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Nitrogen incorporation by epiphytic algae via *Vallisneria natans* using ^{15}N tracing in sediment with increasing nutrient availability

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ABSTRACT: We performed a tracer experiment using the stable isotope ^{15}N to investigate nitrogen incorporation by epiphytic algae via its host plant, *Vallisneria natans*, from the sediment with increasing nutrients in the water column. ^{15}N was added as $^{15}NH_4Cl$ to the sediment, and its fate was traced with increasing nutrient availability in the water column and sediment. Samples of epiphytic algae and *V. natans* were examined for $\delta^{15}N$. The results indicated that with increased nitrogen and phosphorus availability in the water column, the ability of epiphytic algae and the leaves of *V. natans* to utilize nitrogen originating from sediment was significantly decreased ($p < 0.05$), while the ability of the roots of *V. natans* to utilize nitrogen from sediment changed very little ($p > 0.05$). Under the same nutrient levels in the water column, the ability of the roots and leaves of *V. natans* to utilize nitrogen originating from sediment was higher in high-nutrient sediment than that in low-nutrient sediment ($p < 0.05$), while the ability of epiphytic algae to utilize nitrogen from sediment changed very little ($p > 0.05$). Thus, epiphytic algae and the tissues of *V. natans* can be arranged with respect to their accumulated ^{15}N as follows: epiphytic algae < leaves < roots; nitrogen incorporation by epiphytic algae originating from sediment was negligible compared to the tissues of *V. natans* in this experiment. This suggested that when concentrations of nitrogen and phosphorus are >1.0 and 0.1 mg l^{-1} in the water column, respectively, sediment as a nitrogen source is negligible for the growth of epiphytic algae.

KEY WORDS: Nitrogen incorporation · Epiphytic algae · *Vallisneria natans* · ^{15}N labeling · Sediment

INTRODUCTION

Submerged macrophytes are essential for maintaining the health of aquatic ecosystems in shallow lakes (Wetzel 1990, Bornette & Puijalon 2011, Tarkowska-Kukuryk & Mieczan 2017). Submerged macrophytes form a complex, 3-dimensional structure which provides a large surface area for epiphytic algal attachment (Wetzel 1990, Jaschinski et al. 2011). Nutrients are one of the most important abiotic factors influencing the growth of epiphytic algae and their host macrophytes (Poikolainen et al. 1998, Bakker et al. 2010), and the effects of nutrients on the growth of epiphytic algae and aquatic plants have been widely reported (Higgins et al. 2003, Díaz-Olarte et al. 2007, Croel & Kneitel 2011, Smith 2014). In oligotrophic and mesotrophic lakes, rooted submerged macrophytes commonly act as a major nutrient source for epiphytic algae, as the availability of nitrogen and phosphorus in the water column is usu-

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ally low during the growing season of macrophytes. In such systems, epiphytic algae receive most of their nutrients from macrophyte release (Moeller et al. 1988, Wetzel 1990, Pinowska 2001); in addition, nutrients released directly from sediment through rooted macrophytes may account for up to 80% of epiphytic algal nutrients (Moeller et al. 1988, Burkholder & Wetzel 1990, Wetzel 2001). However, in nutrient-rich lakes, the density and biomass of epiphytic algae increase along nutrient availability gradients in the water column, and epiphytic algae can absorb large amounts of nitrogen and phosphorus directly from the water column (Blumenshine et al. 1997, McDougal et al. 1997, Havens et al. 1999). It has also been observed that the community compositions of epiphytic algae attached to living plants are often similar to those on artificial substrates in eutrophic lakes (Cattaneo & Kalff 1978). Therefore, in eutrophic water, some researchers have suggested that macrophytes act as inert substrates, providing a suitable surface for colonization, but not acting as an important source of nutrients for epiphytic algae (Moeller et al. 1988, Quinlan et al. 2008). However, some researchers believe that sediment is still able to provide nutrients for the growth of epiphytic algae by release of the rooted macrophytes in eutrophic water (Albay & Akcaalan 2003), although direct evidence is still lacking. Whether macrophytes pump nutrients from the sediment and release compounds for uptake by epiphytic algae still requires further evaluation.

Therefore, in this study we determined the $\delta^{15}N$ values of epiphytic algae and their host plants *Vallisneria natans*, along with epiphytic algal biomass and the dry weight of the host plants. Our objectives were (1) to evaluate nitrogen incorporation by epiphytic algae via its host plants from sediment with increasing nutrient availability, (2) to examine nitrogen use efficiency of epiphytic algae and submerged plants from the sediment with increasing nutrient availability, and (3) to determine whether macrophytes pump nutrients from the sediment and release compounds for the growth of epiphytic algae in eutrophic water.

MATERIALS AND METHODS

Plant culture and treatments

Vallisneria natans was selected for this experiment as the species is widely distributed in China and used for ecological restoration in eutrophic shallow lakes (Dong et al. 2014, He et al. 2014). Winter buds of *V. natans* were collected from East Taihu Lake (120° 27′ 13″ E, 31° 01′ 18″ N), cultured in plastic containers (50 × 40 × 60 cm) with filtered lake water (40 l) and sediment (10 cm thick) from Taihu Lake, and maintained in a greenhouse. After the plants were approximately 20 cm long and their roots were well developed, the plants were washed free of sediments and transplanted into plastic pots (diameter: 8 cm; depth: 10 cm) containing sediment. Lids with a 5 × 0.5 cm slit for the passage of plant leaves were placed on the pots, each of which contained 3 seedlings. The slits in the partitions were sealed around the base of the plants. Ten pots were then transferred to each aquarium (21 × 31 × 41 cm) with 18 l of tap water. A 2 × 3 factorial design was set up with 3 replicates that consisted of plants grown in 2 types of sediment under 3 levels of nitrogen and phosphorus concentrations in the water column. The 2 types of sediments used were: S1, sand + additional nutrients (7 mg NH_4^+-N kg^{-1} as ^{15}N-labeled NH_4Cl, and 0.7 mg PO_4-P kg^{-1} as NaH_2PO_4) and S2, sand + additional nutrients (14 mg NH_4^+-N kg^{-1} as ^{15}N-labeled NH_4Cl and 1.4 mg PO_4-P kg^{-1} as NaH_2PO_4). The 3 levels of nitrogen and phosphorus concentrations (mg l^{-1}) in the water column were: 1.0, 0.1 (NP1); 2.0, 0.20 (NP2); and 3.5, 0.35 (NP3), respectively, according to a prior experiment. To maintain the initial water column nutrient concentrations and control the growth of phytoplankton over the course of the experiment, half of the culture medium was replaced with fresh culture medium once every 2 d. Total nitrogen and total phosphorus concentrations in the water were measured daily according to the method described by Jin & Tu (1990), and nutrients were added as concentrated solutions (inorganic nitrogen: NH_4Cl and $NaNO_3$ [NH_4^+-N:NO_3^--N = 1:1]; inorganic phosphorus: NaH_2PO_4). To maintain the original water volume, evaporated water was replenished with tap water which contained 1.276 ± 0.021 mg l^{-1} of total nitrogen and 0.021 ± 0.001 mg l^{-1} of total phosphorus. Compared with the total water volume in each container, the extra nitrogen and phosphorus added with tap water were negligible. At the end of the experiment, 4 pots of plants were randomly selected for sampling.

Sample pretreatment for chlorophyll *a* and ^{15}N analyses

Two pots of plant samples were selected at the end of the experiment and then quickly washed with distilled water. The residual water was absorbed with

paper towels. The plant samples were then divided into roots and leaves, dried at 70°C to a constant weight, and ground with a mortar and pestle to a fine powder and stored in clean stoppered polyethylene vials until ^{15}N analysis. The remaining plant samples were utilized for epiphytic algae collection. A soft brush was used to separate epiphytic algae from the leaves of the host plants in 200 ml sterile distilled water. Separated liquid samples containing epiphytic algae were not contaminated by the plant tissues. Leaf surface areas were determined for the leaves of *V. natans* after the separation. Next, 50 ml subsamples were filtered using 47 mm Whatman GF/C glass fiber filters, and the filters with the epiphytic algae were stored at −20°C for chlorophyll *a* (chl *a*) determination. A portion of the remaining sample was centrifuged at 4°C for 20 min at 7000 rpm (5200 × *g*), and the supernatant was decanted away; the pellet was rinsed with deionized water and centrifuged again, and this procedure was repeated 3 times. Then the pellet was freeze-dried, ground, and stored for ^{15}N analysis of the epiphytic algae.

Measurement of epiphytic algal biomass

Epiphytic biomass was determined as chl *a* per unit of leaf surface area. Prepared filters with epiphytic algae were ground to a fine paste with 90% acetone (6 to 8 ml). The paste was transferred to a centrifuge tube, and the mortar was rinsed with 90% acetone 2 or 3 times. The rinse was transferred to the same centrifuge tube and then centrifuged at 4°C for 20 min at 7000 rpm. The top clear liquid was collected in a brown volumetric flask and used for determining chl *a* by using a fluorescence spectrometer (Loftus & Carpenter 1971).

Measurement of nitrogen isotope

The nitrogen isotope composition of the samples was measured by an isotope ratio mass spectrometer (Delta Plus; FinniganMAT) with a typical reproducibility of 0.3‰ for δ^{15} N, which was coupled to an elemental analyzer (EA3000; EuroVector) via a Conflo III interface.

Data treatment and analysis

Data are presented as excess µmol of ^{15}N g^{-1} dry sample (excess ^{15}N; µmol g^{-1}) (Veuger et al. 2007, Zhang et al. 2010) and the percentage of plant N uptake derived from the applied N in the sediment (Ndff; %), which reflects the nitrogen use efficiency of the labeled material (Asagi & Ueno 2009, Wang et al. 2011). The data were analyzed using the following equations:

$$\text{Excess}^{15}\text{N} = \frac{\frac{\text{atom\%}^{15}\text{N}_{\text{sample}} - \text{atom\%}^{15}\text{N}_{\text{control}}}{100} \times \text{µmol of N in sample}}{\text{gram of dry sample}} \quad (1)$$

$$\text{Ndff} = \frac{\text{atom\%}_{\text{sample (plant)}} - \text{atom\%}_{\text{control (plant)}}}{\text{atom\%}_{\text{sample (sediment)}} - \text{atom\%}_{\text{control (sediment)}}} \times 100 \quad (2)$$

$$\text{R} = \frac{\text{atom\%} \times 100}{1 - \text{atom\%} \times 100} \quad (3)$$

$$\delta^{15}\text{N (‰)} = \left(\frac{\text{R}_{\text{sample}}}{\text{R}_{\text{air}}} - 1\right) \times 1000 \quad (4)$$

where $\text{atom\%}^{15}\text{N}_{\text{control}}$ represents the value at the beginning of the experiment, R_{sample} is the $^{15}N/^{14}N$ ratio of the sample, and R_{air} is the $^{15}N/^{14}N$ ratio of atmospheric nitrogen (R_{air} = 0.0036765). All data are presented as means ± SD. Statistical significance was determined using a 2-way ANOVA with sediment nutrient levels and water nutrient levels as the 2 factors; Tukey's HSD test was used for the comparison of means.

RESULTS

Biomass of epiphytic algae and the dry weight of the tissues of *Vallisneria natans* were measured. In this experiment, epiphytic algal biomass significantly increased as the concentrations of nitrogen and phosphorus in the water increased, but no significant difference was found in epiphytic algal biomass in the sediment treatments (Fig. 1A, Table 1). In addition, there was no significant change in the dry weight of the leaves and roots of *V. natans* with the increase of nitrogen and phosphorus in the water column (Fig. 1B,C, Table 1), and the effects of nitrogen and phosphorus in the sediment on the dry weight of the leaves and roots of *V. natans* were also negligible (Fig. 1B,C, Table 1). Results from the 2-way ANOVA showed that there was no significant interaction between the sediment nutrient levels and the water nutrient levels in the dry weight of *V. natans* and the biomass of epiphytic algae, respectively (Table 1).

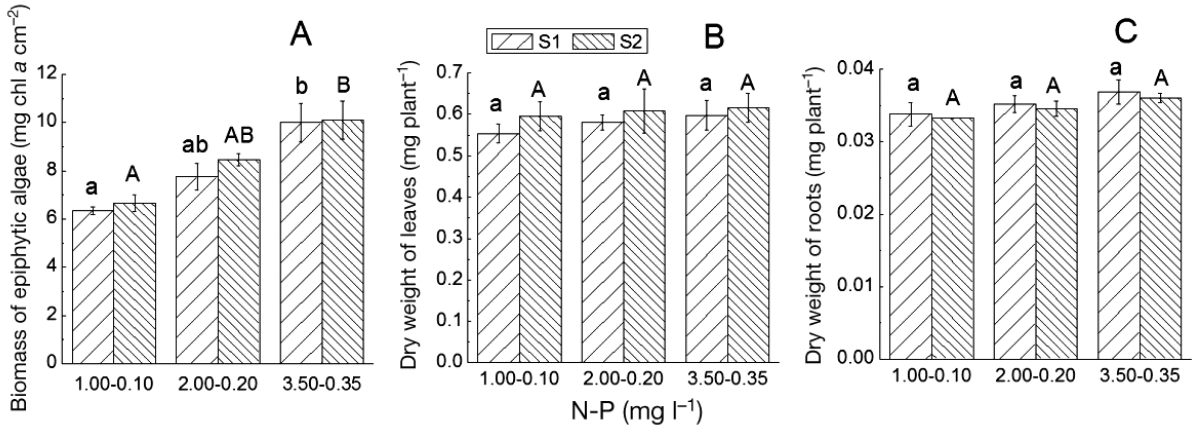

Fig. 1. Mean (±SD) biomass of epiphytic algae and the dry weight of the tissues of *Vallisneria natans*. Bars with the same letter above them are not significantly different (Tukey's HSD; $p < 0.05$); lowercase letters: low nutrient sediment (S1); uppercase letters: high nutrient sediment (S2)

Table 1. *F*- and p-values obtained from 2-way ANOVA testing the effects of nitrogen and phosphorus concentrations in the water column and sediment on the biomass of epiphytic algae and the dry weight of the tissues of *Vallisneria natans*, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$

Parameter	Treatment	*F*	p
Epiphytic algae	Water nutrient	21.19	<0.01**
	Sediment nutrient	0.68	0.44
	Water nutrient × sediment nutrient	0.16	0.86
Leaves of *V. natans*	Water nutrient	5.08	0.05
	Sediment nutrient	0.54	0.49
	Water nutrient × sediment nutrient	0.01	0.99
Roots of *V. natans*	Water nutrient	0.41	0.68
	Sediment nutrient	2.87	0.14
	Water nutrient × sediment nutrient	0.06	0.94

Excess ^{15}N in epiphytic algae and *V. natans*

Excess ^{15}N was observed in both epiphytic algae and the different tissues of its host plant (*V. natans*) (Fig. 2). At the end of the experiment, a considerable reduction in excess ^{15}N was observed in epiphytic algae with increasing nutrients in the water column (Table 2, Fig. 2A). No significant difference was found in excess ^{15}N in epiphytic algae in the sediment treatments (Fig. 2A), and there was no interaction between the sediment

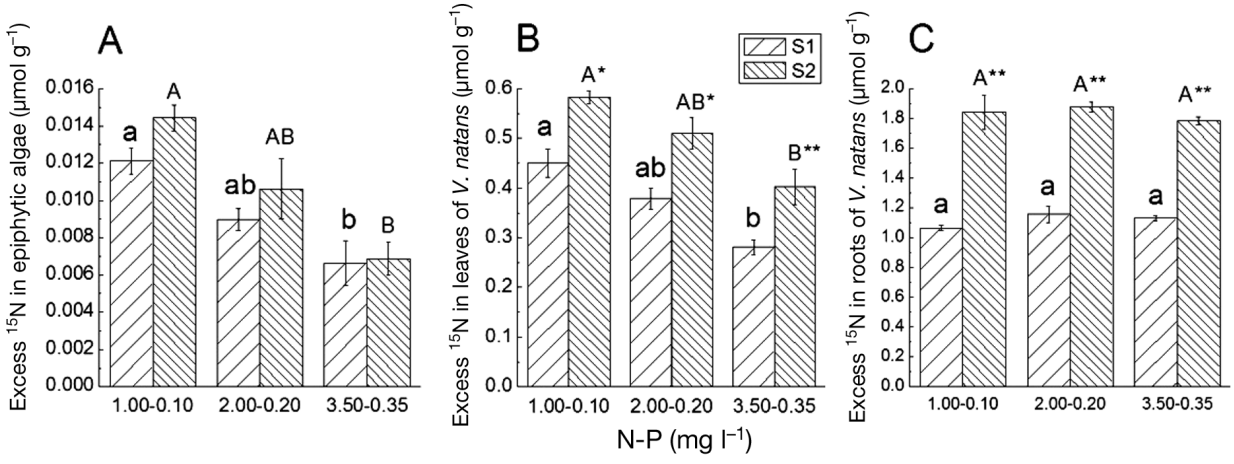

Fig. 2. Excess labeled material (µmol ^{15}N g^{-1}; mean ± SD) in the epiphytic algae and in the tissues of *Vallisneria natans*. Bars with the same letter above them are not significantly different (Tukey's HSD; $p < 0.05$) lowercase letters: low nutrient sediment (S1); uppercase letters: high nutrient sediment (S2). Asterisks indicate significant differences in excess ^{15}N between S1 and S2 (*t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$)

Table 2. *F*- and p-values obtained from 2-way ANOVA testing the effects of nitrogen and phosphorus concentrations in the water column and sediment on excess ^{15}N in epiphytic algae and the dry weight of tissues of *Vallisneria natans*, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$

Parameter	Treatment	*F*	p
Epiphytic algae	Water nutrient	20.89	<0.01**
	Sediment nutrient	2.92	0.14
	Water nutrient × sediment nutrient	0.55	0.60
Leaves of *V. natans*	Water nutrient	27.99	<0.01**
	Sediment nutrient	66.59	<0.01**
	Water nutrient × sediment nutrient	0.23	0.80
Roots of *V. natans*	Water nutrient	0.78	0.50
	Sediment nutrient	238.22	<0.01**
	Water nutrient × sediment nutrient	0.57	0.60

nutrient levels and the water nutrient levels in excess ^{15}N in epiphytic algae (Table 2, Fig. 2A). With increasing nutrients in the water column, the variation tendency of excess ^{15}N in the leaves of *V. natans* was similar to that observed in epiphytic algae, while a significant difference in excess ^{15}N in the leaves of *V. natans* was found in the sediment treatments (Fig. 2B, Table 2). There was also no significant interaction in excess ^{15}N in the leaves of *V. natans* between the sediment nutrient levels and water nutrient levels (Fig. 2B, Table 2). The excess ^{15}N in the roots of *V. natans* was significantly higher in the S2 sediment treatments than that in the S1 sediment treatments (*t*-test, $p < 0.05$), and no significant difference in excess ^{15}N in the roots was found with increasing nutrients in the water column; additionally, there was no interaction between the sediment nutrient levels and the water nutrient levels (Fig. 2C, Table 2). In comparison of epiphytic algae and the leaves and roots of *V. natans*, the lowest accumulations of the labeled material were recorded in epiphytic algae (0.01 to 0.02 µmol $^{15}N\ g^{-1}$), followed by the leaves of *V. natans* (0.27 to 0.60 µmol $^{15}N\ g^{-1}$); the root tissues of *V. natans* had the highest excess ^{15}N (1.05 to 1.96 µmol $^{15}N\ g^{-1}$).

Percentage of epiphytic algae and *V. natans* N uptake derived from the applied ^{15}N in the sediment

The Ndff of the plants decreased with increasing concentrations of nitrogen and phosphorus in the water column, and a significant difference was found in the Ndff of epiphytic algae and the leaves of *V. natans* with increasing concentrations of nitrogen and phosphorus in the water column, respectively (Tables 3 & 4). At the same concentrations of nitrogen and phosphorus in the water column, the Ndff of the leaves and roots of *V. natans* were significantly greater in the S2 sediment treatments than that in the S1 sediment treatments, respectively, while no significant difference was found in the Ndff of epiphytic algae in the sediment treatments. There was no significant interaction between the sediment nutrient levels and the water nutrient levels in the Ndff of the leaves and roots of *V. natans* and epiphytic algae, respectively (Table 3, Table 4). In a comparison of epiphytic algae and the leaves and roots of *V. natans*, the Ndff of epiphytic algae was the lowest (ranging from 0.06 to 0.14%), followed by that for leaves of *V. natans* (2.63 to 5.98%); the Ndff for roots of *V. natans* was the highest, with a range from 11.55 to 19.17%. This showed that the nitrogen use efficiency of epiphytic algae from the sediment was very low, while the nitrogen use efficiency of the leaves and roots of *V. natans* from the sediment was far greater than that of epiphytic algae.

DISCUSSION

The appearance of excess ^{15}N in epiphytic algae and *Vallisneria natans* clearly demonstrated that with increasing concentrations of nitrogen and phosphorus in the water column, the uptake of nitrogen

Table 3. Mean (±SD) percentage of epiphytic algae and *Vallisneria natans* N uptake derived from the applied ^{15}N in the sediment (Ndff). The same superscript letters are not significantly different under the same type of sediment ($p < 0.05$; Tukey's HSD); asterisks indicate significant differences in Ndff between low nutrient sediment (S1) and high nutrient sediment (S2) (*t*-test; $^{*}p < 0.05$; $^{**}p < 0.01$)

N–P ($mg\ l^{-1}$)	Epiphytic algae		Leaves of *V. natans*		Roots of *V. natans*	
	S1	S2	S1	S2	S1	S2
1.00–0.10	0.12 ± 0.01^{a}	0.14 ± 0.01^{a}	4.78 ± 0.30^{a}	$5.98 \pm 0.13^{a*}$	12.98 ± 0.23^{a}	$19.17 \pm 1.21^{a**}$
2.00–0.20	0.09 ± 0.01^{ab}	0.10 ± 0.02^{ab}	3.90 ± 0.23^{ab}	$4.81 \pm 0.30^{ab*}$	12.74 ± 0.62^{a}	$18.67 \pm 0.36^{a**}$
3.50–0.35	0.06 ± 0.01^{b}	0.06 ± 0.01^{b}	2.63 ± 0.14^{b}	$4.10 \pm 0.01^{b*}$	11.55 ± 0.18^{a}	$17.19 \pm 0.26^{a**}$

Table 4. *F*- and p-values obtained from 2-way ANOVA testing the effects of nitrogen and phosphorus concentrations in the water column and sediment on the N uptake derived from the applied ^{15}N in the sediment (Ndff) of epiphytic algae and the tissues of *Vallisneria natans*, respectively. $^{*}p < 0.05$; $^{**}p < 0.01$

Parameters	Treatment	*F*	p
Epiphytic algae	Water nutrient	28.83	<0.01**
	Sediment nutrient	1.44	0.28
	Water nutrient × sediment nutrient	0.26	0.78
Leaves of *V. natans*	Water nutrient	45.58	<0.01**
	Sediment nutrient	48.32	<0.01**
	Water nutrient × sediment nutrient	0.88	0.46
Roots of *V. natans*	Water nutrient	4.49	0.06
	Sediment nutrient	147.77	<0.01**
	Water nutrient × sediment nutrient	0.12	0.90

from the sediment by both epiphytic algae and the leaves of *V. natans* decreased significantly, while the uptake of nitrogen from the sediment by the roots of *V. natans* changed little. Actually, some researchers have reported that epiphytic algae can directly absorb large quantities of nutrients from the water column, and that epiphytic biomass increases along the nutrient availability gradients in the water column (Blumenshine et al. 1997, McDougal et al. 1997). Submerged macrophytes can obtain nutrients from both the water via their shoots and the sediment via their roots; foliar uptake supplied more nitrogen to the plants than did root uptake when ca. 0.1 mg l^{-1} of NH_4^+-N was present in the water (Nichols & Keeney 1976, Madsen & Cedergreen 2002). In this experiment, increased nutrient availability in the water column promoted the growth of epiphytic algae, but did not promote the growth of aquatic macrophytes. In fact, eutrophication generally promotes the growth of aquatic macrophytes and blooms of epiphytic algae due to increased nutrient availability (McDougal et al. 1997, Pizarro et al. 2002, Asaeda et al. 2004). The generally positive effect of increased nutrients on the growth of macrophytes is reversed in the absence of grazers, which have a strong negative effect on epiphyte biomass (Hays 2005, Whalen et al. 2013, Mieczan et al. 2015); blooms of epiphytic algae can also have a negative influence on the growth of its host (Jones et al. 2002, Song et al. 2015). *In situ* observation also indicated that aquatic macrophytes grow slowly and produce little biomass in eutrophic lakes compared with oligotrophic lakes (Rattray et al. 1991).

Those earlier results could account for the results of our research which showed that increased nutrient availability in the water column had no significant effects on the dry weight of the leaves and roots of *V. natans*. In fact, it is also important to understand the reasons for the loss of macrophytes and to determine ecological restoration strategies via the reconstruction of submerged plants in eutrophic lakes. In this experiment, the results showed that epiphytic algae and the tissues of *V. natans* can be arranged according to nitrogen use efficiency from the labeled materials (sediment) as follows: epiphytic algae < leaves < roots; the nitrogen use efficiency of epiphytic algae from the labeled materials (sediment) was negligible in comparison to the tissues of *V. natans* under the conditions of this experiment. It was evident that the water column as the nutrient source for the growth of epiphytic algae predominated in this experiment; in addition, added nitrogen and phosphorus in the sediment would have comparatively little effect on the nitrogen source of the epiphytic algae with high concentrations of nitrogen and phosphorus in the water column; for *V. natans*, both the water column and sediment are important sources of nitrogen in eutrophic lakes.

CONCLUSIONS

In eutrophic lakes, nitrogen incorporation by epiphytic algae via its host plant *V. natans* from the sediment was negligible, and both the water column and sediment were important as nitrogen sources for the growth of the submerged plants. Epiphytic algae and the tissues of *V. natans* can be arranged in the order of their accumulated ^{15}N as follows: epiphytic algae < leaves < roots.

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