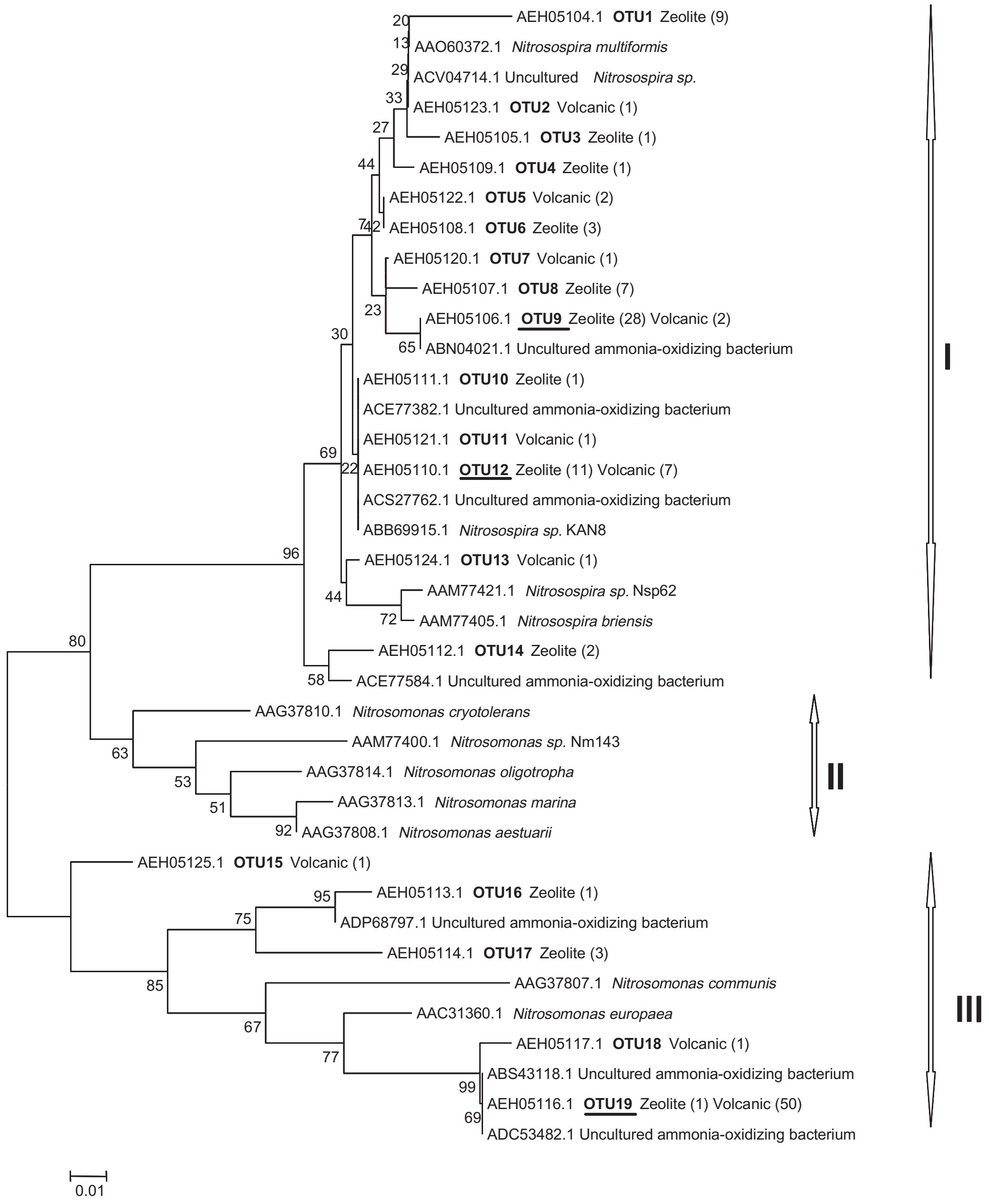

Fig. 4. Neighbor-joining tree constructed using translated *amoA* OTUs and reference sequences. The number in parentheses is the total number of sequences from natural zeolite or volcanic rock that have >97% similarity with representative sequences. The underlined OTUs contain sequences retrieved from both biofilms of natural zeolite and volcanic rock. Bootstrapping was performed 1,000 times.

removal in zeolite-based VFCW was a little higher than that in volcanic-based VFCW. However, no obvious improvement of COD removal was observed among different recirculation ratios. This result may be contributed to two reasons: first, sufficient oxygen diffusion for COD degradation without recirculation and second and more importantly, relatively stable proportion of biodegradable organic compounds in the wastewater. Although great oxygen transfer in VFCWs can improve degradation of organic matters, the recalcitrant organic matters still exist. Previous studies have reported similar COD removal rates [5,7]. For example, the 47% of COD was removed from domestic wastewater in the slag-based vertical wetland, whereas the removal efficiency was 44% in the gravel-based wetland [7].

The concentration of NH_4-N in piggery wastewater is very high. The NH_4-N is adsorbed by binding sites of substrates very quickly. Then nitrification bacteria grown in the biofilm has been thought to promote the NH_4-N transformation. Besides substrate adsorption and microbial transformation, plant uptake and volatilization are another two pathways for NH_4-N removal. The stronger accumulation of NO_2^- and NO_3^- suggests that the ammoxidation is the most important way in our constructed wetlands. In the previous works, the NH_4-N removal of zeolite-based constructed wetlands ranged from 46 to 99% [28,29], whereas that of volcanic-based constructed wetlands ranged from 37 to 89% [12], depending on different wetland designs and operations. In this study, the NH_4-N removals of zeolite-based VFCW were totally higher than that of volcanic-based VFCW under any of the recirculation ratios. This can be partially explained by the different physicochemical characteristics of zeolite and volcanic rock (Table 1). The maximum NH_4-N adsorption of natural zeolite is greatly higher than that of volcanic rock. In addition, the crystalline species of natural zeolite contain Montmorillonite and $KAlSi_3O_8$ that have strong NH_4-N binding capacity, while volcanic rock does not have strong NH_4-N binding capacity. With the increasing recirculation ratio, the NH_4-N removals of both wetland systems were significantly increased, mainly because of the higher oxygen transfer provided by recirculation [30].

The mechanisms of nitrogen removal are manifold and include volatilization, ammonia oxidation, nitrification/denitrification, plant assimilation, and matrix adsorption [7]. NH_4-N is the dominant component of TN in influent piggery wastewater. In this study, strong ammonium oxidation and nitrification produced a large amount of NO_2^- and NO_3^- accumulated in the effluent of both wetlands (Fig. 2). NO_2^- and NO_3^- will be reduced to N_2O or N_2 under suitable condition by denitrification or anaerobic ammonium oxidation. However, the aerobic condition prevailing in the VFCW resulted in low denitrification. The TN removals in our study were not very satisfactory, only ranged from 19.4 to 48.3%, which were comparable to the mean TN removal efficiency of 51 VFCWs (44.6%) investigated by Vymazal [31]. The recirculation condition and substrate species that contributed to higher NH_4-N removal can also improve TN disposal. Additionally, it should be noted that for the purpose of irrigation, reasonable NO_3^- remainder in the effluent can reduce fertilizer requirement [24].

The phosphate compounds are removed mainly via adsorption and precipitation in substrate, plant uptake, and biotic assimilation. PO_4^{3-}-P is the principle component of TP in piggery wastewater. The phosphate retention capacity in constructed wetlands is also variable based on different plants, substrates, and operation conditions. In our study, zeolite-based VFCW performed better than volcanic-based VFCW in removing TP, probably due to different adsorption capacities of natural zeolite and volcanic rock. Likewise, a horizontal subsurface flow constructed wetlands with volcanic tufa as medium substrate can remove 71.8% of COD and 63.8% of NH_4-N, but only 30.5% of PO_4^3-P [32], whereas a VFCW with zeolite as main substrate can remove 64.3% of COD, 44.6% of NH_4-N, and 38.1% of PO_4^3-P [28]. Because of the insufficient contact time, phosphorus removal in VFCW systems is limited [28]. Thus, longer contact time achieved by effluent recirculation improved TP removal of constructed wetlands in this study.

Microbial communities are also involved in the removal of pollutants, including organic compounds degradation, ammonia oxidation, nitrification, and denitrification. For instance, the binding sites for ammonium adsorption in the substrates will be saturated with ammonium after running for a period of time. AOB in the biofilm of substrates can oxidize ammonium to nitrite, and release these binding sites for further adsorption. Thus, ammonium oxidizers are important for long-term sustainable ammonium removal. Therefore, it is very necessary to investigate the diversity and composition of bacterial communities in the biofilms of substrates and their potential role on the performance of wetland systems. Calheiros et al. [21] concluded that substrate type had a considerable effect on the species richness and the structure of bacterial communities. It agreed with our study that the sequences of both total bacteria and AOB between natural zeolite and volcanic rock samples were significantly different ($p < 0.05$). It has been suggested that the level of bacterial diversity in a wastewater treatment facility has a major influence on process stability [33]; the greater the diversity in the treatment plant, the better it would cope with changing conditions. Based on the calculated indices, the diversities of both total bacteria and AOB in biofilm of natural zeolite were higher than those of volcanic rock (Table 2). The better performance of zeolite-based VFCW can be partially related to the greater bacterial diversity of zeolite biofilm.

Phylum *Proteobacteria* is widely distributed in soil and aquatic environments. Some classes of phylum

Proteobacteria are involved in the transformation of nitrogen compounds, such as ammonium oxidization, nitrification, and denitrification. In this study, total bacterial communities of both natural zeolite and volcanic rock were dominated by phylum *Proteobacteria*. Similarly, Bai et al. [9] found that phylum *Proteobacteria* was always the dominant component in zeolite biological aerated filters treating coking wastewater. The β-subclass of *Proteobacteria* contained many functional groups, such as denitrifying bacteria and AOB. In the neighbor-joining tree of β-*proteobacteria*, genera *Alcaligenes*, *Comamonas*, *Burkholderia, Thauera*, and *Rhodocyclus* have been reported as denitrifying bacteria. Of them, genera *Alcaligenes* and *Comamonas* were well studied [34]. Our results indicated that most sequences of β-*proteobacteria* in the biofilm of natural zeolite belonged to genus *Alcaligenes*, while those in the biofilm of volcanic rock belonged to genus *Comamonas*. Genera *Nitrosomonas* and *Nitrosospira* are two kinds of typical ammonia oxidizers. Only sequences of *Nitrosospira* were detected in the biofilm of natural zeolite, whereas only sequence of *Nitrosomonas* was detected in the biofilm of volcanic rock. These results suggest that the compositions of both ammonia-oxidizing and denitrifying bacteria are quite different. Other than phylum *Proteobacteria*, phylum *Firmicutes* and class *Sphingobacteria* were also detected with high frequency. They were less observed in the bacterial community of constructed wetlands [20], since PCR-DGGE which is often employed in recent studies of constructed wetlands cannot embrace most of bacterial composition. Phylum *Firmicutes* is an anaerobic micro-organism. It has been observed as the second dominant group in a photobioreactor treating piggery wastewater [35]. Micro-organisms of this phylum can degrade volatile fatty acids, which make up 80% of total organic carbon in the soluble fraction of swine wastewaters [35]. This phylum was discovered in our constructed wetlands mainly because the influent water was taken from anaerobic lagoon. Additionally, it is noteworthy that phylum *Planctomycete* was found in both biofilms of natural zeolite and volcanic rock. Phylum *Planctomycete* has been reported as an important micro-organism involved in anaerobic ammonium oxidation [36].

The information of 16S rRNA genes clone library cannot give enough evidence to show the difference between AOB communities of natural zeoltie and volcanic rock. Bacterial *amoA* gene libraries were therefore constructed to clarify this problem. According to phylogentic analysis, the neighbor-joining tree was classified into three clusters; all the sequences belonged to either *Nitrosospira*-like or *Nitrosomonas*-like (Fig. 4). Of them, 95.5% *amoA* sequences in biofilm of natural zeolite were grouped into *Nitrosospira*-like, whereas 76.1% *amoA* sequences in biofilm of volcanic rock fell into *Nitrosomonas*-like. This result confirmed our inference that the compositions of AOB between two substrates were different. Gorra et al. [10] used PCR-DGGE to investigate the ammonia-oxidizing communities in different substrates, including gravel, ceramic waste, magnetite, zeolite, and soil mixed marble, and observed no large differences between communities of different substrates and only sequences related to genus *Nitrosospira* were detected. Similarly, *Nitrosospira*-like sequences also dominated in AOB community of natural zeolite in our study. However, all the *amoA* sequences from two sand samples of constructed wetlands were affiliated with *Nitrosomonas*-like linkage [16,37]. Likewise, *Nitrosomonas*-like sequences were the major composition of AOB in the biofilm of volcanic rock in this study. These previous and present studies suggest that substrate type must have posed an impact on ammonia-oxidizing community. The physiochemical characteristics, such as pH, ammonia-binding capacity, and chemical compositions, in these substrates would probably contribute to different communities.

5. Conclusion

Natural zeolite-based VFCW was effective and performed better than volcanic rock-based VFCW in removing nutrients from piggery waster. The recirculation enhances the removals of NH_4-N, TN, and TP, except COD. Therefore, natural zeolite-based VFCW with effluent recirculation is a preferable alternative for the treatment of piggery wastewater. Both total bacteria and AOB in biofilm of natural zeolite were more diverse than those in biofilm of volcanic rock. Phylogenetic analyses further revealed two points: first, genera *Alcaligenes* and *Comamonas* dominated in the β-*proteobacteria* of natural zeolite and volcanic rock, respectively, and second, genera *Nitrosospira* and *Nitrosomonas* dominated in the AOB of natural zeolite and volcanic rock, respectively. The diversity and composition of microbial community in the biofilm of substrate would potentially influence the nutrient removal.

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References

[1] L.S. He, H.L. Liu, B.D. Xi, Y.B. Zhu, Enhancing treatment efficiency of swine wastewater by effluent recirculation in vertical-flow constructed wetland, J. Environ. Sci. (China) 18(2) (2006) 221–226.

[2] X. Dong, G.B. Reddy, Soil bacterial communities in constructed wetlands treated with swine wastewater using PCR-DGGE technique, Bioresour. Technol. 101(4) (2010) 1175–1182.

[3] W.L. Jia, J. Zhang, J. Wu, H.J. Xie, B. Zhang, Effect of intermittent operation on contaminant removal and plant growth in vertical flow constructed wetlands: A microcosm experiment, Desalination 262 (2010) 202–208.

[4] H.M. Wu, W.J. Li, J. Zhang, C. Li, J.Y. Zhang, H.J. Xie, Application of using surface constructed wetland for removal of chemical oxygen demand and ammonium in polluted river water, Desalin. Water Treat. 44 (2012) 142–150.

[5] A. Yalcuk, N.B. Pakdil, S.Y. Turan, Performance evaluation on the treatment of olive mill waste water in vertical subsurface flow constructed wetlands, Desalination 262(1–3) (2010) 209–214.

[6] S.Ç. Ayaz, Ö. Aktaş, N. Fındık, L. Akça, C. Kınacı, Effect of recirculation on nitrogen removal in a hybrid constructed wetland system, Ecol. Eng. 40 (2012) 1–5.

[7] E.A. Korkusuz, M. Beklioglu, G.N. Demirer, Comparison of the treatment performances of blast furnace slag-based and gravel-based vertical flow wetlands operated identically for domestic wastewater treatment in Turkey, Ecol. Eng. 24(3) (2005) 187–200.

[8] W. Jia, J. Zhang, P. Li, H. Xie, J. Wu, J. Wang, Nitrous oxide emissions from surface flow and subsurface flow constructed wetland microcosms: Effect of feeding strategies, Ecol. Eng. 37 (2011) 1815–1821.

[9] Y.H. Bai, Q.H. Sun, R.H. Sun, D.H. Wen, X.Y. Tang, Bioaugmentation and adsorption treatment of coking wastewater containing pyridine and quinoline using zeolite-biological aerated filters, Environ. Sci. Technol. 45(5) (2011) 1940–1948.

[10] R. Gorra, M. Coci, R. Ambrosoli, H.J. Laanbroek, Effects of substratum on the diversity and stability of ammonia-oxidizing communities in a constructed wetland used for wastewater treatment, J. Appl. Microbiol. 103(5) (2007) 1442–1452.

[11] A.I. Stefanakis, C.S. Akratos, G.D. Gikas, V.A. Tsihrintzis, Effluent quality improvement of two pilot-scale, horizontal subsurface flow constructed wetlands using natural zeolite (clinoptilolite), Micropor. Mesopor. Mater. 124(1–3) (2009) 131–143.

[12] J.A.H. Melian, A.J.M. Rodriguez, J. Arana, O.G. Diaz, J.J.G. Henriquez, Hybrid constructed wetlands for wastewater treatment and reuse in the Canary Islands, Ecol. Eng. 36(7) (2010) 891–899.

[13] Y.P. Chen, H.B. Guerra, K.S. Min, Y. Kim, Operation of the vertical subsurface flow and partly submersed stormwater wetland with an intermittent recycle, Desalin. Water Treat. 38 (2012) 378–388.

[14] K. Sleytr, A. Tietz, G. Langergraber, R. Haberl, A. Sessitsch, Diversity of abundant bacteria in subsurface vertical flow constructed wetlands, Ecol. Eng. 35(6) (2009) 1021–1025.

[15] A.M. Ibekwe, C.M. Grieve, S.R. Lyon, Characterization of microbial communities and composition in constructed dairy wetland wastewater effluent, Appl. Environ. Microbiol. 69(9) (2003) 5060–5069.

[16] S.S. Domingos, S. Dallas, L. Skillman, S. Felstead, G. Ho, Nitrogen removal and ammonia-oxidising bacteria in a vertical flow constructed wetland treating inorganic wastewater, Water Sci. Technol. 64(3) (2011) 587–594.

[17] X. Dong, G.B. Reddy, Ammonia-oxidizing bacterial community and nitrification rates in constructed wetlands treating swine wastewater, Ecol. Eng. 40 (2012) 189–197.

[18] N. Park, J. Lee, K. Chon, H. Kang, J. Cho, Investigating microbial activities of constructed wetlands with respect to nitrate and sulfate reduction, Desalin. Water Treat. 1 (2009) 172–179.

[19] C. Kyongmi, Y. Kim, N.I. Chang, J. Cho, Evaluating wastewater stabilizing constructed wetland, through diversity and abundance of the nitrite reductase gene nirS, with regard to nitrogen control, Desalination 264 (2010) 201–205.

[20] C.S.C. Calheiros, A.F. Duque, A. Moura, I.S. Henriques, A. Correia, A.O.S.S. Rangel, P.M.L Castro, Changes in the bacterial community structure in two-stage constructed wetlands with different plants for industrial wastewater treatment, Bioresour. Technol. 100(13) (2009) 3228–3235.

[21] C.S.C. Calheiros, A.F. Duque, A. Moura, I.S. Henriques, A. Correia, A.O.S.S. Rangel, P.M.L. Castro, Substrate effect on bacterial communities from constructed wetlands planted with *Typha latifolia* treating industrial wastewater, Ecol. Eng. 35(5) (2009) 744–753.

[22] L. Iasur-Kruh, Y. Hadar, D. Milstein, A. Gasith, D. Minz, Microbial population and activity in wetland microcosms constructed for improving treated municipal wastewater, Microb. Ecol. 59(4) (2010) 700–709.

[23] J.A. Steele, F. Ozis, J.A. Fuhrman, J.S. Devinny, Structure of microbial communities in ethanol biofilters, Chem. Eng. J 113 (2–3) (2005) 135–143.

[24] M.Y. Sklarz, A. Gross, A. Yakirevich, M.I.M. Soares, A recirculating vertical flow constructed wetland for the treatment of domestic wastewater, Desalination 246(1–3) (2009) 617–624.

[25] American Public Health Association (APHA), Standard methods for the examination of water and waste water, American Public Health Association, Washington, DC, 1998.

[26] B. Wei, X. Yu, S. Zhang, L. Gu, Comparison of the community structures of ammonia-oxidizing bacteria and archaea in rhizoplanes of floating aquatic macrophytes, Microbiol. Res. 166(6) (2011) 468–474.

[27] G.A. Kowalchuk, F.J. de Bruijn, I.M. Head, A.D. Akkermans, J.D. van Elsas, Molecular Microbial Ecology Manual, second ed., Kluwer Academic, Netherlands, 2004, pp. 1321–1344.

[28] A.I. Stefanakis, V.A. Tsihrintzis, Performance of pilot-scale vertical flow constructed wetlands treating simulated municipal wastewater: Effect of various design parameters, Desalination 248(1–3) (2009) 753–770.

[29] T. Saeed, G.Z. Sun, Enhanced denitrification and organics removal in hybrid wetland columns: Comparative experiments, Bioresour. Technol. 102(2) (2011) 967–974.

[30] G. Sun, K.R. Gray, A.J. Biddlestone, S.J. Allen, D.J. Cooper, Effect of effluent recirculation on the performance of a reed bed system treating agricultural wastewater, Process Biochem. 39(3) (2003) 351–357.

[31] J. Vymazal, Removal of nutrients in various types of constructed wetlands, Sci. Total Environ. 380(1–3) (2007) 48–65.

[32] Y. Avsar, H. Tarabeah, S. Kimchie, I. Ozturk, Rehabilitation by constructed wetlands of available wastewater treatment plant in Sakhnin, Ecol. Eng. 29(1) (2007) 27–32.

[33] A.K. Rowan, J.R. Snape, D. Fearnside, M.R. Barer, T.P. Curtis, I.M. Head, Composition and diversity of ammonia-oxidising bacterial communities in wastewater treatment reactors of different designtreating identical wastewater, Fems Microbiol. Ecol. 43(2) (2003) 195–206.

[34] T. Osaka, S. Yoshie, S. Tsuneda, A. Hirata, N. Iwami, Y. Inamori, Identification of acetate- or methanol-assimilating bacteria under nitrate-reducing conditions by stable-isotope probing, Microb. Ecol. 52(2) (2006) 253–266.

[35] E.M. Ferrero, I. de Godos, E.M. Rodriguez, P.A. Garcia-Encina, R. Munoz, E. Becares, Molecular characterization of bacterial communities in algal-bacterial photo bioreactors treating piggery wastewaters, Ecol. Eng. 40 (2012) 121–130.

[36] J.A. Fuerst, E. Sagulenko, Beyond the bacterium: Planctomycetes challenge our concepts of microbial structure and function, Nat. Rev. Microbiol. 9(6) (2011) 403–413.

[37] Q. Zhou, F. He, L. Zhang, Y. Wang, Z. Wu, Characteristics of the microbial communities in the integrated vertical-flow constructed wetlands, J. Environ. Sci. (China) 21(9) (2009) 1261–1267.