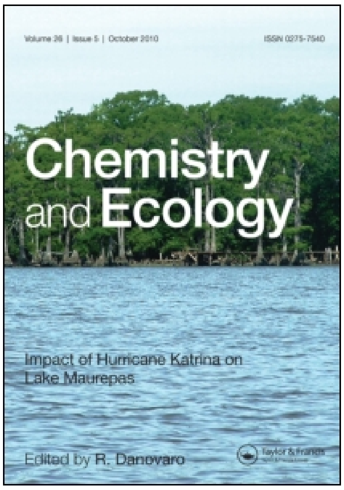

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Impact of influent inorganic nitrogen on nitrate removal efficiency of submerged plant microcosms

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Even though many studies have focused on identifying the effects of NH_4^+-N on various aspects of wetland systems, there have been very few attempts to quantify the overall impact of NH_4^+-N concentration on NO_3^--N removal efficiency. Consequently, this research was conducted to investigate the effects of influent NH_4^+-N (2–10 mgl^{-1}) and NO_3^--N (2–6 mgl^{-1}) concentrations on NO_3^--N removal efficiency and growth inhibition concentrations of *Egeria densa* Planch. Mass balance for NH_4^+-N removal was done to quantify the nitrified NH_4^+-N in order to incorporate total NO_3^--N concentration for the non-linear regression analysis. The NO_3^--N and total nitrogen removal efficiencies were significantly varied at three different NO_3^--N concentrations ($p < 0.05$). Data on oxygen consumption during dark respiration (0.25–0.48 $mgl^{-1}\,hr^{-1}\,g^{-1}$ of dry weight biomass) and final biomass (0.17–0.24 g) variations revealed that the lowest growth inhibition concentration of NH_4^+-N on *E. densa* could be 4 mgl^{-1}. Non-linear regression analysis established a positive correlation ($r^2 = 0.97$) between influent NH_4^+-N concentration and NO_3^--N removal efficiency within the analysed range of NH_4^+-N (0–4 mgl^{-1}). Results suggest that NO_3^--N removal efficiency in submerged plant wetland systems could be improved by adding sufficient amounts of NH_4^+-N to secondary treated wastewater.

Keywords: ammonium; constructed wetlands; mutual interactions; nitrate; regression analysis; removal efficiency

1. Introduction

Wetland treatment is being increasingly recognised as an ecologically viable wastewater treatment method. Better understanding about the interactions among constituents in wastewater is essential for optimising wetland treatment functions. Nitrogen and phosphorus removal mechanisms have been studied with great interest, as they damage the environment in various ways, such as causing eutrophication in surface water bodies. Nitrogen in domestic wastewater is usually added as ammonium, which is a commonly studied constituent, because of its significant impacts on macrophytes such as influences on growth [1], photosynthesis [2,3], as well as inhibitory effects

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at alkaline conditions [4]. Among the factors controlling growth of submerged macrophytes, light, temperature and nutrients are known to be of primary importance [2,5,6].

Effects of NH_4^+-N on NO_3^--N removal efficiency of macrophytes can be considered through various aspects, such as competition for plant uptake and NH_4^+-N inhibition on plant growth and denitrification. Besides the negative impacts, there are positive impacts of NH_4^+-N, especially by stimulating plant growth. Assimilation of NH_4^+-N is considered to be easier than NO_3^--N, due to the relatively lower energy consumption during the assimilation process [4]. It has also been shown that both roots and fronds uptake NH_4^+-N at a higher rate than NO_3^--N [7]. Therefore, the presence of a higher concentration of NH_4^+-N could reduce NO_3^--N removal by plant uptake due to the competition among nitrogen forms, and it may create a negative impact on NO_3^--N removal efficiency of macrophytes.

Most of the species of the higher plants develop severe toxicity symptoms when they are grown with NH_4^+-N as the major nitrogen source. The two major toxicity symptoms identified are chlorosis of leaves and overall suspension of growth. The other visually observable symptom is a lowering of the root:shoot ratio [8]. However, the toxicity of ammonia is dependent on temperature, pH and the plant species itself. The impact of NH_4^+-N on NO_3^--N removal efficiency of macrophytes would be negative, as toxicity causes a comparatively lower growth rate in the plant. In wastewater treatment wetlands receiving high NH_4^+-N concentrations the toxicity effect will not only be on macrophytes, but also on denitrifying bacteria which may result in retarding the denitrification process [2].

On the other hand, the availability of NH_4^+-N in optimum concentrations may result in better growth of submerged macrophytes. Growth of submerged macrophytes will enhance the interactions between plant community and nutrients in wastewater. A dense macrophyte system may increase dissolved oxygen (DO) and pH, eventually creating a linear decline in the rate of photosynthesis [9]. However, increases in pH were found to have no significant impact on dark respiration rates [10]. This asymmetry in DO generation and consumption rates in densely vegetated plants may produce higher fluctuations in DO levels between daytime and night time. Therefore, anoxic conditions can be expected in biofilms, formed either on plants or in the substrate, especially during the latter stages of dark respiration. Subsequently, the availability of NH_4^+-N in optimum concentrations may exert a positive impact on the denitrification process.

Considering all of the above negative and positive implications of NH_4^+-N on NO_3^--N removal, it would be worthwhile studying and establishing a mathematical correlation for them, in order to quantify the resultant impact. Also, a clear understanding on the inhibitory concentrations of NH_4^+-N on *Egeria densa* Planch would be significant as all these interactions would be valid only in non-inhibitory conditions. Therefore, studying the inhibition concentration of NH_4^+-N on *E. densa* is also necessary to formulate a better understanding of the above correlations. Consequently, the objectives of this experiment were as follows: (i) studying the effects of influent NH_4^+-N concentration on NO_3^--N removal efficiency of *E. densa*; and (ii) finding out the NH_4^+-N inhibition concentration on the growth of *E. densa*.

2. Materials and methods

Plants (*E. densa*) for the experiments were collected from Lake Biwa (35° 15′ N, 136° 05′ E) and a nursery was maintained at Saitama University, Japan. The experiment was conducted in an incubator [11], where the lighting was programmed to have a photoperiod of 8 h day^{-1} with a light intensity of 7.5 klx and 0 klx for 16 h day^{-1}. The temperature was kept constant at 25°C.

Each aquarium was supplied with 1 litre of fresh synthetic wastewater as a batch-fed system at four-day intervals, maintaining a six-day hydraulic retention time. Initially, the plant stems of

E. densa selected were the same length (12 cm) and included three double nodes on each. The experiment was conducted for six months in three stages with similar conditions, while varying the influent NO_3^--N concentration as follows: stage 1 – 2 mgl^{-1}, stage 2 – 4 mgl^{-1}, and stage 3 – 6 mgl^{-1} (Figure 1). Eighteen microcosms in 2-litre aquaria were constructed at each stage of the experiment. The bottom 0.5 L of each aquarium was filled with properly washed (soaked and washed manually three times by using tap water to remove the impurities) commercially available river sand to avoid any nutrient supplementation from the substrate.

The wastewater was prepared in the laboratory with a varying concentration of NH_4^+-N as 2, 4, 6, 8 and 10 mgl^{-1} and NO_3^--N as mentioned above (2, 4 and 6 mgl^{-1}). The above concentrations of different forms of nitrogen were consistent with those associated with *Elodea* sp. in order to consider both non-inhibitory and inhibitory conditions of *E. densa* during the experiment [12]. In addition, 1 mgl^{-1} of PO_4^{3-}-P and glucose (15 mgl^{-1} of BOD_5) were added to all aquaria in order to provide other major nutrients. Three replicates were arranged for each NH_4^+-N concentration and controls (aquaria without plants) were maintained at 2, 6 and 10 mgl^{-1} of NH_4^+-N concentration. Consequently, 54 numbers of microcosms were studied during the whole experimental period (Figure 1).

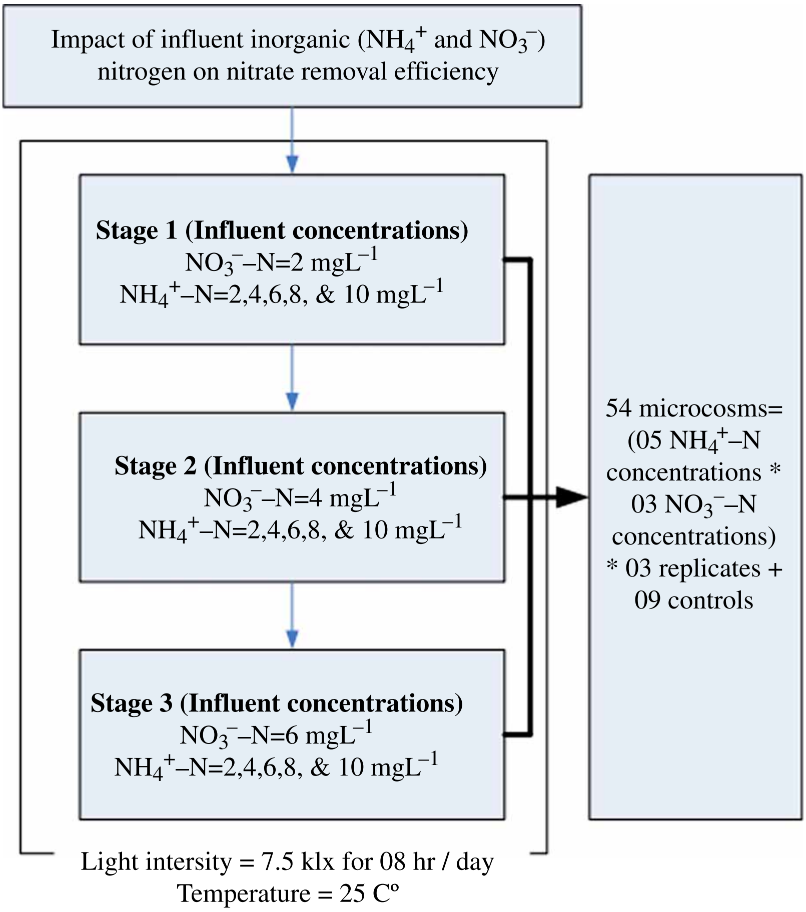

Figure 1. Schematic diagram of the experiment showing three different stages, overall experimental conditions and the number of microcosms.

The concentrations of NH_4^+-N, NO_3^--N, and TN in effluent water were measured [13] using a spectrophotometer (UV-VIS spectrophotometer, Shimadzu, UV mini-1240) at four-day intervals, from the start of the experiment. A sample volume of 150 ml was extracted from each microcosm at 10:00 h on each sampling day, before replenishing the microcosms with fresh wastewater at the same frequency of four-day intervals. The removal efficiency (%) of each parameter in the aquaria was calculated using Equation 1. In addition, temperature, pH, and dissolved oxygen (DO) concentration were also recorded at the same frequency of sampling.

$$\text{Removal (\%)} = \frac{C_{in} - C_{eff}}{C_{in}} \times 100\% \tag{1}$$

where C_{in} = influent concentration and C_{eff} = effluent concentration.

The oxygen consumption of submerged macrophytes during dark respiration was calculated using the difference between DO concentrations measured at 18:00 h on the sampling day and at 10:00 h on the following day, where the light intensity of the incubator was kept at 0 klx. The regression analysis was carried out using Oakdake-Datafit (Version 8.2.79) software. The time averaged concentration of all constituents was considered in all calculations.

The mean and standard deviation of replicates were determined. One-way analysis of variance (ANOVA) model was used to analyse significance of nitrogen removal efficiencies at three different NO_3^--N concentrations. All statistical analyses were performed by using SigmaStat 3.11 software.

3. Results and discussion

3.1. *Effect of influent NH_4^+-N concentration on pH and DO*

The DO concentration showed an increasing trend with increasing NH_4^+-N at lower influent concentrations of NO_3^--N (2 and 4 mgl^{-1}; hereafter, low concentration), while pH remained almost the same in all aquaria during the photoperiod (Figure 2). A similar pattern in DO consumption during dark respiration was observed (Figure 3). This could be due to the increase in plant biomass due to increasing influent NH_4^+-N concentrations, especially during the early stage of the experiment. The maximum plant growth was observed at the highest NH_4^+-N concentration within the first 5 days of the experiment. However, the overall biomass production became highest at the medium concentration of NH_4^+-N (Figure 4). It was also discovered that the increase in DO in water may reduce the photosynthetic rate when excess amounts of NH_4^+-N are present in the influent [9]. Therefore, a higher concentration of influent NH_4^+-N (6–10 mgl^{-1}; hereafter, high concentration) could have resulted in a relatively low photosynthetic rate during the latter part of the experimental period, reducing DO concentrations.

3.2. *Effluent composition*

The concentrations of different forms of nitrogen in effluent are depicted in Figure 5. The effluent NH_4^+-N concentration was less than 0.5 mgl^{-1}, regardless of the influent concentrations when the influent concentration of NH_4^+-N was low. However, the NH_4^+-N removal efficiency of *E. densa* was reduced when the influent concentration was high. The maximum (95.1%) and minimum (77.3%) NH_4^+-N removal efficiencies were obtained at the microcosms, fed with 4 and 10 $mg\,l^{-1}$ influent concentration of NH_4^+-N, respectively. The study suggests that the maximum aquarium capacity for an efficient removal of NH_4^+-N is limited by the averaged NH_4^+-N concentration of 4 mgl^{-1} for an initial dry mass of 120 mg *E. densa* at a microcosm of 1.5 L. The variation of total

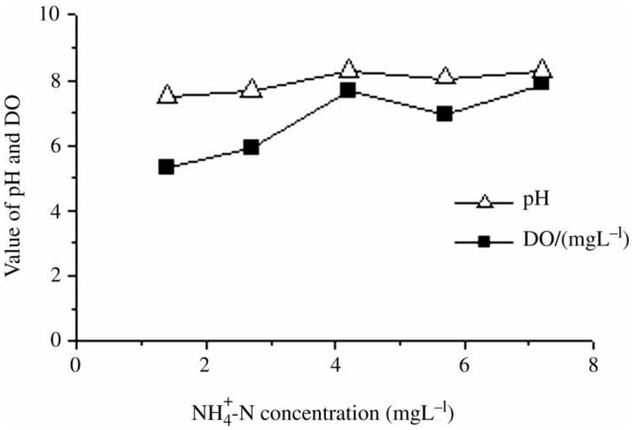

Figure 2. Variation of averaged pH and DO at three different NO_3^--N concentrations (2, 4 and 6 mgl^{-1}) during photosynthesis.

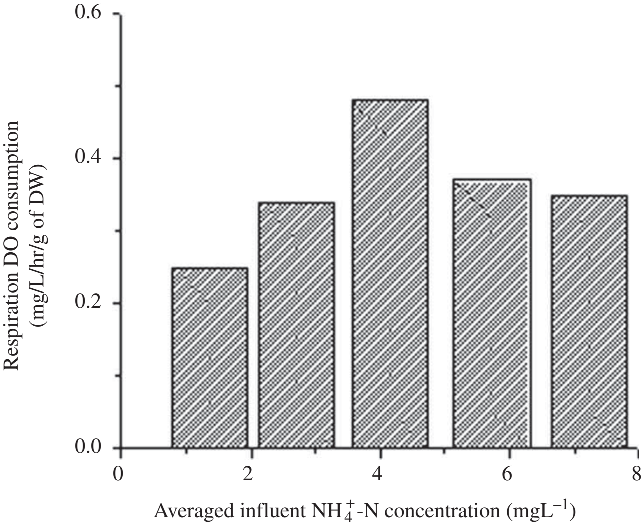

Figure 3. Variation of averaged DO consumption at three different NO_3^--N concentrations (2, 4 and 6 mgl^{-1}) during dark respiration.

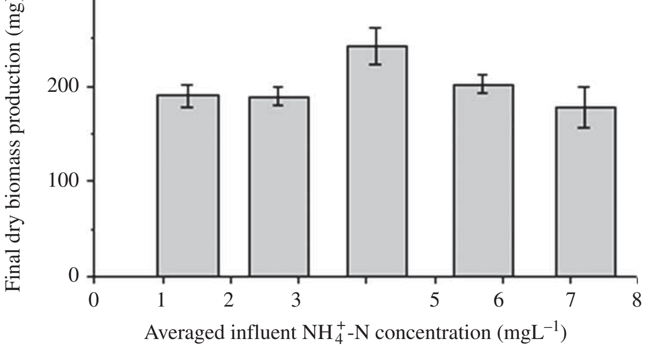

Figure 4. Variation of final dry biomass production at three different NO_3^--N concentrations (2, 4 and 6 mgl^{-1}) with the averaged influent NH_4^+-N concentrations.

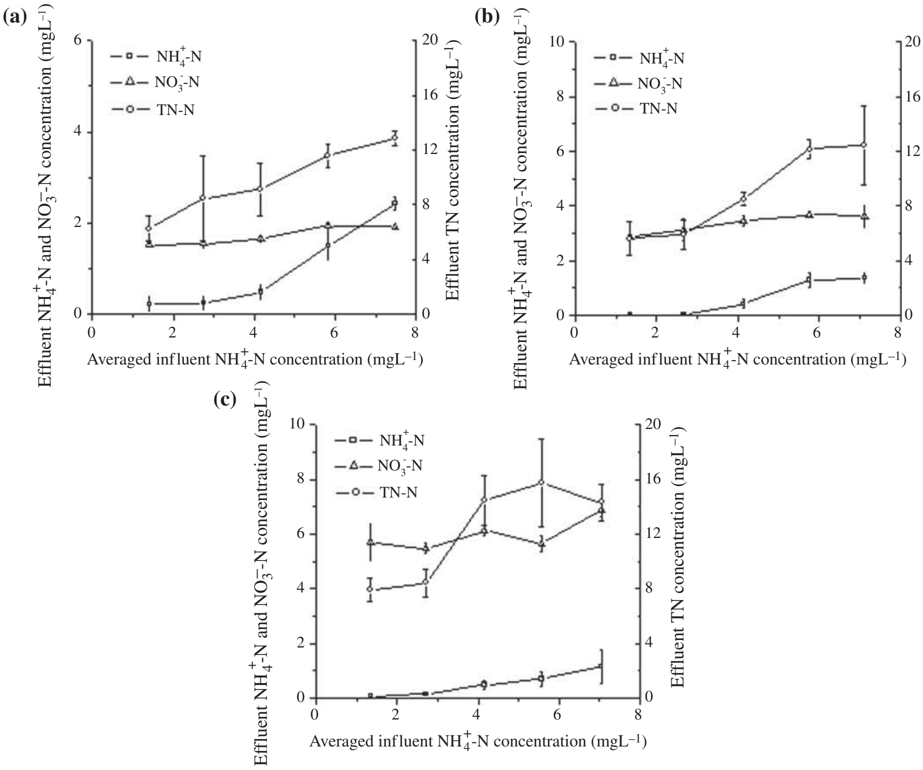

Figure 5. Variation of effluent nitrogen concentrations: (a) at influent NO_3^--N concentration of 2 mgl^{-1}, (b) at influent NO_3^--N concentration of 4 mgl^{-1}, (c) at influent NO_3^--N concentration of 6 mgl^{-1}.

nitrogen (TN) presents the resultant effect from variations of influent inorganic nitrogen. The TN was within the range of 8–12 mgl^{-1} at stage 1 and 2, while it shifted to a 8–16 mgl^{-1} range at stage 3 (Figure 5). Therefore, it can be concluded that TN removal mechanisms were retarded at higher influent NO_3^--N and NH_4^+-N concentrations for the studied microcosms, probably due to plant growth inhibition and malfunction of denitrifying bacteria [2].

The above variation in effluent NO_3^--N concentrations does not express the actual removal capacity of submerged plant systems. The presence of favourable conditions for nitrification during the photoperiod following possible anoxic conditions either at the substrate level or in biofilms, especially in the latter part of non-photoperiod may have expelled a part of the nitrified NH_4^+-N from the system as N_2 gas. Therefore, a simple mass balance study, as follows, on NH_4^+-N removal could be used to explain more clearly the actual efficiency in NO_3^--N removal.

3.3. *Mass balance analysis for NH_4^+-N removal*

Nitrification followed by denitrification, volatilisation, plant uptake and substrate adsorption are the major NH_4^+-N removal mechanisms in a wetland system [14]. In normal ambient conditions, denitrification is probably the most significant pathway of NO_3^--N removal from a wetland system [15]. The loss of NH_4^+-N through volatilisation represented, on average, 20% of the initial concentrations at a similar pH range (7.8–8.4) observed in this experiment [2]. On the other hand, the contribution to TN removal by direct plant uptake was calculated using the difference between initial and final averaged dry weight of *E. densa* at each concentration and the amount of TN contained in the *E. densa* biomass [1]. As adsorption by sediment might

Table 1. Mass balance calculation for nitrified NH_4^+-N and total NO_3^--N (mgl^{-1}).

Averaged NH_4^+-N*	Nitrified NH_4^+-N**			Total NO_3^--N			NO_3^--N removal efficiency (%)		
	S1***	S2	S3	S1	S2	S3	S1	S2	S3
1.37 ± 0.04	0.74	0.90	0.87	2.58	4.51	6.77	41.0	36.6	15.6
2.71 ± 0.04	1.80	1.96	1.86	3.68	5.67	7.68	57.6	44.8	28.8
4.15 ± 0.01	2.60	2.61	2.56	4.45	6.42	8.60	62.6	46.2	28.8
5.72 ± 0.14	2.98	3.13	3.55	4.95	7.02	9.44	61.1	47.7	40.0
7.22 ± 0.23	3.42	4.20	4.35	5.40	8.07	10.65	64.4	55.0	35.3

Note: *Averaged NH_4^+-N = (Influent NH_4^+-N + Effluent NH_4^+-N)/2.
**Nitrified NH_4^+-N = (Influent NH_4^+-N) − (Plant uptake) − (Volatilized NH_4^+-N) − (Effluent NH_4^+-N).
***S1 = 2 mgl^{-1} NO_3^--N; S2 = 4 mgl^{-1} NO_3^--N; S3 = 6 mgl^{-1} NO_3^--N.

contribute less in nitrogen removal at saturated conditions, it was assumed that the nitrogen accumulated in sediment and amount of particulate nitrogen in effluent is negligible for analytical convenience [15]. The results of the mass balance study carried out based on the above hypothesis are presented in Table 1. The averages of influent and effluent NH_4^+-N has been considered for the mass balance calculations, as it assumed that NH_4^+-N concentration was decreased linearly with time in the batch-fed systems. These results were incorporated in non-linear regression analysis to establish a correlation between influent NH_4^+-N concentration and NO_3^--N removal efficiency.

3.4. *NH_4^+-N and NO_3^--N removal efficiency of* **E. densa**

The NO_3^--N removal efficiency of *E. densa* varied between 40–60% at both influent NO_3^--N concentrations of 2 mgl^{-1} and 4 mgl^{-1}, whereas the removal efficiency remained comparatively lower at 6 mgl^{-1} (Table 1). However, the NO_3^--N removal efficiency of *E. densa* reached a peak (32%) at 5.5 mgl^{-1} of NH_4^+-N concentration when influent NO_3^--N concentration was high. This concentration can be understood as the limiting capacity of the studied microcosm for denitrification. Further, the NO_3^--N removal efficiency of *E. densa* increased at low influent NH_4^+-N concentrations, while it remained more or less stable at high concentrations (Table 1). The possible reason for this could be better growth of macrophytes at lower concentrations of NH_4^+-N, creating a higher potential for the formation of anoxic conditions as explained above. This can be confirmed using the data on variation of DO consumption during the dark respiration process (Figure 3), as DO consumption has increased with the influent NH_4^+-N concentration at lower concentrations. Consequently, removal of NO_3^--N by denitrification has increased with increasing NH_4^+-N at lower concentrations.

The one-way ANOVA analysis demonstrated that NH_4^+-N removal efficiency of microcosms at three different NO_3^--N concentrations were not significant ($p > 0.05$). However, the above analysis proved that NO_3^--N and TN removal efficiencies were significantly varied ($p < 0.05$) at three different NO_3^--N concentrations. The NO_3^--N removal efficiency of microcosms was highest at lowest influent NO_3^--N concentration. This could happen because of less competition among NO_3^--N ions on denitrification or plant assimilation at lower concentrations. As an example, the limited organic carbon may control the denitrification process at higher NO_3^--N concentrations [14]. Besides, NO_3^--N reduction is almost always coupled to NH_4^+-N assimilation and contribution from such reactions may have great affect on NO_3^--N removal at its lowest concentrations [8]. Reduction in NO_3^--N removal may simultaneously have influence on TN removal deficiency at higher NO_3^--N concentrations.

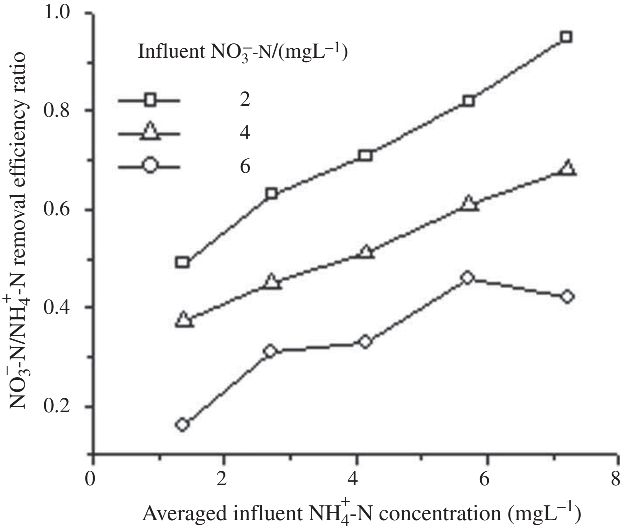

Figure 6. Ratio of removal efficiency (NO_3^--N/NH_4^+-N) variation at different influent NO_3^--N concentrations.

Since nitrification followed by denitrification is the most significant process in nitrogen removal of wetland systems [14], the ratio of NO_3^--N removal efficiency over NH_4^+-N removal efficiency of *E. densa* was evaluated against increasing NH_4^+-N concentration (Figure 6). The ratio reached approximately to 0.9 at stage 1, where the lowest influent NO_3^--N concentration existed. It presents a positive impact by increasing NH_4^+-N concentrations on the NO_3^--N removal mechanism. However, the gradient of the three curves was reduced with increasing influent NO_3^--N, as the positive influence reduced with increasing concentrations (due to its prevailing characteristics in the media). Even though the two curves related to lower concentrations of NO_3^--N (2 and 4 mgl^{-1}) were increased linearly, the curve for the highest concentration (6 mgl^{-1}) peaked due to retardation in dynamics of the denitrification process by predominant dynamics of the nitrification process in media.

3.5. *Nonlinear regression analysis*

The nonlinear regression analysis was carried out using Oakdake-Datafit software [16] to develop the best-fit curve for the correlation between influent NH_4^+-N and NO_3^--N removal efficiency in order to predict the removal efficiency of *E. densa* at lower influent NH_4^+-N concentrations. The effect of influent NH_4^+-N was assumed to show a parabolic variation with the NO_3^--N removal efficiency of *E. densa*, while the effect of influent NO_3^--N was considered linear, based on the trends shown in the experimental results. The model curve matched the data at the low concentration of influent NH_4^+-N as shown by Equation 2 (correlation coefficient = 0.97).

$$NO_3^-\text{-N Removal \%} = \{36.5 + 19.5(NH_4^+\text{-N}) - 2.52(NH_4^+\text{-N})^2 - 7.20(NO_3^-\text{-N})\}. \quad (2)$$

The above equation explains the expected positive correlation between influent NH_4^+-N and NO_3^--N removal efficiency of *E. densa*. The hypothetical influent NH_4^+-N concentration should be less than 7.74 mgl^{-1} for a positive effect on NO_3^--N removal. However, this value is out of the NH_4^+-N concentration range (0–4 mgl^{-1}), used for the regression analysis. Therefore, the above equation confirmed that the influent NH_4^+-N concentration positively affects NO_3^--N removal. Figure 7 presents the 3-D graphical variation of the above equation.

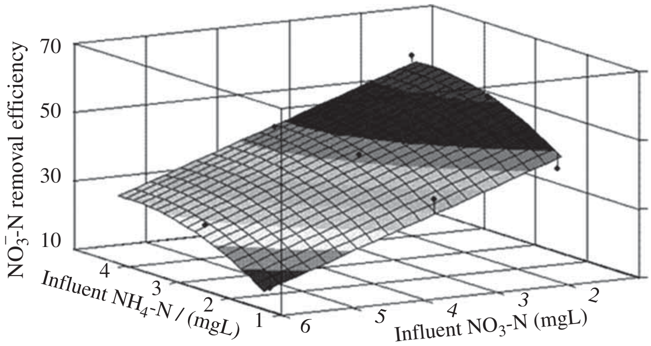

Figure 7. Graphical representation of the model curve developed using nonlinear regression analysis at non-inhibitory NH_4^+-N concentrations of *E. densa*.

3.6. E. densa *growth inhibition*

Short term evidence for plant growth inhibition could be observed through DO consumption during dark respiration (Figure 3). The maximum DO consumption increased with influent NH_4^+-N and reached a maximum at 4 mgl^{-1} in the presence of 2 mgl^{-1} of influent NO_3^--N. However, at higher concentrations of NH_4^+-N ($> 4\,mgl^{-1}$), part of the NH_4^+-N might be converted into NH_3 and inhibit metabolism in *E. densa* [4]. Similar to the pattern shown in DO consumption during dark respiration, final dry mass also recorded its maximum at the mid-level concentrations of NH_4^+-N in influent (Figure 4). These data could be used to explain the long term inhibition effect on the plant. Eventually, it can be concluded that plant inhibition due to freely available NH_3 has affected both short term and long term growth inhibition when the concentration was beyond 4 mgl^{-1}.

4. Conclusions

Influent NH_4^+-N concentration positively affects NO_3^--N removal efficiency of *E. densa* in submerged plant wetland systems when NH_4^+-N concentration is low (0–4 mgl^{-1}). The results of the non-linear regression analysis produced a model equation that expresses the positive correlation, which can be used to quantify the effect of influent NH_4^+-N concentration on NO_3^--N removal. However, the above relationship was not convincing when the influent NH_4^+-N concentration was higher than 4 mgl^{-1}. The lower marginal NH_4^+-N inhibition concentration for *E. densa* could be concluded to be 4 mgl^{-1}. The results suggest that NO_3^--N removal efficiency of *E. densa* can be improved by increasing NH_4^+-N concentrations in the tertiary treatment stage in submerged plant wetland systems.

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