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Original Research

The Fate of Ammonium in Integrated Vertical-flow Constructed Wetlands Using Stable Isotope Technique

Yun Hu[1, 2], Feng He [1]*, Long Wang[1, 2], Jian Sun[1, 2], Tao Huang[1, 2], Zhenbin Wu[1]

[1]State Key Laboratory of Freshwater Ecology and Biotechnology, Institute of Hydrobiology, Chinese Academy of Sciences, Wuhan 430072, China
[2]University of Chinese Academy of Sciences, Beijing 100049, China

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Abstract

The fate of ammonium (NH_4^+) was investigated using the ^{15}N tracer stable isotope technique in integrated vertical-flow constructed wetlands (IVCWs). Two types of IVCW systems were built: unplanted IVCWs (control) and planted IVCWs. The results showed that a high NH_4^+ removal efficiency (95.4%) in the planted IVCWs. Plants not only removed NH_4^+ by direct uptake (13.6±0.7%) but also improved the NH_4^+ removal capacity of IVCWs, compared with the control. Besides plant uptake, microbial conversion was the dominant mechanism of NH_4^+ removal in IVCWs, and a large proportion (75.2-85.6%) of added NH_4^+ may be permanently removed via anammox and nitrification-denitrification processes in IVCWs.

Keywords: ammonium, integrated vertical-flow constructed wetland, ^{15}N tracer, plant uptake, stable isotope technique

Introduction

Ammonium (NH_4^+) is one of the major nitrogen (N) contaminants in natural waters. It can cause serious environmental problems such as eutrophication and toxicity to aquatic organisms [1]. Owing to their low cost, easy operation, and high purification efficiency, constructed wetlands (CWs) have been given much attention for wastewater treatment [2]. However, not all CW types can achieve high N removal efficiency. Vertical-flow constructed wetlands (VFCWs) are beneficial for nitrification, while horizontal subsurface-flow constructed wetlands (HSSF-CWs) can only provide suitable conditions for denitrification [3]. In order to achieve higher N treatment efficacy, a hybrid system integrated vertical-flow constructed wetland (IVCW), consisting of a down-flow wetland unit and an up-flow one in series, was developed. Previous studies paid much attention to N removal efficiency in IVCWs [4-6], but information on quantitatively investigating N transformation forms and removal pathways has only rarely been reported.

The main N removal pathways in CWs are plant uptake, microbial conversion, volatilization, adsorption, and sedimentation [7]. When wastewater flows into the CWs, NH_4^+ can be absorbed by plants and substrate, oxidized to nitrite (NO_2^-), and further oxidized to nitrate (NO_3^-) by nitrifying bacteria. Subsequently, the generated NO_3^- can

*e-mail: hefeng@ihb.ac.cn

be reduced to NO_2^- and converted into gas by denitrifying bacteria. Meanwhile, there is also a non-traditional microbial N removal pathway known as anammox, in which NH_4^+ is directly oxidized to N_2 under anaerobic conditions [8]. However, the estimation of these N removal pathways in IVCWs was difficult because the aerobic-anaerobic zone was complex and a variety of removal mechanisms were combined. A number of methods have been used to investigate N removal pathways, such as physicochemical measurements [9] and mass balance method [10]. Nevertheless, these methods had limitations and cannot be monitored by quantitative N transformation forms. In order to get accurate measurements of the proportions of N removal pathways in IVCWs, ^{15}N tracer technique combined with mass balance method was used in this study.

The fate of NH_4^+ and its removal pathways in IVCWs were conducted using the stable isotope technique to reveal the black box of N forms interconversion. The study also provided information on the relative proportions of plant uptake, microbial conversion, substrate adsorption, and sedimentation.

Materials and Methods

Design and Operation of the IVCWs

Six IVCW systems, each with a down-flow Polyvinylchlorid (PVC) column (diameter = 16 cm, height = 100 cm) and an up-flow column (diameter = 16 cm, height = 100 cm) with a working volume of 12 L were built near Donghu Lake in Wuhan, China. Gravel (φ = 5-8 mm, porosity = 0.4) was filled to a depth of 15 cm, followed by an upper layer of 45 cm with washed river sand (φ = 0-4 mm, porosity = 0.5) for the down-flow and up-flow columns, respectively. Among the six systems, three unplanted ones were set as control group, and the down-flow and up-flow columns of the other three were planted with equal size *Canna indica* L. (*C. indica*) (density = 50 plants/m^2). The schematic representation of the IVCWs is shown in Fig. 1.

The IVCWs were fed with sewage wastewater for one month until the plant roots and microorganisms were well established. Then they were fed with simulated wastewater for two months. The influent simulated wastewater was prepared by tap water with dissolved ammonium chloride (NH_4Cl), and the average concentration of total nitrogen (TN) was 15.4 mg/L, NH_4^+ 15.0 mg/L, NO_3^- 0.4 mg/L, and NO_2^- not detected (Unpublished). Systems were operated in batch mode with a hydraulic retention time (HRT) of 6 d for each batch. Inflow water was added to the top of the down-flow column, and trickled through the up-flow column. The dissolved oxygen (DO) concentrations in unplanted and planted IVCWs along the direction of water flow were 0.16-1.47 and 0.08-1.35 mg/L, respectively. The experiments lasted from May to September, and the mean (±standard deviation; *s.d.*), minimum, and maximum air temperatures were 29.5±3.8, 18, and 38ºC, respectively.

$^{15}NH_4^+$ Trace Experiment

In order to trace the fate of NH_4^+, ^{15}N-labelled NH_4^+ wastewater was used as influent for one batch, which was synthesized with $^{15}NH_4Cl$ (^{15}N atom% = 98, Sigma-Aldrich) in tap water, and the characteristics of the influent were presented in Table 1. Samples of plant and substrate were taken to provide background values before applying ^{15}N-labelled NH_4^+ wastewater. The $^{15}NH_4^+$ trace experiment lasted 6 d.

Sample Collection and Analysis

Water Samples

Water samples (200 mL) were collected from the outlet of the IVCWs every day during trace experiment for analysis of TN, NH_4^+, NO_3^-, and NO_2^-. Each parameter was analyzed according to standard methods [11]. pH value was measured *in situ* with an Orion 5-Star portable conductivity multimeter (Thermo Fisher Scientific Company, USA). Chemical oxygen demand (COD) was measured using a spectrophotometer (DRB 200, Hach, USA).

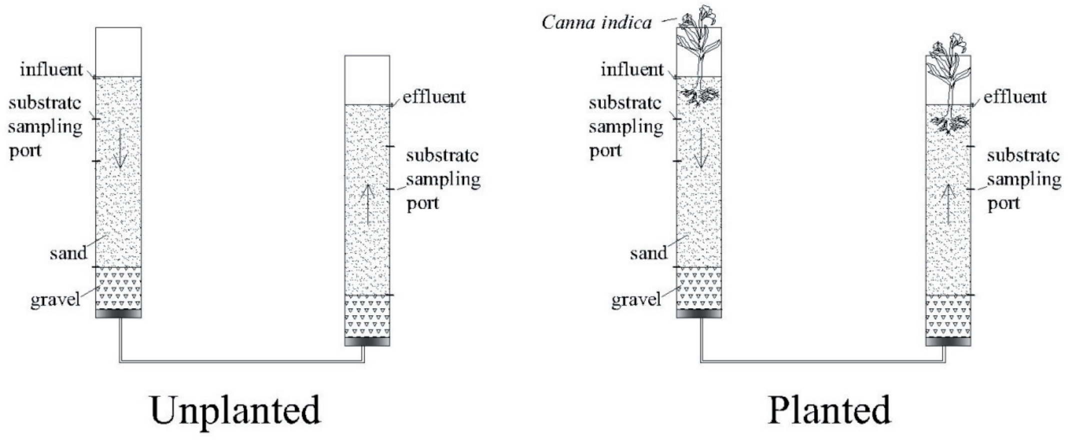

Fig. 1. Integrated vertical-flow constructed wetlands used in this study.

Table 1. Characteristics of the influent of $^{15}NH_4^+$ trace experiment.

pH	COD	NH_4^+–N	NO_3^-–N	NO_2^-–N	TN
	mg·L^{-1}	mg·L^{-1}	mg·L^{-1}	mg·L^{-1}	mg·L^{-1}
7.4±0.1	15.0±1	14.4±0.1	0.4±0.0	n/d	14.8±0.1

Notes: a) Values are means (± *s.d.*, *n* = 3); b) n/d, not detected.

The diffusing method was used to measure the ^{15}N content of NH_4^+ and NO_x^- (NO_3^- and NO_2^-) in water samples [12, 13]. NH_4^+ and NO_x^- were diffused in turn onto acidified GF/D glass fiber filter paper (Whatman) impregnated with 25 µL of 2 M H_2SO_4 and enclosed within PTFE (Millipore LCWP02500) tape, which floated on the water sample. A known volume (100 mL) of sample was put in a 250 mL polyethylene bottle with the addition of 0.2 g MgO. Diffusion bottles were closed immediately and shaken for 6 d at 40ºC on a horizontal shaker (100 rpm). The PTFE tape was removed from the solution and diffusion bottles were left open for 24 h between the NH_4^+ and NO_x^- diffusion period to remove the residual NH_4^+. Then a new tape, 0.2 g MgO, and 0.4 g Devarda's alloy were added into the bottle and shaken for another 6 d. After removal from the solution, the PTFE tapes were opened and the filters were dried. The filters were then packed in tin capsules and analyzed by an elemental analyzer (Carlo Erba NC 2500)-isotope ratio mass spectrometer (EA-IRMS) (Finnigan MAT Delta-plus, Germany).

Plant Samples

The aboveground and belowground parts of *C. indica* were collected from the down-flow and up-flow columns of the IVCWs in the initial and at the end of the trace experiment. After collection, the samples were cleaned with distilled water, weighed wet, dried to constant weight at 50°C, reweighed, and milled into a fine powder. Finally, the ^{15}N content of aboveground and belowground parts of plants were determined on EA-IRMS.

Substrate Samples

To evaluate the changes in ^{15}N content of the substrate, substrate samples were taken from the top (10 cm), middle (20 cm), and bottom (45 cm) sections of the down-flow and up-flow columns in the initial and at the end of the trace experiment, respectively. The samples were dried, ground, and weighed for analysis via EA-IRMS.

Mass Balance Calculations

Emission of ammonia is negligible due to the neutral pH in the influent [14]. The fate of $^{15}NH_4^+$ in IVCWs could be attributed to four parts: plant uptake, substrate adsorption and sedimentation, microbial conversion, and untransformed. A ^{15}N mass balance approach was used to calculate the mass and proportion of added $^{15}NH_4^+$ that were 1) plant uptake, 2) substrate adsorption and sedimentation, 3) untransformed $^{15}NH_4^+$, 4) $^{15}NO_x^-$, and 5) unaccounted $^{15}NH_4^+$. The ^{15}N mass was quantified by the following equation:

$$^{15}N_{\text{mass}} = N_{\text{plant uptake}} + N_{\text{substrate adsorption and sedimentation}} + N_{\text{untransformed}} + N_{\text{microbial conversion}} \quad (1)$$

$$N_{\text{microbial conversion}} = N^{15}_{\text{NOx}-} + N_{\text{unaccounted}} \quad (2)$$

Data Analysis

The amount of ^{15}N in plants, substrate, and water was calculated by subtracting the amount of background ^{15}N. Independent samples t-tests were used to compare significant difference (p < 0.01) of the N removal efficiencies and the proportion of added $^{15}NH_4^+$ in unplanted and planted IVCWs. The statistical analyses were performed by SPSS version 18.0 (IBM, USA).

Results

N Removal Performance of the IVCWs

The N removal performance of the unplanted and planted IVCWs was investigated. As shown in Fig. 2, during the trace experiment TN concentrations decreased from 14.8±0.1 to 0.7±0.1 mg/L, and NH_4^+ concentrations declined from 14.4±0.1 to 0.7±0.1 mg/L in the planted IVCWs. The decline was significant for the first day and relatively constant over the remaining five days. The unplanted IVCWs exhibited a similar variation trend. Additionally, the planted IVCWs were maintained at a very low level of NO_3^- and NO_2^- concentrations compared with the unplanted ones. At the end of the experiment TN and NH_4^+ removal efficiencies can achieve 94.5±0.7% and 95.4±1.0% in the planted IVCWs, which were significantly higher (p < 0.01) than that in the unplanted ones. These results indicated the presence of plant-improved TN and NH_4^+ removal efficiencies in the IVCWs.

Fate of ^{15}N–NH_4^+

To reveal the fate of ^{15}N-NH_4^+ in IVCWs, the same amount of $^{15}NH_4^+$-N (173.1±0.1 mg) was added into the unplanted and planted IVCWs. The mass and proportion of added $^{15}NH_4^+$ were presented in Table 2. 13.6±0.7% of the added ^{15}N was found in plants as a result of plant

Table 2. $^{15}NH_4^+$ mass balance summary[a].

^{15}N balance component	Mass of ^{15}N (mg)		Proportion of added $^{15}NH_4^+$ (%)	
	W1	W2	W1	W2
1. Amount $^{15}NH_4^+$ added	173.1±0.1	173.1±0.1	100±0.1	100±0.1
2. Plant uptake	n/a[b]	23.7±1.3	n/a[b]	13.6±0.7
3. Substrate adsorption and sedimentation	n/d[b]	n/d[b]	n/d[b]	n/d[b]
4. Untransformed $^{15}NH_4^+$	39.9±3.1	7.0±1.5	23.0±1.8	4.1±0.9
5. $^{15}NO_x^-$	3.1±1.0	0.2±0.1	1.8±0.6	0.1±0.0
6. Unaccounted $^{15}NH_4^+$ [1. – (2. + 3. + 4. + 5.)]	130.1±4.0	142.2±2.7	75.2±2.3	82.2±1.6

Notes: a) Values are means (± *s.d.*, $n = 3$); b) n/d, not detected.

uptake. ^{15}N in substrate adsorption and sedimentation were not detected in both unplanted and planted systems. 23.0±1.8% of the added $^{15}NH_4^+$ was untransformed and remained in the unplanted systems at the end of the experiment. Compared with the unplanted systems, the planted IVCWs contained less untransformed $^{15}NH_4^+$. Additionally, the planted systems contained significantly lower $^{15}NO_x^-$ than the unplanted one ($p < 0.01$). Specially, the proportion of transformed and unaccounted-for $^{15}NH_4^+$ was 82.2±1.6% in the planted IVCWs, which was significantly higher ($p < 0.01$) than that in the unplanted ones. The result indicated that the microbial conversion in IVCWs was significantly affected by the existence of plants.

Discussion

$^{15}NH_4^+$ trace experiment results indicate that plant uptake accounted for only a modest proportion of N removal in IVCWs. This is in agreement with previous investigations. Matheson reported that plants assimilated 11-15% NH_4^+ in wetland microcosms [15]. Maltais-Landrya found that the contribution of plant uptake to N removal was less than 20% in CW mesocosms [9]. Besides uptake by plants, the planted IVCWs permanently removed more $^{15}NH_4^+$ than the unplanted ones. This phenomenon was similar to previous studies. Coban demonstrated that plants can enhance NH_4^+ removal efficiency in CWs [16]. Vacca illustrated that plants strongly influenced the microbial community in CWs [17]. Additionally, Gagnon

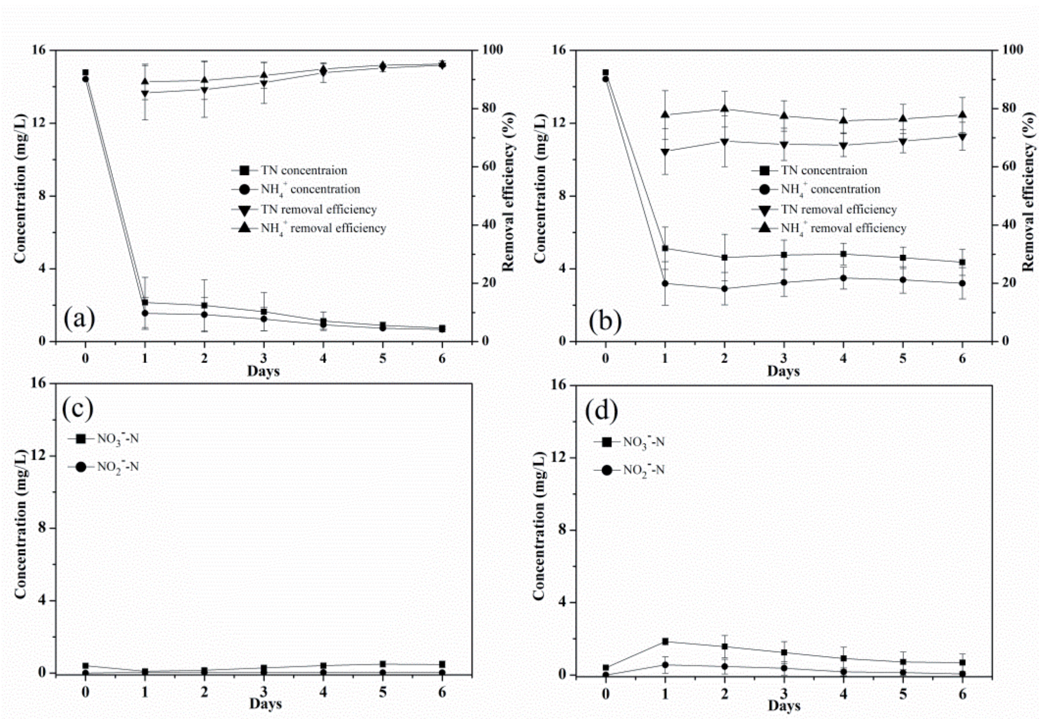

Fig. 2. N removal performance of the IVCWs. (a) and (c) Planted IVCWs; (b) and (d) Unplanted IVCWs. Values are means (Error bars indicate standard deviation, n = 3).

revealed that the root oxygen release of plants influenced microbial diversity and activity [18]. It was supposed that plants could affect microbial conversion to improve N removal efficiency in IVCWs.

The substrate adsorption and sedimentation were not detected because N content in substrate is below the detection limits. Wen et al. demonstrated that the adsorption capacity of gravel and sand was very low, which can be negligible [19]. Additionally, Organic N particulate in the influent is difficult to be decomposed and could be removed through sedimentation in CWs [20]. In our study, the influent contained no organic N particulate. Given the above-considered points, the undetected substrate adsorption and sedimentation could be ignored.

A large proportion of the added $^{15}NH_4^+$ was unaccounted for at the end of the experiment, assuming that the unaccounted fraction was transformed to undetected gas. The ratio was relatively higher than the comparable study [15, 21]. It was shown by Wu et al. that the mean NH_4^+ removal efficiency was 16.5% in VFCWs [22]. Yalcuk and Ugurlu found that NH_4^+ removal efficiencies in VFCWs were 48.9-62.3% [23]. Matheson and Sukias indicated that 36% of the added NO_3^- was unaccounted for and transformed via denitrification to gas [24]. In the present study, 75.2-85.6% of added $^{15}NH_4^+$ was unaccounted for. Thus it can be seen that there are other N removal pathways existing in IVCWs besides the conventional denitrification pathway. Possible reasons for the significant N removal in IVCWs probably rely on the combined effects of the influent and operational conditions. Influent N is mostly NH_4^+ and the C/N ratio was only 1, DO in the IVCWs ranged from 0.08 to 1.47 mg/L, and the average air temperature was 29.5ºC. The suitable growth conditions for anammox were low DO concentration (<2 mg/L), low NO_3^- concentration (<5 mg/L), NH_4^+-rich wastewater, appropriate temperature range (20-43ºC) and pH 6.7-8.3 [25-27]. The phenomenon of high TN removal efficiency in CWs under low C/N ratio and anaerobic conditions may be attributed to anammox [28, 29]. Therefore, the majority of transformed $^{15}NH_4^+$ may be permanently removed through anammox and nitrification-denitrification processes to N_2, NO, and N_2O, and N gas emissions are usually dominated by N_2 [30]. Future studies are likely to detect anammox bacteria in IVCWs.

Conclusion

In this study, ^{15}N tracer technique combined with mass balance method was used to reveal N removal pathways. Integrated analysis confirmed that plant uptake and microbial conversion were the main mechanisms of NH_4^+ removal in IVCWs. The ^{15}N mass balance approach showed that plants not only remove N by direct uptake (13.6±0.7%), but also improve N removal efficiency in IVCW systems compared with unplanted systems. Moreover, a large proportion (75.2-85.6%) of added NH_4^+ may be permanently removed through anammox and denitrification-nitrifiction processes to undetected gas. In future studies, we recommended further exploring the presence of anammox bacteria and investigating the relative N removal pathways in IVCWs.

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