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□ *Ryegrass has increasingly been used in constructed wetlands for treatment of eutrophic wastewater. To properly match plant species with the characteristics of wastewater being treated, it is important to know the performance of plant species under different nitrate/ammonium (NO_3^-/NH_4^+) ratios. We investigated ryegrass (*Lolium perenne* L.) dry matter (DM) production and N accumulation under five NO_3^-/NH_4^+ ratios (100/0, 75/25, 50/50, 25/75, 0/100) in a hydroponic system. The results showed that ryegrass total DM, shoot DM, root DM and nitrogen (N) accumulation were greater under NO_3^-/NH_4^+ ratios of 50/50 and 75/25 than under other NO_3^-/NH_4^+ ratios, indicating that ryegrass can be best used in constructed wetlands for treating wastewater with such NO_3^-/NH_4^+ ratios to achieve high biomass production and efficient removal of N. On the other hand, for treating wastewater with either NO_3^- or NH_4^+ dominate the inorganic N, other plant species that are more adapted to such conditions should be explored.*

Keywords: wastewater, N concentration, dry matter production, root growth, root shoot ratio

INTRODUCTION

Excessive discharge of nutrients into rivers may cause eutrophication and deteriorate ecosystem health (Tylova et al., 2005). In many small cities in China, sewage is still being directly drained into water bodies without treatment (Liu et al., 2009), which has caused serious water pollution and intensified water shortage. Therefore, proper treatment of eutrophic wastewater is crucial for maintaining a healthy environment, clean water supply, and human health. Constructed wetlands (CWs) have emerged as a viable wastewater treatment technology (Liu et al., 2009). In CWs, plant

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nitrogen (N) uptake and denitrification by microorganisms are the two main mechanisms responsible for N removal. Between the two mechanisms, plant uptake of N usually plays a minor role, accounting for 5–23% of the total N removal (Ge et al., 2007). However, plants can greatly contribute to N removal by affecting nitrification and denitrification intensity in the root zone (Stottmeister et al., 2003; Ge et al., 2007). For instance, plant roots can release organic compounds and provide a suitable environment for the microorganisms (Sundaravadivel and Vigneswaran, 2001). In order to achieve efficient N removal from wastewater, plants with high biomass production, especially root biomass production, should be given priority.

Wastewater from different sources has different characteristics, such as different nitrate (NO_3^-)/ammonium (NH_4^+) ratios. The inorganic N composition in wastewater typically has between 0.4 and 99.1% NH_4^+ and between 0.9 and 99.6% NO_3^- (Liu et al., 2009). Plant species may have a preference between NO_3^- and NH_4^+, so species should be carefully selected according to the characteristics of the wastewater (Sundaravadivel and Vigneswaran, 2001). Different NO_3^-/NH_4^+ ratios in the N supply can affect the rate of plant growth as well as biomass allocation between shoot and root (Guo et al., 2002; Bruck and Guo, 2006). Most species grow better and accumulate more N when supplied with a mixture of NO_3^- and NH_4^+ (Ali et al., 2001; Guo et al., 2002). Species such as tea (*Camellia sinensis*) prefer NH_4^+ as the N-source (Ruan et al., 2007), whereas wheat (*Triticum* spp.) is tolerant to NH_4^+ but only to low NH_4^+ concentrations (Cox and Reisenauer, 1973). Many other plants are highly sensitive to NH_4^+ (Ali et al., 2001; Britto and Kronzucker, 2002). For such species, when NH_4^+ is supplied as the sole N source, plants can develop symptoms of toxicity and the root growth can be severely impaired (Lasa et al., 2001; Britto and Kronzucker, 2002).

However, most relevant studies have been focused on cultivated plants (Ali et al., 2001; Lasa et al., 2001; Bruck and Guo, 2006; Ruan et al., 2007), especially vegetables, with emphasis on the economic yield and inorganic N concentrations in plant tissues (which has implications for human health). Although the preference of *Phragmites* and *Glyceria*, two species commonly used in CWs, for NH_4^+ vs NO_3^- have been studied by Tylova et al. (2005), the preference for NH_4^+ vs NO_3^- among other plant species used in CWs for wastewater treatment is poorly understood.

Ryegrass (*Lolium perenne* L.) is a species commonly used in vertical flow CWs (Chang et al., 2004). It can accumulate considerable amounts of nutrients for removal through harvesting (Caicedo et al., 2000). But no detailed studies on the effects of different inorganic N forms on ryegrass for wastewater treatment have been found in the literature. In this study, a hydroponic system was used in order to minimize the transformation of N between the two inorganic forms by microorganisms. The objectives of this study were to (1) determine the impact of NO_3^-/NH_4^+ ratio on total and root biomass production of ryegrass; and (2) investigate the effects of NO_3^-/NH_4^+ ratio on nitrogen uptake and thus nitrogen removal from the culture solution by ryegrass. Results from this study can provide information for ryegrass deployment in CWs.

MATERIALS AND METHODS

Plant Materials and Growth Conditions

The experiment was conducted in the greenhouse (120″05′ E, 30″18′ N) in the College of Life Sciences, Zhejiang University, Hangzhou, in southeast China. Uniform ryegrass seeds were selected and sown on a tray to germinate in distilled water. After about ten days when the first true leaf emerged, ryegrass seedlings were selected for uniformity and transplanted to plastic pots filled with 2 L nutrient solutions with different inorganic N compositions (NO_3^-/NH_4^+ ratios of 100/0, 75/25, 50/50, 25/75, 0/100) at a constant N concentration of 8 mmol L^{-1}. All treatments were replicated four times, for a total of 20 pots in the experiment, with each pot containing six plants. The basal nutrient solution was a modified Hoagland's nutrient solution (Hoagland and Arnon, 1950) that contained the following macronutrients (in mmol L^{-1}): potassium (K^+) 7, magnesium (Mg^{2+}) 2, phosphate (PO_4^{3-}) 1, and calcium (Ca^{2+}) 5 (Table 1). Micronutrients were supplied as follows: 2.86 mg of boric acid (H_3BO_3), 0.08 mg of copper sulfate ($CuSO_4 \cdot 5H_2O$), 0.22 mg of zinc sulfate ($ZnSO_4 \cdot 7H_2O$), 1.81 mg of manganese chloride ($MnCl_2 \cdot 4H_2O$), 0.09 mg of molybdic acid ($H_2MoO_4 \cdot 4H_2O$), and 7.645 mg iron (Fe)- ethylenediaminetetraacetic acid (EDTA) per liter of water (Zhang et al., 2005). The pH of the solution was adjusted to 6.0 ± 0.2 with dilute sodium hydroxide (NaOH) or hydrochloric acid (HCl). Plants were grown with a 14 h photoperiod, at day/night temperatures of 25/15°C and 70–80% relative humidity at an irradiance of *ca.* 280 μmol photon

TABLE 1 Compositions (mmol L^{-1}) of nutrient solutions with different NH_4^+/NO_3^- ratios but the same N concentration (8 mmol L^{-1})

	NO_3^-/NH_4^+ ratio				
	100/0	75/25	50/50	25/75	0/100
Nutrient source	mmol L^{-1}				
KNO_3	4.8	2.8	0.8	2.0	0.0
$Ca(NO_3)_2 \cdot 4H_2O$	1.6	1.6	1.6	0.0	0.0
$CaCl_2 \cdot 2H_2O$	3.4	3.4	3.4	5.0	5.0
$(NH4)_2SO_4$	0.0	1.0	2.0	3.0	4.0
KH_2PO_4	1.0	1.0	1.0	1.0	1.0
$MgSO_4 \cdot 7H_2O$	2.0	2.0	2.0	2.0	2.0
KCl	1.2	3.2	5.2	4.0	6.0

m^{-2} s^{-1}. The volume of culture solution in each pot was maintained at the initial volume of 2 L by adding distilled water every two days. Nutrient solutions were replaced every 10 days.

Measurement and Calculation

After twenty days of growth, fully expanded leaves were selected to determine leaf chlorophyll concentrations. Chlorophyll was extracted using an ethane and ethanol mixture at a volume ratio of 2:1 according to Peng and Liu (1992). Chlorophyll concentration was determined spectrophotometrically in the clear supernatant by measuring the absorbance at 663 and 645 nm for chlorophyll a and b, respectively, using a HP 751 (Hewlett Packard, Shanghai, China) spectrophotometer. Chlorophyll concentrations were calculated using the equations given in Peng and Liu (1992). Before harvest, the rate of net photosynthesis was measured with a portable photosynthesis system (Licor-6400, Licor, Lincoln, NE, USA). Prior to the measurement, the leaves were illuminated at approximately 600 μmol photon m^{-2} s^{-1} for 30 min in a growth chamber.

After 40 days of growth, ryegrass was harvested and divided into shoots (ryegrass has only leaves and root, no stem) and roots. The length of the longest root for each plant was measured. All leaves from each plant were collected and scanned with a scanner (ScanMaker 4900, Microtek International Inc., Cerritos, CA, USA) immediately after harvest. The WinFLORA Pro 2002a software (Regent Instruments Inc., Quebec, Canada) was used to determine leaf area (LA) from the images scanned. Plant shoot and root were dried in a forced air oven at 65°C for 72 h to determine dry matter (DM) yield, referred to as shoot DM, root DM and total DM (the sum of shoot and root biomass). The dried plant material was ground and digested to determine NH_4^+ and NO_3^- concentrations in shoots and roots. From each sample, 0.2 g of material was weighed to analyze nutrient concentrations. The samples were digested by the Kjeldahl method applying sulfuric acid (H_2SO_4) + hydrogen peroxide (H_2O_2). The NH_4^+ and NO_3^- concentrations were determined photometrically at wavelength of 420 nm and 210 nm, respectively (Lu et al., 2004). And total N concentration (TN) equals the sum of NH_4^+ and NO_3^- concentrations.

The following biometric characteristics were calculated following Hunt (1978): root:shoot ratio (R/S, calculated by dividing root DM by shoot DM), and specific leaf area (SLA, leaf area divided by leaf DM). Nitrogen uptake was calculated based on the biomass and concentration data.

Statistical Analysis

Analysis of variance (ANOVA) was performed using the general linear model univariate procedure in SPSS (SPSS 15.0, SPSS Inc., Chicago, IL, USA). The least significant difference was performed on each variable when there was a significant treatment effect from the ANOVA analysis, statistical significance was determined at $\alpha = 0.05$. All data are presented as mean ± standard error (S.E.).

RESULTS

Biomass Production and Allocation

The NH_4^+ to NO_3^- ratio affected the shoot and root growth of ryegrass. Total DM and shoot DM of ryegrass plants were higher under 75/25 and 50/50 NO_3^-/NH_4^+ ratios than under the other three treatments ($P < 0.05$). In addition, total DM or shoot DM were not different between the 100% NO_3^- and 100% NH_4^+ treatments ($P > 0.05$) (Table 2). The ammonium only treatment resulted in root DM on average 68 and 61% lower than the 75/25 and 50/50 treatments ($P < 0.05$), respectively (Table 2). We also found that root DM under the NO_3^- only treatment was also significantly lower than that under the 75/25 treatment, but obviously higher than that under the NH_4^+ only treatment ($P < 0.05$). A near linear decrease of R/S ratio was observed when the proportion of NH_4^+ in the nutrient solution increased (Table 2). The effect of N form on the partition of assimilates between shoot and root is demonstrated in our experiment: only 4.5% of the total DM was allocated to the roots exposed to 100% NH_4^+.

Physiological and Morphological Traits

No difference was found in net carbon dioxide (CO_2) assimilation rate per unit leaf area among the five treatments (Table 2). Leaf area was higher under 75/25 and 50/50 ratios than under 100/0, 25/75 and 0/100 ratios ($P < 0.05$). The chlorophyll concentration increased with the increase in the proportion of NH_4^+ in solutions, while the reverse was true for SLA. Root length of plants decreased with the increasing proportion of NH_4^+. When NH_4^+ reached 100%, root length was the lowest compared with plants in other treatment groups containing NO_3^- ($P < 0.05$) (Table 2).

Tissue N Concentration and Accumulation

A significant increase in root nitrate concentration in plants fed with NO_3^- alone over the other treatments was observed ($P < 0.05$, Figure 1). Generally, the greater the concentration of NH_4^+ in the culture solution, the greater N concentration in plant shoots and roots. N concentration reached peak in plants grown with 100% NH_4^+ (Figure 1).

Nitrogen accumulation mostly occurred in shoots, which accounted for over 90% of the total N accumulation (Figure 2). Ryegrass grown under

TABLE 2 Shoot, root and total dry matter (DM), root/shoot ratio (R/S), leaf area (LA), chlorophyll concentration (Chl), net CO_2 assimilation rate (P_n) of ryegrass (*Lolium perenne* L.) grown under five different NO_3^-/NH_4^+ ratios. Values given are mean ± S.E. (n = 4)

	NO_3^-/NH_4^+ ratio				
Plant trait	100/0	75/25	50/50	25/75	0/100
Shoot DM (g plant^{-1})	1.28 ± 0.08 b	2.22 ± 0.22 a	2.43 ± 0.11 a	1.42 ± 0.29 b	1.47 ± 0.18 b
Root DM (g plant^{-1})	0.14 ± 0.02 bc	0.22 ± 0.02 a	0.18 ± 0.02 ab	0.11 ± 0.01 cd	0.07 ± 0.01 d
Total DM (g plant^{-1})	1.5 ± 0.07 b	2.44 ± 0.24 a	2.61 ± 0.11 a	1.55 ± 0.31 b	1.57 ± 0.22 b
R/S	0.11 ± 0.02 a	0.1 ± 0.01 ab	0.07 ± 0.01 b	0.08 ± 0.01 b	0.04 ± 0.01 c
LA (cm^2)	603.73 ± 47.04 b	972.2 ± 51.65 a	1032.16 ± 39.71 a	599.90 ± 98.11 b	546.03 ± 44.51 b
Chl (mg g^{-1})	1.59 ± 0.12 b	2.04 ± 0.1 ab	2.23 ± 0.08 a	2.26 ± 0.19 a	2.50 ± 0.27 a
P_n (μmol CO_2 m^{-2} s^{-1})	13.84 ± 0.87 a	13.08 ± 1 a	12.89 ± 0.81 a	14.54 ± 0.94 a	12.45 ± 0.54 a
SLA (cm^2 g^{-1})	471.46 ± 14.83 a	437.56 ± 17.66 a	424.54 ± 22.58 ab	378.23 ± 15.32 bc	347.68 ± 9.75 c
Root length (cm)	26.92 ± 2.03 a	27.5 ± 1.65 a	26.28 ± 1.39 ab	21.99 ± 1.96 bc	20.07 ± 0.5 c

Treatment means with different lowercase letters are significantly different ($P < 0.05$) for each listed parameter.

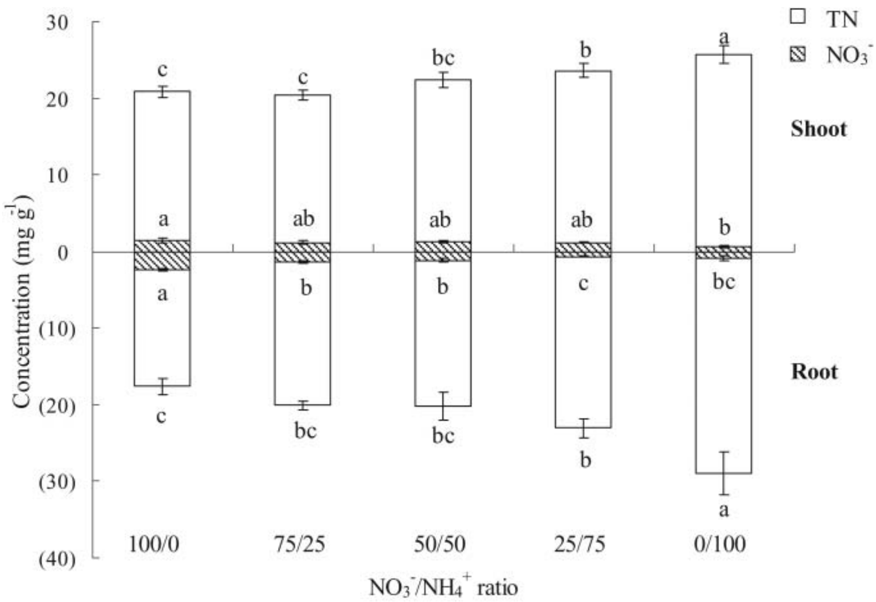

FIGURE 1 Concentrations of TN (open columns) and NO_3^- (dashed columns) in shoots and roots of ryegrass (*Lolium perenne* L.) grown in solutions with different NO_3^-/NH_4^+ ratios. Values given are mean ± S.E. (n = 4). Values with different letters are significantly different ($P < 0.05$) among the treatments for each parameter measured either for root or shoot.

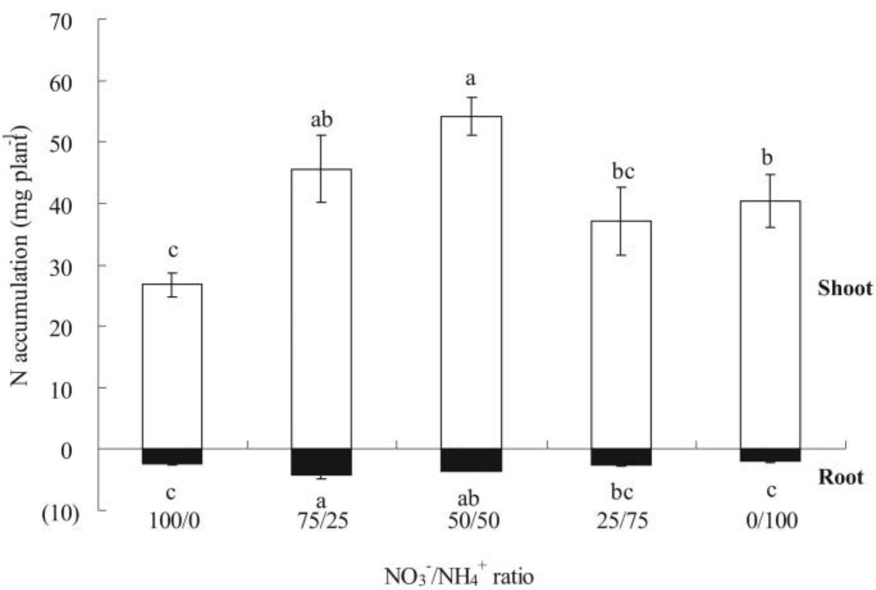

FIGURE 2 N accumulation in shoots (open columns) and roots (solid columns) of ryegrass grown in solutions with different NO_3^-/NH_4^+ ratios. Values given are mean ± S.E. (n = 4). Values with different letters are significantly different ($P < 0.05$) among the treatments for either root or shoot.

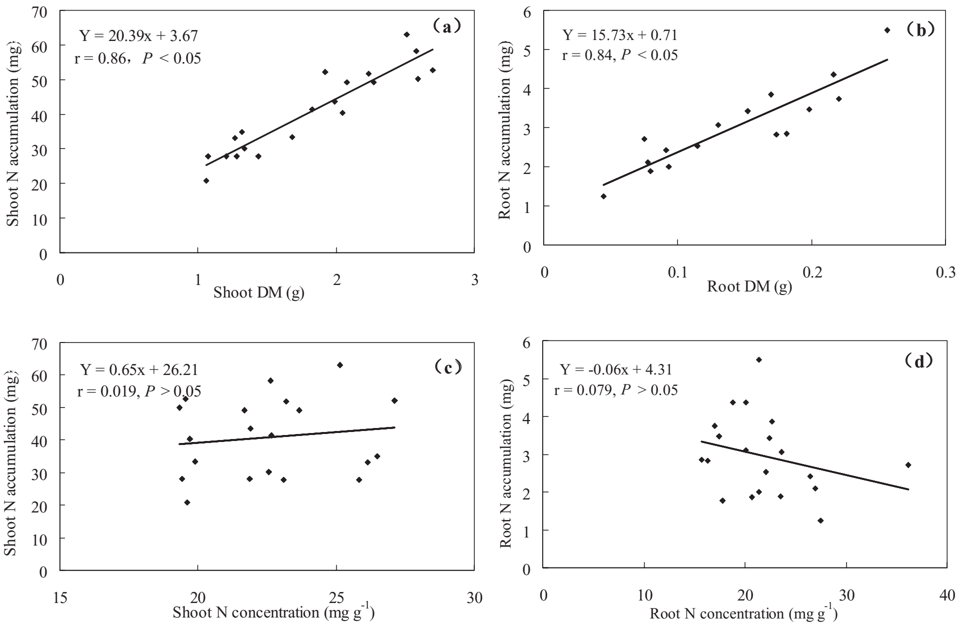

FIGURE 3 Relationships between plant dry mass and N accumulation in ryegrass. [(a) shoot, and (b) root] and relationships between N concentration and N accumulation in ryegrass [(c) shoot, (d) root].

NO_3^-/NH_4^+ ratios of 75/25 and 50/50 had the greatest shoot N accumulation, while the 100% NH_4^+ had the intermediate and 100% NO_3^- had the lowest shoot N accumulation. Positive correlations were found between shoot DM and shoot N accumulation (Figure 3a) and between root DM and root N accumulation (Figure 3b). However, N concentration was not correlated with N accumulation in shoot or root (Figures 3c and 3d).

DISCUSSION

Effects of NO_3^-/NH_4^+ Ratios on Plant Biomass Production

Many previous studies have reported that a mixture of NO_3^- and NH_4^+ supply is better for plant growth compared with NH_4^+ or NO_3^- alone (Ali et al., 2001; Guo et al., 2002; Zhang et al., 2007). In this study total and shoot DM yields were the highest in 75/25 and 50/50 treatments, suggesting that ryegrass prefers close to equal amounts of NO_3^- and NH_4^+ supply in the growth media, with slight preference for NO_3^-. Furthermore, the solutions with NO_3^-/NH_4^+ ratios of 75/25 and 50/50 were conducive to ryegrass's root growth, which may favor the function of microorganisms for improving the efficiency of wastewater treatment. Like many other plant species (Cramer and Lewis, 1993; Frechilla et al., 2001), ryegrass exhibited severe reduction in root growth in the 100% NH_4^+ treatment, implying that ryegrass may not be effective in treating wastewater with sole NH_4^+ in CWs.

Effects of NO_3^-/NH_4^+ Ratios on Biomass Allocation, Physiological and Morphological Traits

Not only dry matter production but also the partitioning of dry matter between shoot and root was affected by the inorganic N ratio in the nutrient solution. Ryegrass R/S ratios were lower in NH_4^+- than NO_3^--fed plants, similar to findings in other experiments (Cramer and Lewis, 1993; Guo et al., 2002; Tylova et al., 2005). For example, Tylova et al. (2005) reported that R/S of *Phragmites* was on average 27% higher under 100% NO_3^- than 100% NH_4^+ treatment, but was 175% higher for ryegrass in this study, which indicated more depressed root growth of ryegrass under sole NH_4^+ supply. This again illustrates that ryegrass does not have an advantage in treating wastewater with 100% NH_4^+.

Our data also indicate significant effects of N form on leaf traits such as leaf area, chlorophyll concentration and SLA. The response of leaf area to different N forms was consistent with that of plant DM in this study. Sole NO_3^- or NH_4^+ supply had restricted leaf expansion, similar to what Guo et al. (2002) reported for French bean (*Phaseolus vulgaris* L.). Based on treatment effects on leaf area, we can infer treatment effects on plant growth. However, the effects of N form on SLA are complex and depend on plant species, the degree of maturity of the leaf, and the environmental conditions (Bruck and Guo, 2006). In this study, from 100% NO_3^- to 100% NH_4^+ the SLA decreased while the chlorophyll concentration increased, suggesting that ryegrass leaves thickened and concentrated chlorophyll under 100% NH_4^+ supply. Ruan et al. (2007) reported that tea plants supplied with NO_3^- displayed yellowish leaves, consistent with the observed decrease in chlorophyll concentrations in the NO_3^- only treatment in this study.

Tissue N Concentration and N Accumulation under Different NO_3^-/NH_4^+ Ratios

Less energy is required in the process of NH_4^+ assimilation (as compared with NO_3^- assimilation) for higher plants (Zhang et al., 2007). A lower NO_3^-/NH_4^+ ratio in the solution led to higher N concentrations in ryegrass, indicating that the increase in NH_4^+concentration enhances the N uptake ability per unit biomass although too much NH_4^+ suppresses plant root growth.

A significantly higher root NO_3^- concentration was found when all N was supplied as NO_3^-, consistent with those reported by Lewis and Chadwick (1983), and Murphy and Lewis (1987). However, NO_3^- concentrations under any of the treatments were lower than the threshold for animal feed

(Niu et al., 2004; Ge et al., 2007), so all harvested biomass can be used as animal feedstock.

Like the performance of wheat (Cox and Reisenauer, 1973), ryegrass fed with NO_3^-/NH_4^+ ratios of 75/25 and 50/50 can achieve the most N accumulation due to its high DM yield rather than high N concentration. The tight relationships between plant shoot DM and N accumulation imply that in wastewater treatment N accumulation was manifested by vigorous plant growth but N concentration was little affected and played an insignificant role in N accumulation. Furthermore, it has been experimentally proven that harvesting plant shoots is an effective way to remove N from wastewater treatment systems (Sundaravadivel and Vigneswaran, 2001; Jiang et al., 2004; Ge et al., 2007).

This study provides information for the application of ryegrass in wastewater treatment systems with different NO_3^-/NH_4^+ ratios; however, further study considering the role of microorganisms that live in the substrate is needed to better understand the interactions between plants and microorganisms and their joint contribution to nutrient removal from wastewater treatment systems.

CONCLUSIONS

This hydroponic study showed that ryegrass preferences approximately equal NO_3^- and NH_4^+ supply, with slight preference for a higher NO_3^- supply. Ryegrass would be a suitable species to use in CWs to treat wastewater with NO_3^-/NH_4^+ ratios between 75/25 and 50/50, as under this condition ryegrass can achieve the highest shoot and root DM, accumulate the highest amount of N, and as a result would be most efficient in removing N from the wastewater by plant harvesting.

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