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ORIGINAL RESEARCH

Responses of Dissimilatory Nitrate Reduction to Ammonium and Denitrification to Plant Presence, Plant Species and Species Richness in Simulated Vertical Flow Constructed Wetlands

Chong-Bang Zhang[1] · Wen-Li Liu[2] · Wen-Juan Han[3] · Ming Guan[1] · Jiang Wang[1] · Shu-Yuan Liu[2] · Ying Ge[3] · Jie Chang[3]

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Abstract This study investigated the effects of plant presence, plant species and their species richness on plant biomass production, pH, dissolved oxygen (DO), oxidation-reduction potential (ORP), denitrification (DNF), dissimilatory NO_3^- reduction to ammonium (DNRA) and two associated bacterial community compositions in thirty vertical flow microcosm wetlands fed with the Hoagland solution, where three plant species richness levels (i.e. unvegetated, monocultured and 4-species polycultured treatment, respectively) were established using four macrophytes. Plant presence increased DO and ORP values, as well as the terminal restriction fragment (TRF) richness and Shannon-Weaver index of the DNRA community and also improved both potential DNF and DNRA rates. The microcosms monocultured with *Cyperus alternifolius* exhibited the greatest DO, ORP, smallest plant biomass parameters and DNF rates among all of the monocultured microcosms, whereas the microcosms monocultured with *Canna glauca* and *Iris pseudacorus* harbored the smallest pH, DO, ORP, the greatest plant biomass parameters and DNRA rates. Compared to both unvegetated and monocultured treatments, the 4-species polycultured treatment was effective in increasing both potential DNF and DNRA rates due to the greatest plant biomass parameters as confirmed by the correlation analysis, but was ineffective in terms of changing both DNF and DNRA community compositions.

Keywords Constructed wetland · Plant species · Plant species richness · Dissimilatory nitrate reduction to ammonium · Denitrification

Introduction

Compared to the natural wetland, which consists of many natural factors, a constructed wetland (CW) is an engineered system designed to remove pollutants from contaminated waters (Truu et al. 2009). Among pollutant purification processes, nitrogen removal is one of most important goals of CW systems. Denitrification (DNF), which often requires an anaerobic condition, is estimated to account for as much as 90 % of overall nitrogen removal of wastewater in CW systems (Faulwetter et al. 2009), until now was regarded as an important nitrogen removal process in most CW systems. Meanwhile, under similar conditions with low oxygen content, there is another nitrate reduction pathway occurring simultaneously in environments such as sediment and soil, i.e., the dissimilatory nitrate reduction to ammonium (DNRA, Woods 1938).

Since Woods (1938) found that the DNRA might occur in common soil bacteria like *Clostridium welchii*, some studies claimed that the DNRA challenged the prevalent view that the denitrification essentially accounts for all NO_3^- dissimilation in anaerobic soils (Stanford et al. 1975). Generally, the DNRA, in contrast to denitrification, may conserve nitrogen in ecosystems such as forest, arable field and grassland because of the reduction of NO_3^- into NH_4^+ which is a less

✉ Chong-Bang Zhang
llhzcb@163.com

[1] School of Life Sciences, Taizhou University, Jiaojiang 318000, China

[2] School of Civil Engineering and Architecture, Taizhou University, Jiaojiang 318000, China

[3] College of Life Sciences, Zhejiang University, Hangzhou 310058, China

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mobile nitrogen compound (Rütting et al. 2011). However, in the CW systems, it is mostly expected that various forms of nitrogen in various wastewaters should be removed to a most extent from CW systems. Therefore, a great DNF rate is favored to nitrogen removal from the CW systems, whereas a great DNRA rate is not favored to the nitrogen removal. Both DNRA and DNF are anaerobic processes, and use dissolved organic carbon compounds as electronic donors, NO_3^- (/NO_2^-) as acceptors, and two processes generating different end products (N-gas versus NH_4^+), so that the relative dominance of the DNRA or DNF directly affects the fate of NO_3^- (/NO_2^-) in a given CW ecosystem (Morrissey et al. 2013). However, until now, most investigations still consider denitrification as the only dissimilatory NO_3^- reduction process in CW systems, while the importance of the DNRA in mediating nitrogen removal of the CW systems has remained unclear.

Plants are an important component in all wetland ecosystems, since plants not only uptake inorganic pollutants, but may also provide oxygen for rhizosphere via aerenchyma, and release dissolved organic carbons, such as sugar and organic acids into substrate matrix to stimulate the microbial activities of rhizospheres (Stottmeister et al. 2003). Also, plant species are different in their anatomical and physiological properties such as root morphology, aerenchyma, photosynthetic rate, exudates and radial oxygen loss (Zhang et al. 2011). On the other hand, some studies also reported that a high plant species richness (i.e. plant species number) not only significantly influenced microbial community structure and activities, but also significantly increased phosphate and inorganic nitrogen removal efficiencies in CW systems (Engelhardt and Ritchie 2001; Zhang et al. 2010, 2011). Therefore, the absence or presence of plants in a given CW system, and the difference in plant species and plant species richness may influence the dominance of the DNRA and DNF communities, since early work has shown that the DNRA is vastly different from the DNF in utilizing organic matter, nitrate and O_2 (Burgin and Hamilton 2007; Zhang et al. 2015).

In terrestrial ecosystems, it is well established that denitrification in ecosystems is generally stimulated by the presence of roots due to the availability of exudates and oxygen sensitivity (Rütting et al. 2011). Meanwhile, a few earlier studies have investigated the direct effect of wetland/freshwater plants on the DNRA in sediments, though these findings are not conclusive. Unlike terrestrial ecosystems, CW systems are often characterized by high pollutant loadings, a special type of filled substrate and continuous watering (Faulwetter et al. 2009). However, in the engineered CW systems, it is unclear how the DNRA and DNF communities respond to the absence or presence of plants, plant species and their different richness patterns.

To clarify the abovementioned questions, thirty simulated vertical flow microcosm wetlands were established on the campus of Taizhou University, located in the Zhejiang Province of eastern China. Four macrophyte species were vegetated in 30 microcosm wetlands following three plant species richness levels: unvegetated, monocultured and 4-species polycultured treatments. The experiment was designed with three goals: (1) to compare plant biomass parameters, pH, DO and ORP values between unvegetated and vegetated microcosms, among four monocultured microcosms and across microcosms with three plant species richness levels; (2) to identify the structures and potential rates of the DNF or DNRA communities following the treatments mentioned above, and (3) to understand the relations of the DNF or DNRA community variants to the plant biomass parameters, pH, DO and ORP values in the CW systems by using correlation analysis.

Materials and Methods

Experimental System Design

In October 2012, thirty vertical flow microcosm wetlands (VFMWs) were established on the campus of Taizhou University (121° 21′ E, 28° 34′ N) in the Zhejiang Province of eastern China. In short, 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), with coarse sand in the moderate 30 cm (diameter: 4–6 mm), and with gravel in the bottom 30 cm (diameter: 50–85 mm) (Liu et al. 2015). In March 2015, the healthy rhizomes of four macrophytes [*Iris pseudacorus* (IP), *Canna glauca* (CG), *Scirpus validus* (SV) and *Cyperus alternifolius* (CA)] were collected from Tianjing plant garden close to Hangzhou City of the Zhejiang Province, in eastern China, and vegetated in a greenhouse. At the end of May, four plant seedlings were transplanted into each VFMW following three plant species richness levels: unvegetated (UNP), monocultured (MONO) and 4-species polycultured treatments (MIX). The UNP or MIX treatment individually occupied five microcosms, while the MONO treatment occupied twenty microcosms in which each macrophyte species shared five microcosm units. All VFMW units were fed with the Hoagland solution (Hoagland and Arnon 1950), and the nutrient loading parameter of the solution was identified as: COD = 132.51 mg L^{-1}, BOD_5 = 79.51 mg L^{-1}, total nitrogen = 79.73 mg L^{-1}, total phosphorus = 34.52 mg L^{-1}, NH_4^+–N = 38.05 mg L^{-1} and NO_3^- –N = 39.63 mg L^{-1}, respectively. All microcosms were operated with a water loading rate (0.2 m^3 d^{-1}), hydraulic retention time of 10 days and empted time of 0.5 days during the entire experiment (Liu et al. 2015). This operation schedule above was repeated from the end of May 2015 to the beginning of August 2015.

pH, Dissolved Oxygen (DO) and Oxidization-Reduction Potential (ORP) Determination in Simulated Wastewater

At the end of August 2015, three plastic pipes (diameter = 4.5 cm and length = 30 cm) were individually inserted into three sites of each microcosm to a depth of 30 cm to determine the pH and ORP of the water by using a pH/ORP waterproof portable meter (Hi98191, HANNA, Romania). To allow water to flow into pipes, holes with 0.5 mm in diameter were drilled in the wall around each pipe. A waterproof portable DO instrument (550A, YSI, USA) was used to detect DO content in the same wastewater pipes. The specific measurements were conducted following manufacturer's instruction.

Sample Collection

After measuring the pH, ORP and DO, the treated water in each microcosm was drained out. Then above- and below-ground plant tissues were collected and cleaned. Finally, two parts of plant tissues were dried to constant weight at 65 °C in an oven, and the dried plant tissues in each microcosm were converted into dried weight m^{-2}.

After collecting the plant tissues, five sub-substrate samples in each microcosm unit were collected down to a 30-cm depth using a sampling spade, since Salomo et al. (2009) showed that the substrate layer across 30 cm depth was the optimal area for the distribution of denitrifying genes in the vertical flow CWs. Five sub-samples from each VFMW unit were mixed into a composite sample, which were sieved (2 mm) and immediately collected in separate Ziploc™ bags. In the experimental room, each composited sample was divided into two parts, with one portion of samples temporarily stored in a 4 °C refrigerator to analyze potential DNF and DNRA rates; and the other portion of samples stored in a −20 °C refrigerator to analyze bacterial community structure parameters associated with DNRA and DNF rates.

Potential DNF and DNRA Rates

Both potential DNF and DNRA rates were determined using the ^{15}N tracing technique. Briefly, 10 g of fresh sand substrate was placed into a 120 mL serum bottle, which was then stopped with silicone rubber stoppers, and flushed with pure N_2 (oxygen-free) by evacuating and refilling it three times. Each sample was in four replicates of bottles. To prevent the N_2O produced by the DNF process from being transformed into N_2, acetylene (10 % v/v) was added to half of all bottles for each sample and equilibrated between the gaseous and aqueous phase for 10 min by shaking the tubes on a rotary shaker (200 rpm). At the same time, the other half of all bottles without the addition of acetylene were used as controls. Next, 1 mL of 1 mM ^{15}N-labeled potassium nitrate solution (99.7 atom %) was added to the bottles. After 6 to 12 h, the incubation (28 °C in the dark) was stopped (Kaspar 1983; Yin et al. 2002). The $^{15}N_2O$ and the abundance of ^{15}N (NH_4^+ and NO_3^-) were determined using the methods suggested by Dong et al. (2009) and Yin et al. (2002). The DNRA and DNF rates were estimated according to the calculation methods in the supplemental material provided by Lu et al. (2013).

Terminal Restriction Fragment Length Polymorphism (T-RFLP) Profiles of DNF and DNRA Communities

The total DNA of each sand sample was obtained by applying the Fast DNA® Spin Kit (MP Biomedicals, USA). Genomic DNA samples were viewed by agarose gel electrophoresis, and DNA quality and quantity were verified by a microvolume spectrophotometer (NanoDrop™-2000, Thermo Fisher, USA). The DNF DNA fragments from the *nirS* gene (encoding a cytochrome cd1 enzyme) were amplified from the total DNA samples using the primer pair cd3aF (5′-GT(C/G)AAC GT(C/G)AAGGA(A/G)AC(C/G)GG-3′) and R3cd (5′-GA(C/G)TTCGG(A/G)TG(C/G)GTCTTGA-3′, Throbäck et al. 2004), yielding approximately 500 bp fragments of the 16S rDNA. The DNRA DNA fragments from the *nrfA* gene (encoding a periplasmic nitrite reductase catalyzing the conversion of nitrite to ammonia) were amplified from total DNA samples using the primer pair *nrfA*-2F (5′-CAC GAC AGC AAG ACT GCC G- 3′) and *nrfA*-2R (5′-CCG GCA CTT TCG AGC CC-3′, Smith et al. 2007), yielding approximately 520 bp fragments of the 16S rDNA. The 5′ end of the forward primers cd3aF or *nrfA*-2F was labeled with FAM dye (6-carboxyfluorescein- N-hydroxysuccinimide ester-dimethyl sulphoxide). PCR reactions and amplifying parameters were described in detail in Throbäck et al. (2004) and Smith et al. (2007), and the PCR products were purified using the QIAquick PCR purification columns (Qiagen Inc., USA).

The TRF richness for the DNF or DNRA community was calculated as the total number of TRFs with a distinct size in a given T-RFLP profile, while the average TRF abundance was computed as the average value of all relative peak area percentages of all TRFs (each relative peak area = the ratio of a specific TRF peak area to the sum of all TRF peak areas in a given T-RFLP profile). The Shannon–Weaver diversity index (H') was computed by using $H_{TRF} = -\sum pi\ (\ln pi)$, where pi is the ratio of a specific TRF peak area to the sum of all distinct TRF peak areas in a given T-RFLP profile, and $\ln pi$ is the natural logarithm for each pi.

Data Analysis

The difference significance of all measurements between treatments was tested using the one-way analysis of variance (ANOVA), and was followed by a least significant difference test (i.e., LSD test at the 0.05 level), if a treatment effect was significant. At the same time, the correlation analysis was used

to test the relationships between microbial parameters and plant biomass productions, pH, DO or ORP by using the Pearson coefficient.

Results

Plant Biomass Production

The above- and below-ground plant biomass parameters changed significantly whether among the microcosms monocultured with four species or across the microcosms vegetated with three species richness levels, as confirmed by one-way ANOVA ($P < 0.05$, Table 1). Both above- and below-ground plant biomass parameters were the greatest in the microcosms monocultured with *C. glauca* (1907.8 and 1073.4 g m^{-2}), and the smallest in the microcosms monocultured with *C. alternifolius* (384.7 and 245.8 g m^{-2}). Across the three species richness levels (Table 1), both above- and below-ground plant biomass parameters were significantly higher in the VFSM systems vegetated with 4 species than in those monocultured VFSM systems ($P < 0.05$).

pH, DO and ORP Patterns

The pH ranged from an average of 6.5 to 7.0 in all microcosms (Table 2), and did not significantly change between the unvegetated and vegetated microcosms (computed as the means of both monoculture and 4-species polyculture), among the monocultured microcosms or across the microcosms with three plant species richness levels ($P > 0.05$). The ORP values ranged from an average of 62.46 to 98.54 -mv in all microcosms (Table 2), and were significantly greater in the vegetated microcosms than in the unvegetated microcosms ($P < 0.05$). Among the monocultured microcosms, the ORP changed significantly ($P < 0.05$), with the microcosms vegetated with *C. alternifolius* showing the largest ORP values, followed by the microcosms vegetated with *C. glauca* and *S. validus*, and the microcosms vegetated with *I. pseudacorus* yielding the smallest ORP values. Conversely, the ORP values did not show a significant change across the microcosms with the three species richness levels ($P > 0.05$). The DO contents ranged ranged from an average of 0.23 to 0.57 mg mL^{-1} in all microcosms (Table 2), followed the ORP patterns between the unvegetated and vegetated microcosms ($P < 0.05$), among the four monocultured microcosms ($P < 0.05$) or across the microcosms with the three plant species richness levels ($P > 0.05$).

DNF and DNRA Community Structural Parameters

The DNF or the DNRA bacterial richness (TRF richness), average TRF abundance and Shannon-Weaver index in a given sample was individually investigated by using the T-RFLP approach. For the T-RFLP optimization, the digestion was individually performed with four restriction endonucleases (*A*luI, *Msp*I, *Rsa*I and *Hae*III). It was found that the DNF digests with *A*luI or the DNRA digests with *M*spI yielded more consistent and representative terminal fragment profiles than with other enzymes, consequently, only data from the *A*luI and *M*spI digestion were used for further analysis.

The TRF richness (50 bp ≤ TRFs ≤ 500 bp), average TRF abundance and Shannon-Weaver index of the DNF community did not exhibit a significant difference between both UNP and vegetated treatments ($P > 0.05$, Fig. 1a, b and c). Conversely, the TRF richness (50 bp ≤ TRFs ≤ 520 bp), average TRF abundance and Shannon-Weaver index of the DNRA community showed a significant difference ($P < 0.05$, Fig. 1a, b and c), with greater values in the vegetated treatments than in the UNP treatments. Meanwhile, both DNF and DNRA communities yielded a similar TRF richness in the UNP microcosms, but the DNRA community showed a greater TRF

Table 1 Plant biomass production in the microcosms with different planting treatments

Plant species richness levels	Above-ground plant biomass (g m^{-2})	Below-ground plant biomass (g m^{-2})
UNP	0^{b}	0^{b}
Vegetated	988.9 ± 120.2^{a}	1149.6 ± 146.3^{a}
IP	884.9 ± 56.2^{a}	640.2 ± 103^{a}
CG	1907.8 ± 103.5^{a}	1073.4 ± 123.5^{a}
SV	564.5 ± 119.1^{a}	382.5 ± 95.2^{a}
CA	384.7 ± 112.7^{b}	245.8 ± 67.4^{b}
UNP	0^{c}	0^{c}
MONO	884.4 ± 149.2^{b}	664.1 ± 131.8^{b}
MIX	1092.9 ± 198.8^{a}	1659.1 ± 180.8^{a}

IP-*Iris pseudacorus*, CG-*Canna glauca*, SV-*Scirpus validus* and CA-*Cyperus alternifolius*; UNP-unvegetated treatment, MONO-monocultured treatment and MIX- 4-species polyculture. In each column, means with different superscript lower-case letters exhibit significant differences between treatments at $P < 0.05$, as shown by LSD test

Table 2 Patterns of pH, dissolved oxygen (DO) and oxidization-reduction potential (ORP) in the microcosms with different planting treatments

Planting patterns	pH	DO (mg mL^{-1})	ORP (– mv)
UNP	6.78 ± 0.2^{a}	0.23 ± 0.08^{b}	93.80 ± 25.6^{b}
Vegetated	6.93 ± 0.2^{a}	0.30 ± 0.07^{a}	89.23 ± 14.2^{a}
IP	6.87 ± 0.1^{a}	0.23 ± 0.01^{b}	95.12 ± 18.1^{b}
CG	6.85 ± 0.3^{a}	0.24 ± 0.05^{b}	84.42 ± 27.9^{ab}
SV	6.88 ± 0.2^{a}	0.29 ± 0.19^{b}	77.70 ± 33.5^{ab}
CA	7.01 ± 0.3^{a}	0.57 ± 0.11^{a}	62.46 ± 17.9^{a}
UNP	6.78 ± 0.2^{a}	0.23 ± 0.08^{a}	93.80 ± 25.3^{a}
MONO	6.90 ± 0.2^{a}	0.33 ± 0.19^{a}	79.93 ± 34.4^{a}
MIX	6.95 ± 0.2^{a}	0.27 ± 0.07^{a}	88.54 ± 33.5^{a}

All abbreviations please see Table 1

richness in the vegetated microcosms than the DNF community ($P < 0.05$, Fig. 1a). On the other hand, compared to the DNRA community, a greater average TRF abundance associated with the DNF community in the UNP or vegetated microcosms was observed, respectively ($P < 0.05$, Fig. 1b).

Among the monocultured microcosms, the TRF richness and Shannon-Weaver index of the DNF or the DNRA communities did not significantly change ($P > 0.05$, Fig. 2a and c), but the average TRF abundance associated with the DNF community significantly changed ($P < 0.05$, Fig. 2b), with the microcosms vegetated with *I. pseudacorus* supporting the greatest average TRF abundance, followed by the microcosms vegetated with *C. glauca* and *S. validus*, and the microcosms vegetated with *C. alternifolius* supporting the smallest average abundance. However, the average TRF abundance associated with the DNRA community did not significantly change among the monocultured microcosms ($P > 0.05$, Fig. 2b). Except for the microcosms vegetated with *C. alternifolius*, a greater TRF richness of the DNRA community in the microcosms vegetated with *I. pseudacorus*, *C. glauca* or *S. validus* than that of the corresponding DNF community was individually observed ($P < 0.05$, Fig. 2a). However, the greater average TRF abundance associated with the DNF community in the microcosms vegetated with *I. pseudacorus* or *C. glauca* than that of the DNRA community was individually observed ($P < 0.05$, Fig. 2b).

Across the microcosms with three plant species richness levels, the TRF richness and Shannon-Weaver index of the DNF community did not significantly change ($P > 0.05$, Fig. 3a and c). In contrast, the average TRF abundance associated with the DNF community was significantly greater in the monocultured microcosms than in the unvegetated and 4-species polycultured microcosms ($P < 0.05$, Fig. 3b). Differently, three parameters of the DNRA community such as the TRF richness, average TRF abundance and Shannon-Weaver index did not significantly change across three plant species richness levels ($P > 0.05$, Fig. 3a, b and c). No significant difference was observed between both DNF and DNRA community parameters such as TRF richness and Shannon-Weaver index across three plant species richness levels ($P > 0.05$), while the average TRF abundance associated with the DNF community

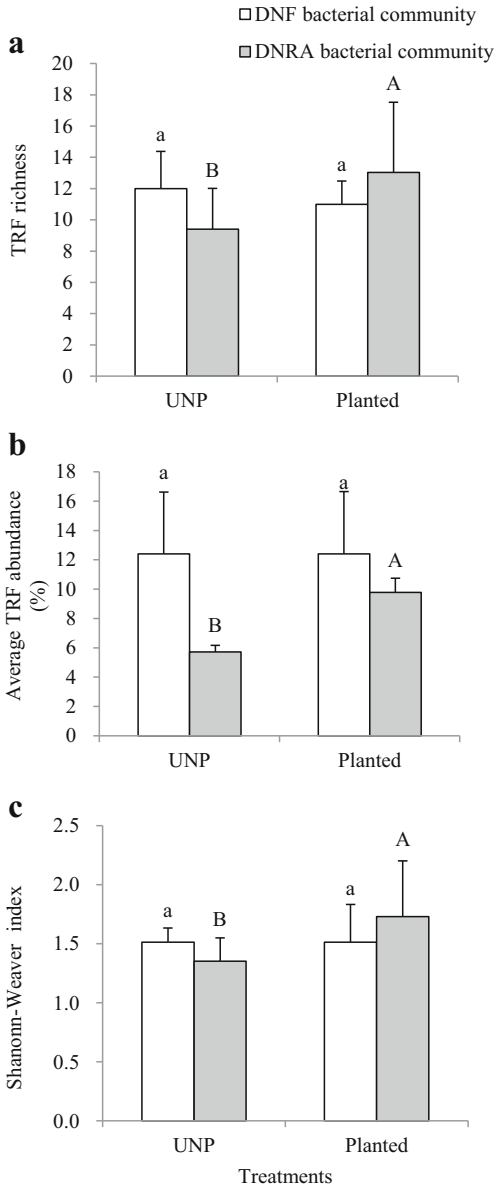

Fig. 1 Comparison of both DNF and DNRA bacterial community structural parameters such as the TRF richness (TRF number in a given community profile, **a**), TRF average abundance (average relative peak area of all distinct TRFs, **b**) and Shannon-Weaver index (**c**) between the unvegetated (UNP) and vegetated treatments (Vegetated). The TRF on the vertical axis represents a terminal restriction fragment of taxonomic assignment in a given DNF or DNRA bacterial community. Each bar represents one standard error ($n = 5$ for the unvegetated microcosms and $n = 25$ for the vegetated microcosms including 20 monocultures and five 4-species polycultures). The different lowercase or uppercase letters on the error bars indicate individually a significant difference of the DNF or DNRA bacterial community parameter at $P < 0.05$ between treatments

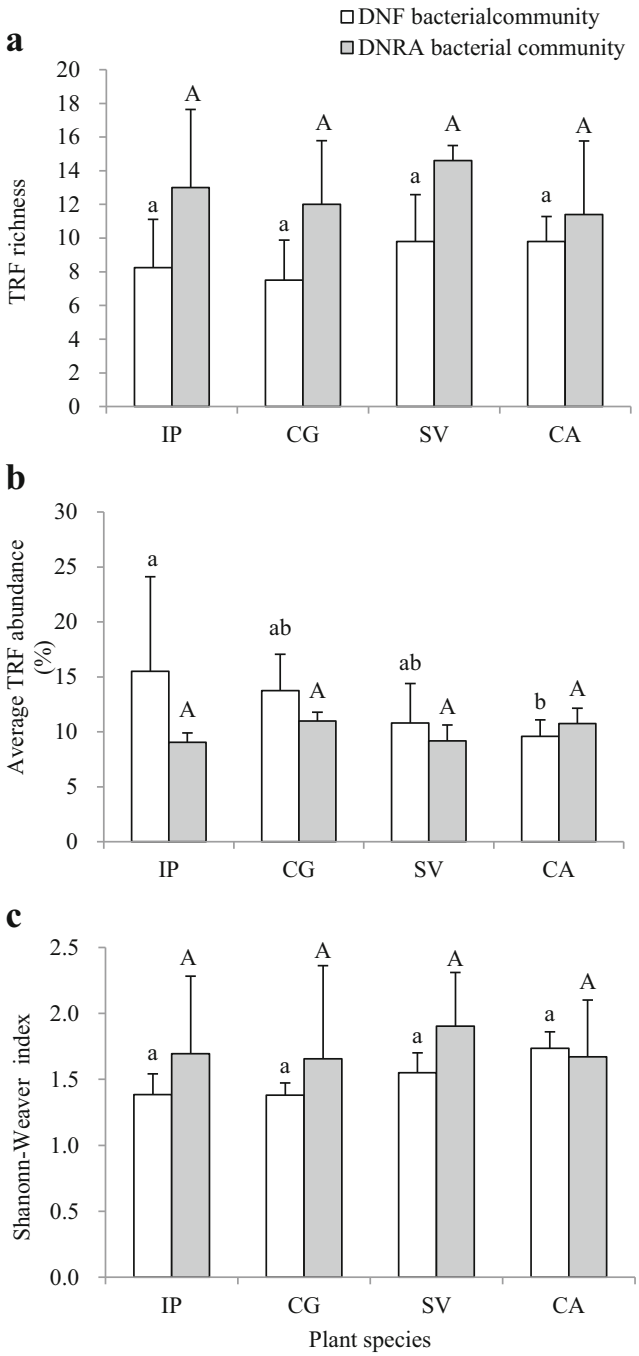

Fig. 2 Comparison of both DNF and DNRA bacterial community structural parameters (**a**, **b** and **c**) among four monocultured treatments. For other explanations, see Fig. 1 and Table 1

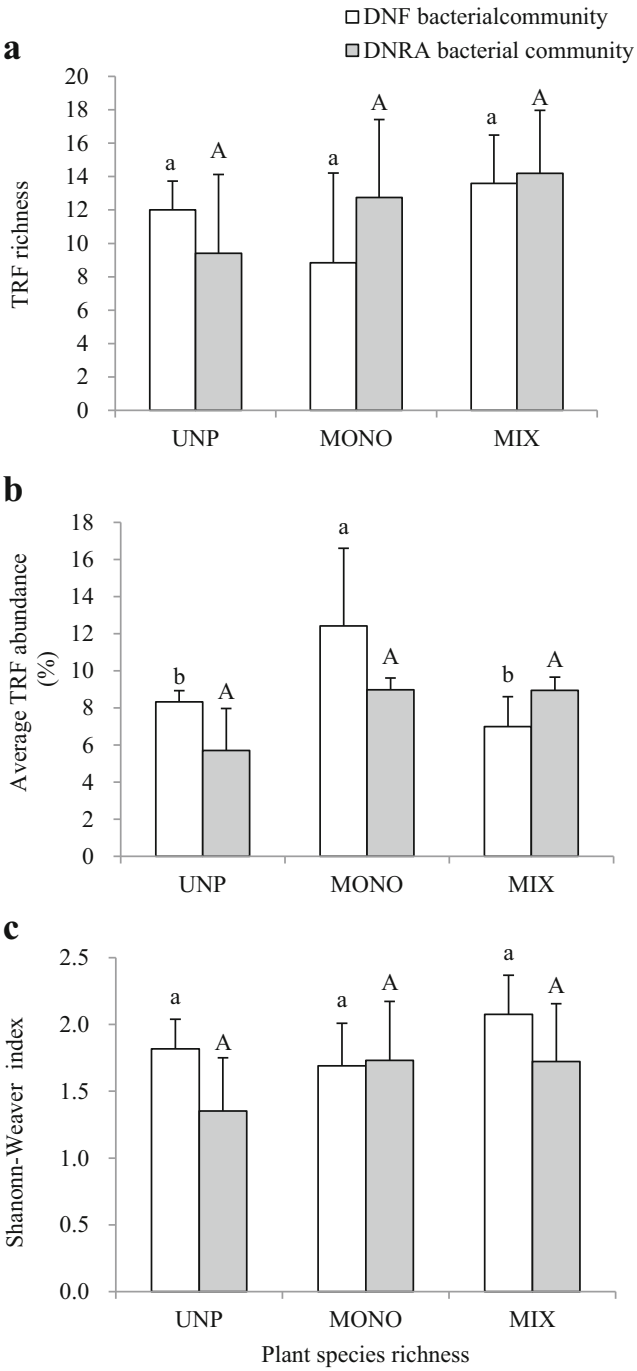

Fig. 3 Comparison of both DNF and DNRA bacterial community structural parameters (**a**, **b** and **c**) across plant species richness levels. MONO and MIX represents the monocultured treatment and 4-species mixed treatment, respectively. For other explanations, see Fig. 1

was greater in the UNP and monocultured microcosms than that of the DNRA community ($P < 0.05$).

Potential DNF and DNRA Rates

The potential DNF rate ranged from an average of 5.56 to 8.30 μg N g^{-1} dw d^{-1} in all microcosms, and was greater in the vegetated microcosms than in the UNP microcosms ($P < 0.05$, Fig. 4a). Among the four types of monocultured microcosms, the microcosms vegetated with *C. alternifolius* showed a significantly smaller DNF rate (5.56 μg N g^{-1} dw d^{-1}, $P < 0.05$) than the other three monocultured microcosms vegetated with *I. pseudacorus*, *C. glauca* and *S. validus* which showed a similar DNF rate (Fig. 4b). At the same time, the potential DNF rate was significantly greater in the 4-species

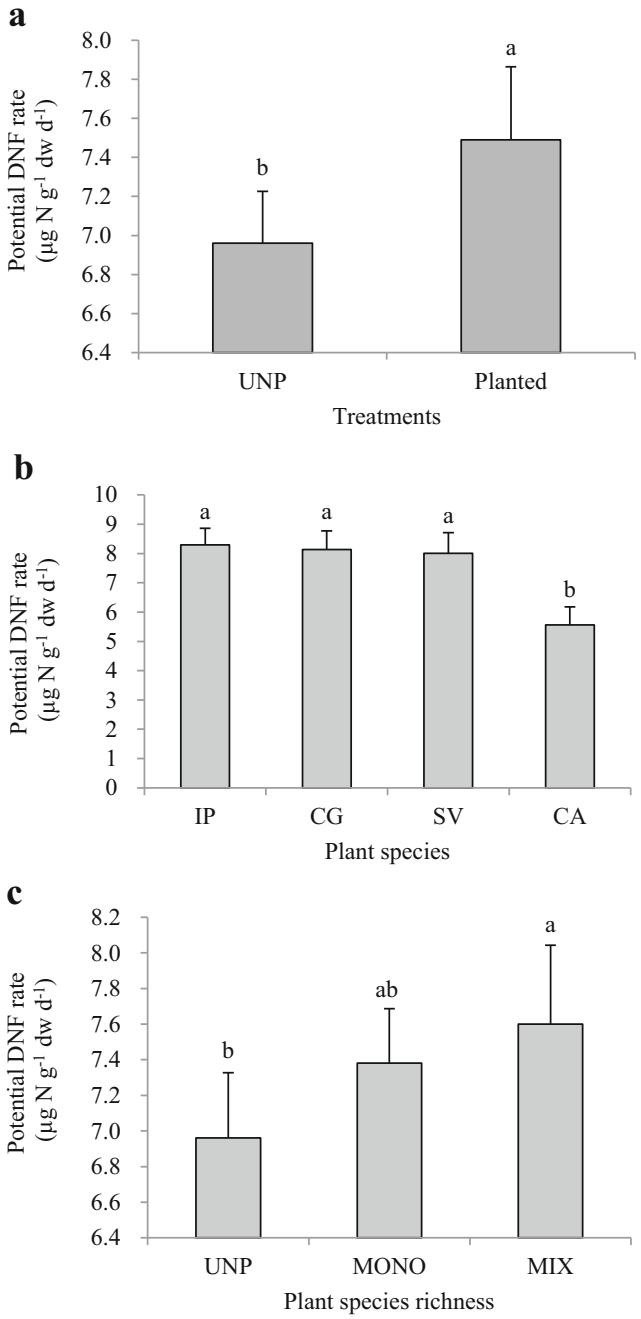

Fig. 4 Comparison of potential DNF rates between both UNP and vegetated treatments (**a**), among four monocultured treatments (**b**) or across three species richness levels (**c**). For other explanations, see Fig. 1

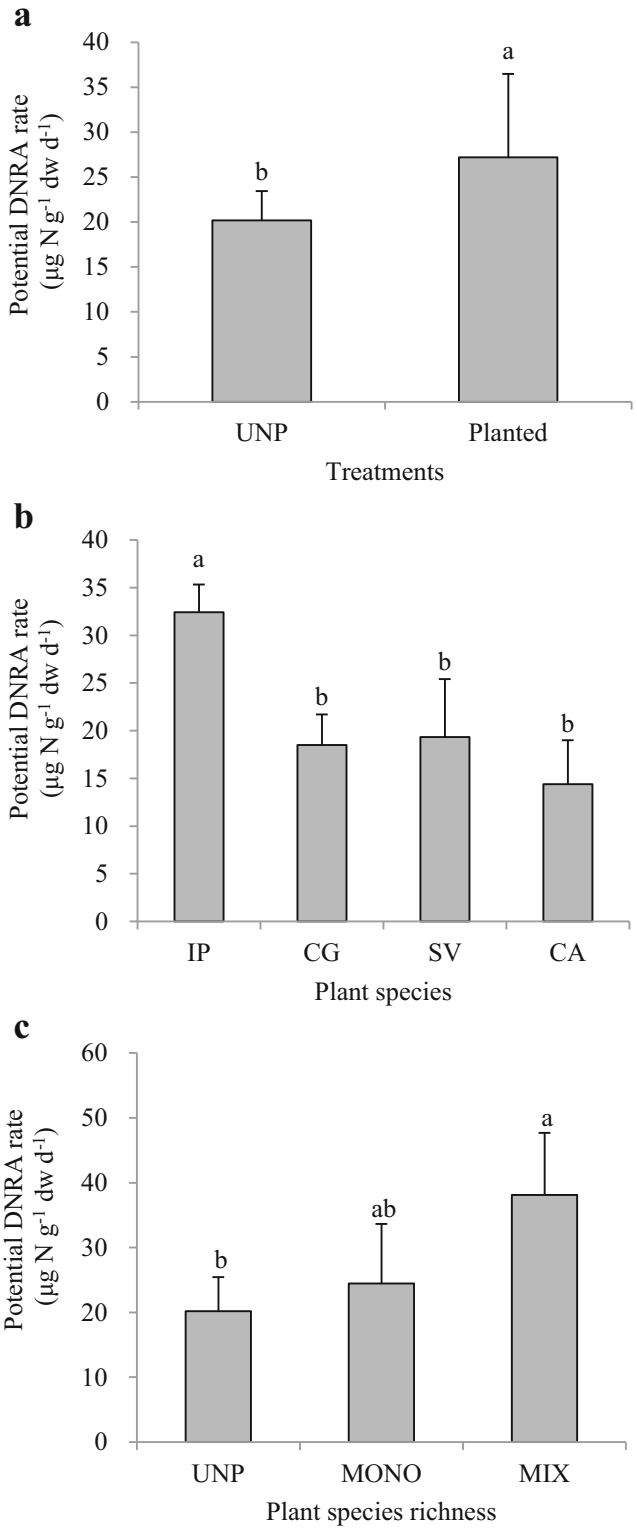

Fig. 5 Comparison of the potential DNRA rates between UNP and vegetated treatments (**a**), among four monocultured treatments (**b**) or across three species richness levels (**c**). For other explanations, see Fig. 1

polycultured microcosms than in the UNP microcosms ($P < 0.05$, Fig. 4c), but no significant difference was observed between the monocultured and polycultured microcosms.

The potential DNRA rate ranged from an average of 14.39 to 38.12 μg N g^{-1} dw d^{-1} in all microcosms, and was significantly greater in the vegetated microcosms than in the UNP ones ($P < 0.05$, Fig. 5a). Among the four monocultured microcosms, the microcosms vegetated with *I. pseudacorus* showed a significantly greater DNRA rate (32.42 μg N g^{-1} dw d^{-1}) than the other three monocultured microcosms which showed similar DNRA rate (Fig. 5b). Like the DNF rate, the potential DNRA rate was significantly greater in the 4-species

polycultured microcosms than in the UNP microcosms ($P < 0.05$, Fig. 5c), but no significant difference was observed between the monocultured and polycultured microcosms. The potential DNRA rate was about 2, 3 and 5 magnitudes greater than the potential DNF rate within each treatment such as the unvegetated, monocultured or 4-species polycultured treatment ($P < 0.05$).

Correlation Analysis of Microbial Parameters to Plant Biomass Production, pH, DO and ORP

As shown in Table 3, among the microcosms vegetated with different plant species, both above- and below-ground plant biomass parameters were positively related to the potential DNF rate ($P < 0.05$), but not significantly related to the DNRA rate ($P > 0.05$). At the same time, the BPB was significantly and negatively related to the DO value ($P < 0.05$). However, both APB and BPB did not significantly influence the DNF and DNRA community structural parameters such as TRF richness, average TRF abundance and Shannon-Weaver index ($P > 0.05$).

Across the microcosms vegetated with three plant species richness levels (Table 4), both above- and below-ground plant biomass parameters were positively related to the potential DNF and DNRA rates ($P < 0.05$), but not related to DNF and DNRA community structural parameters ($P > 0.05$). Meanwhile, the pH was positively related to the DO in the microcosms ($P < 0.05$, Table 4).

Discussion

Plant Biomass Production

Plant species widely differ in their photosynthetic rate, root type and production (Maucieri et al. 2014; Barbera et al. 2015), nutrient uptake and aboveground biomass production (Stottmeister et al. 2003). In the current study, both above- and below-ground biomass parameters significantly changed among the microcosms monocultured with different species. The microcosms planted with *C. glauca* supported the greatest biomass parameters compared to other microcosms monocultured with other species, since *C. glauca* has a relatively greater photosynthetic rate and nutrient uptake rate (Cheng et al. 2009). Like the previous studies, both above- and below-ground biomass parameters significantly increased with the species richness, thus further confirming that a greater species richness may completely support a greater plant biomass production due to the complementary usage of nutrients among diverse plant species (van Ruijven and Berendse 2009; Zhang et al. 2010).

pH, DO and ORP

In general, plant roots are responsible for the substantial changes of the rhizosphere pH by: (1) releasing H^+ or OH^- to compensate for an unbalanced cation–anion uptake at the soil–root interface; (2) root exudation of organic acids and amino acids into the rhizosphere; and (3) the direct release

Table 3 The relationships between microbial parameters and plant biomass productions, pH, DO and ORP in the microcosms monocultured with different plant species

Variables	APB	BPB	pH	ORP	DO	DNF	DNRA	DNF-RICH	DNF-ABUN	DNF-DIVER	DNRA-RICH	DNRA-ABUN	DNRA-DIVER
APB	1.00	0.75**	0.06	−0.04	0.01	0.66**	0.28	−0.25	0.04	−0.19	−0.14	0.17	−0.21
BPB		1.00	−0.01	0.09	−0.51*	0.48*	0.29	−0.38	0.38	−0.33	−0.30	0.18	−0.41
pH			1.00	0.17	−0.09	0.19	−0.09	0.33	−0.31	0.40	−0.10	−0.09	−0.25
ORP				1.00	−0.38	0.21	−0.01	0.02	0.29	−0.18	0.14	0.25	0.04
DO					1.00	−0.15	−0.37	0.15	−0.27	0.21	0.12	−0.36	0.16
DNF						1.00	0.16	0.28	−0.19	0.35	−0.12	−0.24	−0.07
DNRA							1.00	0.13	0.14	0.02	0.17	0.02	0.13
DNF-RICH								1.00	−0.73	0.86**	−0.08	−0.09	−0.02
DNF-ABUN									1.00	−0.29	0.30	0.44	0.21
DNF-DIVER										1.00	−0.24	−0.04	−0.15
DNRA-RICH											1.00	0.33	0.94**
DNRA-ABUN												1.00	0.37
DNRA-DIVER													1.00

APB above-ground plant biomass, *BPB* below-ground plant biomass, *DNF-RICH* denitrifying bacterial richness, *DNF-ABUN* denitrifying bacterial abundance, *DNF-DIVER* denitrifying bacterial diversity, *DNRA-RICH* dissimilatory nitrate reduction bacterial richness, *DNRA-ABUN* dissimilatory nitrate reduction bacterial abundance, *DNRA-DIVER* dissimilatory nitrate reduction bacterial diversity. Other abbreviations please see text and Table 1

Table 4 The relationships between microbial parameters and plant biomass productions, pH, DO and ORP in the microcosms planted with three plant species richness levels

Variables	APB	BPB	pH	ORP	DO	DNF	DNRA	DNF-RICH	DNF-ABUN	DNF-DIVER	DNRA-RICH	DNRA-ABUN	DNRA-DIVER
APB	1.00	0.60**	0.26	−0.19	0.13	0.44*	0.51*	−0.20	0.09	−0.14	0.03	0.24	−0.03
BPB		1.00	0.01	−0.08	−0.25	0.49*	0.42*	0.04	0.06	0.08	0.11	0.19	0.02
pH			1.00	−0.35	0.56*	−0.02	−0.08	−0.08	−0.04	−0.03	0.25	−0.02	0.15
ORP				1.00	−0.33	−0.02	−0.04	0.02	0.01	0.07	−0.30	−0.06	−0.37
DO					1.00	−0.02	−0.36	−0.33	0.16	−0.24	0.17	−0.13	0.24
DNF						1.00	0.02	0.03	−0.08	0.13	−0.04	−0.13	0.01
DNRA							1.00	0.51	−0.19	0.44	0.21	0.02	0.09
DNF-RICH								1.00	−0.79	0.92**	0.05	−0.12	0.00
DNF-ABUN									1.00	−0.77	0.21	0.42	0.19
DNF-DIVER										1.00	−0.05	−0.07	−0.06
DNRA-RICH											1.00	0.33	0.93**
DNRA-ABUN												1.00	0.38
DNRA-DIVER													1.00

All abbreviations please see Table 3

of CO_2 from the roots and indirect effect on the rhizosphere microbial respirations (Rao et al. 2002; Hinsinger et al. 2006). Thereby, the presence/absence of plants, different plant species or plant species richness may influence the pH in the CW systems. However, in the current study, the pH of wastewater at a 30-cm depth of the microcosm system was not significantly affected by the presence of plants, plant species and plant species richness. It is well known that the greater is the pH buffering capacity of a given substrate, the smaller the plant-induced pH changes (Hinsinger et al. 2003). Generally, the sandy substrate exhibits a minimal pH buffering capacity due to smaller organic matter content (Hinsinger et al. 2003). Therefore, the filled sand used in the current CW systems could not greatly affect the plant-induced pH change. This result mentioned above was most possibly attributed to the greater pH buffering capacity in the current Hoagland solution, since the Hoagland solution is not only a nutrient solution, but also is a better buffer solution (Hoagland and Arnon 1950).

Compared to the unvegetated treatments, the vegetated treatment significantly increased both DO and ORP values in the wastewater, thus showing a positive effect of plant growth on the oxygen condition in the current CW systems. This was dominantly related to the oxygen release from the macrophyte roots, since the previous studies found that plants in wetlands can transfer oxygen through the aerenchyma from the atmosphere to the rhizosphere environment (Bezbaruah and Zhang 2004; Jung et al. 2008). The difference in plant species significantly affected both DO and ORP values in the wastewater, with the microcosms monocultured with *C. alternifolius* (CA) harboring greater DO and ORP values than the microcosms monocultured with other species. It was also interesting that the below-ground plant biomass was negatively related to the DO, as confirmed by the correlation analysis. This was possibly related to the developed lysigenous aerenchyma formed in *C. alternifolius* roots (Seago et al. 2005), since the lysigenous aerenchyma has greater gas exchange space than the schizogenous aerenchyma, possibly resulting in a smaller root biomass in the microcosms planted with *C. alternifolius* (Jung et al. 2008). Recently, Lai et al. (2012) and Mei et al. (2014) observed that *C. alternifolius* had a greater radial oxygen loss (ROL rate = 150–157 mM O_2 kg^{-1} root dw d^{-1}) than other macrophytes such as *Acorus calamus, Canna indica, Iris tectorum, and S. validus*, thus supporting the current result.

DNF and DNRA Community Structure

A previous study conducted by Philippot et al. (2002) showed that the *narG*-type nitrate-reducing bacterial community structure was significantly different between the unvegetated and maize-vegetated soils. Similarly, Bremer et al. (2009) reported that the presence of plants affected the composition of the *nirK*-type denitrifier community. In contrast, some studies reported that the presence of plants had no significant impact on the denitrifying bacterial structure (Mounier et al. 2004; Henry et al. 2008; Garcia-Lledo et al. 2011; Chen et al. 2014). In the current study, the DNF community parameters such as the TRF richness, average TRF abundance or the Shannon-Weaver index did not show a significant difference between both unvegetated and vegetated microcosms. This was possibly attributed to a consequence of the combined effects of oxygen and organic carbon compounds on the DNF community structure. On the one hand, the oxygen released from a

plant's roots may inhibit the DNF activity in the rhizosphere environment, and on the other hand, the dissolved organic carbon compounds such as sugars, amino acids and volatile fatty acids secreted from roots may be used as electronic donors of the DNF bacteria (Chen et al. 2014). Also, the insignificantly changed pH of wastewater might be another factor resulting in an insignificant difference of the DNF community structure between both unvegetated and vegetated microcosms, since among several denitrifying genes, the *nirS* gene copy number was mostly influenced by the change in the pH (Čhel et al. 2010). However, Saleh-Lakha et al. (2009) observed that the level of the *nirS* expression was insensitive to the soil pH values that ranged from 6 to 8.

Sharma et al. (2005) showed that the *nirK* gene clones greatly depended on a specific plant species such as *Vicia faba, Lupinus albus and Pisum sativum*. Bremer et al. (2007) found that plant species directly affected the *nirK*-type denitrifier community composition through root exudates. Similarly, Ruiz-Rueda et al. (2009) found that *nosZ* gene genotypes showed a vast difference between two CW systems vegetated with *Typha latifolia* and *Phragmites australis*. These researchers suggested that the difference in the denitrifier community composition might be attributed to the differences in the quality and quantity of root exudates, because a specific exudate may selectively favor some microbial strains over others, thereby altering the microbial community structure (Ruiz-Rueda et al. 2009). Generally, the exudation is a dynamic process depending on plant species, physiological status of the plant, root zone, and nutritional conditions (Bremer et al. 2007). However, in the current study, plant species did not significantly change the TRF richness and Shannon-Weaver index of the DNF community. This might be attributed to the insignificant difference of the wastewater pH values among the microcosms monocultured with different plant species. Besides, we speculated that the high contents of nutrients in the current Hoagland solution possibly discounted the difference in the amount of root exudates released from four different plant roots. Nonetheless, a significantly smaller average TRF abundance of the DNF community was observed in the microcosms vegetated with *C. alternifolius* than those in the microcosms monocultured with other species. This finding might be attributed to the higher DO and ORP values in the microcosms vegetated with *C. alternifolius*, since a great DO or ORP value may inhibit denitrifier growth.

Like plant species, plant species richness did not significantly affect the DNF community composition in the current CW systems either. The current study confirmed previous results in which no influence of plant species composition on soil bacterial diversity was observed (Nunan et al. 2005; Zul et al. 2007). Recently, Prasse et al. (2015) emphasized that environmental factors such as soil temperature, pH and water content were more important in influencing microbial community composition than the plant community composition. Although the correlation was insignificant, our result might mostly be attributed to the pH, DO and OPR patterns in the wastewater, since these factors did not significantly change across three plant species richness levels. However, plant species richness significantly affected the average TRF abundance associated with the DNF community. Unexpectedly, among three species richness levels (unvegetated, monocultured and 4-species polycultured treatments), the greatest average TRF abundance of the DNF community occurred in the monocultured microcosms. This was mainly attributed to the greater average TRF abundance in the microcosms monocultured with highly productive species such as *I. pseudacorus* or *C. glauca* which had greater biomass production than other species.

Several earlier studies investigated the direct effect of wetland/freshwater plants on the DNRA in sediments, though these findings are not conclusive. A higher contribution of the DNRA to the recovery of added $^{15}NO_3^-$ was found in the soil cores containing *P. australis* roots compared to the root-free cores (Nijburg and Laanbroek 1997). In contrast, Dhondt et al. (2003) showed that during the growing season, the DNF bacteria were dominant in a riparian zone, while the DNRA bacteria predominated when plant growth was low. Adversely, another $^{15}NO_3^-$ labelling microcosm study found that the DNRA accounted only for less than 1 % of NO_3^- loss in the wetland soil vegetated with *G. declinata*, while the DNRA accounted for 49 % of the NO_3^- consumption in the the unvegetated wetland (Matheson et al. 2002).

In the current study, the DNRA community parameters such as the TRF richness, average TRF abundance and Shannon-Weaver index in the vegetated microcosms were significantly greater than in the unvegetated microcosms, which showed that the vegetated treatment positively drove development of the DNRA community structure. Philippot et al. (2002) ever pointed out that the presence of roots might alter the abundance of dissimilatory NO_3^- reducers in soils due to a consequence of altered substrate availability. Therefore, the current result was most possibly attributed to the increased carbohydrate contents in the vegetated microcosms, since it is well known that plants may release some dissolved organic carbon compounds such as sugars, amino acids, organic acids and phenolic compounds into the rhizospheric substrate (Bais et al. 2006).

On the other hand, the environmental oxidation state is another principal factor that influences the importance of the DNRA community compared to the DNF (Rütting et al. 2011). The DNRA bacteria often occur under the anoxic condition, thus are regarded as anaerobic bacteria (Schmidt et al. 2011). However, some studies also showed that the DNRA community is less sensitive to the change in the redox condition (Pett-Ridge et al. 2006) and O_2 content than the DNF community (Fazzolari et al. 1998). In the current study, a greater DO or ORP value was observed in the vegetated

microcosms than in the unvegetated microcosms. Therefore, the current result revealed that some facultative aerobic DNRA microorganisms such as bacteria and fungi might dominate the nitrate reducers in the 0-30 cm substrate layer of the CW system. Similarly, Nijburg and Laanbroek (1997) observed that in the presence of reed sweetgrass (*Glyceria maxima*), the DNRA bacteria (53 %) dominated the NO_3^- reducer community in a pot experiment, while denitrifiers dominated in the unvegetated soil (71 %).

Potential DNF and DNRA Rate

Several studies showed that the presence/absence of vegetation had little influence on the spatial and seasonal variations of the DNF activity (Song et al. 2014). However, in a previous study, the addition of maize mucilage resulted in a higher DNF rate but it only found a minor change in the DNF community structure (Mounier et al. 2004). In the current study, higher DO and ORP values were observed in the vegetated microcosms than in the unvegetated microcosms, so our expectation was that the potential DNF rate should be inhibited in the vegetated microcosms, since the DNF community is very sensitive to high oxygen and ORP conditions. Unexpectedly, the potential DNF rate was greater in the vegetated microcosms than in the unvegetated microcosms, showing a positive effect of vegetated treatment on the DNF rate. The cause of the positive effect was mostly attributed to exudates released by plant roots. The current Hoagland solution was characterized by a low carbon content (BOD = 79.51 mg L^{-1}, Liu et al. 2015), so it was possible that the exudates released by plant roots were used as the main origins of electronic donors. Zhai et al. (2013) similarly found that root exudates are potentially important sources of organic C compounds for the denitrification in subsurface flow constructed wetland systems receiving the water with a low loading of BOD_5.

Changes in plant species also significantly affected the DNF rate, i.e., the microcosms monocultured with *C. alternifolius* harbored a lower DNF rate than the microcosms monocultured with three other plant species, thus showing a plant species-specific influence. This was mostly related to the difference of DO and ORP values among microcosms vegetated with different species, since both DO and ORP values in the microcosms monocultured with *C. alternifolius* were greatest among all vegetated microcosms. At the same time, the smallest above- or below-ground plant biomass was also observed in the microcosms monocultured with *C. alternifolius*, thus showing a positive relation of the DNF rate to plant biomass production, as confirmed by the correlation analysis. Similarly, Bastviken et al. (2007) also found a higher denitrifying capacity in the area of a full-scale treatment wetland vegetated with *G. maxima* than in those areas vegetated with other plant species. They attributed their findings to the difference of the organic matter availability among the areas vegetated with different plant species. Ruiz-Rueda et al. (2009) observed that the higher denitrification rates were generally obtained in the CW systems vegetated with *P. australis* than in those CW systems vegetated with *T. latifolia*. They attributed their observation to a plant selective effect on denitrifiers. Based on these findings from published papers above, we speculated that a smaller organic matter availability or the denitrifiers with small denitrification capacity might occur in the microcosms vegetated with *C. alternifolius*.

Across three plant species richness levels, the 4-species polyculture was most effective compared to both UNP and monoculture in improving the DNF rate due to a possible interspecies-complementary effect. This finding was mostly related to the greatest above- and below-ground biomass productions in the current polycultured microcosms as shown by the correlation analysis, since a great biomass production may result in a great amount of root exudates (Bais et al. 2006). Similarly, Coleman et al. (2001) and Fraser et al. (2004) previously reported that the polycultured treatment in the CW systems was more effective in reducing pollutants from wastewater than the monocultured treatment due to the complementary uptake of pollutants and great dissolved organic matter. Conversely, Bachand and Horne (2000) reported that a greater nitrate removal was observed in the 2-species CW systems than in the monocultured systems due to a great denitrification rate. They suggested that the great contribution rate and the quality of organic matter in the 2-species CW systems were the most important factors that drove a great denitrification rate.

Like the DNF rate, the presence of plants also stimulated the potential DNRA rate, thus also showing a positive effect of plant growth on the DNRA rate. However, it is possible that the mechanism behind the greater potential DNRA rate, which occurred in the vegetated microcosms, was not different from that of the DNF rate. First, the greater potential DNRA rate was possibly related to the higher organic matter content in the vegetated microcosms than in the unvegetated microcosms, since researchers consistently claimed that there is always a great DNRA rate in the environments with a higher organic matter content or higher C/NO_3^- ratio (Rütting et al. 2011). On the other hand, the higher DO and ORP values in vegetated microcosms than in the unvegetated microcosms were also important factors in stimulating the DNRA rate, since the previous studies found that the DNRA is less sensitive to variable redox conditions (Pett-Ridge et al. 2006) and O_2 fluctuation than the denitrification (Fazzolari et al. 1998). In addition, several studies investigated the effect of pH on the DNRA, showing that a higher DNRA rate was often associated with pH conditions over the range of 6.2 to 8.2 (Stevens et al. 1998; Schmidt et al. 2011; Zhang et al. 2015). In the current study, the water pH ranged from an average of 6.78 (unvegetated) to 6.93 (vegetated). Therefore, the pH was also

a possible factor resulting in the greater DNRA community parameters in the vegetated treatment, since the pH slightly increased in the vegetated microcosms. Therefore, we concluded that unlike the DNF rate, the improved DNRA rate in the vegetated microcosm was not only related to the ample availability of organic matter, but also related to the higher pH, DO and ORP values.

Plant species also influenced the potential DNRA rate, with *I. pseudacorus* microcosms harboring a greater DNRA rate than the three other monocultured treatments. This result was mostly attributed to the greater below-ground plant biomass production in the microcosms monocultured with *I. pseudacorus* (Bais et al. 2006), as confirmed by the current correlation analysis. Meanwhile, the 4-species polyculture was more effective than the monocultures in improving the potential DNRA rate, which was possibly due to the complementary excretion of exudates among the four plant species. Further, it could be due to the root O_2 release, since the ratio exudates/O_2 could be different compared to other treatments. Like the DNF rate, no significant difference of the DNRA rates between the 4-species polycultured and monocultured microcosms was observed as well, which was attributed to the insignificant change of pH, DO and ORP and plant biomass production between the polycultured and monocultured microcosms.

Conclusion

Effects of plant presence, plant species and their species richness on plant biomass, pH, DO, ORP and the DNF and DNRA communities were investigated in thirty vertical flow microcosm wetlands fed with the Hoagland solution. Plant species and species richness significantly affected above- and below-ground biomass parameters. Meanwhile, plant presence and plant species were important factors that influenced both DO and ORP values in the microcosm wetlands, but plant species richness had no effects on the pH, DO and ORP values. Plant presence diversified the DNRA community composition, and also improved both potential DNF and DNRA rates, but did not influence the DNF community composition. Plant species and plant species richness more dominantly influenced both potential DNF and DNRA rates, but not the DNF and DNRA community compositions. Finally, it may be concluded that plant presence, plant species and species richness are more important for improving the DNF and DNRA community rates than in changing community compositions through mediating DO, ORP and dissolved organic matter. This study suggests that both DNF and DNRA bacteria may employ different ecological strategies in responding to plant presence, plant species and species richness in the CW systems. Because of these microbial community-preferred attributes, the planting pattern during the wetland engineering process should be chosen carefully based on the ecosystem services desired.

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