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Fungal Denitrification Activity in Vertical Flow Constructed Wetlands as Impacted by Plant Species Richness, Carbon, Nitrogen and pH Amendments

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Abstract To control potential fungal denitrification rate (PFDR) in vertical flow simulated wetlands (VFSW) microcosms, thirty VFSW microcosms were established and planted with three plant species richness levels (i.e. unplanted, monoculture, and four-species polyculture treatment), and effects of carbon, nitrogen and pH amendments on the PFDR were investigated using a room-incubating method. Among seven carbon compounds, sodium citrate, glycerol, glucose and sodium succinate were more effective in enhancing PFDRs. These enhanced effects were dependant on a given species richness level. Sodium nitrite mostly stimulated PFDRs to a greater extent than the other three nitrogen compound amendments at any richness level. Treatments with pH 5.6 or 8.4 had significantly greater PFDRs than the treatment with pH 2.8 in the three species richness levels. However, no effect of plant species richness on the PFDR was observed among any carbon, nitrogen and pH amendments. Current results suggest carbon, nitrogen and pH factors should be considered when mediating fungal denitrification in VFSW microcosms.

Keywords Potential fungal denitrification rate (PFDR) · Constructed wetlands · Carbon amendment · Nitrogen amendment · pH amendment · Species richness levels

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Nitrogen (N) pollution has become an increasing environmental problem because of leaching and transport from agricultural, industrial, and domestic sources (Vymazal 2011). Constructed wetlands (CWs) are engineered systems designed to remove pollutants from contaminated waters. They offer a low-cost, low-energy, and low-operational-requirements alternative to conventional treatment system (Faulwetter et al. 2009). Denitrification is one of the most dominant N-removal mechanisms in CWs and is estimated to account for as much as 90% of total N-removal from wastewater (Seo and DeLaune 2010).

The denitrification process driven by bacteria has been studied in CW systems, and has been considered the dominant N-removal pathway (Song et al. 2014). Few studies have considered fungal denitrification as a potential pathway for N-removal in CW systems (Liu et al. 2015). Seo and DeLaune (2010) reported denitrification rates in wetland sediment by fungi and bacteria were 34.3%–35.1% and 1.46%–1.59% of total denitrification, respectively, indicating fungi might be responsible for most of the denitrification under aerobic or weakly reducing conditions.

Some researchers have investigated factors controlling fungal denitrification in soils. McLain and Martens (2006) indicated oligopeptides and NO_2^- mostly improved N_2O production in semiarid soils compared with other N compounds. Herold et al. (2012) found that fungal denitrification potential increased when arable soil pH increased from 4.5 to 7.5. Wei et al. (2014) reported large N_2O emissions occurred after organic fertilizer application onto cropland soil. However, in engineered environments with high pollutant loading, it is uncertain how some factors such as carbon (C), N and pH impact fungal denitrification activity.

It has been suggested that plant roots can not only take up pollutants from wastewater, they can excrete organic matter and oxygen into the micro-environment around rhizospheres,

further influencing microbial activity and function (Zhang et al. 2011). In recent years, polyculture planting (species richness) has been gradually applied to CW design (Zhang et al. 2011; Chang et al. 2014). How plant species richness affects the fungal denitrification in CW systems has been unclear.

Previous studies showed that pH, C, N and plant species richness all are important factors affecting denitrification activity in CW systems (Faulwetter et al. 2009). At the same time, plant roots not only influence substrate pH by excreting some organic acids, they influence substrate C and N contents by excreting some organic matter such as sugar and amino acids or by plant uptake (Zhang et al. 2011). It is possible that plant species richness interacts with C, N and pH in the substrate of CW systems; however these interactions have remained unknown.

The potential fungal denitrification rate (PFDR) in 30 vertical flow simulated wetland (VFSW) microcosms were investigated using three plant species richness levels (unplanted, monoculture and four-species mixture), as well as C, N and pH amendments as fixed factors. Experiments were designed to answer how (1) plant species richness affects PFDR; (2) C, N and pH amendments directly impact fungal denitrification activity in the VFSW microcosms; and (3) interactions between amendments and plant species richness impact fungal denitrification activity.

Materials and Methods

Thirty VFSW microcosms were established at Taizhou University (121°21′E, 28°34′N) in Zhejiang Province. The VFSW microcosm was made from polyethylene plastic [1.2 m (height) × 0.45 m (length) × 0.45 m (wideness)]. The empty-bed volume of each microcosm was approximately 0.24 m^3 and was filled with fine river sand in the top 50 cm (diameter: 1–2 mm), coarse sand in the moderate 30 cm (diameter: 4–6 mm), and gravel in the bottom 30 cm (diameter: 50–85 mm). Wastewater used in the study was a modified Hoagland nutrient solution (Liu et al. 2015).

Planting and operation patterns of the VFSW microcosms were consistent with those described in Liu et al. (2015). Briefly, four macrophytes used commonly in CW systems (*Iris pseudacorus, Canna glauca, Scirpus validus* and *Cyperus alternifolius*) were transplanted into VFSW units following three plant species richness levels: unplanted (UNP); monocultured individually with four different species (MONO); and four-species mixture (MIX). Each planted treatment was repeated in five VFSW units (n = 5, each of the 5 units was considered a replicate) where each unit received four plant seedlings with an equal number of plants assigned to each species (Zhang et al. 2011). The MONO planting included four treatments planted individually with four species, occupying 20 VFSW units, while the MIX treatment consisted of four species, and occupied 5 VFSW units. We considered 20 monocultured plantings as 20 replicates in the statistical analysis. VFSW systems operated with the same water loading rate (0.2 m^3 $days^{-1}$) and hydraulic retention time (10 days) during the entire experiment (Liu et al. 2015). This operation schedule was repeated from May to August 2013.

At the end of August 2013, three sand sub-samples in each VFSW were collected down to 30 cm depth using a spade, since Liu et al. (2015) showed the substrate layer across 30 cm depth was the optimal area for fungal denitrification activity in the VFSM system. Sub-samples in each VFSM were mixed into a composite sample which was sieved (2 mm) and immediately collected in separate Ziploc™ bags. All composite samples were refrigerated at 4°C until analysis of PFDR.

Cycloheximide, which can inhibit protein biosynthesis of fungi but not bacteria, was amended to sand samples to investigate fungal denitrification activity (Herold et al. 2012). The current study utilized a slight modification, i.e. the optimum dose of cycloheximide was 2.8 mg g^{-1} dry weight (dw) according to our pre-experiment (data not shown), rather than 2.0 mg g^{-1} dw as suggested by Herold et al. (2012). The PFDR was calculated by subtracting N_2O production of the substrate with cycloheximide from the N_2O production of the substrate without the inhibitor.

Twenty grams of fresh composited samples were used in all treatments. C amendment incubations were used to measure the potential N_2O production of samples with addition of different C substrates including glucose (GL), sodium succinate (SS), sodium citrate (SC), methanol (ME), ethanol (ET), glycerol (GLY) and sodium acetate (SA), in which potassium nitrate was used as a "inducing substrate" for denitrification activity (10 mg N g^{-1} dw, Castaldi and Smith 1998). The application dose of different C amendments was 5 mg C g^{-1} dw. Similar to the above, different N compounds including aspartic acid (AA), ammonium chloride (AC), sodium nitrite (SNI) and sodium nitrate (SNA) were used to measure the potential N_2O production, The application dose of different N amendments was 10 mg N g^{-1} dw, and all N amendment treatments were simultaneously added with GL to induce heterotrophic activity (5 mg C g^{-1} dw, Seo and DeLaune 2010). At the same time, unamended (UNA) treatments were also included for comparison. The pH effects were proposed with pH = 2.8, 5.6 and 8.4 by addition of 1.0 mol L^{-1} HCl or NaOH solution into sand substrates, and the substrate unamended with HCl or NaOH solution (UNA) was used as the control. All stock solutions were made fresh, and individually applied into the serum bottles with and without cycloheximide in 500 µL of 100 mmol L^{-1} solutions per 10 g of fresh sand sample (Herold et al. 2012). All treatments were performed in triplicate.

All bottles were hermetically sealed with rubber stoppers prior to the addition of acetylene (10% v/v). Sample and solution were gently mixed for 10 min on a roller bed to ensure mixture of sand slurry and solubilization of acetylene. Sample slurries were incubated at 20°C for 8 h, after which, gas samples were drawn from each bottle with a syringe (Herold et al. 2012) and transferred into evacuated gas-tight bags (50 mL, Delin Gas Packaging, Dalian, Northeastern China). N_2O concentrations were determined by using a gas chromatography (Shimadzu GC-14B, Kyoto, Japan) with an ^{63}Ni electron capture detector (ECD) and a 80/100 Poropak Q column (3 m) using an argon-methane mixture (95% argon and 5% methane) as the carrier gas (flow rate 40 mL min^{-1}). Column and detector temperatures were set at 65 and 300°C, respectively. Finally, N_2O data were converted to μg g^{-1} dw of sand sample per day ($days^{-1}$) (Liu et al. 2015).

Statistical differences between the means of PFDR data were tested using two-way ANOVA in which the different amendments and plant species richness were used as fixed factors. Honestly significant differences (HSD) for a multiple mean comparisons were obtained using the Tukey test at $p = 0.05$ level. This statistical analysis was performed with the SPSS statistical software for Windows (version 11.5).

Results and Discussion

Under the same plant species richness level, all C amendments significantly increased PFDRs in VFSWs compared with the UNA [Fig. 1 ($p < 0.05$), Table 1]. Among C amendments, both SC and GLY amendments increased the PFDRs in the UNP treatment, but both GL and SS amendments increased the PFDRs in the MONO treatment. Conversely, only SC increased the PFDR in the MIX treatment (Fig. 1). Under the same C amendment condition, there was no difference in PFDRs between the three plant species richness levels ($p > 0.05$, Fig. 1; Table 1). At the same time, these PFDR patterns across three species richness levels did not show any significant difference among C amendments as shown by two-way ANOVA ($p > 0.05$, Table 1).

Table 1 Two-way ANOVA of the potential fungal denitrification rates using amendments and plant species richness level as fixed factors

Fixed factors	Type III sum of squares	df.	F	Sig.
Carbon amendment (A)	48.31	7	16.85	< 0.01
Species richness (B)	0.04	2	0.05	0.96
A × B	2.37	14	0.41	0.97
Nitrogen amendment (A)	116.81	4	33.32	< 0.01
Species richness (B)	4.55	2	2.60	0.08
A × B	10.21	8	1.46	0.18
pH amendment (A)	0.38	3	1.37	= 0.05
Species richness (B)	0.15	2	0.84	0.78
A × B	0.36	6	1.66	< 0.05

It is known that C compounds act as electronic donors and energy sources during denitrification, so some dissolvable C sources may affect denitrification rate in wetlands (Faulwetter et al. 2009). Studies on effects of C compounds have mainly focused on denitrification driven by bacteria and less so on denitrification driven by fungi. In the current study, seven C compound amendments differently enhanced PDFRs in all VFSW substrates compared with the UNA, showing the importance of dissolved C compounds in mediating fungal denitrification. Except for methanol and ethanol, five other compounds have often been found in root exudates (Haichar et al. 2014). Bais et al. (2006) reported that up to 40% of root exudates derived from photosynthetic product dominantly served as C sources of fungi in soil.

Compared with the UNA, all N amendments significantly increased PFDRs in all VFSWs substrates under the same plant species richness [Fig. 2 ($p < 0.05$), Table 1]. Among four N amendments, SNI increased the PFDR (Fig. 2) in all treatments. Plant species richness did not show a significant

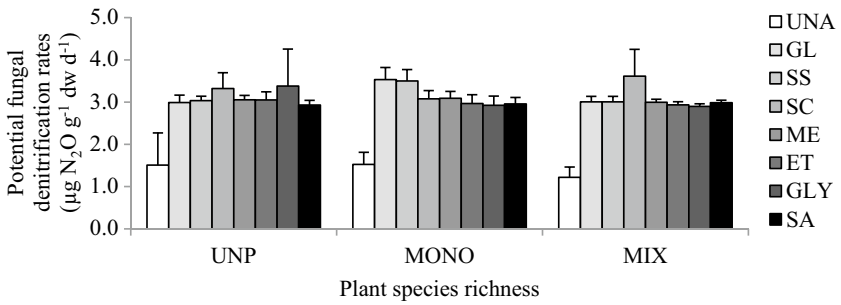

Fig. 1 Mean ± SD potential fungal denitrification rates of various plant species richness as influenced by carbon amendments. *UNA* unamended, *GL* glucose, *SS* sodium succinate, *SC* sodium citrate, *ME* methanol, *ET* ethanol, *GLY* glycerol, *SA* sodium acetate, *UNP* unplanted treatment, *MONO* monocultured treatment, *MIX* four-species mixture. Significant differences tested at $p = 0.05$

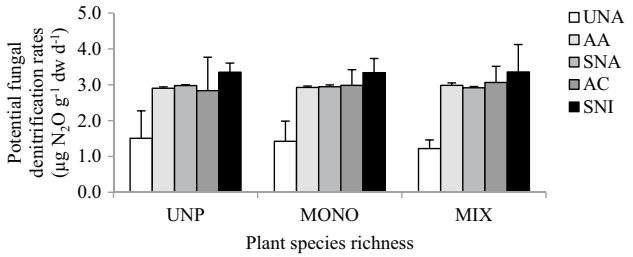

Fig. 2 Mean ± SD potential fungal denitrification rates of various plant species richness as influenced by nitrogen amendments. *AA* arspartic acid, *SNA* sodium nitrate, *AC* ammonium chloride, *SNI* sodium nitrite, include all abbreviations in each figure

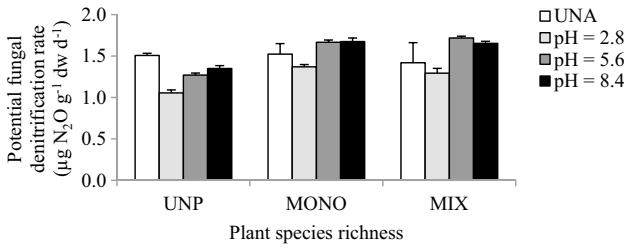

Fig. 3 Mean ± SD potential fungal denitrification rates of various plant species richness as influenced by pH amendments, include all abbreviations in each figure

effect on PFDR in all N-amended substrates ($p > 0.05$, Table 1).

McLain and Martens (2006) evaluated effects of organic N (proteins, oligopeptides, and amino acids) and inorganic N (NH_4^+, NO_3^- and NO_2^-) on fungal denitrification potential, indicating oligopeptides and NO_2^- mostly improved N_2O production potential in soils compared with other N compounds. In the present study, four N amendments enhanced the PFDRs to different extents in the substrates compared with the controls, thus confirming previous results which regard N compounds as the dominant factor for driving fungal denitrification (McLain and Martens 2006). Among four N compounds, only SNI enhanced the PFDR in VFSWs. Shoun et al. (1991) found the fungi *Fusarium oxysporum* and *Gibberella fujikuroi* were able to reduce NO_3^- and NO_2^- into N_2O in a dissimilatory manner, while most other denitrifying fungi seemed to reduce only NO_2^- into N_2O. The current finding was mostly related to the shortening fungal denitrifying pathway by SNI, since the whole fungal denitrifying pathway first passes the transformation of NO_3^- to NO_2^- (Seo and DeLaune 2010).

In the current study, UNA substrate had a pH range of 6.00–6.89. Compared with the pH-unamended treatment, pH amendments (pH = 2.8, 5.6 and 8.4) significantly reduced the PFDRs in UNP treatment ($p = 0.05$, Fig. 3). This result was mostly attributed to the inhibiting effect of higher H^+ or lower H^+ on PFDR under the condition without plants, since Čuhel et al. (2010) showed that higher copy numbers of all denitrification genes were only observed in neutral pH soil. Compared with the pH 2.8 amendment, both 5.6 and 8.4 pH amendments significantly increased the PFDRs in the UNP, MONO and MIX richness levels, indicating that some fungal species with a denitrification activity might be chosen through the significant interaction between pH amendments and species richness ($p < 0.05$, Table 1).

The three different plant species richness levels did not show a significant effect on the PFDRs under the same pH amendment ($p > 0.05$, Table 1). Whether this might be attributed to the shorter duration of the current experiment remains to be elucidated in further work. Baggs et al. (2010) and Čuhel et al. (2010) individually maintained a 24-day and 10-month pH-amendment experiment respectively in which the pH was measured at the end of the experiment. In their experiments, the soil pH was not succesfully achieved due to the high buffering capacity of soil, finally resulting in a return of the pH toward the original pH in a short-term experiment. On the other hand, the significant interaction between pH amendment and plant species richness significantly affected the PFDR ($p < 0.05$, Fig. 3; Table 1). This finding was likely related to the changed pH induced by plant roots, since previous studies demonstrated the monoculture treatment significantly reduced rhizospheric pH (Yang et al. 2012) or the polycultured treatment increased rhizospheric pH (Marschner and Römheld 1983).

Potential fungal denitrification rate was determined using substrate-induced respiration inhibition and acetylene inhibition methods and compared among the substrates amended with seven C compounds, five N compounds and three pH levels. Among seven C amendments, GL and SS enhanced the PFDRs, but it was not depended on plant species richness. Among five N amendments, SNI also enhanced the PFDR, again independent of the plant species richness. However, pH treatments affected the PFDRs differently across substrates and plant species richness levels. Plant species richness did not significantly affect the PFDRs. This is one of the first studies comparing PFDR among substrates amended with multiple C and N compounds and varying pH levels. Results obtained from this study suggest that to enhance the N removal in VFSWs, C, N, and pH factors should be considered when mediating the function of the denitrifying fungi.

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