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Distribution patterns of denitrification functional genes and microbial floras in multimedia constructed wetlands

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ABSTRACT

The present study, a quantitative investigation of distribution patterns of functional gene communities, including Anammox bacteria 16S rRNA, *amoA*, *nxrA*, *narG*, *napA*, *nirK*, *qnorB*, *nosZ*, *nas*, and *nifH* in two multimedia constructed wetland systems (CWs) was conducted. DGGE results showed similar distribution patterns in two constructed wetland groups. *Lactococcus* sp. and *Moraxella* sp. were the dominant organic nitrogen and denitrification flora; *Acinetobacter* sp. were the dominant NO_3^- to NH_4^+ transformation flora; and *Bacillus* sp. and *β-Proteobacteria* sp. were the dominant NH_4^+ removal flora in the two constructed wetland groups. Quantitative real-time PCR results showed the *qnorB* and *nas* functional genes were predominantly enriched in the 15 cm and 60 cm layers of the CW, which was prepared by multi-media packing of 1% zero-valent iron. The *nirK* was predominantly enriched in the 60 cm layer of the CW, which was prepared by multi-media packing of 2% zero-valent iron. Other functional nitrogen transformation genes were predominantly enriched in the 15 cm layer of CWs.

1. Introduction

Constructed wetlands were developed as a sewage treatment method in Germany during the 1970s (US EPA, 1993). The technique was composed of a hierarchical system of fillers, aquatic plants growing on the fillers, and microorganisms deposited on the fillers and aquatic plants. Consequently, a unique man made matrix-plant – microbe ecosystem was established to effectively treat and dispose of wastewater (US EPA, 1993). Wastewater purification mechanisms by constructed wetlands are complex, including matrix precipitation adsorption, ion exchange, plant uptake and microbial degradation, among other factors (Hsu et al., 2011; Vymazal, 2011). Studies have indicated the practice of constructed wetlands had effective removal performance for biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and oils (Ji et al., 2002; Konnerup and Brix, 2010). However, nitrogen removal efficiency showed substantial fluctuations when the wetland was influenced by variable nitrogen and hydraulic loadings, and constructed wetland type (Lin et al., 2002). The North America database documented an average nitrogen removal rate of 44% in wastewater-constructed wetlands (IWA, 2000). Verhoeven and Meuleman (1999) reported the nitrogen removal rate of a classic constructed wetland in Europe was only 35%, and could not exceed 50%, even with optimal design. How to improve nitrogen removal efficiency in constructed wetlands has become a research hotspot in the water science field.

Research has demonstrated that nitrogen removal in constructed wetlands resulted from the combined action of physical, chemical, and biological processes. Denitrification pathways included matrix deposition adsorption, ion exchange, ammonia volatilization, plant uptake, animal nourishment, and microbial nitrogen removal, among other components. Konnerup and Brix (2010) reported the key factors to achieve high-efficient denitrification which included matrix deposition adsorption and microbial transformation. A constructed wetland surface substrate exhibited some degree of adsorption activity, and could absorb dissolved nitrogen in wastewater, particularly reduced form steady-state ammonia, which served a role in blocking and filtering (Davidsson and Stahl, 2000). However, the ion exchange of the substrate active site towards ammonia could not act long-term as the confluence for ammonia removal, because the ammonia adsorbed by the substrate will continue to be exploited (Blankenberg et al., 2008). Different substrates had different adsorption capacities; zeolite, clay, and peat soil (field peaty soil) possessed an increased cation-exchange capacity, which could improve nitrification effects, however gravel cation exchange capacity was poor (Liu and Zhou, 2009; Jenssen et al., 2010; Li et al., 2011). The limitations of natural substrate adsorption capacity resulted in the development of bio-ceramic,

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and other low-cost multi-media packing using waste from industry and agriculture as raw materials. These materials have been verified with suitable nitrogen and phosphorus adsorption properties.

Matrix deposition adsorption was considered a key denitrification factor in constructed wetlands, however a substantial number of studies have reported microbes were responsible for the removal of most nitrogen (89–96%) (Lin et al., 2002; Konnerup and Brix, 2010). Microbial nitrogen removal included, ammonia oxidation, nitrification, denitrification, and anaerobic ammonium oxidation, among other pathways (McDevitt et al., 2000). Nitrification included the following two steps: (i) aerobic ammonium oxidation, where ammonia was oxidized to nitrite; and (ii) oxidation of nitrite, where nitrite was further oxidized to nitrate (Julie et al., 1991). In addition to aerobic ammonia oxidation, anaerobic ammonium oxidation occurred in the natural environment, and wastewater treatment systems. Anaerobic ammonium oxidation oxidized NH_4^+, NO_3^-, or (NO_2^-) to N_2 with NH_4^+ as the electron donor, and NO_3^- or NO_{2-} as the electron acceptor (Mulder et al., 1995). In addition to the presence of aerobic denitrifiers heterotrophic nitrification, microbial nitrogen fixation, and nitrate respiration in aerobic conditions have been widely confirmed (McDevitt et al., 2000).

Denaturing gradient gel electrophoresis (DGGE) has been successfully applied to study ammonia oxidation and denitrifying bacterial diversity, and results have indicated that DGGE was a viable tool to study microbial diversity in environmental samples (Throback et al., 2004). Real-time PCR accurately quantified nitrification and dentrification genes, including *amoA*, *nirK*, and *nosZ* (Henry et al., 2006). However, to date most quantitative studies have only focused on individual functional genes, and the joint-use of PCR-DGGE and real-time PCR has not been reported in denitrification microbes from constructed wetlands, and the distribution patterns of nitrogen transformation functional genes has not been evaluated.

In this study, microbial diversity in two multimedia constructed wetland systems with different nitrogen removal efficiencies were researched by using the qualitative PCR-DGGE and real-time PCR method. Secondly, the distribution patterns of *amoA*, *nxrA*, *narG*, *napA*, *nirK*, *qnorB*, *nosZ*, *nas*, *nifH* were inferred, and the anaerobic ammonium-oxidizing bacteria were identified in the two-constructed wetland ecosystems.

2. Materials and methods

2.1. Multimedia constructed wetland

Two multi-media wetlands systems (each with four parral, total 8 CWs) were established with 5.00 m length × 1.00 m width × 1.70 m depth. The following composition comprised the surface to bottom layer: the rhizosphere layer (0–30 cm), filled with native sandy loam; water distribution layer (30–50 cm), filled with 10 mm particle sized ash; multimedia filter layer (50–130 cm), comprised of permeable filler, and four multi-block layers filled with nested brick, each multimedia block measuring100 cm length × 30 cm width × 13 cm depth. The horizontal and vertical multimedia block spacing was respectively 10 cm and 7 cm apart; and spaces between blocks were occluded with permeable filler. Permeable filler was composed of 10 mm diameter gravel, and 2–4 mm diameter natural clinoptilolite, with a 7:1 volume ratio; multimedia block is mixed by bio-ceramic, 2–4 mm diameter natural clinoptilolite, and 1–2 mm diameter natural clinoptilolite, with a 1:1:1 volume ratio; and the water collection layer level (130–170 cm), was filled with 32 mm ash particles, and 10 mm diameter gravel, with a 2:1 volume ratio. Yellow iris (*Iris pseudacorus* (Iridaceae)) was planted at the constructed wetland surface layer, with a planting density of 25 clusters m^{-2} (the space between clusters was 0.15 cm).

A different treatment between the two-wetland groups was established. Bio-ceramics filled the multimedia block, and the bioceramic material had similar composition with the following main ingredients: fly ash, sawdust, natural clinoptilolite and calcium carbonate. However, bio-ceramic in constructed wetland No. 1 included the addition of 1% metallic iron, and bio-ceramic in constructed wetland No. 2 included the addition of 2% metallic iron. An 8 mm diameter bio-ceramic was used in two constructed wetland systems, and the production process and performance of the product was reported in Ji et al. (2010a). The two constructed wetland systems were established in the water-saving irrigation demonstration base of the Chinese Ministry of Water Resources, Shunyi District, Beijing, China, and used to treat the daily domestic sewage of the base buildings. During the tests, the constructed wetland systems were not under temperature control, and the effluent temperature ranged from 18 to 25 °C.

The commissioning and operation of the two-wetland systems was initiated on May 11, 2009. NH_4^+, NO_3^-, NO_2^-, total nitrogen (TN), and COD transformation efficiencies were analyzed over a 16 week period. The operation was divided into four different phases. Baseline data was measured to determine nitrogen removal efficiencies, including four operation phases. The first was the domestication phase from May 11 to May 31, with hydraulic loading of 10.0 cm d^{-1}. The second was the commissioning phase with 20.0 cm d^{-1} of hydraulic loading from July 1 to July 18. The third was the hydraulic shock loading phase from July 19 to August 31 with hydraulic loading of 40.0 cm d^{-1}. TN shock loading was the fourth phase from September 1 to September 28. During this period, TN loading increased 3.5 times, and hydraulic loading was maintained.

2.2. Sample collection and determination

Throughout the operation, water samples were collected once a week, and measured on-site for NH_4^+, NO_2^-, NO_3^-, TN, COD, pH, dissolved oxygen (DO), and redox potential. A HACH DR2800 multi-function water quality tester was used to measure these parameters applying standard protocols (State Environmental Protection Administration, 2002). The microbial flora in two construced wetland systems was sampled on September 28, 2009. Seven samples from the rhizosphere, water distribution, and water collection layers, and each layer of the four layer multimedia blocks in the multimedia filter layer were collected in the two constructed wetlands. The samples were labeled 15 cm layer, 40 cm layer, 60 cm layer, 80 cm layer, 100 cm layer, 120 cm layer, and 140 cm layer. The sampling column extracted samples from each corresponding layer. The samples were subsequently stored in an ice incubator, and sent to the Laboratory of Environmental Engineering, Peking University. D5625-01 Soil DNA Kits (Omega, USA) were used to extract and purify total genomic DNA from the samples. Extracted genomic DNAs were maintained in a −20 °C freezer until further use, and detected by 1% agarose gel electrophoresis.

2.3. PCR-DGGE (denaturing gradient gel electrophoresis)

2.3.1. PCR (polymerase chain reaction)

The general PCR primers for the 16S rDNA V3 variable region of most bacteria are 338F (5′-TACGGGAGGCAGCAG-3′) and 518R (5′-CCATACGGGAGGCAGGCAG-3′); and the addition of the GC clip CGCCCGCCGCGCGCGGCGGGCGGGGCGGGGGCACGGGGGGCC to the 5′ end of the reverse primer to prepare for denaturing