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Ecophysiological and anatomical responses of *Vallisneria natans* to nitrogen and phosphorus enrichment

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ABSTRACT

Key-words: *nutrient enrichment, photosynthesis, morphological characteristics, anatomical structure,* Vallisneria natans

Here, we describe an experiment using four nitrogen (N) and phosphorus (P) concentrations to investigate the effects of nutrient enrichment on the submersed macrophyte *Vallisneria natans* (tape grass) grown in a sand culture medium. The objective of this study was to examine the influence of nutrient enrichment in the water column on *V. natans*, especially with regard to anatomical structures. The results showed both the absolute growth rate (AGR) and intrinsic efficiency of light energy conversion of PSII (*Fv/Fm*) decreased with increasing nutrient levels. Root morphological characteristics, including the total root length (*L*), root surface area (*SA*), projected root area (*PA*), total root volume (*V*), average root diameter (*AD*), total root length per volume (*LPV*), total tips (*T*) and total forks (*F*), also showed a generally negative relationship with increasing nutrient concentrations. The anatomical structures of stolons and leaves also changed with nutrient enrichment. The shrinkage of aerenchyma and disappearance of starches and chloroplasts were the main structural changes leading to poor growth. These phenomena, especially the anatomical changes, might be the mechanism underlying the effect of nutrient enrichment on *V. natans* growth.

RÉSUMÉ

Réponses écophysiologiques et anatomiques de *Vallisneria natans* à un enrichissement en azote et en phosphore

Mots-clés : *enrichissement en éléments nutritifs, photosynthèse, caractéristiques morphologiques, anatomie,* Vallisneria natans

Nous décrivons ici une expérience utilisant quatre concentrations d'azote (N) et phosphore (P) afin d'étudier les effets de l'enrichissement en éléments nutritifs sur le macrophyte immergé *Vallisneria natans* cultivé dans un milieu de culture sableux. L'objectif de cette étude était d'examiner l'influence de l'enrichissement en nutriments dans la colonne d'eau sur *V. natans*, en particulier en ce qui concerne les structures anatomiques. Les résultats ont montré à la fois que le taux de croissance absolue (AGR) et l'efficacité intrinsèque de la conversion de l'énergie lumineuse du PSII (*Fv/Fm*) ont diminué avec l'augmentation des niveaux d'éléments nutritifs. Les caractéristiques morphologiques racinaires, y compris la longueur totale des racines (*L*), la surface des racines (*SA*), la zone des racines prospectée (*PA*), le volume total des racines (*V*), le diamètre moyen des racines (*AD*), la longueur totale des racines par volume (*LPV*), le totalité des extrémités (*T*) et des ramifications (*F*), ont également montré une relation globalement négative avec

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l'accroissement des concentrations en éléments nutritifs. Les structures anatomiques des stolons et des feuilles ont aussi changé avec l'enrichissement en nutriments. Le rétrécissement de l'aérenchyme et la disparition des grains d'amidon et des chloroplastes sont les principaux changements structurels conduisant à une faible croissance. Ces phénomènes, notamment les changements anatomiques, pourraient être le mécanisme sous-jacent de l'effet de l'enrichissement en éléments nutritifs sur la croissance de *V. natans*.

INTRODUCTION

Eutrophication of lakes is a widespread environmental problem (OECD, 1982), and a consequential decline in macrophytes has been recorded over the past three decades (Burkholder *et al.*, 1992; Perrow *et al.*, 1994; Middleboe and Markager, 1997; Melzer, 1999; Tracy *et al.*, 2003; Qin *et al.*, 2005). Although there has been no consensus in explaining this phenomenon, some incidental stresses of eutrophication have been suggested as the causal factors, including overgrowth epiphytes and macroalgae and the associated light reduction, low sediment redox potential, excessive nutrient supply and an absence of an uptake regulation mechanism for certain species (Sand-Jensen, 1977; Phillips *et al.*, 1978; Burkholder *et al.*, 1994; Terrados *et al.*, 1999; Ni, 2001). The direct effect of high nutrients level in water column has been suggested as an important factor in the disappearance of submerged aquatic plants from eutrophic lakes (Geneviève *et al.*, 1997; Farnsworth and Baker, 2000), for not only the roots, but also the leaves of these plants are encompassed by water.

Numerous studies have shown that excessive nitrogen (N) and phosphorus (P) compounds have strongly negative impacts on macrophytes (Robe and Griffiths, 1994; Tylová-Munzarová *et al.*, 2005) and affect plant morphology, tissue C/N ratios, photosynthesis, density and reproductive strategy (Harlin and Thorne-Miller, 1981; Short, 1987; Sand-Jensen, 1989; van Lent *et al.*, 1995), even though N and P are important sources of nutrition.

With regard to growth, many plants show reductions under elevated ammonium-N (NH_4^+-N) concentrations and develop NH_4^+-N toxicity syndrome (Mehrer and Mohr, 1989). Others studies have found that a high concentration of NH_4^+-N inhibits plant germination and the establishment of seedlings (Barker *et al*., 1970; Westwood and Foy, 1999). When it comes to morphological plasticity, stolon biomass of *Vallisneria spiralis* was significantly higher in nutrient-poor than in nutrient-rich patches; indicating ramets allocate more resources to stolon expansion in nutrient-poor patches. And roots elongated in nutrient-poor patches significantly (Wang and Yu, 2007). Lateral root of *Arabidopsis* elongation is suppressed by both high nitrate and high phosphate availability (Linkohr *et al*., 2002).

The *Fv/Fm* ratio reflects the potential quantum efficiency of PSII and is used as a sensitive indicator of plant photosynthetic performance, with optimal values of approximately 0.83 measured for most plant species (Bjorkman and Demmig, 1987). Lower values indicate that the plant has been exposed to stress or a decline in photosynthetic capacity (Van Kooten and Snel, 1990; Ralph, 1999), and significantly lower photosynthetic rates have been observed in a deficient nutrient treatment compared with natural nutrient treatment (Longstreth and Nobel, 1980; Zhang *et al.*, 2008). In addition, high-nutrient treatments also cause some cellular responses, such as the increase of photorespiration and the generation of excess ethylene and polyamines in plants (Gerendas *et al*., 1997; Barker, 1999). These data are valuable and helpful in unraveling the causes and mechanisms of the high-nutrient-induced decline of macrophytes (Britto and Kronzucker, 2002). However, the effect of nutrients on the anatomical structure of macrophytes is poorly documented and not well understood.

Vallisneria natans, a perennial, submerged plant with a wide geographical range, is a dominant native species in many freshwater habitats in China. It may also be considered as a pioneer species because of its high adaptive capability (Cook, 1990); however, it is largely disappearing because of eutrophication and other factors (Rea *et al.*, 2003). The objective of the present study was to examine the influence of nutrient enrichment in the water column on *V. natans*, especially with regard to anatomical structures.

MATERIALS AND METHODS

> *PLANT CULTURE AND TREATMENTS*

The experiment was conducted in 70-L tanks at TLLER (Taihu Laboratory for Lake Ecosystem Research, Chinese Academy of Sciences) from June to August (growing season). Each tank contained ten weighed vigorous seedlings of *V. natans* (approximately 10 cm in length, without rhizomes) collected in Xukou Bay of Taihu Lake, 20 cm of rinsed sand (approximately 2–3 mm in diameter) and 50 cm culture solution, an adapted Hoagland's solution. According to preliminary experiments, low and small heterogeneity nutrients enrichment didn't bring out anatomical structure differences as *V. natans*'s tolerability. Otherwise, concentration of water total nitrogen (TN) in Meiliang Bay of Lake Taihu is common at 10 $\text{mg}\cdot\text{L}^{-1}$, and there are still some macrophytes live in, including *V. natans*. TN concentrations were designed as 2, 10, 30 and 80 $\text{mg}\cdot\text{L}^{-1}$, hereafter called C (control), L (low), M (medium) and H (high), respectively. The N:P ratio was 20:1 (Wen *et al.*, 2008), with P added as KH_2PO_4. The NH_4^+-N:NO_3^--N ratio was 1:2 using NH_4Cl and KNO_3. The pH of the solution was adjusted to 6.5 using KOH and H_2SO_4. Each treatment contained three replicates. All of the tanks were covered with white gauze and placed outdoors under a canopy that was open during sunny periods and closed during rain.

> *PLANT HARVEST AND GROWTH*

The experiment was conducted for 60 days. After carefully being harvested, the whole plants were rinsed in distilled water and weighed and then separated into leaves, stolons and roots. Sections of leaves (approximately 10 cm from the base) and stolons (approximately 1 cm from the base) were fixed in 2.5% glutaraldehyde solution at 4 °C for evaluation of the anatomical structure. Fresh rinsed roots were kept for morphological analyses. The plant fresh weights were used for the calculation of absolute growth rate (AGR = [final weight – initial weight]/days).

> *PHOTOSYNTHESIS*

The intrinsic efficiency of the light energy conservation of PSII (*Fv/Fm*) was determined with a diving pulse amplitude modulated fluorometer (Diving PAM, Heinz Walz GMBH, Effeltrich, Germany). The measurement was taken *in situ* with intact leaves. Midway between the base and the tip of mature leaves were determined after 30 min dark adaptation with a dark leaf clip (Heinz Walz GMBH, Effeltrich, Germany). Five measurements were taken per replicate.

> *ROOT MORPHOLOGY*

Fresh roots were washed in distilled water and then scanned using an Epson Twain Pro (Japan) to obtain the root images, which were analyzed by WinRHIZO A1600+ (Regent Instruments Inc.) for total root length (*L*), root surface area (*SA*), projected root area (*PA*), total root volume (*V*), average root diameter (*AD*), total root length per volume (*LPV*), total tips (*T*) and total forks (*F*). Seven measurements were made per replicate of each treatment.

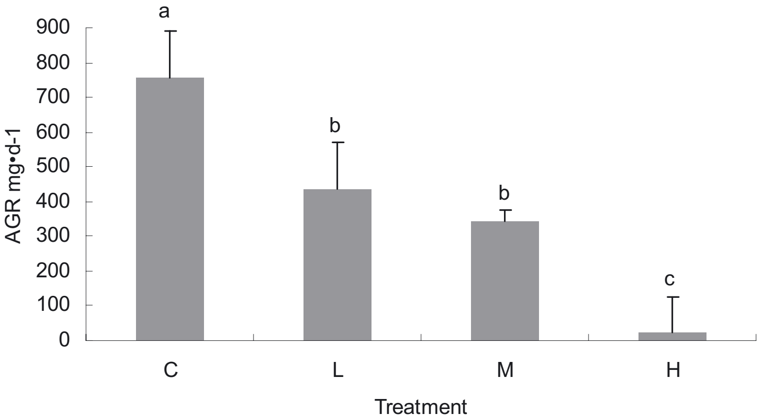

Figure 1
AGR (absolute growth rate) of Vallisneria natans *declined with nutrient enrichment (the error bars represent standard error and different letters identify significant differences (*$p < 0.05$*); C = control, L = low, M = medium, H = high).*

> *ANATOMICAL STRUCTURE*

Scanning electron microscopy and semi-sectioning with embedded resin were used for assessing the anatomical structure. The former procedure was used after dehydration in a graduated series of ethanol, acetone and isoamyl acetate solutions. The samples were then dried using a CPD (Critical Point Dryer) (England), sputtered with gold using an IB-3 sputter coater (Eiko, Japan), scanned using S-4800II FESEM (Field Emission Scanning Electron Microscopy) (Hitachi High-Technologies Corporation, Japan) and photographed.

Samples of the semi-sectioning with embedded resin were also dehydrated in a graduated series of ethanol solutions permuted with propylene epoxide. Resin sections with a thickness of 1 μm were prepared using a microtome (Leica, Germany) and observed under an Olympus BH-2 imaging microscope (Olympus, Japan) equipped with a CCD camera.

> *DATA ANALYSES*

All of the results are reported as means ± standard error (SE). Statistical analyses were performed on a microcomputer using SPSS software (version 16.0). Date was tested for normality and equality of variance to meet the assumptions of parametric statistics, and assumptions were satisfied for all data tested. Least significant difference (LSD) pair-wise comparisons with 0.95 confidence level were applied to parameters showing significant differences among treatments in one-way ANOVA.

RESULTS

> *PLANT GROWTH*

As all of the plants were similar in size at the beginning of the experiment, the final mass and AGR should reflect the relative growth over the course of the entire experiment. Overall, the AGR of *V. natans* decreased with increasing nutrient concentrations (Figure 1). The plants responded well to the control conditions, and the plants under the L and M treatments had a significantly higher AGR than those in the H treatment (ANOVA analysis, $P < 0.05$), under which the plants grew poorly, with some dying.

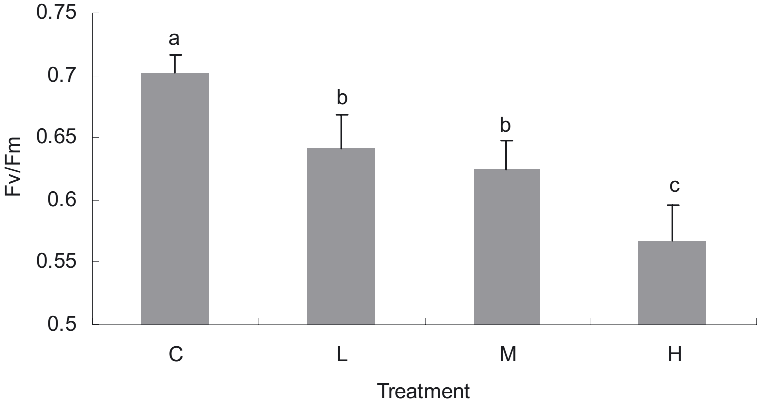

Figure 2
Fv/Fm (the potential quantum efficiency of PSII) values of Vallisneria natans *decreased with nutrient enrichment (the error bars represent standard error and different letters identify significant differences ($p < 0.05$); C = control, L = low, M = medium, H = high).*

> PHOTOCHEMICAL EFFICIENCY

The control plants showed significantly higher *Fv/Fm* values (0.702 ± 0.015) than the other treatments (ANOVA analysis, $P < 0.05$), with the H treatment having the lowest value (0.568 ± 0.028). The variation in the *Fv/Fm* ratio was the same as for the AGR (Figure 2).

> ROOT MORPHOLOGICAL CHARACTERISTICS

There was a negative relationship between the root morphological characteristics and nutrient levels (Table I). The total root length (*L*), root surface area (*SA*), projected root area (*PA*) and total root volume (*V*) in the control treatment were generally higher than those in the other treatments. There were no differences in the total root length (*L*), projected root area (*PA*) and total root volume (*V*) among the L, M and H treatments, and the different nutrient levels did not lead to differences in the average root diameter (*AD*). The root surface area (*SA*), total root length per volume (*LPV*), total tips (*T*) and total forks (*F*) showed the same variation pattern, being significantly higher in the control treatment than in the other treatments, with the L treatment being higher than the M and H treatments.

> ANATOMICAL STRUCTURE OF STOLONS AND LEAVES

The present study showed that the stolons and leaves had the same basic structure in all of the treatments, from external to internal, as follows: EP (epidermis), M (mesophyll) or PA (parenchymatous cells), AC (air chamber) and VB (vascular bundle) (Figure 3). The stolons of healthy plants in the control treatment were developing well at the time of harvest (Figure 3-1). The vascular bundles, surrounded by large air chambers, were also well developed, and starch grains were present. Furthermore, there was a negative relationship between the scale of air chambers and the increasing nutrient levels (Figures 3-1 to 3-4). The starch grains absolutely disappeared in the M and H treatments. In the scanning photographs the structures of starch grains were observed stolons in the control treatment (Figure 3-9-a), whereas there was no starch in the stolons in the H treatment (Figure 3-10-b).

The semi-sections of leaves also showed some degree of plant stress. The leaves of plants in the control treatment were completely differentiated (Figure 3-5), and a great many air chambers were differentiated from mesophyll. Vascular bundles and chloroplasts in the roundish mesophyll cells were clearly observed. There was no obvious difference in the scale of the air chambers with the different nutrient levels, although the number of chloroplasts decreased

Table I
The effects of different nutrient enrichment levels on root morphological characteristics (L: total length, SA: surface area, PA: projected area, V: total volume, AD: average diameter, LPV: total length per volume, T: total tips, F: total forks).

	L/cm	*SA*/cm^2	*PA*/cm^2	*V*/cm^3	*AD*/mm	*LPV*/cm·cm^{-3}	*T*	*F*
C	122.39 ± 28.25a	389.22 ± 46.44a	126.61 ± 10.53a	238.86 ± 3.44a	17.79 ± 2.87a	218.40 ± 39.21a	444 ± 55a	2086 ± 45a
L	87.88 ± 5.87b	307.50 ± 49.67b	88.78 ± 1.70ab	171.36 ± 46.32ab	12.16 ± 2.80a	127.08 ± 43.56b	285 ± 41b	1314 ± 166b
M	64.02 ± 3.92b	141.35 ± 25.39c	53.28 ± 23.99ab	47.83 ± 9.42b	7.26 ± 0.77a	27.27 ± 6.80c	162 ± 28c	277 ± 85c
H	66.07 ± 5.49b	120.15 ± 14.25c	38.90 ± 5.64b	37.76 ± 6.25b	5.82 ± 0.37a	21.55 ± 9.27c	109 ± 16c	137 ± 14c

*Values are the means of seven replicates ± SE. Means with different letters identify significant differences ($p < 0.05$) using one-way ANOVA.

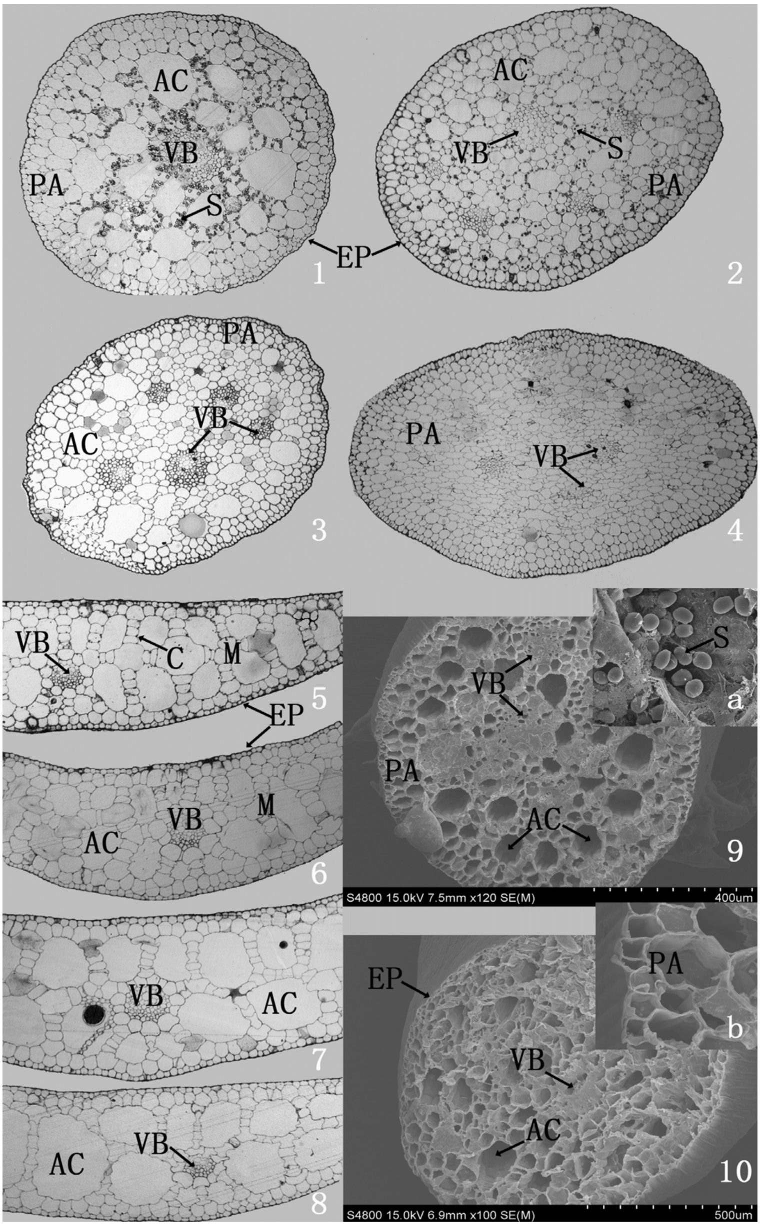

Figure 3
Anatomical structures of stolons and leaves of Vallisneria natans *changed with nutrient enrichment. EP. epidermis; M. mesophyll; PA. paraenchymatous cell; VB. vascular bundle; AC. air chamber; C. chloroplast; S. starch. 1–4. Cross-sections of stolons in C, L, M and H treatments (×100); 5–8. Cross-sections of leaves in C, L, M and H treatments (×100); 9. Scanning of stolons in control (×120); 10. Scanning of stolons in H treatment (×100); a. Local enlargement of 9 (×1100); b. Local enlargement of 10 (×700) (C = control, L = low, M = medium, H = high).*

(Figures 3-5 to 3-8). Moreover, distorted leaf cells were found in plants under the H treatment (Figure 3-8).

DISCUSSION

Enrichment with N and P had a general complex effect on the growth of the submerged plants. Several studies have shown that no significant growth response to N enrichment has been reported (Murray *et al.*, 1992), others have argued that increased nitrogen levels often induce increased growth and biomass (Short, 1987; Van Lent *et al.*, 1995). In other cases, however, decline in macrophytes was observed as a direct effect of increased nitrogen in the form of nitrate (Burkholder *et al*., 1992) and ammonium toxicity to submerged aquatic plants has also been given much attention in a few freshwater aquatic plants (Smolders *et al.*, 1996). Furthermore, mesocosm experiments conducted in Denmark revealed thresholds of TN and TP concentrations of 1.2–2 and 130–200 ug·L^{-1}, respectively, above which there is high probability of losing submerged macrophytes in temperate shallow lakes (Gonzalez Sagrario *et al.*, 2005; Jeppesen *et al.*, 2007). The key factor leading to these confusable conclusions regarding the effects of nutrients on macrophytes has been the total amount of nutrient. Preliminary experiments also showed that under balanced and non-limiting conditions, the growth of macrophytes will not be stimulated if nutrient levels are only slightly increased. In this study, a wide range of nutrient levels have been applied, for macrophytes growth will be affected by high nutrient levels because the tolerance to nutrient stress is limited. The results of plant growth and photochemical efficiency were also approving *V. natans* grow well in the control treatment and be restrained along with the increase of nutrient level.

Morphological plasticity has been viewed as an important adaptive mechanism to changes in nutrient supply for macrophytes. Xie *et al.* (2005) have suggested that the root morphology of *V. natans* varies considerably with the fertility of the sediment in which it grows. A marked effect of the nutrients in the water column on the growth of submerged macrophytes has been reported to be reduced root biomass (Rattray *et al.*, 1991; Madsen and Cedergreen, 2002). Indeed, root morphology can be greatly affected by nutrient availability in the environment and by the nutrient status of the plant (Linkohr *et al.*, 2002). For example, fine and long root length and a high specific root length are closely related to conditions of infertility (López-Bucio *et al.*, 2003). Wang and Yu also found stolon biomass and roots length were higher in nutrient-poor patches significantly (Wang and Yu, 2007). And we just observed the same relationship in the present study. The values of the total root length (*L*), root surface area (*SA*), projected root area (*PA*), total root volume (*V*), average root diameter (*AD*), total root length per volume (*LPV*), total tips (*T*) and total forks (*F*) were highest in the control treatment and decreased gradually from low to high nutrient levels. Morphological plasticity is the ability of an individual organism to alter its morphology in response to environmental conditions. Although the mechanism involved is not clear, we speculate that a feedback effect in which *V. natans* reduces its roots to adapt to high nutrient concentrations could partly account for the observed differences.

In addition to the differences in the AGR, the *Fv/Fm* ratio and the root morphological characteristics associated with nutrient enrichment, there were also changes in the anatomical structure of the stolons and leaves. The results of both scanning electron microscopy and semi-sectioning with embedded resin indicated that the anatomical structure of both the stolons and leaves changed with increasing nutrient levels. It is well known that stress can result in plants that are unhealthy and poorly developed, and associated physiological and anatomical changes include reduced stomatal aperture (Chartzoulakis *et al.*, 1999), the degradation of starch (Goyal *et al.*, 1986; Lloyd *et al.*, 2005) and a reduction of the number of chloroplasts (Nagao *et al.*, 2005). Aerenchyma, a spongy tissue with large air spaces, provides a low-resistance internal pathway for the storage and exchange of gases within plant tissues (Visser *et al.*, 1997; López-Bucio *et al.*, 2003). In our experiment, both the stolons and leaves of healthy plants had well-developed air chambers, but with increasing nutrient levels, these air chambers were reduced in size. Starch is a more efficient C (carbon) storage form than are soluble carbohydrates, and it has been reported that a NH_4^+-N source notably reduced the C storage capacity of *Phragmites australis* rhizomes (Kubín and Melzer, 1996). In our study, starch grains were present in the cells of healthy plants but not in the cells of plants under the higher nutrient treatments. In contrast, starch could be an energy resource that ensures an air supply *via* the aerenchyma. It has been suggested that growth inhibition is related to the luxurious uptake and subsequent incorporation of NH_4^+ in N-rich free amino acids, the assimilation of which requires energy and carbon that cannot then be used for growth (Smolders *et al.*, 1996; Marschner, 1998). We propose that the reduction of the aerenchyma, starch grains and chloroplasts were the significant structural changes that led to the poor growth associated with nutrient enrichment. It is suggested that nutrient enrichment changed the anatomical structure of the macrophyte, *V. natans*, which may be the mechanism underlying the effect of nutrient enrichment on the growth of macrophytes. However, whether these were influenced by NH_4^+-N toxicity or other factors requires further study.

Interestingly, it has been well documented that eutrophication has important roles in the decline of macrophytes (Middleboe and Markager, 1997; Melzer, 1999; Qin *et al.*, 2005). Direct effects of increased water-column nutrients concentrations caused by eutrophication are considered to be the primary factor leading to macrophytes demise (Cambridge and McComb, 1984; Sand-Jensen and Borum, 1991). Other researchers have found that the presence of phytoplankton and periphyton could also influence plant growth by competing for light and nutrients, which might be important factors for the observed effects of eutrophication (Phillips *et al.*, 1978; Roberts *et al.*, 2003). Whereas researches involving large-scale experimental mesocosms described significant decreases in seagrass survival under water column nitrate enrichment that were not associated with algal-imparted light attenuation (Burkholder *et al.*, 1992, 1994). In the present study, we noticed that although the enrichment of nutrients generally led to the suppression of growth, *V. natan* didn't die even in the H treatment, which implied a tolerance of *V. natans* to nutrient stress that was very high, much more than in the field environment. The conclusion was that nutrient enrichment may be an important but not a fatal factor in macrophytes decline in the field circumstance.

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