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Research Article

RESEARCH ARTICLE

Impact of plant harvest management on function and community structure of nitrifiers and denitrifiers in a constructed wetland

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One sentence summary: Plant harvest treatment could change subsequent plant development and associated microenvironments in a constructed wetland, and affecting the function and community structure of nitrifiers and denitrifiers.

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ABSTRACT

Plant harvest is one of the most important management practices in constructed wetlands. In this study, we evaluated the impact of harvesting *Phragmites australis* (Cav.) Trin. ex Steudel on the activity and community structure of nitrifiers and denitrifiers in a free-water surface constructed wetland. The nitrifiers were targeted using bacterial and archaeal-*amoA* that encode ammonia monooxygenase, and the denitrifiers were targeted using *nirK* and *nirS* that encode the nitrite reductase. The community structures were evaluated using denaturing gradient gel electrophoresis. The potential nitrification and nitrate reduction rates were shown to be significantly higher in the harvested plant rhizosphere than in a non-harvested control plot. The potential nitrification rate positively correlated with the potential nitrate reduction rate and influenced the community structure of *nirK*. In addition, plant canopy developed differently after harvest and simultaneously changed the microclimate beneath the plant community. These results suggest that plant harvest management could change subsequent plant development and associated microenvironments, thereby affecting the function and community structure of nitrifiers and denitrifiers. Our study highlights the importance of plant harvest management within constructed wetlands to regulate the functions of nitrification and denitrification.

Key words: harvesting reed; PCR-DGGE; nitrification; denitrification

INTRODUCTION

Constructed wetlands (CWs) have been utilized worldwide to improve water quality, including remediation of high concentrations of nitrogen and phosphorus arising from sewage, domestic discharge and agricultural drainage. Nitrogen assimilation by plants and microbial nitrification and denitrification are the major nitrogen removal processes in CWs (Vymazal 2007).

Nitrification includes two aerobic steps, namely the oxidation of ammonium (NH_4^+) to nitrite (NO_2^-), which is further

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oxidized to nitrate (NO_3^-). The initial step of ammonia oxidization to hydroxylamine is catalyzed by the enzyme ammonia monooxygenase. Therefore, the phylogeny of *amoA*, which encodes the α subunit of ammonia monooxygenase, functions as a powerful molecular tool for analyzing indigenous ammonia-oxidizing bacterial (AOB) and ammonia-oxidizing archaeal (AOA) communities (Rotthauwe, Witzel and Liesack 1997).

Denitrification is a microbial respiratory process wherein soluble nitrogen oxides (NO_3^- and NO_2^-) are reduced to gaseous products (NO, N_2O and N_2). The reduction of NO_2^- to NO, a key process in denitrification, is catalyzed by the enzyme nitrite reductase, which are of two types both used to target denitrifiers: a Cu-containing enzyme encoded by *nirK* and a cytochrome cd_1 enzyme encoded by *nirS* (Braker *et al.*, 2000).

Nitrogen cycling depends on a close relationship between plants and microbes wherein root exudates from plants provide labile organic carbon compounds, which serve as energy and carbon sources for denitrifying microorganisms (Nguyen 2003). Oxygen release from roots of submerged vascular plants in wetlands creates oxidized conditions that stimulate nitrification (Bodelier *et al.*, 1996; Risgaard-Petersen and Jensen 1997). Several studies have shown that changes in the activity, composition and abundance of denitrifiers (Hallin and Lindgren 1999; Ruiz-Rueda, Hallin and Bañeras 2009; García-Lledó *et al.*, 2011; Bañeras *et al.*, 2012) may be plant specific. Therefore, it is important to select the optimal plant species, in terms of the plant–microbial interactions, when designing CWs for nitrogen removal.

In addition to the selection of appropriate plant species, plant harvest is important for the management of CWs. Standing old and dead culms of reed have a great impact on the microenvironment. The shading effect of the canopy is substantial in reed stands; therefore, harvesting reed was observed to change water temperature (Granéli 1989). Harvesting mature vegetation changed the light conditions enhancing algal photosynthesis and increased wind velocity promoting gas exchange between water and atmosphere (Hansson and Granéli 1984), and nitrification rates were increased due to elevated concentrations of dissolved oxygen in open water (Sartoris *et al.*, 2000; Thullen, Sartoris and Walton 2002). Vegetation coverage is well known to have a great potential for controlling the function, abundance and community structure of nitrifiers and denitrifiers (Woldendorp 1962; Hallin and Lindgren 1999 Nguyen 2003; Ruiz-Rueda, Hallin and Bañeras 2009; García-Lledó *et al.*, 2011; Bañeras *et al.*, 2012). Nevertheless, the effects of plant harvest management on nitrifiers and denitrifiers remain largely unknown. A better understanding of the effects on nitrification and denitrification processes is crucial for designing CWs for optimal nitrogen removal conditions.

The present study aimed to explore how the activity and community structure of nitrifiers and denitrifiers in the rhizosphere of reed (*P. australis*) respond to plant harvest at different periods of the annual growth cycle. We hypothesized that variation in the timing of plant harvest may impact subsequent plant development and meditate changes in the microenvironment and availability of root exudates such as oxygen energy and carbon sources within the rhizosphere. We further hypothesized that the changes would alter the function and community composition of nitrifiers and denitrifiers. Therefore, we determined the chemical characteristics and community structure of nitrifiers and denitrifiers in the rhizosphere by denaturing gradient gel electrophoresis (DGGE) of major functional genes in the nitrification (bacterial-*amoA*, archaeal-*amoA*) and denitrification pathways (*nirS*, *nirK*) at various stages of plant development following harvest.

MATERIALS AND METHODS

Study site and plant harvest

Samples were obtained from a free-water system CW located in Jinning, Kunming, China, (24° 46′ N, 102° 44′ E) with a surface area of 0.23 km^2. Effluent from the Nanchong River is discharged into lake Dianchi, which has become the largest eutrophic lake in Yunnan Province because of the recent economic and population growth. Agriculture in this area is a major contributor of wastewater and contains a high concentration of nitrate nitrogen (NO_3^--N) (>10 ppm) that flows into lake Dianchi during the rainy season (Tanaka *et al.*, 2013). The predominant vegetation is *P. australis*, cattail (*Typha sp.*) and Manchurian wild rice [*Zizania latifolia* (Griseb.) Turcz. ex Stapf]. Three 5.0 × 5.0-m plots of *P. australis* were mapped of which two were subsequently harvested on 26 January (Jan-harvest) and 30 March 2013 (Mar-harvest), with the non-harvested plot being retained as a control. Plants were harvested by cutting at a height of 40 cm above the ground level to avoid exposing the cut end of the stem to flooding because this has been found to negatively impact growth (Burian and Sieghardt 1979).

Plant canopy structure analyses

The canopy structure and light distribution within the canopy were determined on 20–21 March, 22–24 May and 22–23 July 2013 by applying the stratified clipping method described by Monsi and Saeki (1953). Two 1.0 × 0.5-m quadrats were marked in reed stands of each experimental plot (Jan-harvest, Mar-harvest, control). The vertical light distribution was measured using the LUX/FC Light Meter (TM-201, TENMARS, Taiwan). After measurement of light intensity, all the plants within the quadrat were cut with a sickle at the ground level. Plants were cut into 0.4-m segments along the stem, keeping plant and leaf inclinations as natural as possible. The shoot height varied from 2.0 to 5.6 m; therefore, the segments with 0.4 m length were considered reasonable. The plant segments were placed into polyethylene bags for transport to the laboratory where the cut segments were sorted as new stems, old stems, leaves, inflorescences and weed. Leaf sheaths were included in the stem fraction. Green leaves were separated from dead leaves. The leaf areas of green leaves and dead leaves were measured by imaging of digital camera pictures using Photoshop CS6 (Adobe systems, CA, US), and the leaf area index (LAI, leaf area per unit ground surface area) was calculated. The dry weight (DW) was determined after oven drying at 70°C for at least 3 days.

Light intensity was assumed to attenuate through the leaf canopy following the Beer–Lambert law: $I = I_0 \exp(-K \times F)$, where I is the shaded light intensity under the cumulative LAI F, I_0 is the original incoming light intensity and K is the extinction coefficient. We evaluated the relative light intensity at the ground level according to this formula.

Sampling of rhizosphere

Rhizosphere samples were collected on 11 April and 6 July 2013 from the Jan-harvest, Mar-harvest and control plots. The April samples represent spring conditions corresponding to the early growing season (75 and 12 days after Jan-harvest and Mar-harvest, respectively). The July samples represent summer conditions corresponding to the late growing season (161 and 98 days after Jan-harvest and Mar-harvest, respectively). The surface sediment of *P. australis* formed a root mat structure of

approximately 2-cm depth, from which the rhizosphere was sampled using a 3-cm-diameter core sampler. Three replicate composite samples were collected from each treatment. We first randomly sampled five cores in a selected area within one square meter, and then mixed the upper 2-cm rhizosphere parts to make a composite sample. The three specific areas within one square meter were always selected within visually homogeneous pure stands with uniform shoot density in each plot. The composite samples were manually homogenized using sterile spatulas and scissors.

Rhizosphere chemical analysis

Rhizosphere samples were analyzed for NO_3^--N, nitrite nitrogen (NO_2^--N) and ammonium nitrogen (NH_4^+-N) by 2 M KCl extraction from fresh samples. NH_4^+-N concentration of the extract was determined using the indophenol blue method according to APHA (1998). NO_2^--N concentration was determined by colorimetric method according to APHA (1998). NO_3^--N within the extract was reduced to NO_2^- using the cadmium–copper column method according to Mulvaney (1996), and subsequently determined as described above. NH_4^+-N, NO_3^--N and NO_2^--N concentrations were measured using the 4802 UV/Vis Double Beam Spectrophotometer (Unico, Shanghai, China). Rhizosphere samples were measured for water-soluble organic carbon (WSOC) content. WSOC was extracted by modifying the method of Burford and Bremner (1975), wherein 20 ml of distilled water was added to 10 g of fresh sample and extracted by gentle shaking for 15 min in a stoppered 50-ml polyethylene centrifuge tube. The tubes were centrifuged at 5000 rpm for 10 min, and each supernatant was filtered through a 0.45-μm filter. Organic carbon content in the filtrate was measured using a Shimadzu TOC Analyzer (TOC-L CSH, Shimadzu, Kyoto, Japan) held in Laboratory of Soil Science, Kyoto University (Kyoto, Japan).

Potential nitrification and nitrate reduction activity

Each rhizosphere sample was analyzed for potential nitrate and nitrite reduction rates as an indication of denitrification by modifying the method of Ruiz-Rueda, Hallin and Bañeras (2009) and Bañeras *et al.* (2012). Approximately, 10 g of fresh rhizosphere sample, including roots, was placed in a 250-ml flask and diluted in 48 ml of sterile distilled water. After adding the rhizosphere sample and sterile distilled water, the flasks were flushed with N_2 gas for 10 min and agitated for 30 min before the addition of 2 ml of 1000 mg l^{-1} KNO_3^-. The samples were incubated at 25°C with continuous agitation (150 rpm) for 8 h, with 1 mL of the liquid phase being removed at hourly intervals for NO_3^--N and NO_2^--N determination.

The potential nitrification activity was measured as nitrite and nitrate production rates. Incubations were performed in a similar manner as those for nitrate and nitrite reduction assays but under aerobic conditions. The rhizosphere homogenates were diluted with $(NH_4)_2SO_4$ to a final concentration of 210 mg l^{-1} NH_4^+-N, and liquid aliquots were collected hourly over a 24-h period. Samples for the analysis of NO_3^--N and NO_2^--N concentrations (1 ml) were removed from the homogenates, centrifuged for 2 min at 12 000 g, and filtered through a 0.20-μm filter. NO_3^--N and NO_2^--N concentrations were analyzed by high-performance liquid chromatography (HPLC) (PIA-1000, Shimadzu, Kyoto, Japan) using a 2 × 150-mm Shim-pack IC-A3 (S) analytical column. Rates were calculated from the first 5 h of incubation using linear-decay kinetics and standardized in terms of DW.

DNA extraction and PCR conditions

DNA was extracted from 0.25 g of each rhizosphere sample using the Power Soil TM DNA Isolation Kit (MO BIO Laboratories Inc, Carlsbad, CA, USA), following the manufacturer's protocol. PCR for the amplification of *nirS* was performed with the primer pair cd3aF (Michotey, Mejean and Bonin 2000) and R3cd (Throbäck *et al.*, 2004), while that for the amplification of *nirK* was performed with the primer pair F1aCu and R3Cu (Hallin and Lindgren 1999). These primer pairs have relatively high specificity and broad host coverage (Throbäck *et al.*, 2004). PCR for the amplification of bacterial-*amoA* was performed with primers amoA-1F and amoA-2R (Rotthauwe, Witzel and Liesack 1997), while that for the amplification of archaeal-*amoA* was performed with primers of Arch-amoAF and Arch-amoAR (Francis *et al.*, 2005). The GC clamp (5′-CCGCCGCGCGGCGGGCGGGGCGGGGGCACGGGG- 3′) described previously (Muyzer *et al.*, 1997) was added to the 5′ end of R3cd, R3Cu, amoA-2R and Arch-amoAR.

The PCR reaction mixture (50 μl) contained 1.25 U of TaKaRa Ex Taq HS DNA polymerase (TaKaRa Bio, Shiga, Japan), 1 × Ex Taq Buffer (TaKaRa Bio), 0.2 mM dNTPs, 0.5 μM each of the forward and reverse primers, 25 μg of BSA and 1 μl of sample DNA. Amplification reactions were performed in the Applied Biosystems 2720 Thermal Cycler for *nirK*, *nirS*, bacterial-*amoA* and archaeal-*amoA* (Applied Biosystems, California, USA), under previously described PCR conditions (Rotthauwe, Witzel and Liesack 1997; Throbäck *et al.*, 2004; Francis *et al.*, 2005). The detection and size of the amplified fragments were determined by agarose (1.0%) gel electrophoresis and UV transillumination following ethidium bromide staining.

DGGE analysis for *nirS*, *nirK* and *amoA*

DGGE was performed using the NB-1480A DGGE System (Nihon Eido, Tokyo, Japan) with an acrylamide concentration of 8% and a denaturing gradient of 35–65% for *nirS*, *nirK* and bacterial-*amoA*. DGGE was performed with an acrylamide concentration of 7% and a denaturing gradient of 25–55% for archaeal-*amoA*. Fixed volumes of PCR reaction mixture were applied, and electrophoresis was performed for 13 h at 60 V. Subsequently, the gels were stained with SYBR Green I (Generay Biotech, Shanghai, China), and a digital image of the gel was obtained with Chemi-Doc XRS (Milano, Bio-Rad, Italy). DNA bands were detected using the software Quantity One 4.6.2 (Milano).

Data analysis

All statistical analyses were performed on R version 2.15.1 (R Development Core Team 2013). Differences in the chemical and biological parameters among the three harvest treatments were analyzed using two-way analysis of variance (ANOVA) to compare the April and July rhizosphere samples. One-way ANOVA was performed to check for quantitative differences between samples within the same sampling time. A P-value < 0.05 was considered to be statistically significant. The differences were tested using Tukey's test. Relationships between the rhizosphere chemical parameters, potential nitrification and denitrification activities were explored using Pearson correlation analysis. Differences in regressions between sampling times were tested by analysis of covariance (ANCOVA).

Relationships between the differences in the composition of the *nirS*, *nirK*, bacterial-*amoA* and archaeal-*amoA* communities and differences in single variables (chemical parameters and

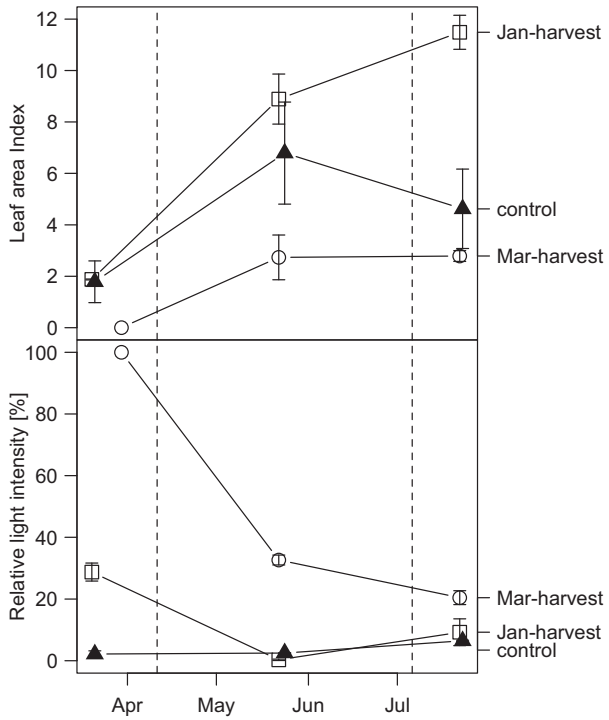

Figure 1. Seasonal variation in the LAI and relative light intensity of *P. australis* at the ground level in Jan-harvest, Mar-harvest and control stands. Bars and broken lines indicate mean standard deviation (SD) and rhizosphere sampling date, respectively.

biological variables) among the samples were determined by correlating dissimilarity matrices generated by the Bray–Curtis distance measure. For this purpose, the Mantel test was used with Monte Carlo simulations (999 randomizations).

The digitalized DGGE banding profiles were aligned for samples using a complete linkage clustering algorithm (Ishii, Kadota and Senoo 2009) and were used to perform non-metric multidimensional scaling (NMS) for the graphical representation of community relationships among samples. NMS was constrained to two dimensions with a random starting configuration for 100 iterations using BiodiversityR Package (Kindt and Coe 2005). To qualify the relationships between the environmental factors and community composition of nitrifiers and denitrifiers among the samples, the normalized chemical and biological variables were incorporated into analysis through the use of ordinations, where variables were combined into a secondary matrix and correlated with the NMS axes. The correlation between variables and NMS axes were represented as vectors to indicate the direction and strength of the correlation. Permutation tests ($n = 1000$) were performed to determine the significance of vector fits with NMS axes using the vegan package (Oksanen *et al.*, 2008).

RESULTS

Characteristics of the *P. australis* community

Canopy development and the relative light intensity shift of *P. australis* from 20 March to 24 July 2013 are shown in Fig. 1. The relative light intensity at the ground level was evaluated using the Beer–Lambert law, and different trends of development among harvest treatments were determined. The LAI reached the maximum of 11.5 ± 0.9, 2.8 ± 0.3 and 6.8 ± 2.8 in Jan-harvest, Mar-harvest and control plots, respectively. The dry matter production was relatively high for the Jan-harvest and low for the Mar-harvest plot (data not shown). Accordingly, the light intensity at the ground level showed different seasonal changes (Fig. 1B). LAI from the Mar-harvest plot showed lower values than that from the Jan-harvest plot. Although LAI in the control plots gradually increased, the relative light intensity was consistently low, varying between 2.1 and 6.4%.

Chemical and potential activity characteristics of rhizosphere samples

The chemical characteristics are shown in Table 1. The result of two-way ANOVA showed significant differences in NH_4^+-N and WSOC between the two sampling times and significant differences in NO_3^--N according to harvest treatment; however, no interactions between sampling times and harvest treatments were observed in any chemical parameters.

The average values of nitrite and nitrate production and reduction rates are shown in Fig. 2. Sampling time had no significant influence on nitrite and nitrate production and reduction rates; however, harvest treatment had a significant impact according to the result of a two-way ANOVA test. The results of the Tukey's test showed that the nitrite and nitrate production rates were significantly higher in the Jan-harvest and Mar-harvest samples than in the control samples ($P < 0.05$). The nitrate and nitrite reduction rates were significantly higher in the Mar-harvest samples than in control samples ($P < 0.05$).

To identify potential contributors to the changes observed in the rhizosphere activity, the chemical and potential activity characteristics of the rhizosphere samples were subjected to Pearson correlation analyses. A significant correlation between nitrite and nitrate production rates and nitrate and nitrite reduction rates was observed by analyzing all data ($P = 0.004$, $r = 0.65$).

Table 1. Chemical characteristic of rhizosphere samples. Data correspond to mean values and SDs of three replicate samples.

Sampling time	Harvest treatment	Chemical characteristics			
		NO_2^--N (mg N g DW^{-1})	NO_3-N (mg N g DW^{-1})	NH_4^+-N (mg N g DW^{-1})	Water-soluble organic carbon (mg g DW^{-1})
April 2013	Jan-harvest	0.0045 ± 0.0028	0.059 ± 0.041	0.26 ± 0.04	0.18 ± 0.01
	Mar-harvest	0.0015 ± 0.0011	<0.001	0.16 ± 0.08	0.14 ± 0.04
	Control	0.0012 ± 0.0008	<0.001	0.25 ± 0.07	0.17 ± 0.02
July 2013	Jan-harvest	0.0048 ± 0.0025	0.026 ± 0.008	0.57 ± 0.16	0.41 ± 0.08
	Mar-harvest	0.0028 ± 0.0001	0.036 ± 0.018	0.37 ± 0.07	0.27 ± 0.05
	Control	0.0022 ± 0.0004	0.013 ± 0.003	0.33 ± 0.05	0.31 ± 0.11

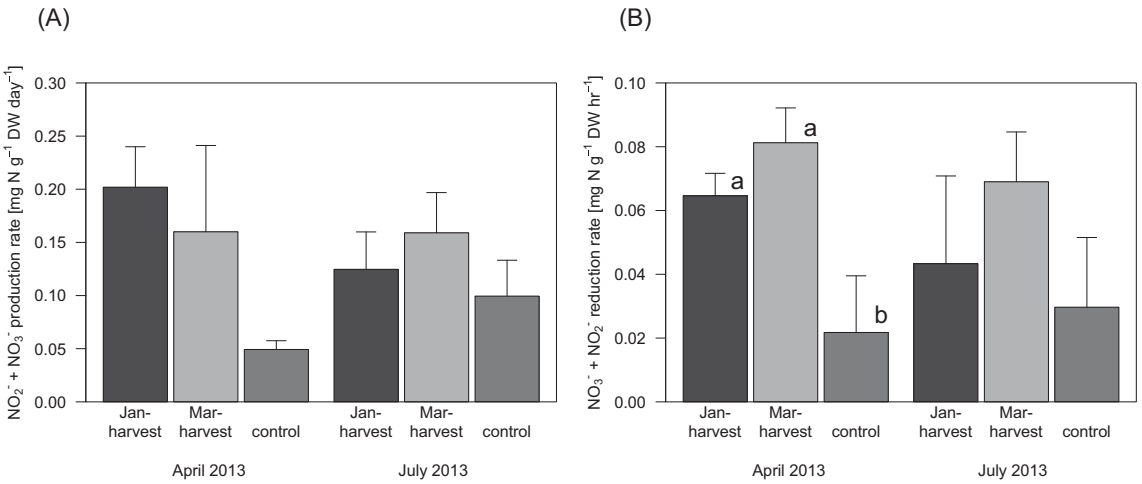

Figure 2. Potential nitrate and nitrite production rates **(A)** and potential denitrification rates **(B)** from April and July rhizosphere samples. Rates are expressed as means and SDs of three replicates. Different letters above the bars indicate significant differences ($P < 0.05$) between samples within each sampling date according to Tukey's test.

Approximately 42% of the variance in the nitrate and nitrite reduction rate was explained by the nitrification rate. The slopes and intercepts were not significantly different between the two sampling times according to the result of ANCOVA.

Relationships between rhizosphere parameters and nitrifier and denitrifier communities

NMS ordinations of DGGE profiles are shown in Figs 3–6. NMS ordinations of *nirK*, *nirS* and archaeal-*amoA* clearly revealed seasonal community variations and differences between each harvest treatment (Figs 3, 4 and 6), while that of bacterial-*amoA* revealed neither community shifts between sampling times nor differences due to the harvest treatment (Fig. 5).

The result of Mantel tests and permutations testing for significance of vector fit to the NMS axes in ordinations are shown in Table 2. The Mantel tests between environmental parameters and dissimilarity matrices and the permutation tests of vector fit to the NMS ordination indicated similar results. The band diversity of *nirK* and *nirS* within rhizosphere communities did not correlate with nitrate and nitrite reduction rates, but the *nirK* band diversity was significantly affected by nitrite and nitrate production rates, according to the result of Mantel tests (Table 2). In addition, the diversity of *nirK* bands had significant relationships with NO_3^--N and NO_2^--N concentrations, and that of *nirS* bands had significant relationships with NO_3^--N concentrations and WSOC according to the results of Mantel and permutation tests. The result of Mantel test also revealed significant relationships with NO_3^--N and NO_2^--N concentrations in the band diversity of bacterial-*amoA* and archaeal-*amoA*.

DISCUSSION

In general, potential nitrification and denitrification activities were higher in the harvest treatment plots than in the non-harvested control plot according to the result of a two-way ANOVA test and the following Tukey's test ($P < 0.05$). This indicates that plant harvest treatment is a key determinant of the capacity of nitrogen removal in CWs. Differences in plant development between harvest occasions over the growth season were observed, and the relative light intensity was higher in the Jan-harvest and Mar-harvest plots than in the control in the early growing season (Fig. 1). Light intensity affects ground temperature, as reported by Granéli (1989) who found that winter harvest resulted in an increase in water temperatures. Several researchers reported that temperature is one of the most important factors for enhancing the activity and for influencing the community structure of nitrifiers (Avrahami, Liesack and Conrad 2003; Urakawa *et al.*, 2008) and denitrifiers (Braker, Schwarz and Conrad 2010; Song *et al.*, 2012). This may provide a partial explanation for the higher potential nitrite and nitrate production and reduction rates observed in the harvested plots. We also observed a significant difference in potential nitrate and nitrite reduction activity between harvest and control plots within April samples but not within July samples (Fig. 2B). Furthermore, the April sampling time revealed that the nitrite and nitrate production activities were slightly higher in the Jan-harvest area (0.20 $\pm$ 0.04 mg N g^{-1} DW day^{-1}) than in non-harvested control (0.05 $\pm$ 0.01 N g^{-1} DW day^{-1}) although this difference was not significant ($P = 0.058$). This coincided with a shift of the relative light intensity at the bottom (Fig. 1), indicating that the effect of plant harvest on the microbial activity in July was weaker than that in April. Furthermore, several researchers reported that oxygen released from aquatic macrophytic roots into the surrounding sediment promotes the coupled nitrification–denitrification rate (Bodelier *et al.*, 1996; Risgaard-Petersen and Jensen 1997). Because it is known that the main aerating mechanism in *P. australis* and other aquatic macrophytes depends on the temperature, solar radiation and stage of plant development (Armstrong and Armstrong 1990; Caffrey and Kemp 1991), harvesting and the regrowth of plant may induce consecutive change of this dynamic system and affect oxygenation of the rhizosphere. This may also have influenced the function and community structure of nitrifiers and denitrifiers, as we discuss later. Our results suggest that plant harvest may be an effective way to improve nitrification and denitrification during the early growing stage until the maturity of canopy. However, our assessment of nitrification and denitrification was based on the potential enzyme activity. Thus, *in situ* measurements of nitrification and denitrification are required to evaluate the contribution to nitrogen removal in CWs. The effect of harvesting on both plant nutrient uptake and microbial processes (nitrification and denitrification)

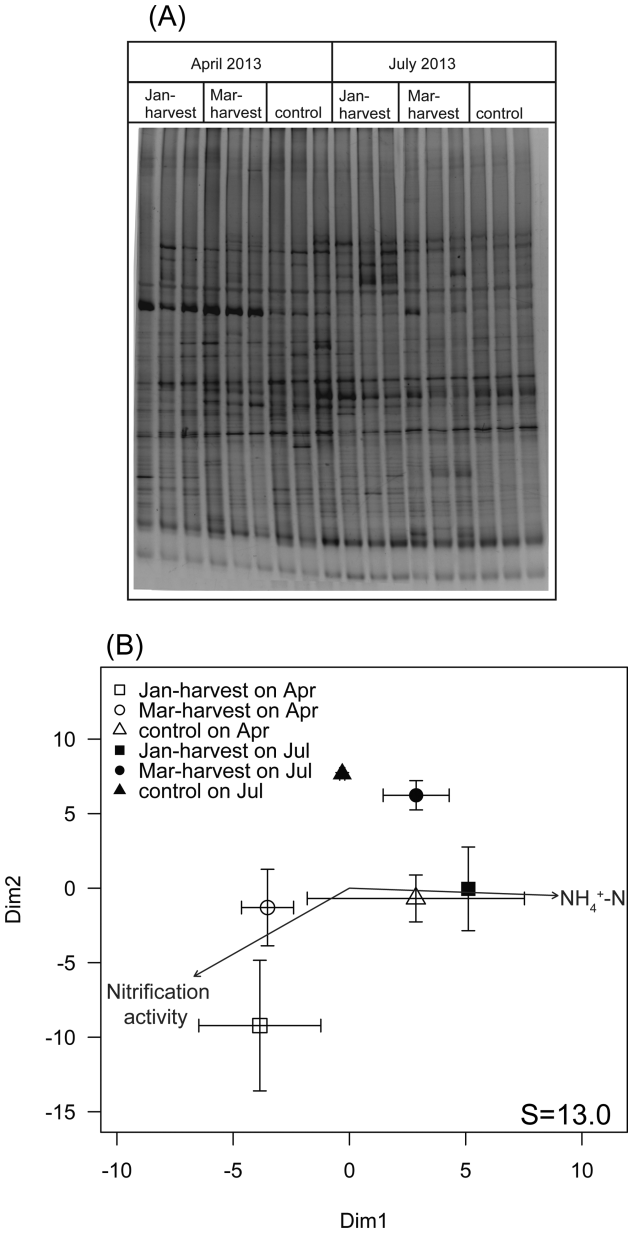

Figure 3. DGGE profile **(A)** and NMS ordinations of *nirK* **(B)** with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors. Correlations with *P*-values of <0.1 are shown as vectors. The arrow lengths are proportional to the strength of the correlation, and the vector orientation indicates the directions in which they have the maximum correlation with the ordination configuration. Stress values (*S*) for all ordinations are indicated. Means and SDs of three replicates are represented by symbols and bars, respectively. Open symbols refer to samples collected in April 2013 and filled symbols to samples collected in July 2013. Different shapes indicate plant harvest treatments (squares: Jan-harvest; circles: Mar-harvest; triangles: control).

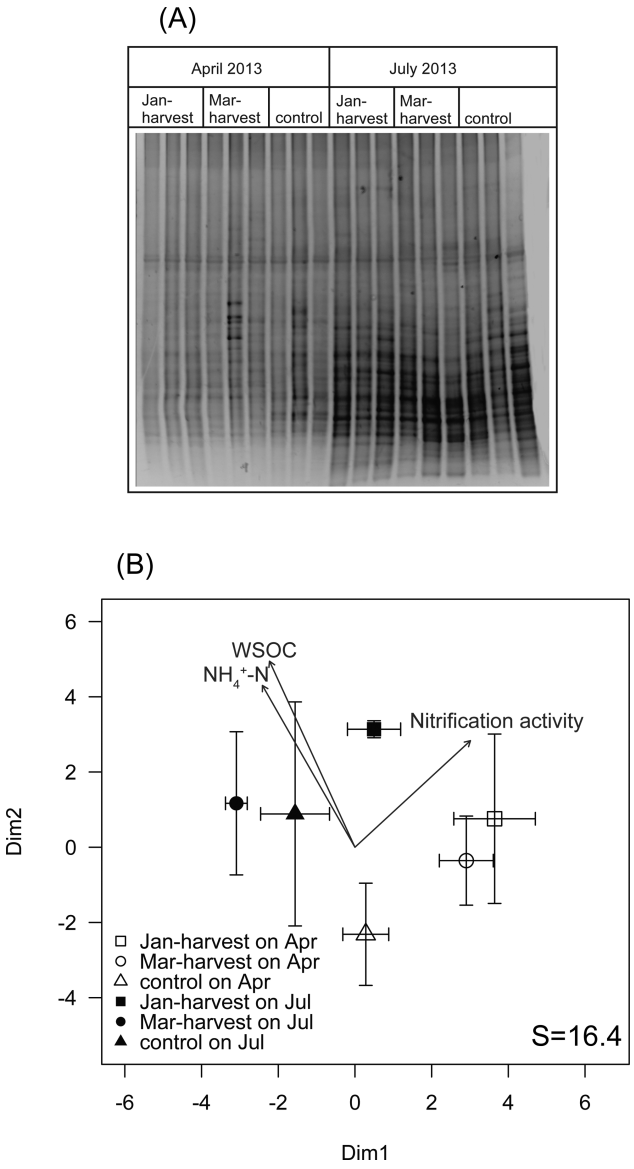

Figure 4. DGGE profile **(A)** and NMS ordinations of *nirS* **(B)** with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors. Correlations with *P*-values of <0.1 are shown as vectors. The arrow lengths are proportional to the strength of the correlation, and the vector orientation indicates the directions in which they have the maximum correlation with the ordination configuration. Stress values (*S*) for all ordinations are indicated. Means and SDs of three replicates are represented by symbols and bars, respectively. Open symbols refer to samples collected in April 2013 and filled symbols to samples collected in July 2013. Different shapes indicate plant harvest treatments (squares: Jan-harvest; circles: Mar-harvest; triangles: control).

should be quantitatively estimated to optimize vegetation management (e.g. timing and frequency of harvesting) for the nitrogen removal.

Most studies have been unable to elucidate the link between the structure and function of denitrifiers (Rich and Myrold 2004; Boyle *et al.*, 2006; Song *et al.*, 2012), although the denitrification activity has been found to correlate with the abundance of the denitrifier community (Philippot *et al.*, 2009; Enwall *et al.*, 2010). In the present study, we were unable to demonstrate direct relationships between the potential nitrate reduction activity and the band diversity of *nirK* and *nirS* (Table 2). However, the potential nitrification activity showed a significant correlation with community structures of *nirK* and the potential nitrate and nitrite activity. Accordingly, we suggest the possibility that plant harvest and the subsequent plant regrowth affects the oxygen availability in the rhizosphere. This would mean that the dissimilatory reduction of nitrite and nitrate is accompanied by the use of oxygen as electron acceptor in rhizosphere. In turn, this means that a correlation between the reduction of the nitrogen oxides and the community composition of denitrifier is not only

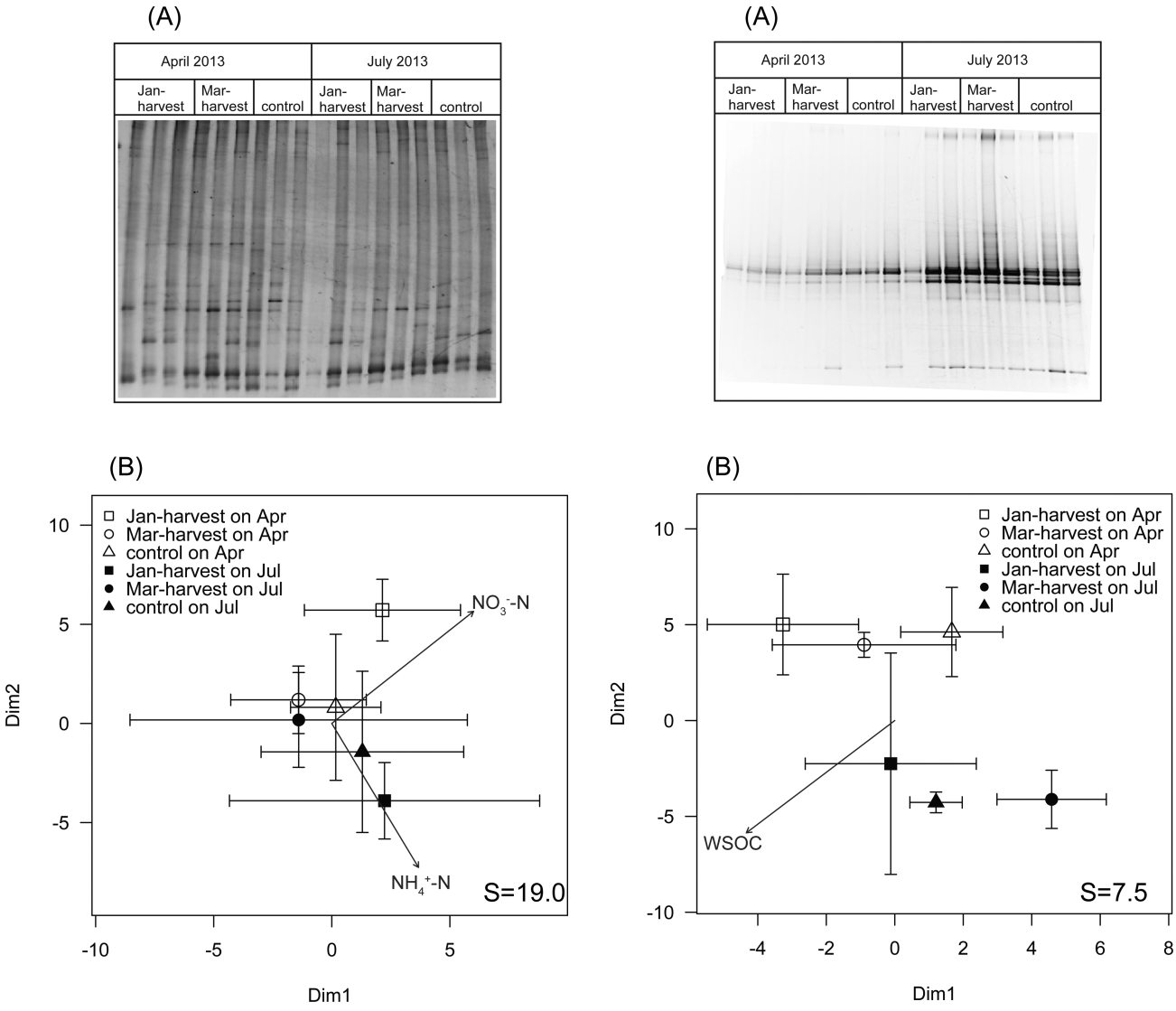

Figure 5. DGGE profile **(A)** and NMS ordinations of bacterial-*amoA* **(B)** with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors. Correlations with *P*-values of <0.1 are shown as vectors. The arrow lengths are proportional to the strength of the correlation, and the vector orientation indicates the directions in which they have the maximum correlation with the ordination configuration. Stress values (*S*) for all ordinations are indicated. Means and SDs of three replicates are represented by symbols and bars, respectively. Open symbols refer to samples collected in April 2013 and filled symbols to samples collected in July 2013. Different shapes indicate plant harvest treatments (squares: Jan-harvest; circles: Mar-harvest; triangles: control).

Figure 6. DGGE profile **(A)** and NMS ordinations of archaeal-*amoA* **(B)** with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors. Correlations with *P*-values of <0.1 are shown as vectors. The arrow lengths are proportional to the strength of the correlation, and the vector orientation indicates the directions in which they have the maximum correlation with the ordination configuration. Stress values (*S*) for all ordinations are indicated. Means and SDs of three replicates are represented by symbols and bars, respectively. Open symbols refer to samples collected in April 2013 and filled symbols to samples collected in July 2013. Different shapes indicate plant harvest treatments (squares: Jan-harvest; circles: Mar-harvest; triangles: control).

governed by their nitrate and nitrite reduction capacity, because most denitrifiers prefer to use oxygen under aerobic conditions (Averill 2007). Our results indicate that plant harvest has a potential to promote nitrification and subsequently enhance denitrification. The weak correlation of the band diversity of denitrifiers with the nitrification potential may be coupled to the effect of the presence of oxygen promoting nitrification, which may also have affected the denitrifiers and thereby resulting in shifts in the denitrifier community structure and especially so for the *nirK* populations. In this context, it should be noted that the potential nitrate and nitrite reduction rates used as an indication of denitrification in this study may also include assimilatory and dissimilatory nitrate and nitrite reduction to ammonium by microorganisms and plants (Tiedje *et al.*, 1982). This would further corroborate possible correlations between the nitrate and nitrite reduction rates and the band diversity of denitrifiers.

WSOC did not correlate with the potential denitrification activity at any of the sampling occasions, although WSOC is usually considered a limiting factor for denitrification in various environments (Burford and Bremner 1975; Jahangir *et al.*, 2012), and plant root exudates containing labile carbon compounds have been observed to stimulate denitrification in the rhizosphere (Woldendorp 1962; Nguyen 2003). This suggests that in the present study site, the temperature regime resulting from changes in the light intensity and oxygen availability may affect microorganisms in the rhizosphere, resulting that nitrification

Table 2. Mantel tests and results of permutation testing for significance of vector fit to NMS axes in ordinations.

Parameter	Mantel tests (r)				Vector fit in NMS ordination (R^2)			
	nirK	*nirS*	Bacterial-*amoA*	Archaeal-*amoA*	*nirK*	*nirS*	Bacterial-*amoA*	Archaeal-*amoA*
NO_2^--N	0.33*	0.09	0.34*	0.39***	0.18	0.15	0.24	0.21
NO_3^--N	0.49**	0.19*	0.30*	0.40**	0.20	0.05	0.33*	0.14
NH_4^+-N	0.03	0.10	0.29*	0.20	0.26	0.39*	0.32	0.15
WSOC	0.07	0.17*	0.07	0.18	0.23	0.47**	0.13	0.34*
Nitrite + nitrate production rate	0.22*	0.11	−0.02	0.11	0.26	0.28	0.16	0.23
Nitrate + nitrite reduction rate	−0.02	−0.10	−0.06	−0.08	0.16	0.07	0.09	0.08

*$P < 0.05$; **$P < 0.01$; ***$P < 0.001$.

might be a key limiting factor for denitrification rather than WSOC. However, as noted above the nitrate and nitrite reduction may include also a dissimilatory reduction to ammonium, which is the dominating pathway at high levels of easily degraded organic compounds (Tiedje *et al.*, 1982). The shift in the band diversity of *nirS* significantly correlated with WSOC and ammonium concentrations, which may be an indication of that this pathway is in operation, since some denitrifiers have this capacity as well (Samuelsson 1985) (Table 2). A seasonal difference would be expected since there was a difference in WSOC observed for the two sampling times.

The DGGE profiles indicate relatively low diversities in bacterial and archaeal-*amoA* (Figs 5A and 6A). The observed band diversity of bacterial-*amoA* was not affected by plant harvest, while the archaeal-*amoA* showed the changes (Figs 5B and 6B). No significant relationship was observed between the band diversity of archaeal-*amoA* and the potential nitrification activity. Recently, the presence of AOA has been demonstrated in various environments, revealing that the abundance of AOA was higher than that of AOB in freshwater sediments (Herrmann, Saunders and Schramm 2009), natural wetland sediments (Sims *et al.*, 2012) and soil ecosystem (Leininger *et al.*, 2006). Nicol *et al.* (2008) found that different AOB and AOA phylotypes are selected by specific pH values and that differences in phylotype abundance are reflected by different contributions to ammonia oxidizer activity. Available ammonia is also a major factor for selecting certain types of rhizosphere-associated ammonia oxidizers (Bollmann, Bär-Gilissen and Laanbroek 2002; Herrmann, Saunders and Schramm 2009). Similarly, different ammonia-oxidizing communities, especially of AOA, may be selected in the changing microenvironments resulting from harvest, and introduce new specific phylotypes as contributors to the nitrification activity. Several researchers reported that the majority of AOB in CWs were phylogenetically affiliated to *Nitrosospia* species (Gorra *et al.*, 2007) or *Nitrosomonas* species (Ruiz-Rueda, Hallin and Bañeras 2009). Furthermore, Hatzenpichler (2012) reported three AOA phylogenetic groups (*Nitrosopumilus*, *Nitrosotalea* and *Nitrososphaera* cluster) to be present in freshwaters and sediments. However, to further develop this picture and elucidate the role of the AOA as well as the AOB, simultaneous analyses of taxonomic composition and gene expression for 16S rRNA and functional genes are required. Physicochemical assessment of rhizosphere responsible for nitrifier and denitrifier populations are also essential to provide feedbacks for improvement of CWs managements.

In conclusion, we observed no direct links between the community structure and activity of denitrifiers but demonstrated that plant harvest enhanced the coupled nitrification–denitrification activity and caused shifts in the band diversity of *nirK*, *nirS and* archaeal-*amoA*. Our results suggest that plant harvest could influence subsequent plant development and the microenvironment, thereby impacting the function and community structure of nitrifiers and denitrifiers. Our findings suggest that it is important to evaluate the effect of plant harvest management not only on the capacity of nitrogen uptake by plant regrowth, but also on nitrification and denitrification activities in order to optimize sustainable and effective nitrogen removal of CWs.

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