

Studies on reed (*Phragmites*) roughage production from lakeshore
vegetation for the optimization of nitrogen cycling in the basin of Lake
Dianchi, Yunnan, China

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Chapter 1

Introduction

1.1 Background

Nitrogen (N) is one of the most essential nutrients in most ecosystems (LaBauer and Treseder, 2008). After the invention of the Haber-Bosch process in the 20th century, a large amount of N became available as chemical fertilizer. After World War II, European, North American and Japanese agriculture were the first beneficiaries of inexpensive nitrogenous fertilizer (Smil, 2011). By the 1960s, increasing N applications made it possible to achieve high yields with new short-stalked wheat and rice cultivars planted in the low-income countries of Asia and Latin (Smil, 2011). Consequently, it has significantly increased the amount of food production and has contributed to population growth. However, the inefficient overuse of chemical fertilizer may have resulted in surplus nutrient accumulation in soils, which may erode or leach into surface and ground water or enter the atmosphere (Vitousek et al., 1997). Phosphorus (P) is also one of the crucial nutrients for agriculture. After the 1950s, increasing applications of P fertilizer would either remain in the soils or be transferred to surface waters by erosion or leaching (Carpenter et al., 1998).

By the mid-20th century, eutrophication problems had been widely recognized by people who lived near lakes because of increased algal scums, macrophyte growth, and periodic fish kills (Schindler, 2006). For instance, Lake Biwa, the largest lake in Japan, has suffered severe eutrophication because of an increasing population since the 1970s. Although the frequency of red tides has been reduced with the introduction of sewage systems and the improvement of its efficiency, the chemical oxygen demand and NO_3 and PO_4 concentrations in the surface layer water have not been substantially improved due to N and P loads originating from agriculture (Nakano et al., 2008). Indeed, the management of reducing point sources, including domestic and industrial wastewater, has quickly contributed to the control the eutrophication. However, it is difficult to control loading from a non-point source (NPS) such as agriculture.

China, the world's most populous country, has become the largest user, as well as the largest producer of chemical fertilizer (FAO, 2015). Consequently, the inputs of anthropogenic N and P have sharply increased and have caused serious issues in the eutrophication of lakes, rivers, and estuaries. In general, the direct causal relationship between agriculture and

eutrophication remains unclear, and it is difficult to evaluate the environmental loads quantitatively. For that reason, water quality has not been improved although there is a pressing need to solve the issues of eutrophication.

1.2 Impact of intensive agriculture on eutrophication of Lake Dianchi

Lake Dianchi has become the largest eutrophic lake in the Yunnan Province of China, and has been listed in China's 'Three Important Lakes Restoration Act.' Population growth, increasing applications of fertilizer, and the changes in cropping intensity and cropping varieties have been observed since the 1990s due to Chinese economic reform in Kunming, Yunnan Province, China (Statistical Bureau of Yunnan, 1985–2005, 2007–2010). The eutrophication of this lake is serious as algal blooms break out almost every summer, and the lake water cannot be used for drinking water. Sun et al. (2010) reported that approximately 1/3 of the total nitrogen (TN) and 1/4 of the total phosphorus (TP) were provided by NPS pollution in Lake Dianchi. Therefore, NPS pollution from agriculture or rural areas is the key point in the recovery of Lake Dianchi (Feng et al., 2008).

Plastic greenhouse cultivation is rapidly expanding in China because of its high economic benefit (Cheng et al., 2011). In the coastal area of southeast Lake Dianchi, wide areas for paddy rice-broad bean cropping systems were converted to multiple vegetable and flower fields with greenhouses in the 2000s. The primary land use of northern area of Lake Dianchi is a populated city, and the southern area is agricultural land. The land use distribution of the southeast coastal area of Lake Dianchi is shown in Figure 1-1. Most of the artificially covered areas are greenhouses. The previous paddy rice-broad bean cropping system was less dependent on external inputs of N and P fertilizer than the present multiple cultivation of vegetables and flower. After 15 to 20 years of land use change from a paddy rice-wheat double cropping system to continuous vegetable land around Lake Taihu in Jiangsu, China, nitrate N leaching and P runoff from vegetable lands might have a high potential to induce eutrophication of the lake (Cao et al., 2004). Furthermore, the amount of applied chemical fertilizer around Lake Dianchi is higher than the national average level in China (Duan et al., 2003).

The number of domestic animals has also increased recently in this area (Statistical Bureau of Yunnan, 1985–2005, 2007–2010). It is assumed that the excretions of domestic animals might have an indirect impact on the water system in this study area because the excreta are sold to neighboring farmers and applied as manure in their fields. In the southeast coastal areas of Lake Dianchi, the N and P outputs through animal manure onto cropland are higher

than those inputs into the livestock production systems from the local cropland (Anzai et al., 2016). Consequently, the inputs of N and P as chemical and organic fertilizer have enormously increased at Lake Dianchi.

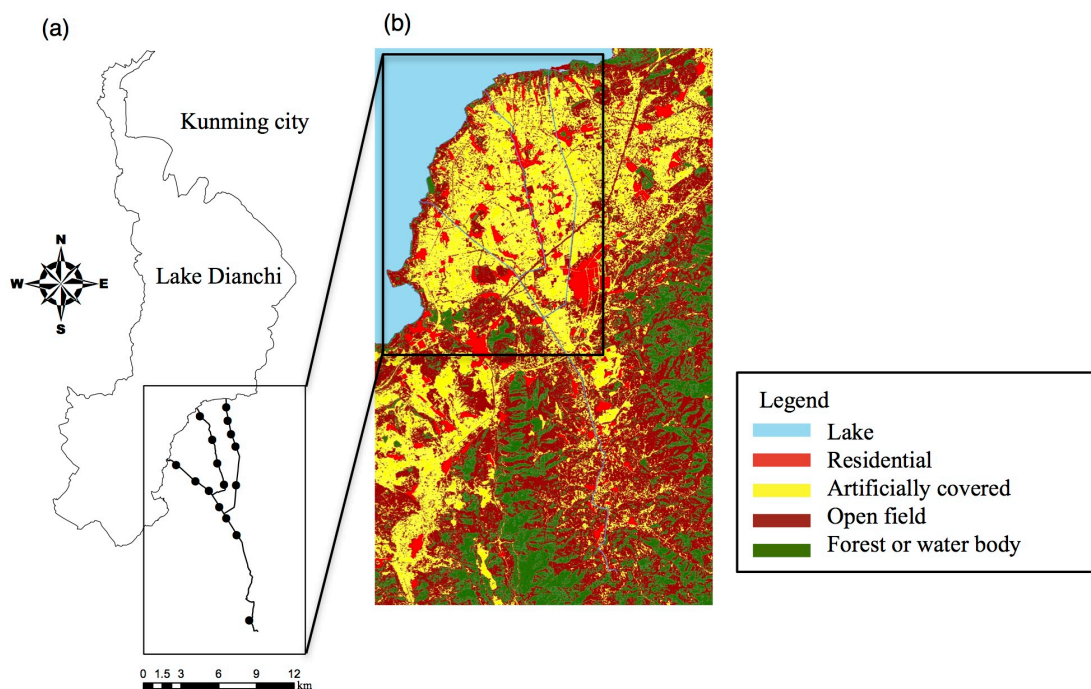


Figure 1-1 Map of Lake Dianchi and the location of the study site for the water survey (a), land use distribution of the southeastern coastal area (b).

Note: Solid lines represent river watercourse. Black solid points represent water sampling sites.

Water quality was surveyed at a river that may be affected by agricultural land use, as shown in Figure 1-1. Mean TN and nitrate N (NO_3^- -N) concentrations were observed having a comparatively strong correlation to the 3-day cumulative precipitation before the sampling date, as shown in Figure 1-2. It was suggested that increases in TN and NO_3^- -N concentration might have resulted from agricultural runoff or the percolation of pollutants in the soil by precipitation, which is due to the high soluble mobility of nitrate and the high NO_3^- -N percentage of TN. Thus, the excess of nutrients may accumulate in the soil via the overuse of fertilizer, which in turn may be released to the water system during rainfall events.

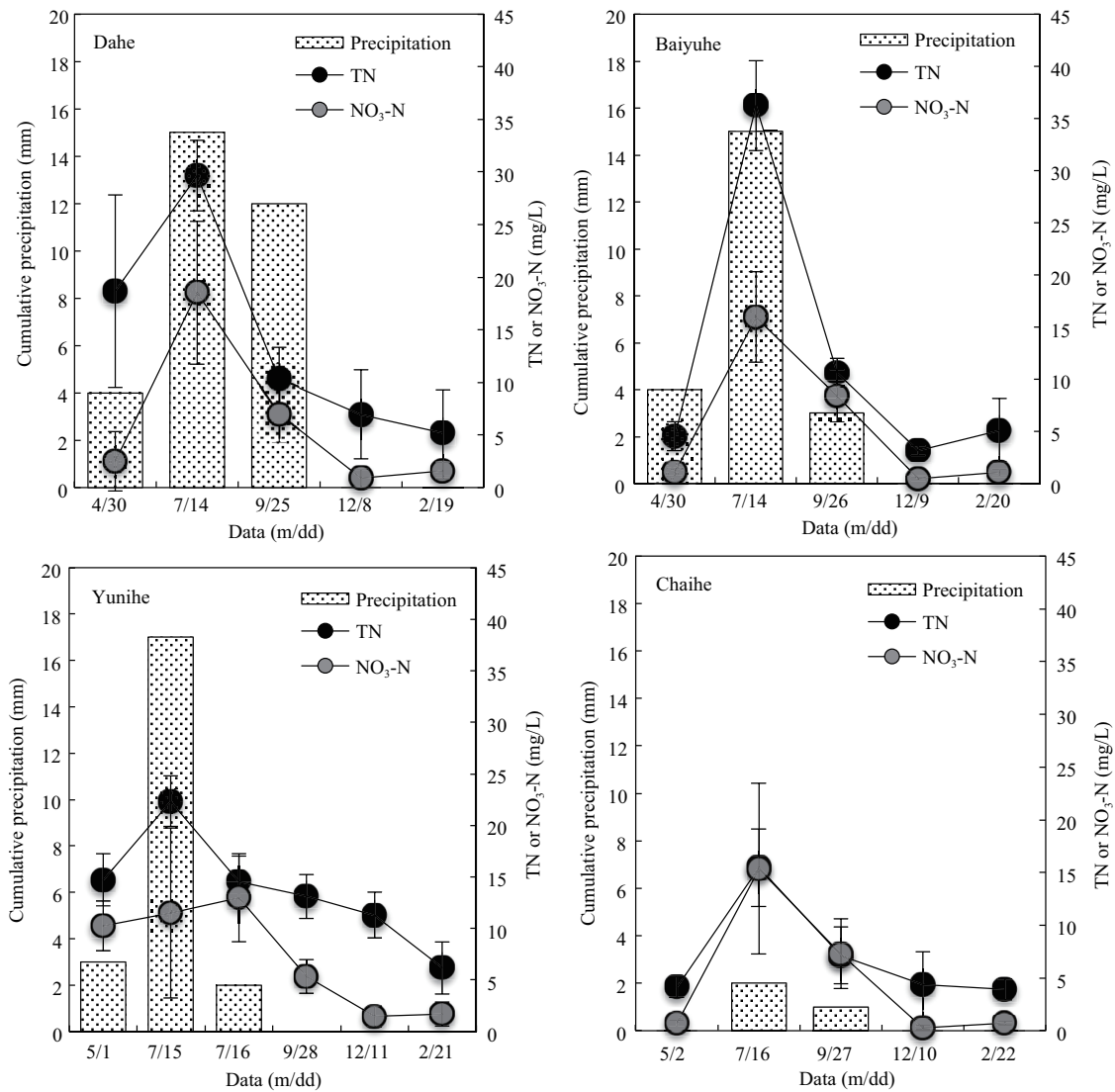


Figure 1-2 Relationships between the 3-day cumulative precipitation before sampling and water quality variables, total nitrogen (TN), and nitrate nitrogen (NO₃-N).

1.3 Approaches to the solution

A diagram of N dynamics at Lake Dianchi is shown in Figure 1-3. As noted above, the recent changes in land use and the livestock industry have evoked unbalanced N cycles at the study site. A large amount of feed or chemical fertilizer is imported from the outside boundary. To tackle the issue of eutrophication at Lake Dianchi, there are two primary approaches: (1) reducing external nutrient inputs by improving nutrient use efficiency on farmland and (2) removing excessive nutrients within the system boundary. For the former approach, the amount

of applied chemical fertilizer can be reduced by half of the local conventional application rate without vegetable yield loss, as documented by Wang et al. (2016). For the latter approach, the local government have begun to introduce artificially created riparian zones or constructed wetlands (CWs) along the coastal areas of Lake Daichi. However, the vegetation in the CWs has not been harvested because of the capital costs of harvesting, although several researchers have reported that plant harvesting could improve nutrient removal, especially in the productive region (Álvarez & Bécares, 2008). Increasing the utilization of local feed material in animal production can facilitate nutrient cycling within a system (Anzai, 2016). Therefore, we hypothesized that harvested biomass in CWs can be used as roughage, and it may enhance the inner nutrient cycles because harvesting could improve nitrogen removal from the CWs and contribute to the reduction of livestock dependence on external feed. In the present study, we assumed that reed (the genus *Phragmites*) growing in the CWs could be a great potential source for roughage because of the dominant growing area (approximately 38 ha), as shown in Figure 1-4.

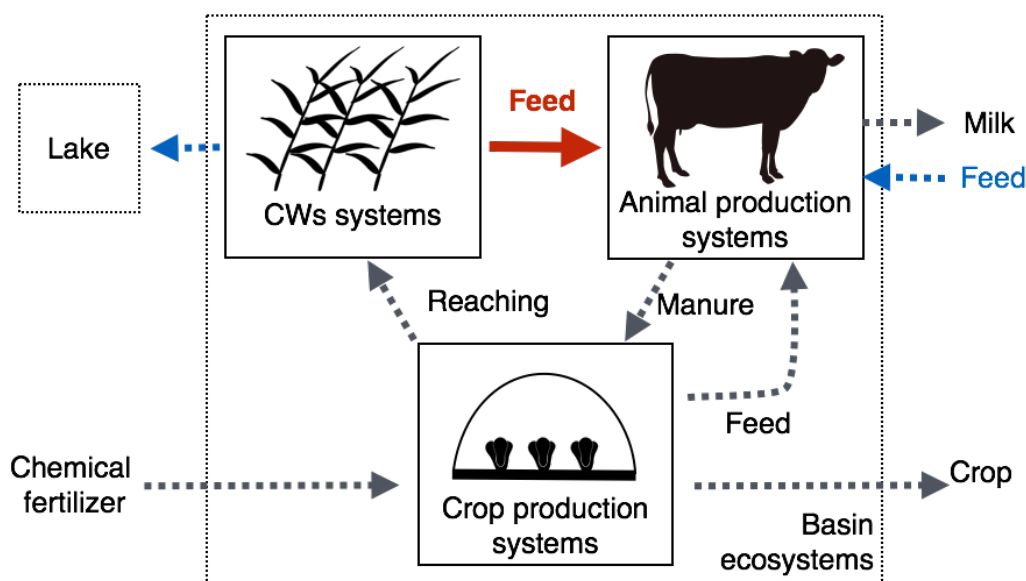


Figure 1-3 Flow diagram of nitrogen in the Lake Dianchi basin.

Note: Red and blue arrows represent the increased and reduced N flow by reed roughage production, respectively. CWs indicate constructed wetlands.

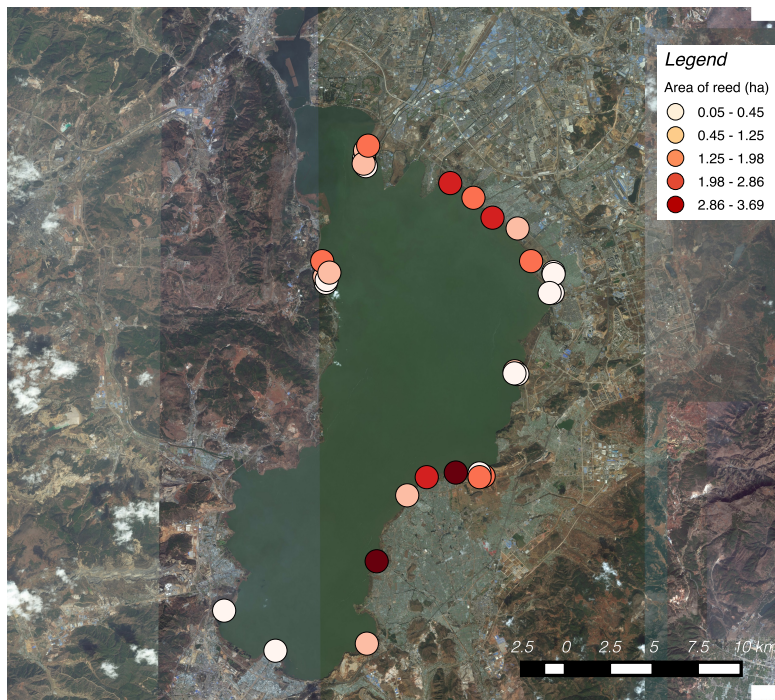


Figure 1-4 Distribution of *Phragmites* species around Lake Dianchi.

1.4 Research objectives

The objectives of this study were to evaluate reed (*Phragmites* sp.) management for roughage production and N removal at CWs from an agronomic, genetic, and environmental perspective. The structure of this thesis is shown in Figure 1-4. In Chapter 2, the effects of plant harvesting on canopy structure and nutritive value of common reed (*P. australis*) were analyzed to understand the basic information on reed roughage production. The plant canopy developed differently after harvest and simultaneously changed the microclimate beneath the plant community. In Chapter 3, the impact of harvest management on the function and community structure of microbes responsible for the N cycle was also investigated to understand whether harvest management has a positive effect on microbial N removal. During field research at Lake Dianchi, I found that *P. australis* and *P. japonicus* were the dominant species. *Phragmites* species originating from different habitats exhibited both intraspecific and interspecific differences in morphological traits and heading time that may be genetically determined. In Chapter 4, phylogenetic analyses of the *Phragmites* species were performed in southwest China (Yunnan, Sichuan, Guizhou, Chongqing, and Guangxi Province). In Chapter 5, the effects of harvest timing and frequency on yield and nutritive value were evaluated in the two *P.*

japonicus communities with different heading timings and genotypes. In Chapter 6, I discuss and answer the following two questions: (1) is harvesting a necessary and feasible practice; (2) what should be done to improve the management for reed roughage production at CWs in terms of N removal, biomass production, and nutritive quality?

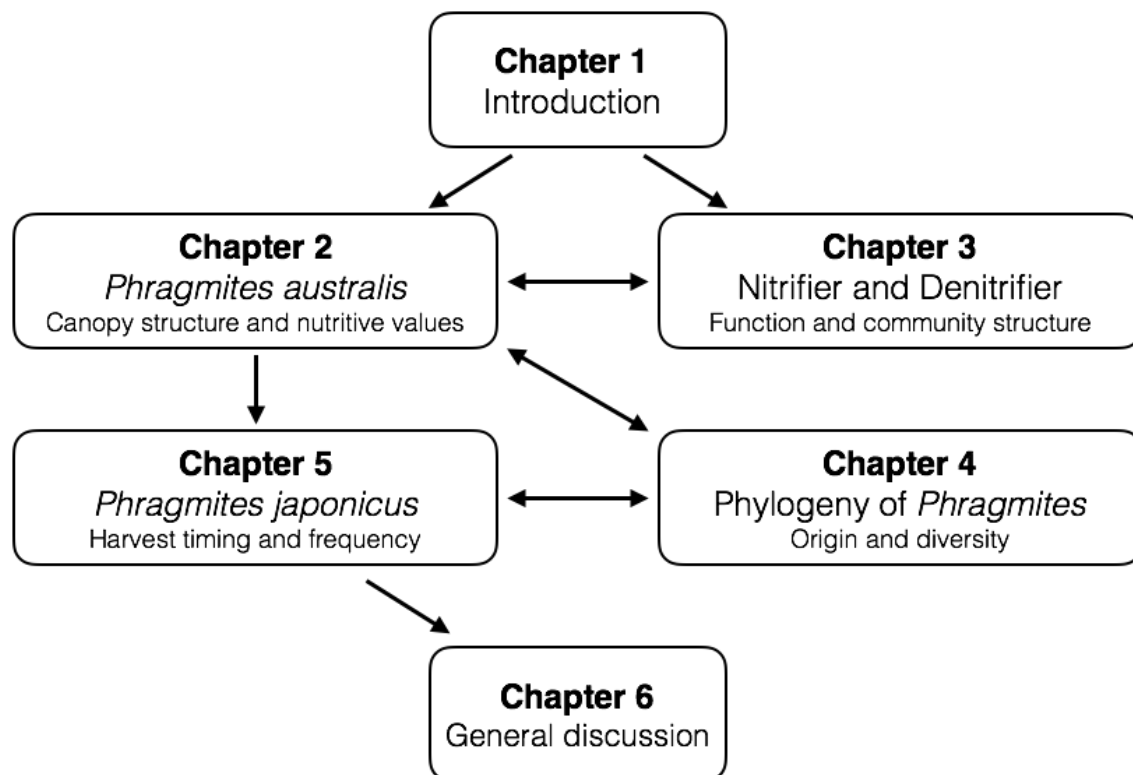


Figure 1-5 Structure of this thesis.

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Chapter 2 Timing of harvest of *Phragmites australis* (CAV.) Trin. ex Steudel affects subsequent canopy structure and nutritive value of roughage in subtropical highland

2.1 Introduction

Common reed [*Phragmites australis* (CAV.) Trin. ex Steudel] is a cosmopolitan perennial emergent macrophyte species mainly distributing in lakesides, along rivers and in marshes. The reed has a high ability of biomass production and dominating of aquatic habitats, whose introduction and rapid expansion can threaten natural ecosystems (Saltonstall et al., 2010). On the other hands, the reed is the most frequently used plant species worldwide in constructed wetlands (CWs) for wastewater treatment because of its high biomass production and nutrient uptake. CWs provide high filtration of pollutants from wastewater arising from sewage, domestic discharge, and agricultural drainage with low investment and operation costs. Currently, CWs vegetated with reed is an attractive technology for removal of metal (Gikas et al., 2013) and BTEX (Ranieri et al., 2013) as well as practice for removing nutrients.

Harvesting aboveground biomass is a recommended practice for control and/or managing the growth (Asaeda et al., 2006) and improving nitrogen (N) removal of CWs in productive areas (Álvarez and Bécares, 2008; Koottatep and Polprasert, 1997). On the other hand, biomass waste produced by CWs has become a problem in many places (Kadlec and Wallace, 2009; Liu et al., 2012). As a consequence of difficulties of capital cost of harvesting, plant harvest is not favored for nitrogen removal (Crites and Tchobanoglous, 1998). It is necessary to find uses for harvested biomass, as well as new sources of income for local communities, when macrophyte cultivation is applied for ecological restoration (Köbbing et al., 2013).

Increases in the demand for livestock products have been driven largely by human population growth, income growth and urbanization (Thornton, 2010). Thus, human population growth throughout developing countries increases demand for feed all over the world. Encouraging bioenergy production could directly compete with forage-livestock production (Sanderson and Adler, 2008). Moreover, unstable production of feed has been caused by recent abnormal weather. Therefore, exploitation of alternative feed resources is necessary to meet the increasing demand. In recent years, the reed has attracted increasing interest for its potential as high-quality roughage because of its high N content, neutral detergent fiber (NDF), potassium, and magnesium (Baran et al., 2002; Kadi et al., 2012). From the viewpoint of potential large areas of reed bed (Guo et al., 2013) and the increasing number of CWs for wastewater treatment

(Kadlec and Wallace, 2009), the reed could be a valuable source of roughage.

Efforts are required to manage the growth of *P. australis* and the nutrient values for roughage. The timing of the harvest of aboveground biomass of reed is one possible strategy, since it affects the annual rhizome resource allocation and aboveground regrowth (Asaeda et al., 2006). It has the potential to reduce subsequent regrowth because rhizome reserves are reallocated from the aboveground biomass (Karunaratne and Asaeda, 2004; Kühl et al., 1997). Several studies have reported that winter harvest promotes subsequent aboveground regrowth (Granéli, 1989; Hansson and Granéli, 1984). On the other hand, contradictory reports have found that winter harvest does not affect aboveground biomass (Bjorndahl, 1985). Karunaratne and Asaeda (2004) reported that early summer harvest negatively affected regrowth, whereas Güsewell (2003) showed early summer harvest had no effect on biomass production. Furthermore, Fogli et al. (2014) reported that winter and summer harvesting only slightly decreased aboveground biomass in the riparian community, did nothing in the intermediate community, but significantly diminished biomass in fen meadows. Thus, the quantity of aboveground biomass production affected by the timing of harvest varies with locations and conditions.

To determine the most efficient vegetation management for sustainable utilization of reed bed, it is essential to understand the effect of harvest timing on aboveground biomass production. In addition, standing reed litter can have a direct detrimental effect on the reed itself, probably due to the creation of shade (Granéli, 1989). Thus, harvesting may influence canopy structure and morphology. It is also important to make clear the effect of harvesting on development of canopy structure for a better understanding of subsequent biomass production. The canopy structure has been investigated by several researchers, which just sampled once for the growing season (Hirose and Weger, 1995; Hirtreiter and Pitts, 2012). Currently, little is known about seasonal changes of canopy structure.

As noted above, the timing of harvest is well known to affect subsequent biomass production. However, it remains unclear how the timing of harvesting affects the nutritive values of reed, total digestible nutrients (TDN), crude protein (CP), etc. We hypothesized different timings of harvest would affect the seasonal dynamic of nonstructural carbohydrate between shoots and rhizomes, and subsequently affect the nutritive values of roughage.

The present study aimed to explore the effective timing of harvest for managing the growth, and for using the regrown biomass as roughage for ruminants. To investigate the management implications of the different harvest times, we reaped reed shoot biomass in winter

(January), spring (March), and early summer (May). Our final goal was to contribute to the establishment of vegetation management strategies able to improve both sustainable utilization of reed bed and nutritive values of roughage.

2.2 Materials and Methods

2.2.1 Study site

Because of recent economic and population growth, Lake Dianchi has become the largest eutrophic lake in the Yunnan province of China, and has been listed in China's 'Three Important Lakes Restoration Act'. According to monitoring data from 2005 to 2012, annual concentrations of total N ranged from 1.82–3.01 mg L⁻¹, and those of total phosphorus ranged from 0.13–0.20 mg L⁻¹ in the main water body of Lake Dianchi (Zhang et al., 2013). To resolve the eutrophication of the lake, a large number of CWs were established along the lakeside. Samples were obtained from a free-water system CW of Lake Dianchi in Jinning, Kunming, China (24° 46' N, 102° 44' E) with a surface area of 0.23 km² (Figure 2-1). Effluent from the Nanchong River is discharged into the lake. Agriculture in this area is a major contributor of wastewater, which contains a high concentration of nitrate N (>10 mg N L⁻¹) and flows into the lake 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]. The water depth was 0 m, but the ground remained wet from February to May. The water depth increased from late June, reached a maximum of 0.6 m in August, and then decreased again from October onward. The water depth thus changed seasonally from 0 to 0.6 m at *P. australis* communities.

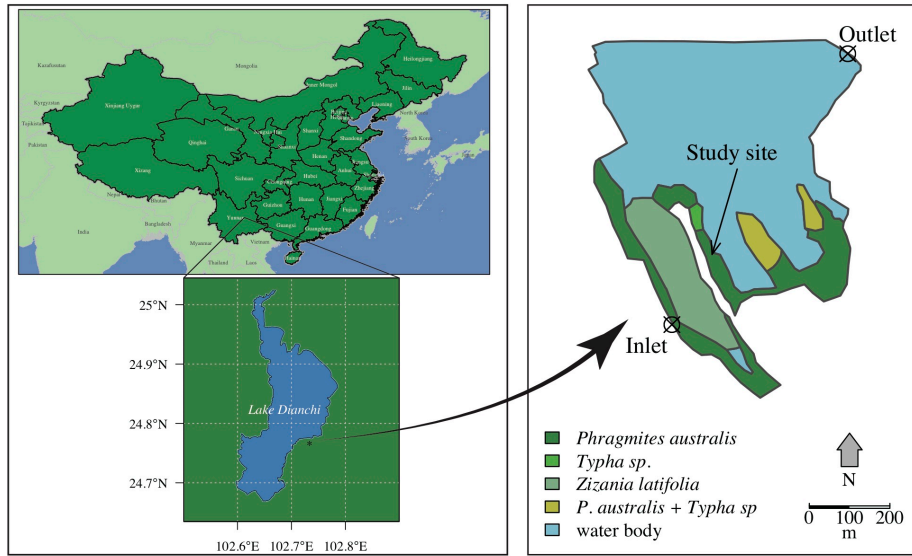


Figure 2-1 Location of the study site.

Lake Dianchi lies at an altitude 1880 m above sea level, with subtropical highland climate in Köppen's classification. Hourly precipitation and air temperature data were obtained from the weather station (WeatherHawk Station, Campbell Scientific, USA) located in Xiaozhai village (E24° 41' N, 102° 43' E). Monthly cumulative rainfall and mean daily temperatures are shown in Figure 2-2. The rainy season occurs from May to October, and annual rainfall was 654 mm in 2013. The mean annual temperature was 16.3 °C, and the mean daily temperature reached a maximum of 23.6 °C on 26 June and a minimum of 0.3 °C on 16 December. Mean daily temperature was relatively high from May to July.

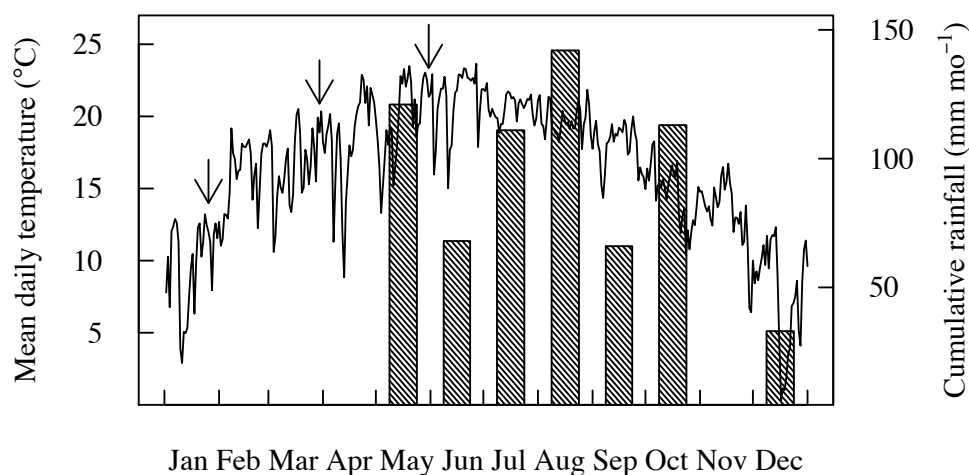


Figure 2-2 Monthly cumulative rainfall and mean daily temperature.

Note: Arrows represent the timing of harvest treatments (Jan-harvest, Mar-harvest, and May-harvest).

2.2.2. Plant harvest, sampling, and canopy structure analyses

The scheme for harvesting and sampling is shown in Figure 2-3. In this study, harvest just represents the mowing treatment, and it does not mean sampling. Plots of *P. australis* 5.0 × 5.0-m in size were mapped and subsequently harvested on January 26 (Jan-harvest), March 30 (Mar-harvest), and May 31 (May-harvest) 2013, while an additional non-harvested plot (non-harvest) was retained. Jan-, Mar-, and May-harvests represent winter, spring, and early summer harvesting, respectively. We assumed that practical harvest management was applied at a height of 40 cm for roughage use. Thus, plants were cut at a height of 40 cm above the ground.

Harvest treatment	Jan 26	Mar 20-21	30	May 22-24	31	Jul 22-23	Sep 19-20	Nov 3-4
Jan harvest	H	S (50 DAH)		S (110 DAH)		S (170 DAH)	S (230 DAH)	s
Mar harvest			H	S (50 DAH)		S (110 DAH)	S (170 DAH)	s
May harvest					H	S (50 DAH)	S (110 DAH)	s
nonharvested	s	S		S		S	S	s

Figure 2-3 The scheme of harvesting and sampling in 2013.

Note: The different characters represent the timings of harvest and sampling (H: harvest time; s: sampling time; S: sampling time when the stratified clipping method was applied in the days after harvest). The gray areas indicate regrowth periods after each harvest.

Three replicate samples of the aboveground standing biomass within a 0.5 m² (1.0 × 0.5 m) frame were taken in the non-harvest plot on 26 January and in each experimental plot on

3-4 November 2013. Samples were always taken from previously unsampled new quadrats by cutting with a sickle at ground within a visually homogeneous pure stand with uniform shoot density.

Canopy structure and light distribution within a canopy were determined on 20–21 March, 22–24 May, 22–23 July, and 19-20 September 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, Mar, May, and non-harvest). The relative light intensity was measured using a 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 ground level. Plants were cut into 0.4-m segments along the stem, keeping plant and leaf inclinations as natural as possible. The plant segments were placed into polyethylene bags for transport to the laboratory, where cut segments were sorted as stems, leaves, inflorescences, ears and weeds. As for samples gathered 50 days after harvest (DAH), the stems were classified into old and new stems by appearance. The old stems existed before the harvest, and the new ones emerged after. Leaf sheaths were included in the stem fractions. Green leaves were separated from dead leaves. The leaf areas of green leaves were measured using a digital camera and image analyzer software (Photoshop CS6, Adobe systems, CA, US), and leaf area index (LAI) was calculated. The dry weight (DW) was determined after oven drying at 70 °C to a constant weight. Dried plant materials were ground until they were fine enough to pass through a 2-mm sieve, and their N contents were determined with an indophenol method after Kjeldahl digestion.

Light intensity was assumed to attenuate through the leaf canopy following the Beer–Lambert law (Monsi and Saeki, 1953):

$$I = I_0 \exp (-K \times F) \quad (1)$$

where F is the cumulative LAI from the top of the canopy, I is the shaded light intensity under F , I_0 is the original incoming light intensity, and K is the extinction coefficient. K was estimated from the slope in the linear regression after logarithmic transformation of equation 1.

2.2.3. Analyses of nutritive values

To analyze nutritive values, we prepared plant samples obtained by the stratified clipping method. Briefly, stratified plants above a 40-cm height were mixed and homogenized according to their weight ratio. Plant samples were then analyzed for NDF, acid detergent fiber (ADF),

acid detergent lignin (ADL), neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP), ether extract (EE), and crude ash. The NDF and ADF contents were expressed exclusive of residual ash (Van Soest et al., 1991) without α -amylase for the NDF analysis. Crude ash was determined by combustion in a muffle furnace at 600 °C for 2 h. Nitrogen content was calculated by summing up N contents within each stratified sample, and CP was calculated by multiplying the concentration of N by a factor of 6.25. NDICP and ADICP content were analyzed in neutral and acid fiber fractions, respectively. Concentrations of TDN were calculated for each sample from the summative equation of feeds at maintenance (NRC, 2001):

$$\text{TDN} = \text{tdNFC} + \text{tdCP} + \text{tdNDF} + (\text{tdFA} \times 2.25) - 7 \quad (2)$$

$$\text{tdNFC} = 0.98 \times \{100 - [(\text{NDF} - \text{NDICP}) + \text{CP} + \text{EE} + \text{Ash}]\} \quad (3)$$

$$\text{tdCP} = \text{CP} \times \exp(-1.2 \times \text{ADICP} / \text{CP}) \quad (4)$$

$$\text{tdNDF} = 0.75 \times \{(\text{NDF} - \text{NDICP}) - \text{ADL}\} \times \{1 - [\text{ADL} / (\text{NDF} - \text{NDICP})]^{0.667}\} \quad (5)$$

$$\text{tdFA} = \text{EE} - 1, \text{ if } \text{EE} < 1 \text{ then } \text{tdFA} = 0 \quad (6)$$

2.3 Results

2.3.1. Characteristics of the *P. australis* growth

Seasonal variation of aboveground biomass, N standing stock, and green LAI are shown in Figure 2-4. Growth of *P. australis* had seasonality at the experimental location, and in January the biomass is represented by old shoots from previous year growth. The images of the stands are shown in Figure 2-5. Aboveground biomass in Jan-harvest plot increased to a maximum of $4503 \pm 218 \text{ g m}^{-2}$ on 22 July (170 DAH). Aboveground biomass in Mar-harvest and May-harvest plots increased to maximums of 1329 ± 122 and $1447 \pm 196 \text{ g m}^{-2}$ on 22 July and 20 September (110 DAH), respectively. Aboveground biomass in the non-harvest plot remained relatively stable. The N standing stock showed similar trends to the aboveground biomass. However, the differences among experimental plots were relatively small. At the end of May, green LAI reached a maximum of 8.3 ± 1.4 , 2.6 ± 1.3 and 5.2 ± 2.3 in the Jan-, Mar-, and non-harvest plots, respectively.

The effect of the different harvesting times on aboveground biomass and N, which could be removed by the second harvest within a year, is shown in Table 2-1. The aboveground biomass and N that could be removed by the second harvest were estimated by the maximum biomass and N content above 40 cm from ground level on the assumption that each plot (Jan-,

Mar-, and May-harvest) would be harvested again during the duration of the experiment. The estimated aboveground biomass and N were relatively higher in the Jan-harvest than in the Mar- and May-harvest plots.

Table 2-1 The effect of different harvest times on aboveground biomass and nitrogen, which could be removed by the second harvest within a year

Treatment	Sampling time	Day after harvest	Aboveground biomass (g m ⁻²)	Aboveground nitrogen (g N m ⁻²)
Jan-harvest	22-Jul	170	3339 ± 125	43.04 ± 4.01
Mar-harvest	22-Jul	110	745 ± 165	12.45 ± 1.66
May-harvest	20-Sep	110	1030 ± 194	17.06 ± 3.32

The aboveground biomass and nitrogen that could be removed by the second harvest were estimated by the maximum biomass and nitrogen content above 40 cm from ground level on the assumption that the second harvest was applied within a year. Values are means ± standard deviation (n = 2).

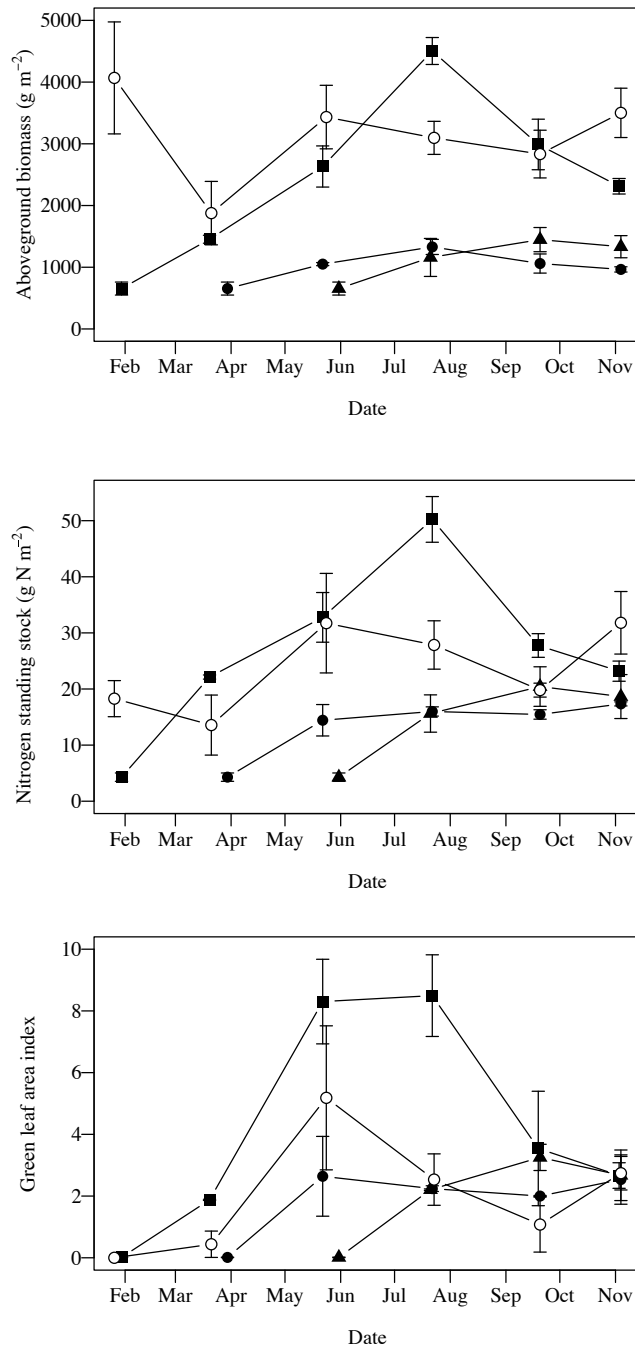


Figure 2-4 (a) Seasonal variation of aboveground biomass; (b) nitrogen standing stock; and (c) green leaf area index.

Note: Bars indicate standard deviation. Filled symbols refer to samples collected in harvested plots, and different shapes indicate plant harvest treatments (squares: Jan-harvest; circles: Mar-harvest; triangles: May-harvest). Open symbols refer to samples collected in non-harvest plot.

(a)



(b)



Figure 2-5 Images of: (a) Jan-harvest and non-harvest plots in 1 March 2013; and (b) old shoots from previous year and the new shoots emerged from the old shoots.

2.3.2. Canopy structure

The productive structure is shown in Figure 2-6. The canopy height at 50 DAH of the Jan-harvest plot was relatively short, although those of the other plots were more than 3 m. Most of the leaves were distributed in the upper layer in the Jan-harvest at 170 and 230 DAH, and a similar pattern was observed in the non-harvest plots at the end of May. On the other hand, leaves were distributed evenly in the Mar- and May-harvest plots. N concentration of leaves was higher in the upper layers at 50, 110, and 170 DAH, and that of stems also showed a similar trend at 50 and 110 DAH. Basal biomasses (0-40 cm) were higher in the Jan-harvest plot than in other plots. Old and new stem biomasses at heights from ground level to 40 cm at 50 DAH are shown in Fig. 2-7. New stem biomass was relatively higher in the Jan-harvest plot than in the Mar- and May-harvest plots.

K values as indicated by the slope of each regression analysis are shown in Table 2-2. In the Jan-harvest plot, K values at 170 and 230 DAH were relatively lower than at 50 and 110 DAH. In the Mar- and May-harvest plots, the range of K values exhibited little variation. In the non-harvest plot, K values showed large variations from 0.41 to 1.66.

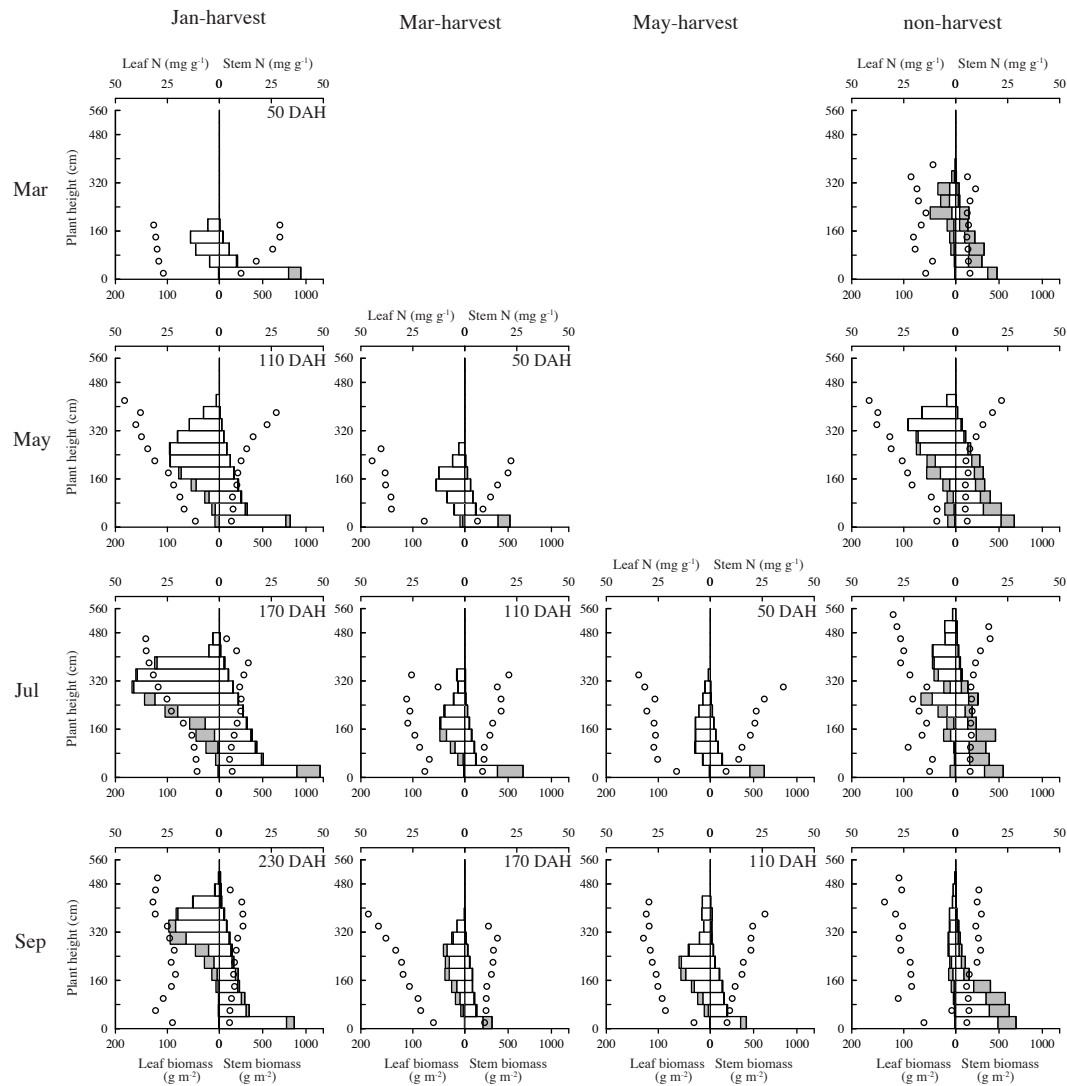


Figure 2-6 Vertical distribution of dry matter and nitrogen content.

Note: Leaves (left) and stems (right) are shown in opposite directions in each figure. Plant height is scaled to the y-axes (cm). White and black-filled areas indicate the dry weight of live and dead tissues, respectively. Circles indicate nitrogen content.

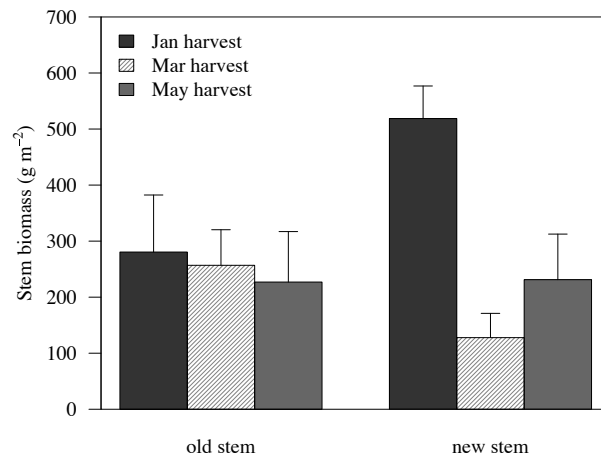


Figure 2-7 Old and new stem biomass at the height from the ground level to 40 cm on 50 days after harvest.

Table 2-2 Seasonal variation of light extinction coefficients (K)

Treatment	Days after harvest	Sampling time	K
Jan-harvest	50 DAH	20-Mar	0.69
	110 DAH	22-May	0.46
	170 DAH	22-Jul	0.15
	230 DAH	19-Sep	0.15
Mar-harvest	50 DAH	22-May	0.56
	110 DAH	22-Jul	0.35
	170 DAH	20-Sep	0.41
May-harvest	50 DAH	21-Jul	0.58
	110 DAH	20-Sep	0.68
non-harvest	-	21-Mar	1.66
	-	24-May	0.41
	-	23-Jul	0.46
	-	20-Sep	0.84

K values were determined in the linear regression analyses after logarithmic transformation of equation 1.

2.3.3. Nutritive values

Nutritive values are listed in Table 2-3. Concentrations of CP and TDN declined across sampling dates, while those of NDF, ADF, and ADL increased with sampling date in harvested plots. In the non-harvest plot, concentrations of each value remained relatively stable, although those of TDN ranged from 39.3–44.0%. The results showed that concentrations of NDF in the Jan-harvest ($78.7\pm0.0\%$) plot were relatively higher than in the Mar ($72.8\pm0.2\%$) and May-harvest plots ($71.9\pm2.2\%$) at 110 DAH. Meanwhile, concentrations of ADF in the Jan-harvest plot ($38.7\pm0.3\%$) were relatively lower than in the Mar ($43.6\pm0.7\%$) and May-harvest plots ($45.7\pm0.1\%$) at 50 DAH, and those in the Jan-harvest plot ($52.2\pm0.3\%$) were relatively higher than in Mar-harvest plot ($45.2\pm0.8\%$) at 110 DAH. The concentrations of TDN in the Jan-harvest plot ($60.3\pm0.5\%$) were relatively higher than in the Mar ($52.2\pm0.9\%$) and May-harvest plots ($49.8\pm0.2\%$) at 50 DAH, and those of truly digestible NFC in the Jan-harvest plot ($15.4\pm0.3\%$) were also relatively higher than in the Mar ($8.7\pm0.5\%$) and May-harvest plots ($8.6\pm0.8\%$) at 50 DAH. The result also showed seasonal differences in CP and ADL between 50 and 110 DAH, and differences in crude ash among occasions of harvest.

Total digestible nutrients and truly digestible components are shown in Figure 2-8. Although concentrations of truly digestible NDF remained stable, those of truly digestible CP declined across sampling dates. Concentrations of truly digestible nonfiber carbohydrate (NFC) were less than 10% in most of the plots during the experiments; however, that of truly digestible NFC was 15.7% in the Jan-harvest plot at 50 DAH.

Table 2-3 Chemical compositions and total digestible nutrient concentrations (%) in the aboveground tissues of different harvest treatment and sampling time

Harvest treatments	Days after harvest	Sampling time	Crude Protein	Crude ash	Neutral detergent fiber	Acid detergent fiber	Acid detergent lignin	Ether extract	Total digestible nutrient
Jan-harvest	50	20-Mar	15.0±0.4	7.7±0.2	66.6±0.4	38.7±0.3	2.9±0.2	1.5±0.2	60.3±0.5
	110	22-May	9.7±0.1	7.9±0.1	78.7±0.0	52.2±0.3	9.4±2.0	0.8±0.6	46.0±2.2
	170	22-Jul	8.0±0.3	7.6±0.1	78.0±0.6	55.1±0.5	9.4±0.1	1.0±0.3	45.4±0.6
	230	19-Sep	6.9±0.1	7.4±0.7	82.0±0.1	56.9±1.6	10.8±0.1	0.2±0.0	42.8±0.3
Mar-harvest	50	22-May	13.5±0.7	11.3±0.2	68.2±0.8	43.6±0.7	4.7±0.3	1.3±0.4	52.2±0.9
	110	22-Jul	10.0±0.2	11.7±0.0	72.8±0.2	45.2±0.8	8.2±0.5	1.3±0.1	45.5±0.6
	170	20-Sep	10.5±0.7	10.2±0.7	76.7±0.4	50.6±1.3	9.1±0.5	1.0±0.3	44.3±0.1
May-harvest	50	21-Jul	12.6±0.1	11.3±0.0	70.9±0.7	45.7±0.1	6.0±0.1	0.9±0.1	49.8±0.2
	110	20-Sep	10.4±0	11.0±0.4	71.9±2.2	48.4±2.3	9.0±0.6	1.5±0.1	45.2±1.5
non-harvest	-	21-Mar	4.4±0.4	9.5±0.5	82.3±1.4	59.2±1.6	11.5±0.4	0.4±0.1	39.3±0.1
	-	24-May	6.2±0.6	7.0±0.2	79.6±0.8	56.7±3.6	10.0±0.4	0.4±0.3	44.0±0.7
	-	23-Jul	5.9±0.2	6.1±1.0	79.3±1.6	59.9±0.1	12.0±0.4	1.3±0.2	42.9±0.3
	-	20-Sep	4.7±0.3	5.2±0.3	84.3±0.5	61.4±0.9	13.4±0.1	1.0±0.0	39.5±0.1

Values are means ± standard deviation (n = 2).

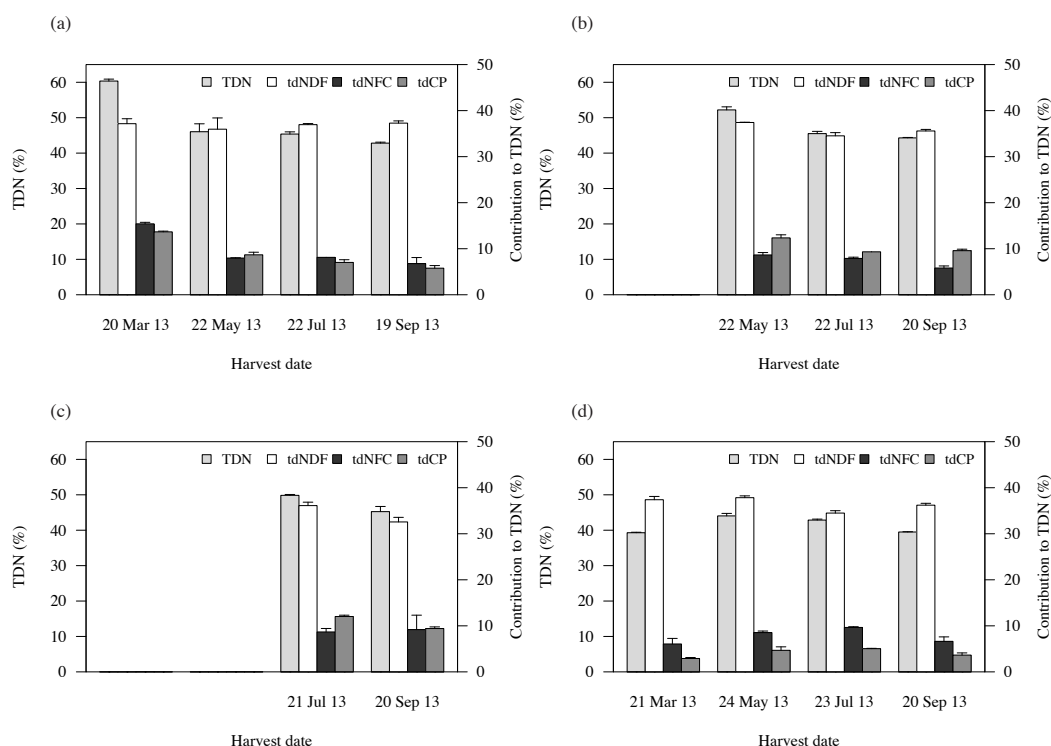


Figure 2-8 Total digestible nutrients and truly digestible components of: (a) Jan-harvest; (b) Mar-harvest; (c) May-harvest; and (d) non-harvest.

Note: Total digestible nutrients (TDN) were estimated using the summative equation of feeds at

maintenance (NRC, 2001). Truly digestible nonfiber carbohydrate (tdNFC), truly digestible crude protein (tdCP), and truly digestible neutral detergent fiber (tdNDF) are scaled to the secondary y-axes.

2.4 Discussion

2.4.1. Biomass production and N uptake

The results of this study showed that the timing of harvest substantially affects the regrowth of aboveground biomass in the plant *P. australis*. Aboveground biomass in the Jan-harvest plot was relatively higher than in the Mar- and May-harvest plots; it continued to increase to 170 DAH in the Jan-harvest, and to 110 DAH in the Mar-, May-, and non-harvest plots. Our results correspond with those of previous studies in temperate regions that found aboveground biomass production was increased by winter harvest (Hansson and Granéli, 1984; Granéli, 1990). Production of aboveground biomass was reported to decrease significantly in the current and following year due to a June harvest, but no significant difference was observed after harvest in July or August (Weisner and Granéli, 1989; Asaeda et al., 2006). The translocation of nonstructural carbohydrates and mineral nutrients from shoots to rhizomes begins immediately after the foliar structure has been established (Granéli et al., 1992). Therefore, harvesting in the early growing stage results in the scarcity of reserves of carbohydrates and mineral nutrients in rhizomes, and has a serious effect on subsequent shoot growth.

In the present study site, sufficient nonstructural carbohydrates might have been translocated from rhizome to shoot in the Jan-harvest plot. Rhizomes were short of nonstructural carbohydrates in the Mar- and May-harvest plots because storage had already been exhausted for new shoot emergence in February, and the assimilated product was used not for shoot growth but mainly for rhizome reserves after the foliage structure was established. In the early growing stage (50 DAH), aboveground biomass rapidly increased (Figure 2-4), and basal aboveground biomass (0-40 cm) was also extremely high in the Jan-harvest plot, probably due to the new shoot emergence (Figure 2-7). This result also supported the theory that vigorous early regrowth in the Jan-harvest plot was the result of translocation of nonstructural carbohydrates from rhizomes. Furthermore, the timing of heading was delayed in the Mar- and May-harvest plots compared to the Jan and non-harvest plots (data not shown). This result also suggested that initial growth restricted by shortages of rhizome reserves might negatively affect vegetative growth, and that insufficient vegetative growth can delay the timing of heading. Overall, even though our data were obtained under subtropical highland climate, those are

similar to the previous studies of temperate regions.

The N standing stock of aboveground biomass reached a maximum of 50.2 ± 4.0 g N m^{-2} in the Jan-harvest plot at 170 DAH; the maximum N standing stock in the Mar- and May-harvest plots were generally lower than in the Jan and non-harvest plots. However, the differences in N standing stock among harvest treatments were not distinct by November, because the newly assimilated N in shoots was also translocated to rhizomes by the end of the growing stage (Zhao et al., 2013). This indicated that an annual harvesting in January might not have a great effect on improving N removal in CW, even though the highest N standing stock was observed in the Jan-harvest plot. The aboveground N can be removed by the second harvest, which showed a maximum of 43.04 ± 4.01 g N m^{-2} in the Jan-harvest plot at 170 DAH (Table 2-1). Therefore, a large amount of N can be removed in a January harvest if the plot is harvested twice within a year at a time when the N standing stock reaches its maximum. Karunaratne and Asaeda (2004) reported that harvesting at certain stages when rhizome reserves have already been replenished does not affect subsequent regrowth. Moreover, concentration of N in the plants also might depend on the chemical characteristics of the wastewater. To establish a sustainable strategy, further research is required to elucidate the effect of a second harvest on subsequent regrowth and that of water quality of wastewater on N uptake.

2.4.2. Canopy structure

Productive structure showed the different trends of developing canopies among harvest plots. The canopy height was relatively high in Mar- and May-harvest plots at 50 DAH. Haslam (1970) reported that the cumulative temperature was correlated with canopy height because temperature has a positive effect on the elongation of internodes, but does not affect the number of nodes. The cumulative temperature from 0–50 DAH was 837, 1017, and 1089 °C for the Jan-, Mar-, and May-harvest plots, respectively. Temperature seems to be one of the major factors affecting canopy height. In the non-harvest plot in March, canopy height was 360 cm, relative light intensity even at the height of 200 cm was approximately 10%, and the K value was 1.66. Karunaratne et al. (2003) reported that standing dead and old culms had a greater effect on shading than leaves in the early growing stage, and equation 1 seems inadequate to estimate K values during the early growth stages without a harvest. Since serious internal competition for light resources possibly occurred in the non-harvest plot, the aboveground biomass remained relatively stable, but the canopy height was compelled to lengthen.

In the Jan-harvest plot, K values were relatively small at 170 and 230 DAH, and most

of the leaves were distributed in the upper layer of the canopy (Figure 2-6). Hirose and Werger (1995) reported that the effect of stems on light attenuation might be negligible in the upper layers, but would increase with increasing depth in the canopy. Our results indicated that K values might be somewhat affected by the shading effect of stems even in the early growing stage, and that K values decreased with a decline in the influence of stems due to the change in the vertical distributions of leaves. Furthermore, when high leaf distribution in the upper layer was observed in the Jan-harvest plot at 170 and 230 DAH, the aboveground biomass and LAI showed extremely high values (Figure 2-4). This indicated that the vertical distribution of leaves became steeper with denser and more productive stands to improve light attenuation, probably due to the increasing light interception. K values indicate differences in canopy structure as leaf size, leaf angle, and leaf transmissibility (Monsi and Saeki, 1953; Oikawa, 1977). Similarly, vertical biomass allocation of leaves and stems might be another important factor affecting light attenuation in dense and productive stands.

2.4.3. Nutritive value

In the present study, we demonstrated a high possibility of using reed as roughage for ruminants because of its relatively high concentrations of TDN in the early growing stage (>50%). According to the Nutrient Requirements of Dairy Cattle (NRC, 2001), the nutritive values of reed are similar to those of sorghum (TDN, 54.4%; CP, 9.4%; NDF, 64.8%; Lignin, 6%). Generally, concentrations of TDN were relatively high at 50 DAH in all harvested plots, and then declined drastically to approximately 45% at 110 DAH. These values were similar to those of the non-harvest plot. Similar results were found in a previous study, where TDN concentrations showed a sharp decline when duration of growth exceeded 40 days (Asano et al., 2014). In addition, the results showed that the concentrations of TDN and those of truly digestible NFC in the Jan-harvest plot were relatively higher than in the Mar- and May-harvest plots at 50 DAH. This may reflect that higher concentrations of truly digestible NFC at 50 DAH are associated with an earlier harvest time, which is one of the reasons why the concentration of TDN was relatively higher in the Jan-harvest plot. Again, this may have been due to a large amount of nonstructural carbohydrate, including NFC, being translocated from rhizomes, as discussed above.

The unique nature of truly digestible NDF was found to maintain relatively static values during our experiment (Figure 2-8). Although concentrations of NDF increased with sampling dates on each plot, those of ADL also increased (Table 3). Equation 5 showed that

truly digestible NDF is calculated from the balance between NDF and ADL. This resulted in stable values of truly digestible NDF irrespective of growing stage. On the other hand, truly digestible CP steadily decreased as the season advanced. Therefore, truly digestible CP was generally the main contributor to concentrations of TDN. Moreover, we observed vertical, non-uniform distribution of N in the canopy structure for leaf biomass until 170 DAH, and for stem biomass until 110 DAH (Figure 2-6). No such trend was observed at 230 DAH, corresponding with the onset of senescence (Figure 2-4). The leaf biomass is mainly redistributed in the upper canopy positions in the productive stands, as noted above. Overall herbage cell wall concentration increases as the leaf-to-stem ratio shifts towards a greater proportion of stem (Jung, 2012). These results indicated that the upper layer of harvested biomass is appropriate for roughage. Harvesting is a recommended operation treating diluted wastewater, especially in productive areas since the efficiency of CWs was reduced in non-harvested wetland during the winter and early spring period due to the vegetation decomposition (Álvarez and Bécares, 2008). In this context, harvesting in early growing stage (e.g., 50 DAH) is not desirable in CWs since we cannot expect high N removal, even though it is applicable in natural habitats without necessity of nutrient removal. However, it may contribute not only to high N removal but also to both the reduction of litter decomposition and production of high-quality roughage if aboveground biomass is harvested after reaching its maximum, and just the upper layer were used for roughage. Therefore, cutting height can also be an important strategy. It is thus desirable to harvest shoots twice within a year after the first harvest in winter. This indicated a probability that the high nutrient removal efficiency and high-quality roughage production could be compatible by considering the frequency and timing of harvest, cutting height. It should be noted, however, that this study was conducted for only one annual cycle. Continuous harvesting practices could affect the growth negatively, and thus reduce the efficiency of nutrient removals of CWs in a long terms perspective. Moreover, accumulations of heavy metal in foliar tissues depend on the seasonal translocation from root and rhizome systems (Ranieri et al., 2013), which may also be influenced by the period of harvesting. Further long-term investigation is required to understand the impact of harvesting on subsequent biomass, nutrition, and heavy metal dynamics between shoot and rhizome for balancing phytoremediation and roughage use. More detailed studies, metabolism tests using ruminants, and postharvest treatments to improve digestibility are also necessary in order to put reed to practical use as roughage.

2.5 Conclusions

This study showed the potential use of reed as high-quality roughage, especially when harvested in the early growing stage. In agreement with previous findings, aboveground biomass production was promoted by harvesting in winter, probably due to sufficient rhizome reserves. Similarly, relatively high concentrations of TDN were found in the Jan-harvest plot in the early growing stage, presumably due to the sufficient supply of NFC from the rhizomes. Therefore, efficient timing of harvest is an important strategy in controlling nutritive values of roughage. On the other hand, CP is also a strong contributor to high TDN values. The vertical distribution of leaves showed a relatively steep biomass gradient, with more productive stands in the upper layers, and N concentration also showed a steady gradient until the onset of senescence. Therefore, careful management of cutting height and ensuring that only the upper portions of plants are used as roughage could be another way to improve both N removal and nutritive values for roughage in the late growing stage.

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Chapter 3 Impact of plant harvest management on function and community structure of nitrifiers and denitrifiers in a constructed wetland

3.1 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 oxidized to nitrate (NO_3^-). The initial step of ammonia oxidation 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 et al., 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 et al., 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 et al., 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 et al., 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 mediate 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.

3.2 Materials and Methods

3.2.1 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 km². 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 (NO₃⁻-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).

3.2.2 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.

3.2.3 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.

3.2.4 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).

3.2.5 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 et al. (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 $1000 \text{ mg l}^{-1} \text{KNO}_3^-$. 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 $(\text{NH}_4)_2\text{SO}_4$ to a final concentration of $210 \text{ mg l}^{-1} \text{ NH}_4^+\text{-N}$, and liquid aliquots were collected hourly over a 24-h period. Samples for the analysis of $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations (1 ml) were removed from the homogenates, centrifuged for 2 min at 12 000 g, and filtered through a $0.20\text{-}\mu\text{m}$ filter. $\text{NO}_3^-\text{-N}$ and $\text{NO}_2^-\text{-N}$ concentrations were analyzed by high-performance liquid chromatography (HPLC) (PIA-1000, Shimadzu, Kyoto, Japan) using a $2 \times 150\text{-mm}$ 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.

3.2.6 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 et al., 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 et al., 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'-CCGCCGCGCGGCGGGCGGGGCGGGGCACGGGG-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), $1 \times$ 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 et al., 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.

3.2.7 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 ChemiDoc XRS (Milano, Bio-Rad, Italy). DNA bands were detected using the software Quantity One 4.6.2 (Milano).

3.2.8 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 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 et al., 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).

3.3 Results

3.3.1 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 Figure 3-1. 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 (Figure 3-1b). 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%.

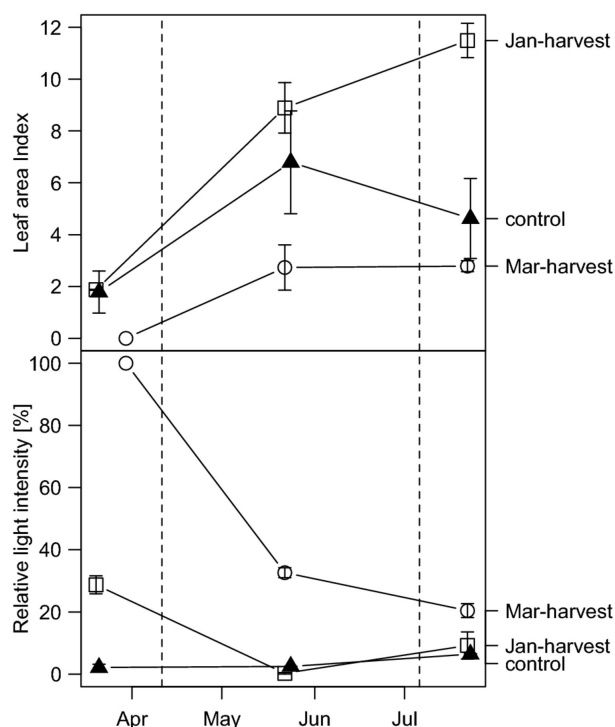


Figure 3-1 Seasonal variation in the LAI and relative light intensity of *P. australis* at the ground level in Jan-harvest, Mar-harvest and control stands.

Note: Bars and broken lines indicate mean standard deviation (SD) and rhizosphere sampling date, respectively.

3.3.2 Chemical and potential activity characteristics of rhizosphere samples

The chemical characteristics are shown in Table 3-1. 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.

Table 3-1 Chemical characteristic of rhizosphere 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)
Apr 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
Jul 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

Data correspond to mean values and standard deviations of three replicate samples.

The average values of nitrite and nitrate production and reduction rates are shown in Figure 3-2. 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 ($P = 0.004$, $r = 0.65$). 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.

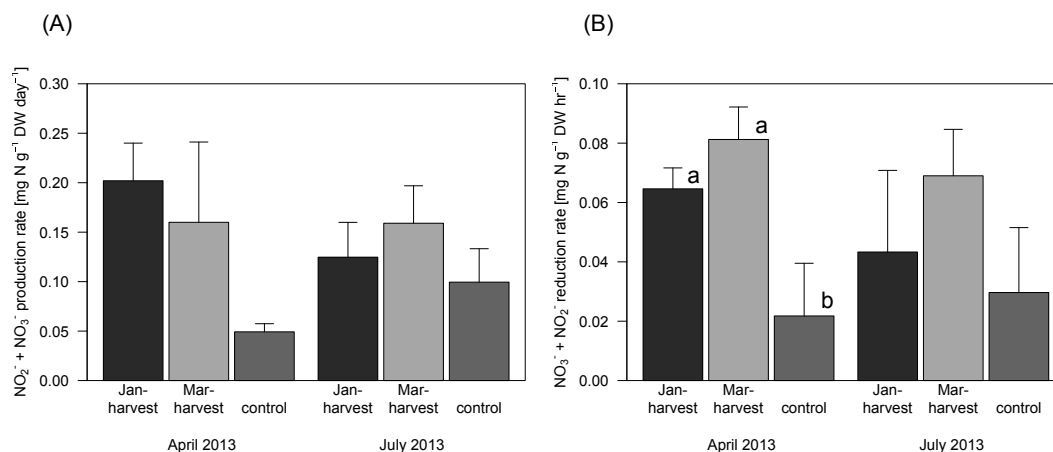


Figure 3-2 Potential nitrate and nitrite production rates (A) and potential denitrification rates (B) from April and July rhizosphere samples.

Note: 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.

3.3.3 Relationships between rhizosphere parameters and nitrifier and denitrifier communities

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

The result of Mantel tests and permutations testing for significance of vector fit to the NMS axes in ordinations are shown in Table 3-2. 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 3-2). 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*.

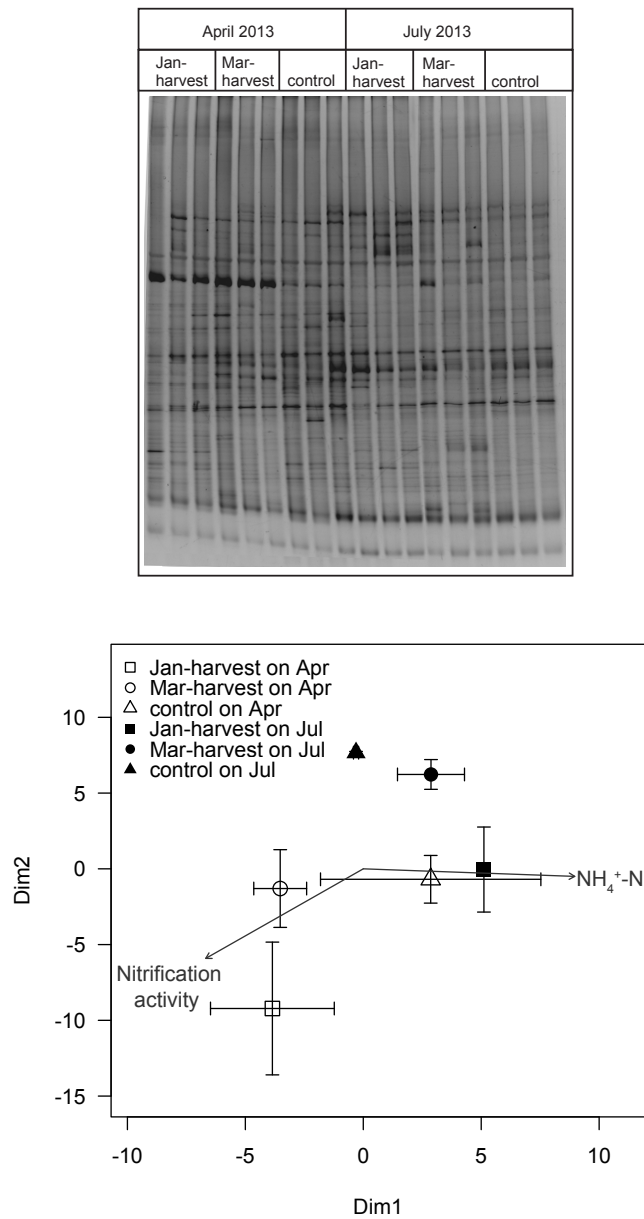


Figure 3-3 DGGE profile (A) and NMS ordinations of *nirK* (B) with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors.

Note: 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).

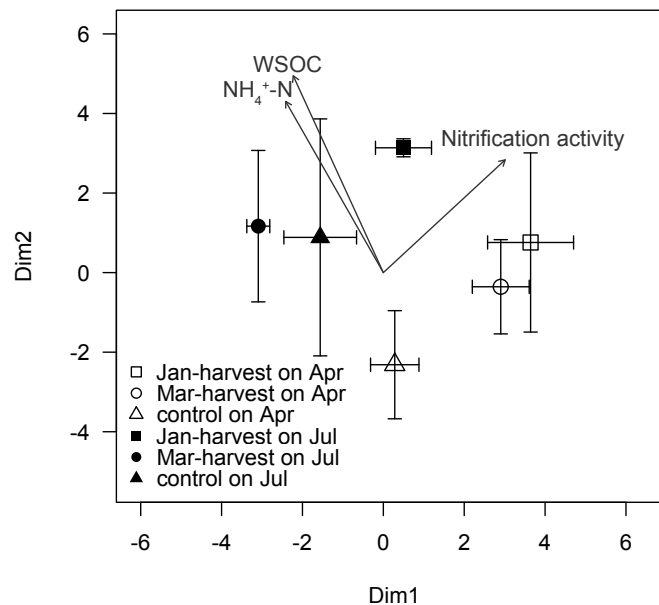
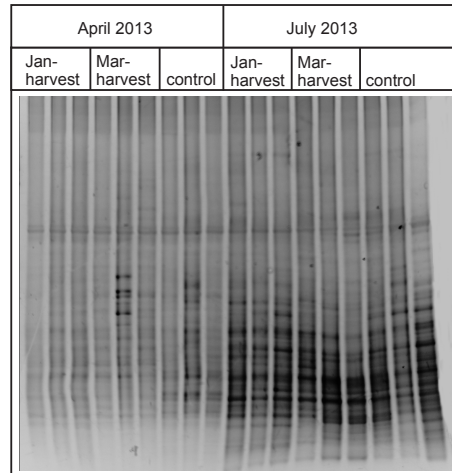


Figure 3-4 DGGE profile (A) and NMS ordinations of *nirS* (B) with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors.

Note: 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).

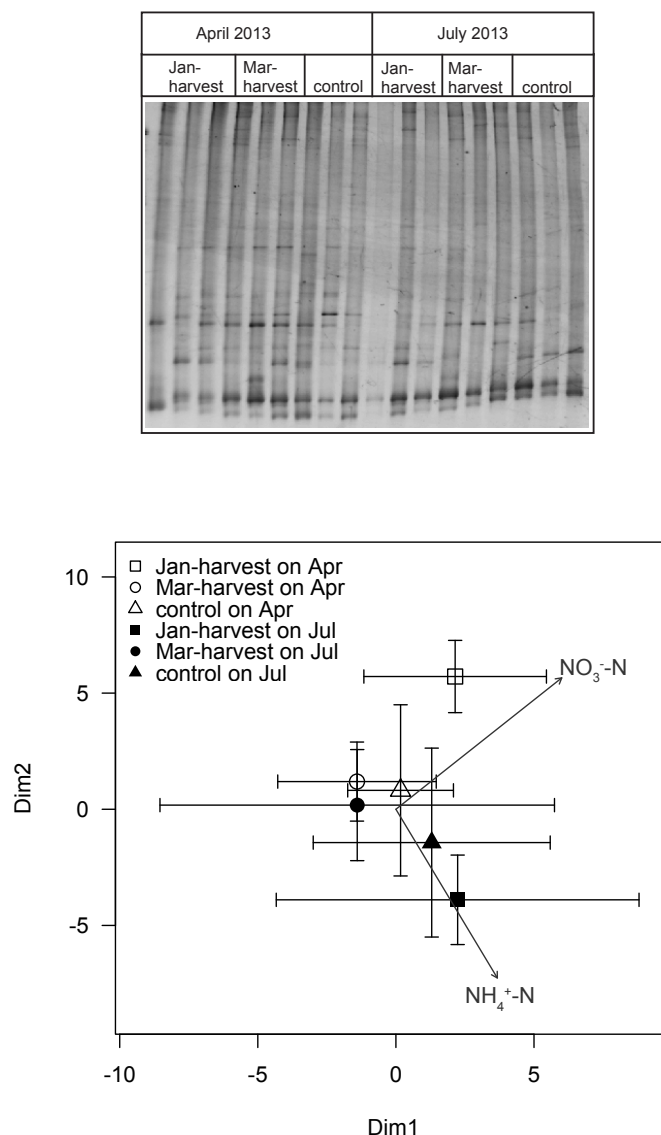


Figure 3-5 DGGE profile (A) and NMS ordinations of bacterial-*amoA* (B) with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors.

Note: 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).

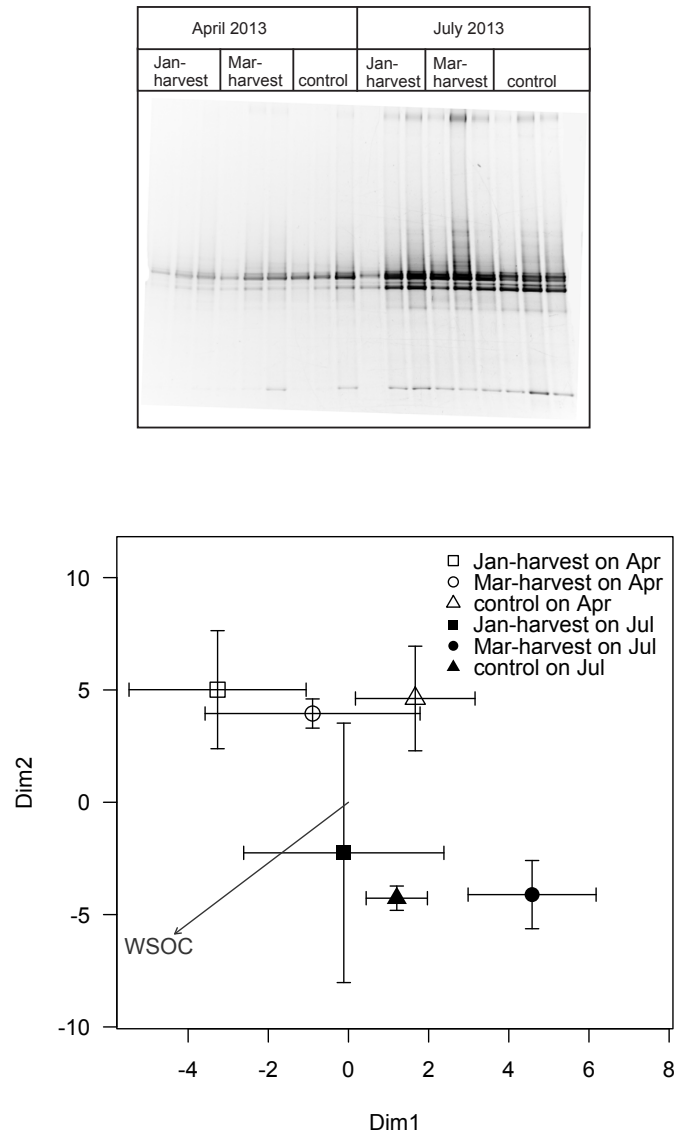


Figure 3-6 DGGE profile (A) and NMS ordinations of archaeal-*amoA* (B) with the biological and chemical parameters from the rhizosphere correlated with the axes as vectors.

Note: 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).

Table 3-2 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)			
	<i>nirK</i>	<i>nirS</i>	bacterial- <i>amoA</i>	archaeal- <i>amoA</i>	<i>nirK</i>	<i>nirS</i>	bacterial- <i>amoA</i>	archaeal- <i>amoA</i>
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

3.4 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 (Figure 3-1). 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 et al., 2003; Urakawa et al., 2008) and denitrifiers (Braker et al., 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 (Figure 3-2b). Furthermore, the April sampling time revealed that the nitrite and nitrate production activities were slightly higher in the Jan-harvest area ($0.20 \pm 0.04 \text{ mg N g}^{-1} \text{ DW day}^{-1}$) than in non-harvested control ($0.05 \pm 0.01 \text{ N g}^{-1} \text{ DW day}^{-1}$) although this

difference was not significant ($P = 0.058$). This coincided with a shift of the relative light intensity at the bottom (Figure 3-1), 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) 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 3-2). 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 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 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 3-2). 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* (Figures 3-5A and 3-6A). The observed band diversity of bacterial-*amoA* was not affected by plant harvest, while the archaeal-*amoA* showed the changes (Figures 3-5B and 3-6B). 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 et al., 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,

et al., 2002; Herrmann et al., 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 *Nitrosospira* species (Gorra et al., 2007) or *Nitrosomonas* species (Ruiz-Rueda et al., 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.

3.5 Conclusions

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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Chapter 4 Phylogenetic analyses of the genus *Phragmites* in southwest China

4.1 Introduction

The species delimitation has attracted considerable attention from systematic biologists because taxonomy is a fundamental problem for evolutionary biology (Cracraft, 1983). Traditionally, taxonomy was mainly based on morphological characters. However, the traditional approaches have been with the new tidal wave of data emerging from molecular techniques in recent years (Karp et al., 1996). Recent molecular approaches have documented intraspecific variations in chloroplast DNA has frequently attributed to a phenomenon known as ‘chloroplast capture’, in which the chloroplast genome of one morphologically identifiable species was captured from another species (Bain and Jansen, 1996; Bobola et al., 1996; Soltis et al., 1991). Such genetic exchange can occur between well-established, morphologically distinct species (Schaal et al., 1998). Therefore, phylogenetic analysis by molecular approaches rather than classical taxonomy based only on morphology is very powerful for species delimitation.

The genus *Phragmites* is a cosmopolitan emergent macrophyte. *Phragmites* species are tall perennial grasses that predominantly occur in shallow freshwater, littoral lake zones, blackish wetlands, and along rivers, and they reproduce sexually and asexually. The four *Phragmites* species *P. australis* (Cav.) Trin. ex Steud., *P. japonicus* Steudel., *P. karka* (Retz.) Trin. ex Steud., and *P. mauritanus* Kunth are recognized currently according to the World Checklist of Selected Plant Families (WCSP, 2016). Among the *Phragmites* species, *P. australis* represents a cosmopolitan species with considerable variations in morphology and ploidy level (3x, 4x, 6x, 7, 8x, 10x, 11x, and 12x; Clevering and Lissner, 1999), and it occurs in a wide range of climatic and habitat conditions.

Although each allopatric *Phragmites* species (*P. australis*, *P. karka*, and *P. mauritanus*) has shown species-specific morphological traits, Clayton (1967) suggested that *Phragmites* species cannot be readily separated by any single morphological characters because of the overlap in morphological characteristics near the boundary between two species where introgression may well occur. Thus, to explore the genetic diversity and evolutionary scenarios of the genus *Phragmites*, molecular studies have been conducted using a range of molecular markers in recent years. Saltonstall (2002) developed haplotype analyses based on chloroplast DNA (cpDNA) sequences using worldwide *Phragmites* samples and herbarium specimens, and the results demonstrated that cryptic and invasive *P. australis* with haplotype M introduced

from the Eurasian continent displaced the native species in North America. This invasion is also supported by studies using microsatellite markers, although the hybridization of native and introduced *P. australis* has been considered impossible because of the low levels of sexual reproduction and different flowering times between these two lineages of *P. australis* (Saltonstall, 2003). Intraspecific hybridization, however, has been demonstrated using artificial cross-pollination (Meyerson et al., 2010). Then, Saltonstall et al. (2014) revealed field intraspecific hybridization between native and introduced *P. australis* using both cpDNA and microsatellite markers in North America. Saltonstall et al. (2016) further indicated the dominant distributions of hybrids between native and introduced *P. australis* in Vegas, USA. Moreover, interspecific hybridization and back-crossing between different *Phragmites* species (*P. mauritanus* and *P. australis*) are detected by combinations of cpDNA, microsatellites, and Amplified Fragment Length Polymorphisms (AFLPs) in Gulf Coast of North America (Lambertini et al., 2012a). Ongoing introduction of non-native *P. australis* with haplotype L from Europe to North America is also indicated by combinations of cpDNA sequences and the variations in microsatellite regions (Meyerson and Cronin, 2013).

In addition to North America, several phylogeographic studies of *Phragmites* species have been conducted in the other continents and regions. Lambertini et al. (2012b) suggesting that the climatic barriers in the Mediterranean region hinder the dispersal of tropical and subtropical genotypes into the temperate regions in Europe or the breeding barriers maintain the Mediterranean genotypes isolated from temperate *P. australis* and from *P. frutescens*. Hurry et al. (2013) identified 4 haplotypes (AE, AF, AG, and P) based on cpDNA in *P. australis* of Australia, which may be likely derived from a frequent haplotype in Asia. Colin and Eguiarte (2016) identified a high level of diversity in haplotypes (e.g., BH, BN, BU, etc.) in Mexico, suggesting that climatic changes during the Pleistocene played an important role in the demographic expansion of the populations constituting the different genetic groups of *P. australis*. Previous molecular works have revealed that the mechanisms of expansion, genetic diversification, and interspecific hybridizations of *Phragmites* might be associated with numerous factors, such as the invasion of non-native lineages, geological and reproductive isolations, and eco-climatic changes.

An et al. (2012) investigated the haplotype distribution of *P. australis* in China and suggested that distinct genetic differentiation occurred between eastern-northeastern and western-northwestern regions; however, the distribution and genetic diversity *Phragmites* species have not been explored in southwest (SW) China. SW China is a biodiversity hotspot

with overlapping global biodiversity conservation priority systems; thus, it is designated as a highly vulnerable region of high irreplaceability (Brooks et al., 2006). SW China is a significant area of plant endemism, including the eastern fringe of the Yunnan-Guizhou Plateau and the Tibetan Plateau (Hengduan Mountains *sensu lato*) (López-Pujol et al., 2011). The large vertical height range has an elevation difference of 6663 m and creates a variable microclimate in Yunnan Province of SW China (Yang et al., 2004). The southern part of the mountainous areas in China, including the Hengduan Mountains, are hypothesized to represent areas of former glacial refugia during the Quaternary period because of relatively stable environmental conditions. This probably allowed endemism to sustain large assemblages of a variety of living forms throughout glacial/interglacial cycles; thus these mountainous areas are the center of endemism (López-Pujol et al., 2011). The highly heterogeneous topography of the mountains favors the emergence of new lineages that likely evolved as a result of microallopatric speciation (Hewitt, 2000; Tzedakis et al., 2002).

In addition, constructed wetlands (CWs) for treating wastewater have rapidly increased in China, particularly since the late 1990s (Liu et al., 2009). The macrophytes primarily used in CWs include *Phragmites*. However, the artificial propagation patterns of *Phragmites* by planting still remain largely unknown, although the human-assisted transport and urban development may have influenced the current pattern of phylogeographic relationships of *Phragmites* in Europe (Lambertini et al., 2012b) and in North America (Lambert et al., 2016).

Here, SW China has the varied topography creating climatic and biological diversity, and non-native *Phragmites* may be introduced in the CWs of SW China in recent years. Thus, there may be native cryptic lineages in SW China, and invasive lineages may have been introduced from the outside of SW China. The objectives of the study was to 1) explore the distribution and of *Phragmites* in SW China, and evaluate the phylogenetic relationships between different *Phragmites* lineages, including hybridization events in SW China to different novel lineages might reflect geographic isolation; and 2) assess whether the artificial dispersal of *Phragmites* species influence native genetic diversity through the replacement of non-native lineages. For comparisons with previous global molecular studies, we analyzed haplotypes based on the sequencing of two non-coding regions of chloroplasts (Saltonstall, 2002). AFLPs were used to investigate the genetic background.

4.2 Materials and Methods

4.2.1 Plant materials

We randomly explored 8643 km in total across Yunnan, Sichuan, Guizhou, and Guanxi provinces and Chongqing. A total of 44 plant samples were collected in SW China. Habitats were observed in restricted riversides and lakeshores of flat areas or wetlands on plateaus because almost all these areas present steep slopes in SW China. We did not observe native species in Guizhou Province, which has an extremely complicated karst topography. For the molecular analyses, fresh leaf samples were collected for individuals from each locality, placed in a plastic bag, stored at 2°C during the transfer, and then stored at -80°C in the laboratory. We determined whether the populations were wild or artificially planted after interviewing the local people. However, we were not able to determine whether the artificially planted populations were just transplanted from native populations.

4.2.2 DNA extraction

Genomic DNA was extracted from fresh leaves using Plant DNA Isolation Reagent (TaKaRa, Shiga, Japan) according to the manufacturer's protocols. The DNA concentration was subsequently measured using a 4802 UV/Vis Double Beam Spectrophotometer (Unico, Shanghai, China). The DNA samples were stored at -20°C until further use.

4.2.3 Chloroplast DNA sequences

Two chloroplast intergenic spacers were PCR amplified using the primer pairs *trnT* (UGU) “a”-*trnL* (UAA) “b” (Taberlet et al., 1991) and *rbcL-psaI* (Saltonstall, 2001). The PCR mixture (25 µL) contained 0.625 U of TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan), 1× Ex Taq Buffer (TaKaRa Bio), 0.2 mM dNTPs, 0.2 µM each of the forward and reverse primers, and 10 ng of template DNAs. The PCR assay was performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, California, USA). The program included 35 cycles of DNA denaturation at 95°C for 30 s, annealing at 56°C (*trnT-trnL*) or 48°C (*rbcL-psaI*) for 1 min, and extension at 72°C for 1 min. The PCR products were purified using an ExoSAP-IT for PCR Product Clean-UP kit (Affymetrix, California, USA). The purified PCR products were sent to the Sangon Company (Shanghai, China) and sequenced directly in both directions on an ABI 3730xl DNA Analyzer (Applied Biosystems) using the same primer pairs used for the PCR amplification.

4.2.4 Sequence data analysis

The sequences were aligned using ClustalW (with the default parameters) implemented in the MEGA 6.06 program (Tamura et al., 2013). We downloaded the *trnT-trnL* and *rbcL-psaI* sequences of *P. australis*, *P. karka* (Saltonstall, 2002; Meadows and Saltonstall, 2007), and *P. japonicus* (Chu et al., 2011; Lambertini et al., 2012b) from NCBI and included these sequences in the alignment. The two mono-nucleotide repeat regions in *trnT-trnL* that showed intrahaplotype length variations were excluded from the haplotype analyses following Saltonstall (2002).

Novel haplotypes were identified based on the presence of non mono-nucleotide repeat insertions and deletions (indel) in the sequences that did not deposited in GenBank, and new names were assigned according to the alphabetic coding system by Saltonstall (2002, 2016). Repetitive regions enable a more in-depth examination of evolutionary processes, such as gene flow, founder effects, genetic drift and population bottlenecks, at multiple geographical scales (Saltonstall and Lambertini, 2012). Thus, variations in the mono-nucleotide repeat regions of both *trnT-trnL* and *rbcL-psaI* were classified according to Saltonstall (2016). The locus haplotypes are indicated by the capital letters “T” and “R”, which represent *trnT-trnL* and *rbcL-psaI*, respectively; a second number, which represents the haplotypes with sequences previously deposited in GenBank (Saltonstall, 2002; Chu et al., 2011); and lowercased letters, which represents variations in microsatellite regions (e.g., T5a, T5e, T5d, R9b, R9c, etc.). The new sequences were deposited in GenBank (accession number: KX185520–KX185523). Combined haplotype were determined by combining locus haplotypes without considering the variations in microsatellite regions (e.g., locus haplotype T4 + R4 = combined haplotype M).

We assessed the genetic relationships between haplotypes by obtaining parsimony networks (Templeton et al., 1992), as implemented in the TCS program software (Clement et al., 2000), with a 95% connection probability limit and treating each gap as a single evolutionary event. The parsimony network was constructed using the sequence data obtained in the present study, in combination with sequences from the previous studies by Saltonstall (2002), Meadows and Saltonstall (2007), Chu et al. (2011), and Lambertini et al. (2012b).

4.2.5 AFLP

AFLPs were assessed following Lambertini et al. (2006; 2012a) with several modifications. Briefly, restriction digestion was performed in a 40-μL reaction containing 500 ng of genomic

DNA, 2.5 U each of EcoRI (New England Biolabs, Ipswich, Massachusetts, USA) and MseI (New England Biolabs, Ipswich, Massachusetts, USA), and 1X CutSmart buffer (New England Biolabs, Ipswich, Massachusetts, USA). We incubated the restriction reaction at 37°C for 4 h, which was followed by denaturing at 70°C for 15 min. A 10-μL aliquot of the ligation reaction containing 5 pmol EcoRI and 50 pmol MseI adapters, 1 U of T4 ligase (New England Biolabs, Ipswich, Massachusetts, USA), and 1X ligation buffer (New England Biolabs, Ipswich, Massachusetts, USA) was added to the restriction reaction. We incubated the reaction at 16°C for 3 h, which was followed by denaturing at 65°C for 10 min. Restriction digestion, ligation, and PCR assays were performed using an Applied Biosystems 2720 Thermal Cycler (Applied Biosystems, California, USA). The digested DNA was diluted 4 times prior to pre-amplification and selective amplification.

Three primer combinations (E-ACT+M-CTT, E-CAG+M-ATG, E-CGT+M-CAG) were used for selective amplifications as described in Lambertini et al. (2006; 2012a). The PCR mixture (20 μL) contained 0.5 U of TaKaRa Ex Taq (TaKaRa Bio, Shiga, Japan), 1× Ex Taq Buffer (TaKaRa Bio, Shiga, Japan), 0.2 mM dNTPs, 0.2 μM each of the forward and reverse primer, and 1 μL of the 4-times diluted DNA product. For pre-amplification, the program included 20 cycles, each with 30 s DNA denaturation at 95°C, 1 min primer annealing at 56°C and 1 min DNA extension at 72°C. For selective amplification, the program included 95°C for 30 s; 65°C for 30 s, with the temperature decreased by 0.7°C/cycle for 12 cycles; 72°C for 1 min; and then 23 cycles at 95°C for 30 s, 56°C for 30 s and 72°C for 1 min.

Electrophoresis was conducted on an NB-1480A system (Nihon Eido, Tokyo, Japan) with an acrylamide concentration of 6% for 360 min at 100 V with 50-500 bp external markers. Subsequently, the gels were stained with SYBR Gold (Invitrogen, California, USA), and a digital image of the gel was obtained using Chemi-Doc XRS (Milano, Bio-Rad, Italy).

4.2.6 AFLP data analysis

The fragments were manually checked using chromatograms generated with the software Image Lab 5.2.1 (Milano, Bio-Rad, Italy). Peak height data were converted as a binary matrix, and the presence or absence of fragments were further assessed using the R package AFLPScore version 1.4b (Whitlock et al., 2008) and a relative threshold of 20% the mean peak height for each locus. In total 107 AFLP polymorphic fragments were scored.

A phylogenetic tree was constructed using the UPGMA method (Nei and Li, 1979) with 1,000 bootstrap resampling, which implemented in the R Package ‘poppr’ version R 3.2.4

(Kamvar et al., 2014).

To identify cryptic population structure, and to detect migrants or admixed individuals, the binary matrix of AFLP was analyzed with software Structure 2.3.4 (Pritchard et al., 2000; Falush et al., 2003). The number of inferred groups was evaluated at values of K ranging from 1 to 10. Ten replicate runs were performed for each value of K . The burnin consisted of 100,000 MCMC reps, followed by 100,000 MCMC reps after the burnin. The other parameters were left at defaults. We used admixture model and performed clustering not using any information on origins and haplotypes, under the F model, which assumes that the allele frequencies correlated. Structure harvester (Earl and vonHoldt, 2012) was used to determine the best value of K .

4.3 Results

4.3.1 cpDNA sequencing

A total of 3 locus haplotypes were identified in the *trnT-trnL* sequences, which is consistent with previously identified locus haplotypes (T1, 4, and 5; Saltonstall, 2002). The *trnT-trnL* sequence showed that T1 and T5 were in a majority of the samples (36.4 and 61.4%, respectively) (Table 4-1). There were 2 types of mono-nucleotide variations in T1, i.e., T1b and T1d; T1d was a novel locus haplotype (Table 4-2; GenBank accession KX185523). There were 5 types of mono-nucleotide variation in T5 (T5a, T5d, T5e, T5g, and T5h), consistent with the sequences of Saltonstall (2016) (Table 4-2). Four previously identified *rbcL-psaI* haplotypes (R3, 5, 9, 18; Saltonstall, 2002; An et al., 2012), and a novel locus haplotype (R25; GenBank accession KX185520) was identified in this study. The frequency of R3, 5, and 9 were 29.5, 40.9, and 25.0% respectively, which were primarily detected in the *rbcL-psaI* sequences (Table 4-1). There were two types of previously identified mono-nucleotide variations in R5 (R5b and R5d), and two types of novel variations in R9 (R9b and R9c) (Table 4-3; GenBank accession KX185521, KX185522).

Table 4-1 Sampling locations and haplotypes identified by chloroplast DNA (cpDNA).

Microsatellite variations in cpDNA are indicated with a small letter following the *trnL-trnT* and *rbcL-psaI* sequences according to Saltonstall (2016).

Ref.ID	Location	Latitude	Longitude	Altitude (m) ^a	Habitat	Origin	<i>trnL-trnT</i>	<i>rbcL-psaI</i>	Haplo type	AFLP
ChA 1	Yuzhong, Chongqing	29.551	106.566	183	Riverside	Native	T5a	R3	I	x ^c
GuA 1	Huaxi, Guiyang, Guizhou	26.447	106.674	1097	CW ^b	Planted	T1b	R5b	P	x
GvA 2	Jiangnan, Nanning, Guangxi	22.812	108.313	70	Riverside	Native	T5d	R9c	U	x
SiD 1	Renhe, Panzhihua, Sichuan	26.475	101.753	1104	Freshwater stream	Native	T5a	R3	I	x
SiD 2	Renhe, Panzhihua, Sichuan	26.475	101.753	1104	Freshwater stream	Native	T5a	R9b	U	x
SiU 2	Jiuzhaigou, Ngawa Tibetan and Qiang Autonomous Prefecture, Sichuan	33.210	103.901	2197	Freshwater stream	Native	T4b	R18	BB	x
SiW 1	Xichang, Liangshan, Sichuan	27.846	102.292	1515	Roadside	Native	T5a	R3	I	
SiW 2	Yanyuan, Liangshan, Sichuan	27.281	101.300	2829	Freshwater swamp	Native	T1b	R5b	P	x
SiW 3	Yanyuan, Liangshan, Sichuan	27.719	100.870	2704	Roadside	Native	T1b	R5d	P	x
SiW 4	Yanyuan, Liangshan, Sichuan	27.646	101.830	1737	Freshwater stream	Native	T5a	R3	I	x
SiW 5	Xichang, Liangshan, Sichuan	27.842	102.293	1512	CW	Planted	T5a	R3	I	x
YuA 1	Yilaing, Kunming, Yunnan	24.994	103.186	1551	Freshwater stream	Native	T5e	R9b	U	x
YuA 2	Yilaing, Kunming, Yunnan	24.975	103.161	1541	Freshwater stream	Native	T5e	R9b	U	x
YuA 3	Chenggong, Kunming, Yunnan	24.845	102.863	1960	Upland	Native	T5e	R3	I	x
YuA 4	Fuming, Kunming, Yunnan	25.439	102.672	1668	Upland	Native	T5e	R3	I	x
YuA 5	Songming, Kunming, Yunnan	25.272	103.109	1896	Natural wetland	Native	T1b	R5d	P	x
YuA 7	Jinning, Kunming, Yunnan	24.683	102.629	1890	CW	Planted	T1b	R5d	P	x
YuA 8	Jinning, Kunming, Yunnan	24.685	102.630	1892	CW	Planted	T5h	R9b	U	x
YuA 9	Jinning, Kunming, Yunnan	24.680	102.622	1891	CW	Planted	T1b	R5b	P	x
YuA 10	Xishan, Kunming, Yunnan	24.873	102.660	1890	CW	Planted	T5e	R9b	U	x
YuA 11	Xishan, Kunming, Yunnan	24.874	102.660	1890	CW	Planted	T1b	R5d	P	x
YuA 12	Guandu, Kunming, Yunnan	24.922	102.737	1890	CW	Planted	T5e	R9b	U	x
YuA	Xishan, Kunming, Yunnan	24.997	102.626	1891	CW	Planted	T1b	R5d	P	x

13	Yunnan									
YuA 14	Guandu, Kunming, Yunnan	24.938	102.681	1888	CW	Planted	T1b	R5d	P	x
YuA 15	Chenggong, Kunming, Yunnan	24.868	102.787	1889	CW	Planted	T5e	R3	I	x
YuE 1	Chuxiong, Chuxiong, Yunnan	25.059	101.587	1773	Freshwater stream	Native	T5e	R9b	U	x
YuE 2	Nanhua, Chuxiong, Yunnan	25.203	101.253	1854	Riverside	Native	T5e	R3	I	
YuF 1	Chengjiang, Yuxi, Yunnan	24.609	102.951	1982	Sandy lakeside	Native	T5h	R9b	U	x
YuG 1	Mengzi, Honghe, Yunnan	23.436	103.323	1284	Lakeside	Native	T5e	R9b	U	x
YuG 2	Mengzi, Honghe, Yunnan	23.355	103.419	1309	Upland	Native	T5a	R3	I	x
YuK 1	Menghai, Xishuangbanna, Yunnan	21.762	100.280	1044	Orchard irrigation ditch	Native	T5g	R5d	Q	x
YuK 2	Jinghong, Xishuangbanna, Yunnan	22.003	100.804	540	Riverside	Native	T5d	R25	AT	x
YuL 1	Dali, Dali, Yunnan	25.750	100.145	1971	Freshwater stream	Native	T5e	R3	I	x
YuL 2	Heqing, Dali, Yunnan	26.616	100.214	2201	Roadside	Native	T1b	R5d	P	x
YuL 3	Jianchuan, Dali, Yunnan	26.488	99.947	2198	Wheat field edge	Native	T5e	R3	I	x
YuL 4	Dali, Dali, Yunnan	25.944	100.099	1969	CW	Planted	T1d	R5d	P	x
YuN 1	Mang, Dehong, Yunnan	24.171	98.142	786	Riverside	Native	T5e	R3	I	
YuN 2	Mang, Dehong, Yunnan	24.161	98.107	791	Freshwater stream	Native	T5e	R9b	U	x
YuP 1	Yulong, Lijiang, Yunnan	26.878	100.155	2448	Natural wetland	Native	T1b	R5d	P	x
YuP 2	Yulong, Lijiang, Yunnan	27.020	100.078	1843	Upland	Native	T5a	R5d	Q	x
YuP 3	Ningliang, Lijiang, Yunnan	27.670	100.805	2706	Sandy lakeside	Native	T1b	R5b	P	x
YuP 4	Yulong, Lijiang, Yunnan	26.861	100.147	2446	Natural wetland	Native	T1b	R5d	P	x
YuR 1	Xianggelila, Diqing, Yunnan	27.890	99.659	3274	Barley field edge	Native	T1b	R5b	P	x
YuR 2	Xianggelila, Diqing, Yunnan	27.505	99.817	3144	Pasture	Native	T1b	R5b	P	x

^a Altitude data were obtained from Google earth, ^bconstructed wetland, ^c"x" indicates markers analyzed for each sample.

Table 4-2 Variations of mono-nucleotide repeat regions in *trnT-trnL* cpDNA sequences. Base pairs represent the location of each poly-A microsatellite.

Haplotype	bp 190	bp 767	bp 989	GenBank accession
T1b	11	10	10	KP994330; GU338049
T1d	11	10	9	KX185523
T4b	10	9	10	AY016327
T5a	9	11	10	
T5d	10	10	10	
T5e	10	11	10	
T5g	11	10	10	
T5h	11	11	10	AY016328

Table 4-3 Variations of mono-nucleotide repeat regions in *rbcL-psaI* cpDNA sequences. Base pairs represent location of each poly-A microsatellite.

Haplotype	bp 179	bp 201	bp 974	GenBank accession
R3	10	8	9	AY016334
R5b	10	8	11	KP994332
R5d	10	8	10	AF457382; GU338046
R9b	10	8	9	
R9c	10	8	11	
R18	10	8	10	JF503248
R25	10	8	9	KX185520

Combinations of two locus haplotypes revealed 6 previously identified combined haplotypes (I, P, Q, U, AT, and BB) in the present study (Table 4-1). Haplotype I and U were the most frequent types occurring in native *Phragmites* (Table 4-4). Haplotype I and U are shared among subtropical species, such as *P. frutescens*, *P. mauritanus*, and *P. karka* (Lambertini et al., 2012b); moreover, haplotype I is shared between *P. australis* and its close variations (e.g., BH, BN, BU, etc.) in Mexico (Colin and Eguiarte, 2016) (Table 4-4). A parsimony network was constructed as shown in Figure 4-1. Haplotype Q represented a group that included not only *P. australis* but also the tropical and subtropical species *P. karka* and *P. mauritanus* (Saltonstall and Lambertini, 2012). Haplotype AT was obtained from a subtropical area of Xishuangbanna, Yunnan Province in the present study. Haplotype AT were previously identified from tropical species *P. karka* (Lambertini, unpublished), and showed a close relationship with haplotypes I and U (Figure 4-1).

Table 4-4 Frequency of native and planted *Phragmites* haplotypes

Origin	Total number of samples	Number of Haplotype					
		AT	BB	I	P	Q	U
putative native <i>Phragmites</i>	32	1	1	11	9	2	8
putative planted <i>Phragmites</i>	12	0	0	2	7	0	3

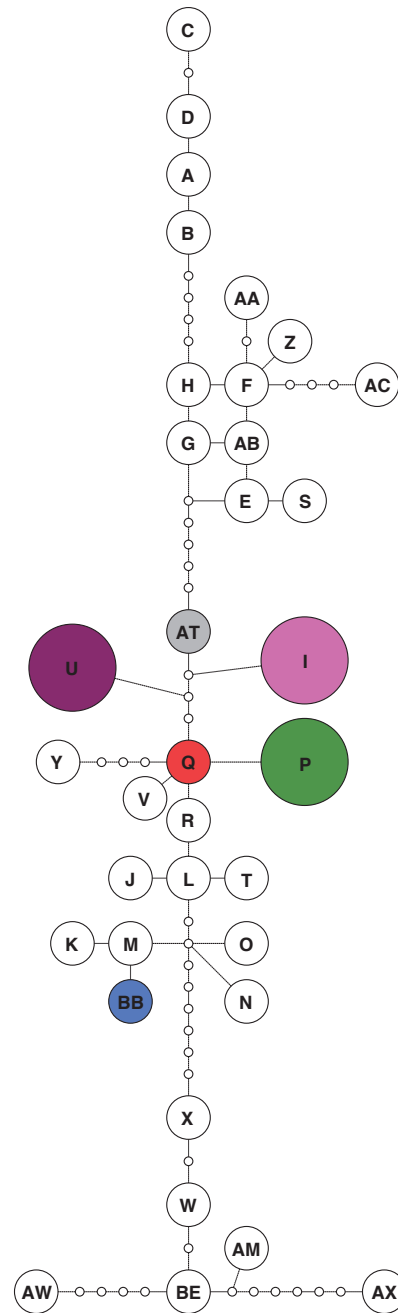


Figure 4-1 Parsimony network of *Phragmites* species haplotypes based on two chloroplast DNA sequences obtained in the present study and previous studies (Saltonstall, 2002; Meadows and Saltonstall, 2007; Chu et al., 2011; Lambertini et al., 2012b).

Note: Unlabeled nodes indicate inferred haplotypes that were not identified in this analysis. Loops in the network reflect homoplasy in the number of repeats in certain indels. The haplotypes identified in southwest China are indicated in color.

Haplotype P was also frequently detected (35.6%) in both Native and planted *Phragmites*. Haplotype P was previously identified in *P. australis* obtained from Asia, Australia, and North America (Saltonstall, 2002; Chu et al., 2011; An et al., 2012; Hurry et al., 2013; Lambert et al., 2016). The distribution of haplotypes is shown in Figure 4-2. Only native *Phragmites* with haplotypes AT, I, Q, and U was observed at lower altitudes (less than 1800 m). However, only native *Phragmites* with haplotype P was observed in the Hengduan Mountains at altitudes more than 2400 m above sea level.

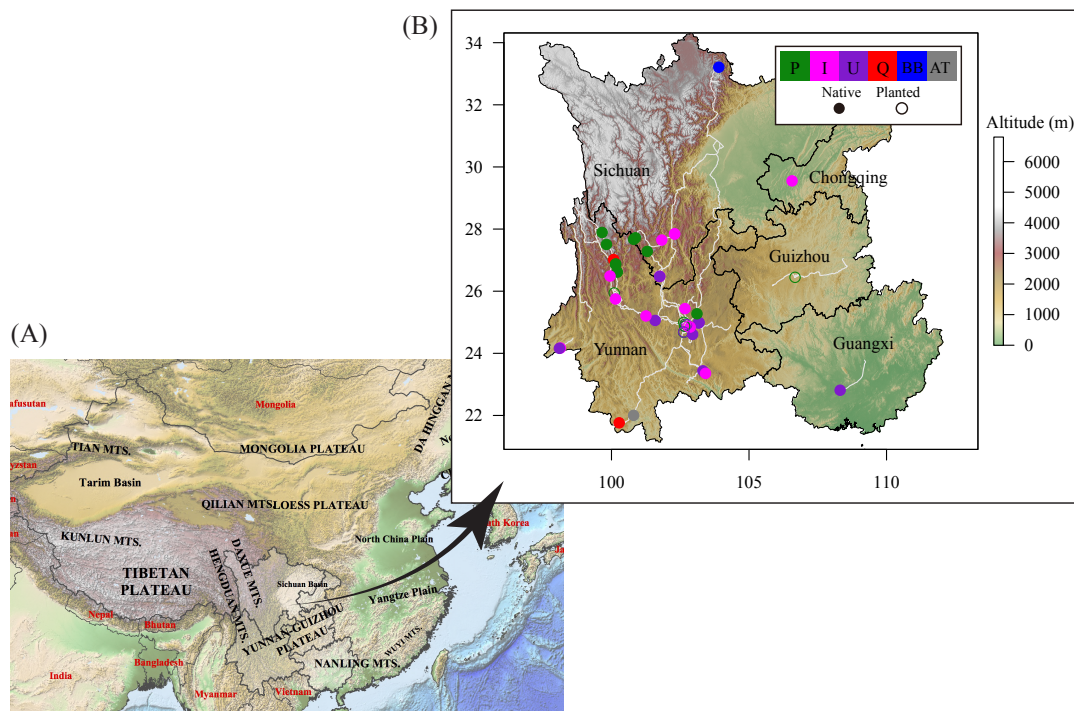
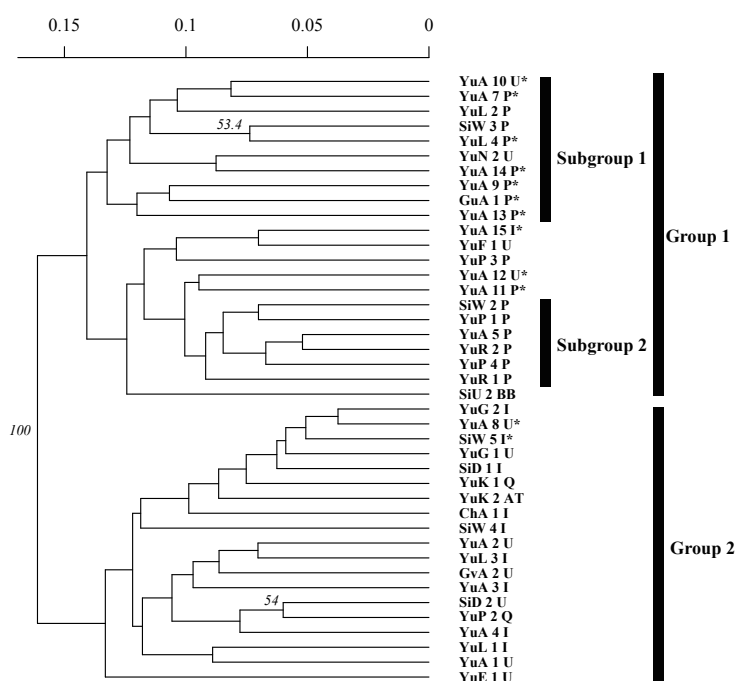


Figure 4-2 Topographic map of China with major names of mountain ranges, plateau, basin and plains (A). Distribution of *Phragmites* haplotypes overlaid on the elevation map in southwest China (B).

Note: The digital elevation maps were obtained from ETOPO1 (Amante and Eakins 2009) for map A and the SRTM 90 m Database of CGIAR (<http://www.cgiar-csi.org/>) for map B. Different shapes indicate three *Phragmites* species (squares: *P. australis*, circles: *P. japonicus*, and triangles: *P. karka*). Filled symbols indicate native samples, and open symbols indicate samples planted in constructed wetlands. The colors of the symbols indicate the haplotypes (green: P, light violet: I, violet: U, red: Q, blue: BB, gray: AT). White lines indicate the route explored in the present study.

The AFLPs produced 107 polymorphic fragments. The number of scored fragments was almost corresponded with the previous results presented by Lambertini et al. (2006, 2012a). We observed two haplotype-specific loci in AT, and I and Q. A UPGMA tree based on the AFLPs is shown in Figure 4-3. The two groups were well supported by the bootstrap value (100%). Group 1 is primarily composed of either native or planted *Phragmites* with haplotype P, whereas Group 2 is primarily composed of native *Phragmites* with haplotypes I, U, Q, and AT. Although the other groups were supported by weak bootstrap values (<50%), the clades were largely classified according to haplotypes or origins. Subgroup 1 primarily consisted of planted *Phragmites* with haplotype P. Subgroup 2 included native *Phragmites* samples with haplotype P and was primarily observed in plateau regions at altitudes more than 2400m, as described above.



Note: The numbers on branches are bootstrap percentages. The terminals are labeled with the reference IDs of the samples and haplotypes as shown in S1. Terminal labels with asterisks indicate the samples planted in constructed wetlands.

Structure analysis of AFLPs identified $K=2$ as the most reliable estimate (Figure 4-4). Bar plot of Q values (proportion of ancestry estimated by Structure) for 41 individuals from SW China is shown in Figure 4-5. The order of individuals was sorted by haplotype to facilitate detection of the ancestry admixture. The clustering is strongly relevant to the haplotypes. *Phragmites* with haplotype P and BB had a pure ancestry (cluster 1). Although most of *Phragmites* with haplotype I, U, Q, and AT had another type of pure ancestry (cluster 2), several individuals with haplotype I and U apparently had mixed, or alternative (cluster 1) ancestry.

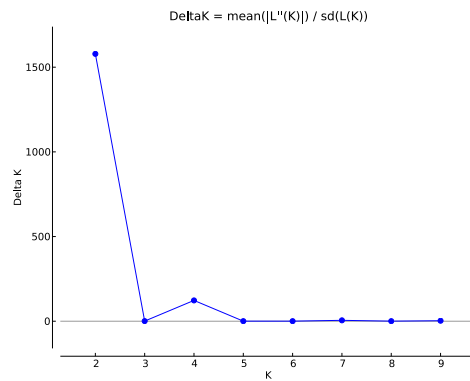


Figure 4-4 DeltaK plot for the determination of the best value of K .

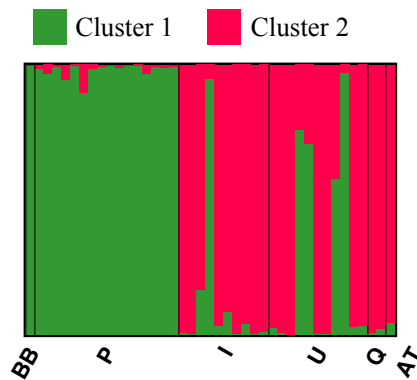


Figure 4-5 Bar plot of Q values (proportion of ancestry estimated by Structure) for 41 individuals from SW China.

Note: F_{st} of cluster 1 is 0.211, and that of cluster 2 is 0.184. The order of individuals was sorted by haplotype to facilitate detection of the ancestry admixture. The parameter USEPOPINFO were not used for the analysis.

4.4 Discussion

4.4.1 Relationships among genotypes

We found geographical distribution of different haplotypes. Native *Phragmites* with haplotypes AT, I, Q, and U distributed at elevations less than 1800m. We also observed native *Phragmites* with haplotype P primarily distributed in the Hengduan Mountains at higher elevations more than 2400m. Haplotypes AT, I, Q and U were previously identified not only from *P. australis* but also from subtropical and tropical species including *P. frutescens*, *P. mauritanus*, and *P. karka* (Lambertini et al., 2012b; Saltonstall and Lambertini, 2012), as mentioned above. Furthermore, haplotype P were previously identified from cosmopolitan species *P. australis* mainly in temperate zones (Saltonstall, 2002; Chu et al., 2011; An et al., 2012; Hurry et al., 2013; Lambert et al., 2016). These results indicated that haplotypes AT, I, Q, and U represent a tropical lineage, and haplotype P is common lineage. This idea was further supported by the results of the UPGMA tree (Figure 4-3). A distinct genetic difference between groups that were primarily composed of haplotypes P (Group 1), and were composed of haplotype AT, I, Q and U, was observed (Group 2). Furthermore, the result of the UPGMA tree also indicated the phylogenetic group of native common lineage primarily obtained from the Hengduan Mountains (Subgroup 2) is different from that of planted common lineage obtained from CWs. Therefore, these two native lineages might be geographically isolated by the Hengduan Mountains in SW China.

Haplotypes O, M, L, P, and K were previously detected in *P. australis* samples from east, northwest, and northeast China, and the wide distribution of haplotype O indicated that it is a primitive haplotype in China (An et al., 2012). The widely distributed dominant haplotype O, however, was not detected in SW China in the present study. In addition, haplotypes I and U were not identified in the other areas of China in a previous study (An et al., 2012). Therefore, the *Phragmites* species of SW China might further have been geographically isolated from the surroundings by the Yunnan-Guizhou Plateau. Although two lineages are inferred in SW China as mentioned above, several samples from tropical lineage with haplotype I and U (YuA 10, YuN 2, YuA 15, YuF1, and YuA 12) were nested within Group 1 (Figure 4-3). The result of structure analysis also supported the two types of lineages, and further indicated that the five individuals with haplotype I and U apparently had mixed, or largely belonged to different ancestry (Figure 4-5). Overall, these results suggested that the putative hybrids might have been resulted from crossings between maternal tropical and paternal common lineages, and

back-crossing of the hybrids with the paternal common lineage.

4.4.2 Impact of artificial dispersal

High frequency of haplotype P was detected from *Phragmites* planted in CWs (Table 4-4), and these samples formed Subgroup 1 in the UPGMA tree (Figure 4-3). These results indicated that most of the planted *Phragmites* might have been introduced from an area outside SW China, such as East China (e.g. North China Plain), where *P. australis* with haplotype P is a dominant type, and these findings are consistent with An et al. (2012). Furthermore, three individuals (YuA 10, YuA 12, and YuA 15) of the five putative hybrids between tropical and common lineages originated from CWs in Kunming, Yunnan province. These results partially indicated possible hybridizations between native tropical lineage in SW China and common lineages introduced from the outside of SW China. However, the process of hybridization events was still largely uncertain because our study did not focus on genetic structure between populations. Further research should be oriented towards exploring phylogeographic relationships using wider population samples and molecular markers, and the reproductive characteristics and environmental responses to understand the propagation scenarios in SW China.

The biodiversity in Yunnan Province, SW China, is under considerable pressure because of the recent intensification of land use and expansion of the human population (Yang et al., 2004; Willson, 2006). Habitats of native reeds have also been destroyed by recent urbanization (e.g., YuE1, SiD1, and SiD2 have been ruined because of bank protection work). Although the subsequent propagation of artificially introduced *Phragmites* has not been observed, *Phragmites* propagation and crossing with native *Phragmites* should be traced because of the increasing likelihood of the impact of artificially introduced populations on native *Phragmites* diversity. In addition, *Phragmites* species have attracted increasing interest because of their potential as a bioremediation tool for metal removal (Gikas et al., 2013), a material for biofuel (Liu et al., 2012), and a high-quality roughage for ruminants (Tanaka et al., 2016a, b). Each genotype population showed different morphological and physiological traits (Tho et al., 2016), and *P. australis* from different origins demonstrated varying degrees of tolerance to heavy metals (Ye et al., 1997). Thus, superior lineages of *Phragmites* species might be selected for human use in the future. In this context, the conservation of native *Phragmites* and continuous research on its genetic diversification and introgressions via hybridization and the impacts of non-native populations are required.

4.5 Conclusions

The present study improves our understanding of the phylogenetic relationships of *Phragmites* lineages in SW China. The results demonstrate that *Phragmites* in SW China are largely classified into two types, representing tropical and common lineages. The lineages may be geographically isolated not only within SW China but also with surroundings because of the diverse topographic characteristics of this region. Furthermore, the results also indicated several putative hybrids between the lineages. Further research should be oriented towards exploring phylogeographic relationships using wider samples and molecular markers, and the reproductive characteristics and environmental responses to facilitate understanding of delimitation and evolutionary history of the *Phragmites* lineages.

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Chapter 5 Effect of *Phragmites japonicus* harvest frequency and timing on dry matter yield and nutritive value

5.1 Introduction

Recent economic and human population growth has increased the demand for livestock products (Thornton, 2010). Meanwhile, serious issues concerning animal feed production have arisen. The bioenergy sector could directly compete with forage-livestock production (Sanderson and Adler, 2008). Forage production systems cannot be widely adapted due to competition for land with food crop production (McIntire and Debrah, 1986), and an overall slowdown in the expansion of agricultural land, including pasture, is expected (Bruinsma, 2003). Consequently, human population growth throughout developing countries is increasing the demand for feed all over the world; consequently, the exploitation of alternative feed resources is a matter of great urgency.

Phragmites is a cosmopolitan clonal plant found throughout the world. Within the genus, *P. australis* (CAV.), Trin. ex Steudel has attracted attention for its potential use as roughage because of its high contents of crude protein (CP), neutral detergent fiber (NDF), and total digestible nutrients (TDN) (Baran et al., 2002; Kadi et al., 2012; Asano et al., 2015; Tanaka et al., 2016). Furthermore, *Phragmites* species have been used as important vegetation for constructed wetlands (CWs) to treat nutrient-rich wastewater. Since the first full-scale CW systems were put into operation during the late 1960s, there are currently more than 50,000 CWs in Europe and more than 8,000 CWs in North America (Vymazal, 2005; Vymazal, 2011). CWs are also gaining popularity for cost-effective wastewater management in developing countries, and the number of CWs has increased in Southern and Central Africa and Asia (Kadlec and Wallace, 2009). Conversely, anthropogenic influences, including nutrient enrichment, have often fueled the increasing expansion of *P. australis*, which has become a serious issue in many regions (Findlay et al., 2003; Güsewell, S., 2003; Kettenring et al., 2011). Given the increasing habitat area of *Phragmites*, it could be a valuable source of roughage. Although harvesting aboveground biomass is a recommended practice for improving nitrogen (N) removal in productive areas (Álvarez and Bécares, 2008; Koottatep and Polprasert, 1997), the capital costs have interfered with plant harvest (Crites and Tchobanoglous, 1998). Thus, roughage production by harvesting *Phragmites* is expected to play an important role in not only supplying feed but also recovering the harvesting costs.

Multiple harvest of reed is more effective than single harvest in removing N and P

(phosphorus) (Hernández-Crespo et al., 2016; Suzuki et al., 1989). Tanaka et al. (2016) suggested that high nutrient removal efficiency and high-quality roughage production could be compatible when considering the frequency and timing of harvest. The harvest timing could negatively affect subsequent regrowth because it hinders the annual rhizome reserves allocation from the aboveground biomass (Asaeda et al., 2006; Fogli et al., 2014; Karunaratne and Asaeda, 2004; Köhl et al., 1997); therefore, continuous harvesting could reduce the efficiency of nutrient removal in CWs from a long-term perspective. Furthermore, insufficient reloading of reserve carbohydrates in the rhizome could result from harvesting during the growing season and could subsequently reduce the nutritive value of shoots due to the minimal translocation of reserve carbohydrates from the rhizome (Tanaka et al., 2016). However, little is known about the effect of harvest frequency on dry matter yield, nutrient value, and N removal efficiency.

An appropriate management strategy, including a combination of timing and frequency of harvesting, should be established for sustainable roughage production and high-efficiency N removal in CWs. Therefore, we first aimed to explore the effect of harvest timing on the yield and nutritive value; second, we estimated the effect of harvest frequency on the yield, nutritive value, and N removal; and finally, we considered the implications for better CW management. In the present study, we annually reaped *P. japonicus* Steudel from one to three times and investigated the yield and nutritive value of the harvest.

5.2 Materials and Methods

5.2.1. Study site

Lake Dianchi is the largest freshwater lake in the Yunnan Province of China and has had a serious water eutrophication problem since the 1970s. According to monitoring data from 2005 to 2012, the annual concentrations of total N ranged from 1.82 to 3.01 mg L⁻¹, and those of total P ranged from 0.13 to 0.20 mg L⁻¹ in the main water body of Lake Dianchi (Zhang et al., 2013). To mitigate the eutrophication of the lake, a large number of CWs were established along the lakeside. Samples were obtained from the following two different free-water system CWs: from the east side (E) and west side (W) of Lake Dianchi (Figure 5-1). Site E (24° 52' N, 102° 47' E) was situated at a distance from the lakeshore, where the maximum water depth did not exceed 10 cm. Site W (24° 52' N, 102° 39' E) was located near the lakeshore, where the water was relatively deeper than at Site E, and the water depth reached a maximum of 38 cm in winter. Agriculture is a major land use at both sites. The predominant vegetation in the coastal areas of Lake Dianchi is *P. japonicus*, cattail (*Typha sp.*), and manchurian wild rice [*Zizania latifolia*

(Griseb.) Turcz. ex Stapf]. *Phragmites japonicus* is morphologically similar to *P. australis*, but it has a unique ability to generate epigeal stolones and is distributed over a wide range in Asia.

Hourly precipitation and air temperature data were obtained from the weather station (WeatherHawk Station, Campbell Scientific, USA) in Kele village (E24° 52' N, 102° 47' E). The distance from weather station to site E and W was 0.7 and 13.5 km, respectively. The mean daily temperatures and precipitation are shown in Figure 5-2. The rainy season occurs from May to October, and annual rainfall was 980 mm in 2015. The mean annual temperature was 17.5 °C, and the mean daily temperature reached a maximum of 24.7 °C on May 31 and a minimum of 2.7 °C on January 10.

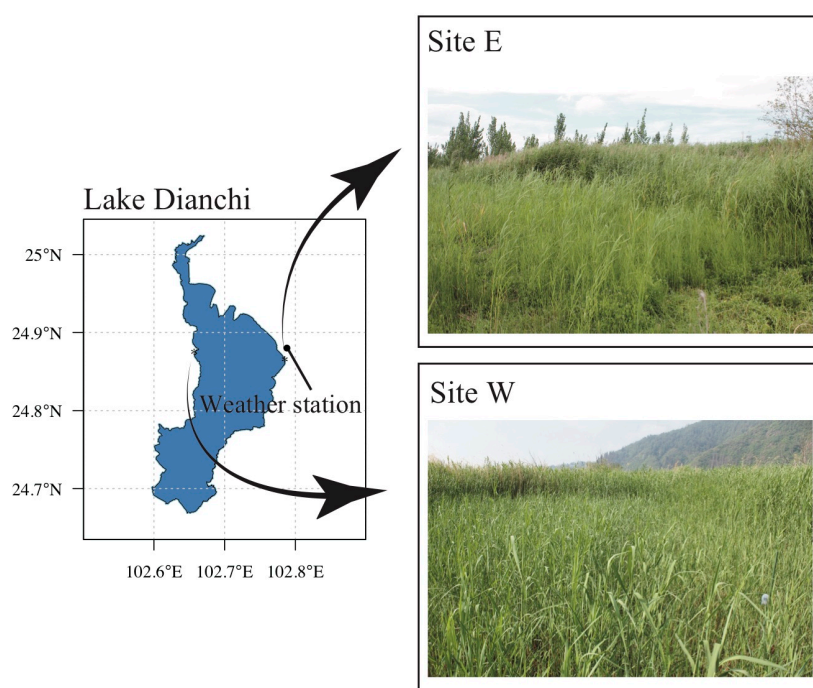


Figure 5-1 Location of the study site.

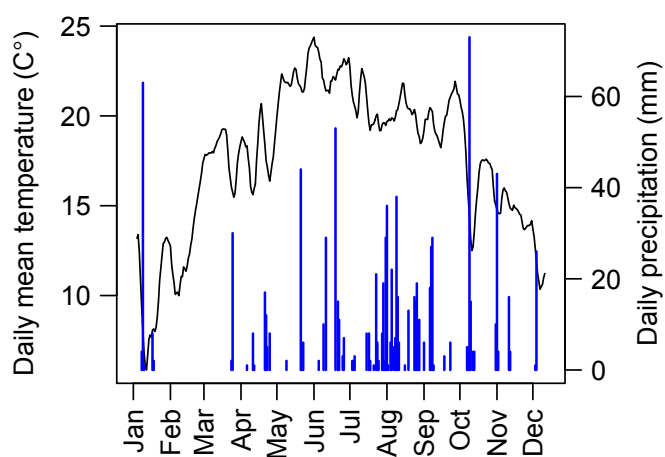


Figure 5-2 Mean daily temperature and precipitation.

Note: The black solid line represents the mean daily temperature, and the blue solid lines represent the daily precipitation. The mean daily temperature was calculated as a simple moving average at 7-day intervals.

5.2.2. Harvest management

Four treatments with different harvesting frequencies were carried out in monospecific stands of *P. japonicus*. Each plot area was 64 m² (8 m × 8 m). The harvesting schedule is shown in Table 5-1. In this study, harvest just represents the mowing treatment, and it does not mean sampling.

Table 5-1 The harvest method

Treatment	Removal of dead biomass on Feb 3–5, 2015	Harvest on Apr 11–13, 2015	Harvest on Jun 12–15, 2015	Harvest on Aug 13–14, 2015	Removal of dead biomass on Jan 29 – Feb 1, 2016
HF0	○	-	-	-	○
HF1	○	-	-	○	○
HF2	○	○	○	-	○
HF3	○	○	○	○	○

HF0: no harvest, HF1: single harvest at 6 months, HF2: two harvests at 2 and 4 months, HF3: three harvests at 2, 4, and 6 months. The symbol of “○” and “-” represents the presence and absence of harvesting treatment at the time, respectively.

The four harvesting frequencies included a single harvest at 6 months (HF1), two harvests at 2 and 4 months (HF2), three harvests at 2, 4, and 6 months (HF3), and no harvest as the control (HF0). Dead aboveground biomass has a great effect on shading, and may cause a serious internal competition for light resources (Tanaka et al., 2016). The quality of harvested roughage may also be degraded by dead aboveground biomass. Thus, dead aboveground biomass was removed from all treatments at sites W and E on February 3 and 5 of 2015, respectively. Furthermore, the dead aboveground biomass was removed on January 29 and February 1 of 2016 at sites E and W, respectively. We assumed that practical harvest management was implemented at a specified height above the surface water level. Thus, the plants were harvested or removed at a height of 10 and 40 cm above the ground at sites E and W, respectively.

5.2.3. Plant sampling and analysis

Three replicate samples of aboveground standing biomass within a 0.25 m² (0.5 × 0.5 m) frame were cut with a sickle at ground level and sampled monthly from March to August of 2015. Immediately before the removal of dead biomass on January 29 and February 1 of 2016 (Table 5-1), the samples of aboveground biomass were also collected. To investigate the effects of harvest frequency on aboveground growth in the following year, the aboveground biomass was collected in April and June of 2016. Sampling was always performed within visually homogeneous stands with uniform shoot density. The plant samples were placed into polyethylene bags for transport to the laboratory.

The samples of shoots were divided into two parts at the cutting height of 10 and 40 cm from the base at site E and W, respectively, and then upper part was sorted as harvested or removed biomass. Harvested biomass was regarded as roughage sample collected from March to August of 2015, and was then classified as dead or live tissues and then being sorted as stems, leaves, and ears. Removed biomass represents dead biomass assumed to remove in January 29 and February 1 of 2016 at sites E and W, respectively. The dry weight was determined after oven drying at 80 °C to a constant weight. The dried plant materials were ground until they were fine enough to pass through a 2-mm sieve.

The plant samples of harvested biomass (stems and leaves) were analyzed for NDF, acid detergent fiber (ADF), acid detergent lignin (ADL), CP, neutral detergent insoluble crude protein (NDICP), acid detergent insoluble crude protein (ADICP), ether extract (EE), and crude ash. The NDF and ADF contents were expressed exclusive of residual ash (Van Soest et al., 1991) using α -amylase for the NDF analysis. The ADL, CP, EE, and crude ash content were

determined according to the Association of Official Analytical Chemists (AOAC, 1990). The contents of NDICP and ADICP were analyzed in neutral and acid fiber fractions, respectively (Licitra et al., 1996). The concentrations of TDN were calculated for each sample from the summative equation of the feeds at maintenance of dairy cattle (NRC, 2001) as follows:

$$\text{TDN} = \text{tdNFC} + \text{tdCP} + \text{tdNDF} + (\text{tdFA} \times 2.25) - 7 \quad (1)$$

$$\begin{aligned} \text{Truly digestible nonfiber carbohydrate (tdNFC)} \\ = 0.98 \times \{100 - [(\text{NDF} - \text{NDICP}) + \text{CP} + \text{EE} + \text{Ash}]\} \end{aligned} \quad (2)$$

$$\text{Truly digestible CP (tdCP)} = \text{CP} \times \exp(-1.2 \times \text{ADICP}/\text{CP}) \quad (3)$$

$$\begin{aligned} \text{Truly digestible NDF (tdNDF)} \\ = 0.75 \times \{(\text{NDF} - \text{NDICP}) - \text{ADL}\} \times \{1 - [\text{ADL}/(\text{NDF} - \text{NDICP})]^{0.667}\} \end{aligned} \quad (4)$$

$$\text{Truly digestible fatty acids (tdFA)} = \text{EE} - 1, \text{ if } \text{EE} < 1 \text{ then } \text{tdFA} = 0 \quad (5)$$

The yield of TDN was estimated by multiplying the TDN concentration by the DM yield. The N yield was calculated by multiplying the N content by the DM yield using each dataset of harvested biomass from 2015 and the removed dead biomass from 2016 in the four treatments (HF0, HF1, HF2, and HF3) and integrating those values.

5.2.4. Data analyses

All of the statistical analyses were performed using R version 3.2.4 (R Development Core Team, 2016). To understand how the TDN components (tdNFC, tdCP, tdNDF, and tdFA) explain the seasonal variations of TDN concentration, the relative importance of the TDN components was analyzed using a multiple regression analysis. The TDN was used as a dependent variable, and the TDN components were used as independent variables. All of the variables were standardized prior to the multiple regression analysis. The relative importance of each TDN component to the TDN variation was estimated as a proportion of each correlation coefficient. The differences in each TDN component, DM and TDN yield within HF3, and the aboveground biomass among the different levels of harvest frequency were analyzed using one-way analysis of variance (ANOVA). The differences were tested using Tukey's test. Differences among the means were considered statistically significant at $P < 0.05$.

5.3 Results

5.3.1. Effect of harvest timing on yield and nutritive value of fist crop

The seasonal variations in the TDN concentration, and the DM and TDN yield of first crop are shown in Figure 5-3. Similar seasonal variations were observed between sites E and W. However, there was a one-month delay in the response of all parameters (TDN concentrations, DM and TDN yield) to the duration of growth at site W in comparison to site E. Sharp declines in TDN concentrations were observed from approximately 30 to 90 days, and from 60 to 120 days of growth duration at site E and W, respectively. The DM and TDN yields reached a plateau at approximately 60 and 90 days of growth duration at site E and W, respectively. The appearance of ears at site E (17 May 2015) occurred one month earlier than at site W (15 June 2015).

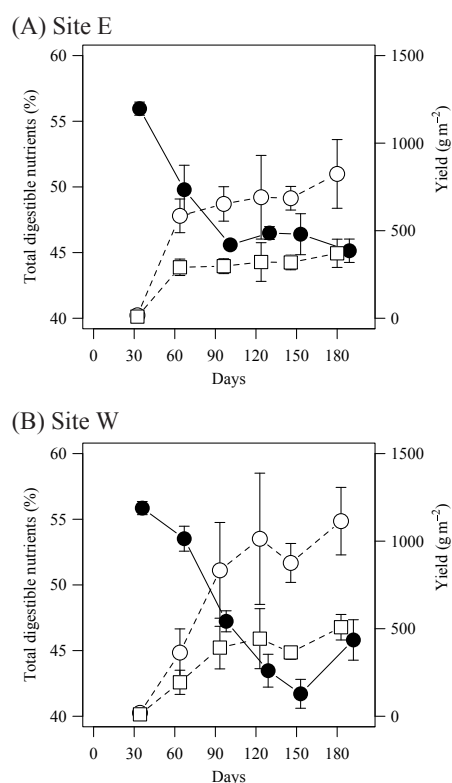


Figure 5-3 Seasonal variations of total digestible nutrient (TDN) concentrations, dry matter (DM) and TDN yield of first crop at site E (A), and at site W (B).

Note: Values of no harvest plot (HF0) are used to represent the seasonal variations of fist crop. Bars indicate standard deviations. Filled circles refer to TDN concentrations, and open circles and squares indicate DM and TDN yields, respectively. TDN yield was estimated by multiplying the TDN concentration by the DM yield.

The seasonal variation of TDN concentration and its components of leaf and stem of first crop are shown in Table 5-2. Comparing within the same growth duration, the TDN concentration of the leaves was always higher than that of the stems. As for the leaf samples, the relative importance of tdNFC, tdCP, and tdNDF to the variations in TDN were almost equivalent to each other (approximately 30%) at both sites. However, the relative importance of the tdNFC of the stem samples was relatively higher (approximately 40–50%) than the other TDN components. Generally, the tdFA was not a great contributor to the variations in the TDN concentrations (<15%).

Table 5-2 Seasonal variation of TDN concentrations and its components of leaves and stems of first crop

Locality	Part	Days	tdNFC ^a		tdCP ^b		tdNDF ^c		tdFA ^d		TDN ^e	
			% DM ^f	RI to TDN (%) ^g	% DM	RI to TDN (%)	% DM	RI to TDN (%)	% DM	RI to TDN (%)	% DM	
Site E	Leaf	67	19.4 ± 2.5		14.7 ± 0.3		29 ± 1.8		0.1 ± 0.2		56.3 ± 1.7	
		101	22.1 ± 1.2		10.8 ± 0.2		23.8 ± 0.8		0.8 ± 0.1		51.5 ± 0.5	
		130	17.7 ± 1.5	26.6	10.1 ± 0.5	30.8	28.1 ± 1.6	29.4	1.1 ± 0.1	13.1	51.3 ± 0.9	
		153	20.3 ± 1.6		9.9 ± 0.6		27 ± 2.2		1.3 ± 0.1		52.9 ± 2.6	
		189	19.1 ± 0.5		7.8 ± 0.7		26.5 ± 1.1		1.2 ± 0.1		49.2 ± 0.2	
	Stem	67	10.5 ± 2		5.3 ± 0.6		37.1 ± 1.6		0.3 ± 0.4		46.7 ± 2	
		101	8.2 ± 1.3		2.9 ± 0.9		38.2 ± 0.3		0 ± 0		42.3 ± 0.8	
		130	12.7 ± 2	43.7	1.3 ± 0.9	26.9	37.2 ± 0.8	22.6	0 ± 0	6.8	44.2 ± 0.9	
		153	8.8 ± 2.5		2.4 ± 0.1		38.8 ± 1		0 ± 0		42.9 ± 1.5	
		189	13.6 ± 1.2		1.2 ± 0.3		35.5 ± 0.2		0 ± 0		43.3 ± 1.2	
Site W	Leaf	67	12.4 ± 0.8		14.4 ± 1.1		34.4 ± 1		1 ± 0.3		56.4 ± 2	
		98	15.9 ± 2.2		10.9 ± 1		26.9 ± 2.7		2.2 ± 0.2		51.6 ± 1.6	
		129	10 ± 2.4	25.0	10.8 ± 1.8	27.8	32.2 ± 0.9	34	1.8 ± 0.3	13.2	50 ± 0.7	
		153	12.5 ± 0.4		9.8 ± 0.5		29 ± 2		0.9 ± 0.2		46.3 ± 2.4	
		192	13.1 ± 0.3		7.2 ± 0.9		32.3 ± 2.2		1.5 ± 0.3		48.9 ± 2.1	
	Stem	67	13.9 ± 1.7		5.6 ± 1.4		39.8 ± 0.5		0.1 ± 0.1		52.4 ± 1.9	
		98	9.9 ± 1		2.9 ± 0.6		39.7 ± 1.6		0 ± 0		45.5 ± 0.6	
		129	8.3 ± 1.3	53.4	1.2 ± 0.2	24.8	37.9 ± 1.4	19.3	0 ± 0	2.5	40.4 ± 2.1	
		153	2.8 ± 0.9		2.2 ± 0.1		41.2 ± 0.7		0.2 ± 0.1		39.6 ± 0.8	
		192	11.4 ± 1.7		0.8 ± 0.3		39.1 ± 0.8		0 ± 0		44.2 ± 1	

Values are the means±standard deviations of no harvest plot (HF0) (n=3) because they correspond to the seasonal variations in TDN and its components of first crop.

^aTruly digestible nonfiber carbohydrate; ^btruly digestible crude protein; ^ctruly digestible neutral detergent fiber; ^dtruly digestible fatty acids; ^etruly digestible nutrients; ^fdry matter; ^gthe relative importance to TDN was estimated as a proportion of each correlation coefficient using a multiple regression analysis.

5.3.2. Effect of multiple harvesting on yield, nutritive value, and N yield

The nutritive value, DM and TDN yield of HF3 are listed in Table 5-3. At site E, the TDN concentration remained relatively constant (approximately 49%), which was likely to be balanced by variations of tdNFC, tdCP, and tdNDF. Meanwhile, at site W, the TDN concentration of the first harvest was significantly higher ($53.5 \pm 1.0\%$) than that of the second ($47.7 \pm 0.8\%$) and third harvest ($50.7 \pm 0.6\%$), and the tdNFC and tdCP also exhibited a similar trend. The ratio of leaf to stem increased on the order of first, second, and third harvest at both sites. The highest DM yield was found in the first harvest at site E ($585 \pm 96 \text{ g m}^{-2}$). However, the second harvest showed the highest DM yield at site W ($635 \pm 110 \text{ g m}^{-2}$).

The N yields of different harvest frequencies are shown in Figure 5-4. The N yield increased with the harvest frequencies at both sites. The removed dead biomass in winter exhibited a weak contribution to the total N yield. At site E, the N yield of HF3 ($17.3 \text{ g N m}^{-2} \text{ yr}^{-1}$) was approximately 5 times higher than that of HF0 ($3.8 \text{ g N m}^{-2} \text{ yr}^{-1}$). At site W, the N yield of HF3 ($22.6 \text{ g N m}^{-2} \text{ yr}^{-1}$) was approximately 10 times higher than that of HF0 ($2.2 \text{ g N m}^{-2} \text{ yr}^{-1}$).

Table 5-3 Nutritive value, dry matter and total digestible nutrients yield of the three-harvest treatment (HF3)

Locality	Harvest	Date	Days	tdNFC ^a % DM	tdCP ^b	tdNDF ^c	tdFA ^d	TDN ^e	Leaf/ stem ^f	DM ^g yield g m ⁻² yr ⁻¹	TDN yield
Site E	1st	13 Apr 15	67	13.3 b	8.4a	34.5b	0.3a	49.8a	0.48b	585a	291a
	2nd	15 Jun 15	63	17.3 a	5.4b	32.8b	0.0a	48.5a	0.52b	216b	104b
	3rd	13 Aug 15	59	9.7 c	9.0a	37.7a	0.0a	49.4a	0.75a	147b	73b
	Mean (Total) ^h			13.5	7.6	35.0	0.1	49.2	0.58	(948)	(467)
Site W	1st	11 Apr 15	67	13.4a	8.2a	38.2b	0.3b	53.5a	0.41b	364b	194a
	2nd	12 Jun 15	62	8.8b	6.3a	38.6b	0.5b	47.7c	0.63a	635a	302a
	3rd	14 Aug 15	63	6.4c	7.6a	42.0a	0.8a	50.7b	0.81a	339b	171a
	Mean (Total)			9.6	7.3	39.6	0.5	50.6	0.62	(1337)	(668)

Values are the means (n=3). Different small letters indicate significant differences (P<0.05)

^aTruly digestible nonfiber carbohydrate; ^btruly digestible crude protein; ^ctruly digestible neutral detergent fiber; ^dtruly digestible fatty acids; ^etruly digestible nutrients; ^fratio of leaf to stem, based on dry matter weight; ^gdry matter; ^hmean or total values of 1st to 3rd harvest.

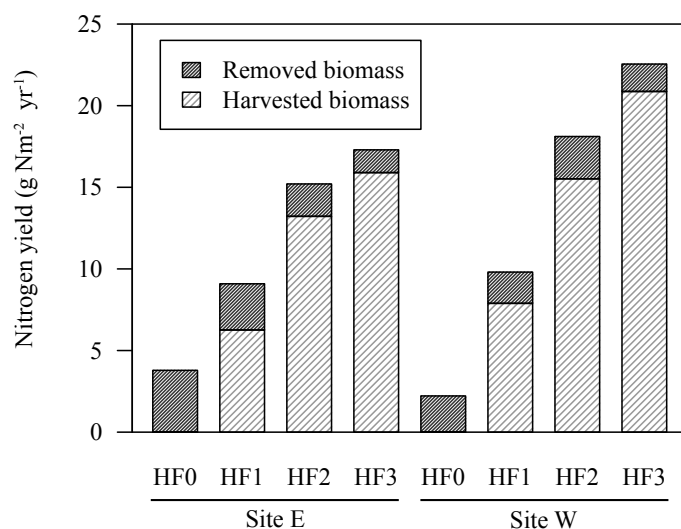


Figure 5-4 Nitrogen (N) yields of different harvest frequencies.

Note: The N yield of removed biomass represents the amount of N via removing dead aboveground biomass that is not regarded as roughage production in January 29 and February 1 of 2016 at sites E and W, respectively. The N yield of harvested biomass represents the amount of N via harvesting aboveground biomass that is regarded as roughage production in 2015 according to the four treatments of different harvest frequencies at two experimental sites, as shown in Table 5-1.

5.3.3. Effect of harvest frequency on the following growth

The aboveground biomass, N content and N yield from April and June 2016 are shown in Table 5-4. In general, the aboveground biomass declined across harvest frequency in the following year. The aboveground biomass of HF1 was slightly lower than that of HF0, but the result of Tukey's test showed that there were always no significant differences between them. The results also indicated that the aboveground biomass of HF3 was significantly lower than that of HF0 at both sites in both seasons ($P < 0.05$), excluding the case of site W on June 16, 2016. There were no large differences in the N content between treatments. Overall, the N content of site W was higher than that of site E. The variations in N yield were similar to those of aboveground biomass.

Table 5-4 Aboveground biomass, N content and N yield in April and June 2016.

Location	Sampling time	Treatment	Aboveground biomass		Nitrogen content		Nitrogen yield	
			g m ⁻²		% DM		g N m ⁻²	
Site E	14 Apr	HF0	387	a	1.66	NS	6.44	a
		HF1	313	ab	1.52		4.73	ab
		HF2	177	b	1.78		3.15	ab
		HF3	139	b	1.74		2.53	b
	16 Jun	HF0	1132	a	0.83	NS	9.39	a
		HF1	764	ab	0.90		6.82	ab
		HF2	590	b	0.87		4.99	b
		HF3	383	b	0.95		3.70	b
Site W	15 Apr	HF0	759	a	1.85	ab	13.92	a
		HF1	445	ab	1.74	b	7.67	b
		HF2	257	ab	1.86	ab	5.31	b
		HF3	215	b	2.22	a	4.78	b
	18 Jun	HF0	1394	NS	1.35	NS	18.72	NS
		HF1	1098		1.20		12.93	
		HF2	978		1.30		12.81	
		HF3	642		1.39		8.85	

Different letters indicate significant differences ($P < 0.05$). NS represent no statistical significance among treatment.

5.4 Discussion

5.4.1. Effect of harvest timing

Several important characteristics of *P. japonicus* for roughage production can be identified from our results. The TDN concentration exhibited relatively high values in the early growing stage, and it sharply declined across the duration of growth (Figure 5-3), which is consistent with previous reports of *P. australis* (Asano et al., 2015; Tanaka et al., 2016). Meanwhile, we observed different timing in the sharp decline of TDN and the rate of the increase in the DM yield, and they were one month earlier at site E than at site W. This corresponded to the timing of appearance of ear. These results indicate that drastic changes in the TDN concentration and biomass production might be associated with the transition from the vegetative to the reproductive phase. The difference in heading time between the two experimental sites may be genetically determined because there is a distinct difference in the genetic background based on chloroplast DNA sequences between the two populations of *P. japonicus* (Tanaka, in prep.). Thus, the heading time might be one of the important characteristics required for selecting a lineage of *Phragmites* that is suitable for roughage production.

The relative importance of tdNFC in the stems accounted for approximately 50% of the variations in TDN concentrations, although tdNFC showed relatively smaller values than the tdNDF (Table 5-2). Granéli et al. (1992) reported that a decrease in the nonstructural carbohydrate (NSC) of *P. australis* rhizome during spring corresponded to shoot production until the establishment of foliar structure, and the NSC of the rhizomes were reloaded by translocation from shoots during the summer. Therefore, our finding indicated that the timing or quantities of accumulation of assimilated NSC in the stems and the translocation of NSC between aboveground stems and the belowground rhizome might be important characteristics for nutritive value determination.

5.4.2. Effect of harvest frequency

The ratio of leaf to stem increased on the order of the first, second, and third harvest (Table 5-3), which is probably due to the increasing ratio of tillers that emerged from the stubble, with shorter internodes to shoots that emerged from the rhizomes. The TDN concentration of the leaves is higher than that of the stems (Table 5-2), as noted above. Nevertheless, no increase in the TDN of the whole plant was observed corresponding to the order of harvest, which resulted

from a decline in the TDN concentration of the leaves at the second and third harvest (data not shown). Forage digestibility is exacerbated by elevated temperatures, such as increasing lignification of plant cell walls (Van Soest, 1982). The cumulative temperature during the growth of the second and third harvest was approximately 200 °C higher than that of the first harvest, and this may be a partial explanation for the decline in the leaf TDN. Consequently, the TDN concentration of the whole plant samples remained relatively constant at site E or showed declines in the second and third harvest at site W.

Our study demonstrated that N yield can be substantially improved by increasing the harvest frequency (Figure 5-4). However, it was not clear whether the harvest frequency could improve the N removal efficiency of whole CW systems in the present study. Vymazal (2007) determined that the amount of N removed via harvesting is quite low and usually does not exceed 10% of the inflow load for secondary treatment systems, and N removal via harvesting may be important when loading is low. Further field-scale investigations, such as nutrient input-output analysis, are required to understand the contribution of multiple harvesting on the N-removal efficiency of CWs.

In the HF3 plot, the DM yield exhibited different trends between sites E and W (Table 5-3). The DM yield gradually declined on the order of first, second, and third harvests at site E. However, the DM yield of the second harvest exhibited a significantly higher value at site W. The early summer harvest of *P. australis* was reported to negatively affect subsequent biomass production, whereas it did not affect production when harvested in the later seasons (Weisner and Granéli, 1989; Asaeda et al., 2006). The translocation of nonstructural carbohydrates and mineral nutrients from the shoots to rhizomes begins immediately after the foliar structure has been established (Granéli et al., 1992). Therefore, harvesting aboveground biomass during growing seasons might result in a shortage of rhizome reserves and mineral nutrients, which would negatively affect subsequent shoot growth. Such different trends in the DM yield between the two sites may be due to different degrees of shortage in rhizome reserves, which are indispensable for vigorous shoot emergence. Furthermore, production of aboveground biomass and N yield in the following year declined across harvest frequency (Table 5-4). These results indicate that harvest frequency may negatively affect subsequent regrowth and that harvesting practice remarkably reduced biomass production at HF3 in the following year. Thus, an extremely high frequency of harvesting may not be a sustainable and efficient practice from a long-term perspective. More detailed studies focusing on rhizome growth and the allocation of reserves should be undertaken to identify an appropriate harvest

frequency to optimize N-removal efficiency and roughage production. Furthermore, accumulations of heavy metal in foliar tissues are determined by the seasonal translocation from root and rhizome systems (Ranieri et al., 2013a,b), which may also be influenced by the period of harvesting. Further research is required to understand the impact of harvesting on heavy metal dynamics between shoot and rhizome when considering roughage production at CWs used for wastewater treatment.

5.4.3. Implication for management

To obtain a larger amount of high-quality roughage, it is necessary to explore an appropriate harvest timing and frequency. The mean TDN concentration of HF3 was approximately 50%, and the total DM yields were 948 and 1337 g m⁻² yr⁻¹ at site E and W, respectively (Table 5-3). The TDN concentration of *P. japonicus* is equivalent to that of rice straw (43–54%), as reported by Drake et al. (2002). Furthermore, the DM yield of *P. japonicus* is superior to the mean DM yield of rice straw in Asia (500 ± 120 g m⁻²), as reported by Witt et al. (1999). Given the high availability of rice straw for feeding ruminants and the feed deficits in Asian countries (Devendra and Sevilla., 2002), multiple harvestings of *Phragmites* species are a potential alternative to rice straw. Furthermore, Anzai et al. (2016) reported that most of the feed types, including rice straw, are imported from outside of the area, and a large amount of manure is applied within the area; therefore, livestock production systems have the potential for high N and P loads in the basin of Lake Dianchi. Thus, our results indicate that multiple harvesting could contribute not only to enhancing N-removal from CWs but also to reconstructing environmentally harmonious nutrient dynamics in the Lake Dianchi basin. However, the DM yield estimated in the present study represented annual performance, and our results also showed the drastic decline in biomass production in the following year. Long-term multiple harvesting at high frequency may result in low DM yield and nutrient-removal efficiency in CWs. Not only the harvest timing and frequency but also the combinations of further practices, including partial harvesting adapted for Napier grass cultivation as suggested by Sekiya et al. (2015), should be taken into account to develop appropriate and sustainable vegetation management to achieve high N-removal efficiency and roughage production in CWs. For that purpose, long-term investigation is required to understand the impact of harvesting on biomass and nutritive value because our results obtained by annual investigation are restrictive to lead to a conclusion on the best management practices. Furthermore, our results indicated that aboveground biomasses, N content and yields of site W were generally higher than those of site

E (Figure 5-4; Table 5-4). The difference in growth between two CWs is probably not only due to the genetic difference as noted above, but also due to the difference in water depth of their habitats. In the present study, harvest management was implemented at a specified height above the surface water level, and the plants were harvested at a height of 10 and 40 cm above the ground at sites E and W, respectively. Consequently, biomass of stubble after harvest management remained more at site W than at site E, which may have affected shoot regeneration. We also observed that shallower water depth increased more serious weed competition. Moreover, plant growth also might depend on the nutrient load (e.g. N, P, and potassium) of wastewater. The quality and rate of inflow water could be variable during wet season because wastewater primarily originates from agricultural runoff after heavy rainfall events at both sites. Thus, habitat environment, including water depth and nutrient dynamics of sediment and wastewater, should be taken into account for the further research.

5.5 Conclusions

We demonstrated that earlier timing of harvesting substantially enhanced the nutritive value of *P. japonicus*, which is consistent with previous research on *P. australis*. The sharp decline in the TDN concentration and the rate of increase in the DM yield were associated with heading timings, and the seasonal variations were largely influenced by carbohydrate accumulation in the stems; therefore, such characteristics are very important to select novel lineages for planting in CWs. Our data suggest that harvesting three times annually at intervals of approximately 60 days contributed to greatly improving the N and DM yields without decreasing the nutritive value, but this harvesting negatively affected growth in the following year. Furthermore, the harvesting practice reduced biomass production in the following year, especially in the plot with a high frequency of harvesting. Therefore, not only combinations of timing and frequency of harvest but also other management practices, including partial harvesting, may be required to optimize CW performance and roughage production. However, fluctuating environmental factors, including water depth and water quality of wastewater, could lead to variability in biomass performances. Thus, our conclusions on specific recommendations of harvest management are restrictive because our 1-year monitoring data is not sufficient to draw firm conclusions.

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Chapter 6

General discussion

This thesis intended to reveal the feasibility of harvesting reeds of CWs from a perspective of roughage production and nutrient management. Chapter 2 and 3 highlighted the importance of plant harvest management, which plays a crucial role in the improvement of nitrogen removal from CWs. Harvesting *P. australis* from CWs might contribute not only by promoting plant N uptake and removal (Chapter 2) but also by improving microbial N removal processes such as coupled nitrification–denitrification by changing the microenvironment under the canopy (Chapter 3). Harvest timing is a crucial factor affecting biomass performances and quality of roughage due to the possibility of seasonal changes in the physiological interaction (sink–source relationships) between aboveground (shoots) and belowground biomass (rhizomes). Furthermore, most of the leaves were allocated in the upper layer of the canopy, and the N concentration of the young shoot tissues (leaves and stems) of the upper layers exhibited higher values. Thus, the nutritive value of the shoots was inferred to be higher in the upper layers. Considering that new shoot tissues will be formed until the transition from vegetative to reproductive growth and that the lignification of the lower stems declines the nutritive value, heading characteristics were assumed to be an important factor affecting TDN. On the other hand, a vegetation survey indicated that not *P. australis* but *P. japonicus* was the dominant *Phragmites* species around Lake Dianchi, and each population showed different heading timings. Therefore, it was necessary to establish the best harvest management in light of the *Phragmites* lineages. Before proceeding to study the effects of harvest timing and frequency on productivity and nutritive value, genetic backgrounds within the genus *Phragmites* in southwest China were analyzed by comparing them with other global molecular research in order to understand the genetic diversity that might determine phenotypic characteristics (Chapter 4). *Phragmites* species are classified into two lineages including tropical and common types. The tropical lineage primarily consisted of native *Phragmites* with haplotypes I, Q and U at relatively low altitudes, and the common lineage consisted of native *Phragmites* from the Hengduan Mountains or artificially planted *Phragmites* with haplotype P. According to the results in Chapter 4, I selected two locations of *P. japonicus* with differences in genetic background and heading time to investigate the effects of harvest timing and frequency on yield and nutritive value (Chapter 5).

In the present chapter, the results are summarized and discussed to answer the

following two questions: (1) is harvesting a necessary and feasible practice, and (2) what should be done to improve the management?

Is harvest necessary and feasible practice?

In general, plant harvesting is a recommended practice of CWs to improve nutrient removal, especially in productive areas. First, the results from Chapter 2 and 5 support this idea. Plant harvesting contributes to N removal from the system boundary, and it also promotes subsequent growth if the plant harvesting management (e.g., timing, frequency, cutting height) is well considered, which is noted in the next section. Second, plant harvesting management shed light on the potential positive impacts harvesting may have on microbial N removal processes in Chapter 3. Plant harvesting might change light conditions beneath the stands and rhizosphere functioning. Accordingly, it might positively affect microbial communities and their function, specifically, coupled nitrification–denitrification. The enhancement of this process is especially needed under the highly reduced state of CWs. Nitrate N of wastewater could be easily denitrified near the inlet of CWs, and the remaining ammonium N could be immobilized by microorganisms or absorbed by the plants afterwards. Therefore, these findings highlighted that ammonium N removal could be improved by coupled nitrification–denitrification associated with plant harvesting management. However, further studies using field-scale quantitative analyses must be completed to support the feasibility of this effect.

Intensive agriculture is one of the primary contributors to the eutrophication of Lake Dianchi. The overuse of chemical fertilizer and manure in agriculture is a principal factor in water eutrophication. Considering that feed production needs fertilization and local livestock farmers largely depend on the external feeds, harvesting reed for roughage production is a possible practice because it might optimize agricultural nutrient cycling within the basin of Lake Dianchi. As shown in Chapter 2 and 5, the nutritive value of either *P. australis* or *P. japonicus* is equivalent to that of the conventional feeds. Furthermore, several local minority people sometimes feed cows and yaks with *P. japonicus* at the Diqing Tibetan Autonomous Prefecture and buffalo with *P. karka* at the Dehong Dai and Jinpo Autonomous Prefecture, Yunnan, China. Here, the amount of annual DM yield from multiple harvests is estimated at approximately 433 t DM y⁻¹, according to the results of dry matter yields (11.4 t DM ha⁻¹ yr⁻¹) that were obtained from three harvests at 2, 4, and 6 months (Chapter 5) in the dominant areas of *Phragmites* (approximately 38 ha) (Chapter 1). Harvesting can improve the roughage self-efficiency rate from 62.3 to 67.7% (DM basis) and decrease external feed by 1.24 kg N ha⁻¹

yr⁻¹ if external rice straw (N concentration: 0.8% DM basis) were replaced with harvested reed in the southeast agricultural coastal areas of Lake Dianchi (2799 ha). This value is equivalent to approximately 2.8% of the current external feed for cattle (44.1 kg N ha⁻¹ yr⁻¹) (Anzai et al., 2016). The contribution of harvested *Phragmites* is likely to be a small fraction for reducing N inputs. However, there is still room for introducing artificially created riparian zones and CWs around Lake Dianchi, and some new CWs are actually under construction. Thus, there is a prospect for increasing the areas of *Phragmites* in the basin of Lake Dianchi.

The results from the evaluation of feed production costs and selling price are shown in Table 6-1. The costs and selling price were estimated using the data from interviewing local farmers. The cost of reed roughage production is three times higher than the selling price of conventional forage. Thus, the local government has to subsidize reed harvesting. However, this also means that roughage production by harvesting reeds at CWs can compensate for a part of the capital costs of harvesting. Eventually, a clear reason for reed roughage production is needed. For example, the costs of nutrient removal by plant harvesting are lower than the costs of nutrient management by introducing wastewater treatment plants. Further studies, including better management for improving yields and quality, the development of harvest machinery, and the design of CWs appropriate for the harvester, are required in terms of cost reduction. Thus, to answer the question “Is harvesting a necessary and feasible practice,” the answer is partially yes in the case of Lake Dianchi. To resolve the remaining challenges as noted above, reed roughage production is expected to be applicable not only in Lake Dianchi but also in other places due to the wide distribution of *Phragmites* species with high environmental adaptability.

Table 6-1 The cost of reed roughage production and selling price of the local conventional roughage.

		CNY kg ⁻¹ (DM)
Cost of reed roughage production	Labor [†]	3.0
	Transportation	>0.1
	Total	3.0
Selling price of rice straw		1.0

[†] Labor cost: 150 CNY d⁻¹, labor output per person: 100 m⁻², Dry matter yield: 0.5 kg m⁻² (Mean values of first harvest from Chapter 5).

What should be done to improve the management?

The feasibility of roughage production by harvesting reeds was discussed in the previous section. Next, there remains the question “What is the best management for improving yield, quality of reed roughage and nutrient removal?” As shown in Chapter 2, cutting height is a crucial practice because the higher the cut, the better the nutritive quality of harvested biomass. However, it may also negatively affect subsequent regrowth because several tillers emerge from the remaining stubbles immediately after harvest. Another important strategy is harvest timing and frequency, as described in Chapter 5. If the harvest intervals are short with high frequency, it will retard plant growth year to year. Accordingly, those three strategies determine not only immediate yield but also subsequent yield and nutritive value; namely, it affects the sustainable productivity of reed roughage. This thesis provided evidence that multiple harvests might substantially improve yield, nutritive value, and N removal at CWs. Plant responses to the management strategy differed from case to case depending on genetic and environmental factors. Thus, this thesis presents a series of noteworthy points but cannot conclude the best management strategy.

From the aspect of genetic resources, this study noted the influence of the shift in growth stages, namely, the heading time growth stage (Chapter 2 and 5). Furthermore, there might be the other genetically determined phenotypes that are crucial for roughage use, including the ratio of aboveground to belowground biomass, photosynthetic capacity, and culm thickness. Thus, it is important to select a superior lineage before creating CWs. From the environmental aspect, water level might have a considerable effect on plant growth as suggested in Chapter 5. Furthermore, nutrient content of wastewater and sediment is also a crucial factor, although this was not tested in the thesis. Therefore, research on gene-environment interactions from various perspectives is necessary to help us conclude what the best management practice is.

References

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List of publications

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Tanaka, T.S.T., Irbis, C., Kumagai, H., Inamura, T. (2016) Timing of Harvest of *Phragmites australis* (CAV.) Trin. ex Steudel Affects Subsequent Canopy Structure and Nutritive Value of Roughage in Subtropical Highland. *Journal of Environmental Management* 166: 420–428.

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