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RESEARCH ARTICLE

Temporal variation of nitrogen balance within constructed wetlands treating slightly polluted water using a stable nitrogen isotope experiment

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Abstract Slightly polluted water has become one of the main sources of nitrogen contaminants in recent years, for which constructed wetlands (CW) is a typical and efficient treatment. However, the knowledge about contribution of individual nitrogen removal pathways and nitrogen balance in constructed wetlands is still limited. In this study, a stable-isotope-addition experiment was performed in laboratory-scale constructed wetlands treating slightly polluted water to determine quantitative contribution of different pathways and temporal variation of nitrogen balance using $Na^{15}NO_3$ as tracer. Microbial conversion and substrate retention were found to be the dominant pathways in nitrogen removal contributing 24.4–79.9 and 8.9–70.7 %, respectively, while plant contributed only 4.6–11.1 % through direct assimilation but promoted the efficiency of other pathways. In addition, microbial conversion became the major way to remove N whereas nitrogen retained in substrate at first was gradually released to be utilized by microbes and plants over time. The findings indicated that N_2 emission representing microbial conversion was not only the major but also permanent nitrogen removal process, thus keeping a high efficiency of microbial conversion is important for stable and efficient nitrogen removal in constructed wetlands.

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Introduction

Large amounts of slightly polluted water such as agricultural, fishing, and sewage treatment plants drainage discharged directly into aquatic ecosystems has led to excessive input of nitrogen over recent years (Wu et al. 2013), which is difficult and tedious to control (Reinhardt et al. 2006). Excess nitrogen discharge has been identified as a primary cause of water environment deterioration and ecosystem degeneration problems (Moffat 1998), among which eutrophication is one of the most serious threats to aquatic life and human health which induces noxious blooms and seasonal hypoxia (Smith et al. 1999; Smith 2003).

Varieties of treatment technologies have been used in many countries worldwide to reduce nitrogen inputs, such as aerated biological filters (Meda and Cornel 2010), riparian buffer strips (Huang et al. 2013), ecological ditches (Patterson and Cooper 2007), and wetlands. As a low-cost alternative to conventional sewage treatment, constructed wetlands have been paid more and more attention to, especially in the practice of slightly polluted water treatment in urban and rural areas (Wu et al. 2013; Yang et al. 2014).

In constructed wetland system, nitrogen brought by wastewater is transformed and removed through complicated conversion pathways, including plant and microbial assimilation, nitrification and denitrification, decomposition, volatilization, sedimentation, and adsorption, etc. (Kadlec et al. 2005), three of which are the dominant nitrogen removal processes: plant uptake, sorption

by substrate, and microbial transformation to N_2 and N_2O (Liu et al. 2014). Due to this complexity, most previous studies mainly focused on the total nitrogen removal performance of constructed wetlands (Gu and Dreschel 2008; Saeed and Sun 2013), whereas knowledge is still limited about the quantitative contribution of each process to nitrogen removal. In addition, these nitrogen removal processes are not synchronous—sorption and microbial transformation are rapid while plant assimilation is slower. Thus, we hypothesize that contribution of each nitrogen removal process (plant uptake, microbial transformation, and sorption by substrate) in constructed wetlands changes over time, which means the main factor influencing nitrogen removal efficiency differs at different times.

Stable N isotope labeling methods has become a prime tool of N cycling study within aquatic ecosystems (Faulwetter et al. 2009; Li et al. 2010; Payne et al. 2014) from laboratory microcosm (Matheson et al. 2002; Tanner et al. 2005) to the pilot scale (Matheson and Sukias 2010). A stable N isotope addition experiment uses ^{15}N tracer and mass spectrometry to obtain information about the cycling and fate of nitrogen in the studied system, which can also be applied into quantitative determination of contribution of each nitrogen removal process in constructed wetlands.

Therefore, to confirm the hypothesis, $^{15}NO_3^-$ tracer was added once into the laboratory-scale constructed wetland systems before ^{15}N enrichments in plant, substrate, and emitted gas were measured at prescribed time intervals. Study findings provide important information on the different contributions of nitrogen removal pathways in wetland ecosystem.

Materials and methods

Experimental design and operation

Before the stable-isotope-addition experiment, laboratory-scale constructed wetlands designed in 60× 40×60 cm polymethyl methacrylate cuboid had been running in sequential batch mode with a hydraulic retention time (HRT) of 7 days beginning from September 2013 and running stably from the sixth month to August 2014. Experiments were conducted in triplicate with three treatment units. The lower layer in each experimental unit was filled with washed zeolite (particle size <5–7 mm, mainly SiO_2 69.58 %, Al_2O_3 12.2 %, CaO 2.59 %, and others) and the upper one thoroughly mixed soil. Native macrophytes in similar size, *Iris sibirica* and *Elodea nuttallii*, were planted in constructed wetlands as emergent and submerged plant in density of 85 and 480 plants m^{-2}, respectively. Each wetland unit contained 75 L slightly polluted water and the water depth was controlled 15 cm above the substrate surface. The simulated slightly polluted water used as inlet water was composed of $C_6H_{12}O_6$, $(NH_4)_2SO_4$, KH_2PO_4, KNO_3, and other macronutrients (Huett et al. 2005; Wu et al. 2013) and dissolved by tap water. The main chemical variables of inlet water were as follows: NH_4^+-N 8±0.3 mg L^{-1}, NO_3-N 7±0.2 mg L^{-1}, COD 75±3.5 mg L^{-1}, TP 1.5±0.1 mg L^{-1}. N concentration, redox potential, and pH were measured.

^{15}N tracer addition

Considering that the ^{15}N abundance cannot be accurately measured if it is higher than 10 %, the inlet water was isotopically enriched by adding $Na^{15}NO_3$ ^{15}N, Sigma-Aldrich) at a concentration of 593 μg ^{15}N L^{-1}. At the first day of stable-isotope-addition experiment, the isotopically enriched water of 75 L was fed once from inlet into each wetland system and then the system was kept still standing.

Sampling and pretreatment

Water, plant, sediment, and gas samples were taken at days 0, 1, 2, 3, 6, 9, 12, and 19 after ^{15}N addition. Water samples were collected at three sample points distributed in the top, middle, and bottom layer of wetlands, respectively, using 250-mL HDPE bottles prewashed with acid. Samples of *I. sibirica* and *E. nuttallii* were collected manually with scissors. At the final day of the experiment, a destructive sampling was implemented to calculate the total biomass of plants while *I. sibirica* was separated into roots and overground part. Sediment samples were taken at three sample points sectioned at 5-cm intervals (0–5, 5–10, and 10–15 cm) in each wetland unit. All water, plant, and sediment samples were stored at 5 °C immediately after collection and sent to the laboratory for treatment. Water samples were pretreated by distillation and acidification into powder according to the previous studies (Feast and Dennis 1996; Zhang et al. 2012). Plant and sediment samples were washed with deionized water, dried at 65 °C for 48 h, weighed, and finally ground into fine powder using a ball grinder. The sample powder was stored securely in centrifuge vials prior to stable isotope analysis.

Gas samples were collected using three closed PMMA chambers inserted into the substrate of each wetland, in which the vegetation was included. Gases emitted from the wetland units were trapped by the chamber and then transferred to glass vials previously evacuated via double-sided needles. The three gas sample replicates were collected at 0

and 2 h after chambers were sealed at the same time of the day between 8:00 a.m. and 10:00 a.m., and then sent for measurement of N_2O flux, N_2, and N_2O isotopic compositions separately.

Gas measurement and ^{15}N determination

The concentration of N_2O was determined by gas chromatography (Agilent 4890) equipped with flame ionization detector (FID) and electron capture detector (ECD). Gas flux was calculated using the following formula:

$$J = \frac{dc}{dt} \bullet \frac{M}{V0} \bullet \frac{P}{P0} \bullet \frac{T0}{T} \bullet H$$

where $\frac{dc}{dt}$ is the slope of the gas concentration curve variation along with time; M is the mole mass of each gas; P is the atmospheric pressure in the sampling site; T is the absolute temperature during sampling; V_0, T_0, and P_0 are, respectively, gas mole volume, air absolute temperate, and atmospheric pressure under standard conditions; and H is the height of chamber above the water surface.

The total amount of N_2 was calculated based on the chamber volume, density of N_2, plus N_2 dissolved in water which can be determined using the following equation (Zhang et al. 2009):

$$m = \left[\left(1.15 \times V_g\right) + \left(1.15 \times V_1 \times a\right)\right] \times 10^6$$

where m is the total amount of N_2 (mg), 1.15 is the density of N_2 (kg m^{-3}) at 25 °C and standard pressure, V_g (m^3) is the chamber volume, V_1 (m^3) is the water volume, and a is the Bunsen correction coefficient (0.0143 at 25 °C). The air pressure change in the chamber was small during the 2-h treatment; therefore, the N_2 production was ignored in this process.

Stable nitrogen isotope analysis of all samples was performed at Stable Isotope Laboratory in Nanjing Normal University (Nanjing, China). The N_2O and N_2 isotopic abundance were determined by direct measurement of $^{44}[N_2O]/^{45}[N_2O]$ for N_2O and $^{28}N_2/^{29}N_2$ and $^{28}N_2/^{30}N_2$ mass ratios for N_2 using a Finnigan MAT 253 mass spectrometer (Zhang et al. 2009). The water, plant, and sediment isotopic abundance were determined using a Europa Integra continuous flow isotope ratio mass spectrometer (Europa Scientific, Seron, Cheshire, UK) coupled with an in-line elemental analyzer.

The nitrogen isotope ratio was expressed as the conventional delta (δ) notation, defined as the per mill (‰) deviation from the following isotope standard:

$$\delta^{15}N\left(\frac{0}{00}\right) = \left(\frac{^{15}N/^{14}N_{\text{sample}}}{^{15}N/^{14}N_{\text{standard}}} - 1\right) \times 1000$$

All results are presented with respect to the international standard of atmospheric nitrogen (AIR, N_2).

Statistical analysis

Statistical analysis was performed using SPSS version 18.0 software. The timely differences in contributions of each nitrate removal pathway in wetland units were analyzed by one-way ANOVA test. The significant differences were accepted at the 0.05 level.

Results and discussion

^{15}N enrichment in plants

Figure 1 showed $\delta^{15}N$ values of *I. sibirica* (emergent plant) and *E. nuttallii* (submerged plant) during the 19-day experiment. Both increased steadily after ^{15}N addition, while ^{15}N content in *E. nuttallii* is ten times as in *I. sibirica*. The destructive sampling at the last day of the experiment found that ^{15}N enrichment in underground part of *I. sibirica* is twice as in its overground part, but still much lower than in *E. nuttallii*.

Both *E. nuttallii* and *I. sibirica* displayed a significant ^{15}N enrichment throughout the whole experiment, likely due to the depletion of ^{15}N from the wastewater (each $P<0.05$), which indicated the occurrence of nitrogen assimilation in the two plant species. The maturity of plants and growth of roots with time promoted the efficiency of plant uptake (Bastviken et al. 2009).

The results suggested that *E. nuttallii* had a higher nitrogen assimilation efficiency than *I. sibirica*, which may result from the fact that nitrogen can be assimilated by all types of submerged plant biomass while emergent plant is limited with root assimilation. These findings were consistent with previous studies using ^{15}N-addition experiment (Gu and Dreschel 2008). In addition, the results indicated that ^{15}N assimilated by emergent plants is more likely to be stored in roots (Li et al. 2010); therefore, harvest of the overground plant part cannot

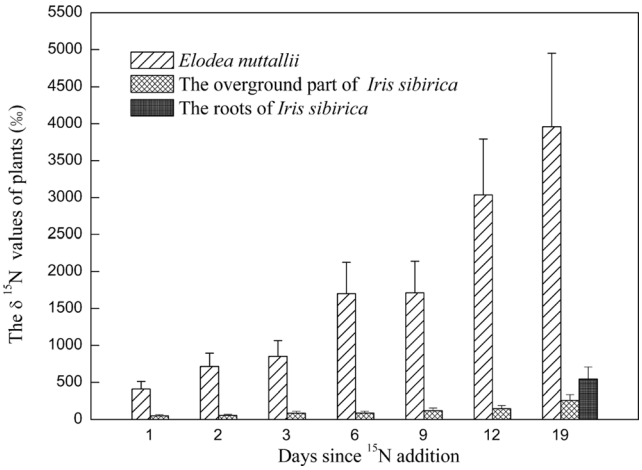

Fig. 1 Time series of $\delta^{15}N$ values of *Elodea nuttallii* and *Iris sibirica* during the 19-day experimental period

be considered an efficient way to remove nitrogen from wastewater treated by constructed wetlands. However, roots cannot be harvested unless destructing the whole system; thus, selection of different plants for their optimized combinatorial effect maybe a better alternative.

^{15}N enrichment in sediment

$\delta^{15}N$ value of sediment at depth of 0–15 cm throughout the whole experiment was displayed in Fig. 2. ^{15}N enrichment in sediment was detected since the first sampling at day 1 and increased slowly until day 12 when a sharp rise turned out on the growth curve. Compared to day 1, there was a 1.8- and 3.7-fold increase in ^{15}N content of sediment at days 12 and 19, respectively.

The sudden increase during the last week of the experiment indicated that ^{15}N content in sediment may reach a higher level in longer term. Actually, there are complex mechanisms influencing the ^{15}N storage in substrate. Zhou et al. (2011) reported that nearly 2.0 to 5.7 % of total ^{15}N content in soil was contributed by soil microbial biomass. Thus, microbial and periphyton assimilation also contribute to nitrogen removal in substrate besides sorption and interception by wetland packings, which suggested that the sudden increase in growth curve may due to the enhancement of nitrogen assimilation by microorganisms in soil. In addition, Zhou et al. (2011) reporting that low infiltration rate was more beneficial to nitrogen retention in soil than high infiltration rate and Wozniak et al. (2008) finding no ^{15}N enrichment in the marl soil of an oligotrophic freshwater marsh indicated that water infiltration rate and substrate type are also significant influence factors of ^{15}N enrichment in wetland substrate. In this study, the mode of still standing after once feeding and utilization of thoroughly mixed soil guaranteed the satisfaction of those two conditions, otherwise ^{15}N content in sediment maybe much lower.

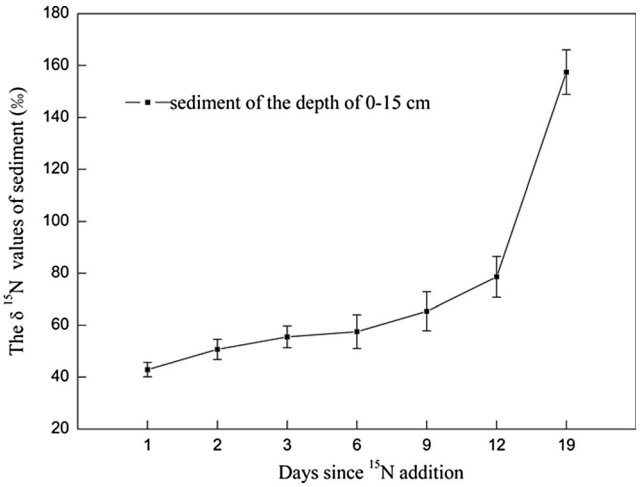

Fig. 2 Time series of $\delta^{15}N$ values of the depth interval of 0–15 cm sediment during the 19-day experimental period

^{15}N enrichment in gases

Figures 3 and 4 demonstrated the variation of ^{15}N fluxes emitted in the form of N_2 and N_2O, respectively (denoted as N_2-^{15}N and N_2O-^{15}N below for convenience). N_2-^{15}N flux showed a slow declining trend during the first 6 days of the experiment. At day 9, it suddenly dropped to half of that at day 6 and kept that level until the last day. A similar trend was found in N_2O-^{15}N flux, which presented a relatively slow decrease from 22.57 $\mu g\,^{15}N\,m^{-2}\,h^{-1}$ at day 1 to half of that at day 6, and declined suddenly to less than 2 $\mu g\,^{15}N\,m^{-2}h^{-1}$ at day 9 which was kept to the final day. However, the amount of N_2-^{15}N flux was averagely 60 times as N_2O-^{15}N flux, while the variation of the two fluxes were significantly correlated ($P<0.01$).

The results indicated that the decreased trend of N_2-^{15}N and N_2O-^{15}N flux was also significantly correlated with the decrease of nitrate concentration and COD/TN ($P<0.05$). Therefore, the sudden decrease of ^{15}N emission may be a result of a reduction of denitrification rate caused by nitrate loss in wastewater. Another influence factor may be the decrease of COD/TN, which can lead to a reduction of carbon source for microbes and thus make their reaction less active. Actually in most denitrification processes, organic compounds are used as electron donors and oxidized forms of inorganic nitrogen are used as electron acceptors (Faulwetter et al. 2009), which makes availability of carbon source and NO_3^- fundamental prerequisites for production of N_2. Additionally, as a potent greenhouse gas, N_2O has been paid much attention to, which is generated by incomplete denitrification where the final step of denitrification is unable to be performed due to lack of the nitrous oxide reductases (Nos) gene (Abell et al. 2010; Philippot et al. 2011). Nevertheless, its emission flux is quite

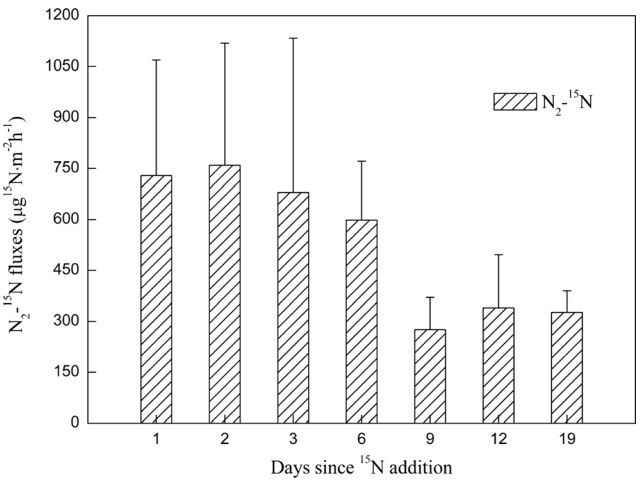

Fig. 3 N_2-^{15}N fluxes during the 19-day experimental period

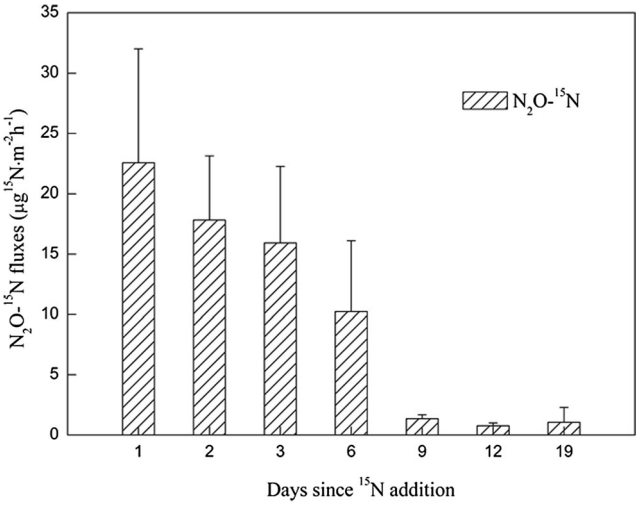

Fig. 4 N_2O-^{15}N fluxes during the 19-day experimental period

low—in this study, it was only approximately 1 $\mu g\,^{15}N\,m^{-2}h^{-1}$ during the last week of the experiment. On the whole, emission as nitrogenous gas represents the complete and permanent nitrogen removal from constructed wetlands. In order to maintain the high efficiency of this pathway, external carbon source can be added to ensure the stabilization of COD/TN ratio and thus ensure the stabilization of denitrification rate.

Quantification and ^{15}N balances variation in the wetlands

Table 1 and Fig. 5 show the fate of ^{15}N during the experiment and the temporal variation of quantitative accumulation of ^{15}N in different nitrogen pools. In this study, it was assumed that the unknown part of ^{15}N loss remained in zeolite substrate, which was difficult to determine unless the whole wetland system was destroyed. In addition, ammonia volatilization was negligible in this study due to the neutral pH and low $^{15}NH_4^+$ concentration in the water (Liu et al. 2014).

The contribution of plant uptake to nitrogen removal increased from 4.94 % at day 1 to 11.12 % at day 19, mainly due to the increase of ^{15}N enrichment in submerged plant from 2.28 to 6.75 %, while the contribution of emergent plant remained at around 2.3 % until it suddenly reached 4.37 % at the final day of the experiment. The results indicated that contribution of plant uptake to ^{15}N removal in this experiment was lower than some previous reports (Harrison et al. 2012; Kadlec et al. 2005; Wu et al. 2013). Actually, the contribution of plant uptake is significantly influenced by plant species and density, nitrogen component, competition of denitrification, nitrogen concentration, and other physical and chemical factors (Bastviken et al. 2009; Chen et al. 2014). Thus, the relatively low nitrogen removal contribution of plant in this study may be caused by many complicated factors, especially by the type of nitrogen component because nitrate is usually not the preferred source nitrogen for plants compared with ammonia (Lee et al. 2009). Additionally, although plant contributed only a small part to nitrogen removal through direct assimilation, it was not the only way plant makes influence. The existence of the plants can also promote nitrification and denitrification by increasing the availability of carbon compounds from root exudates, influencing the oxygen conditions and creating microenvironments with oxic-anoxic zones around the roots (Garcia-Lledo et al. 2011).

Emission of nitrogenous gas and retention in substrate made over 90 % of contribution to nitrogen removal in constructed wetlands, rising from 24.38 to 79.93 % and dropping from 70.66 to 8.94 %, respectively, during the experiment. Findings of previous studies also agreed that they were the dominant pathways in nitrogen removal, but there were some differences in concrete values. Chen et al. (2014) reported similar results demonstrating that denitrification and sedimentation burial, respectively, contributed 54–94 and 1–46 % to the N removal in constructed wetlands using stable nitrogen isotope analysis; Erler et al. (2010) also supported the results by finding that 40.8 % of the added ^{15}N lost in the form of N_2, with 30.8 % in sediments after 157 days of ^{15}N addition.

Transformation to nitrogenous gas represents the contribution of microbial conversion, which is the net nitrogen loss

Table 1 The accumulation of added ^{15}N in various nitrogen pools during the 19-day experimental period

	Water	N_2		N_2O		Emergent plant		Submerged plant		The upper substrate		Unknown
	mg	mg	%	mg	%	mg	%	mg	%	mg	%	%
Added	44.45											
Day 1	31.09	3.157	23.65	0.09776	0.73	0.3556	2.66	0.3041	2.28	0.01256	0.094	70.57
Day 2	24.17	6.447	31.81	0.1750	0.86	0.4691	2.31	0.5472	2.69	0.01484	0.073	62.24
Day 3	18.39	9.392	36.05	0.2441	0.94	0.5792	2.22	0.6283	2.41	0.01625	0.062	58.31
Day 6	10.32	17.17	50.29	0.3771	1.10	0.8074	2.36	1.239	3.63	0.01683	0.049	42.55
Day 9	4.497	20.75	51.94	0.3947	0.98	0.8922	2.23	1.403	3.51	0.01912	0.048	41.28
Day 12	1.604	25.16	58.73	0.4046	0.94	0.9015	2.10	2.374	5.54	0.02301	0.054	32.62
Day 19	0.0337	35.06	78.95	0.4367	0.98	1.942	4.37	2.996	6.75	0.04608	0.10	8.84

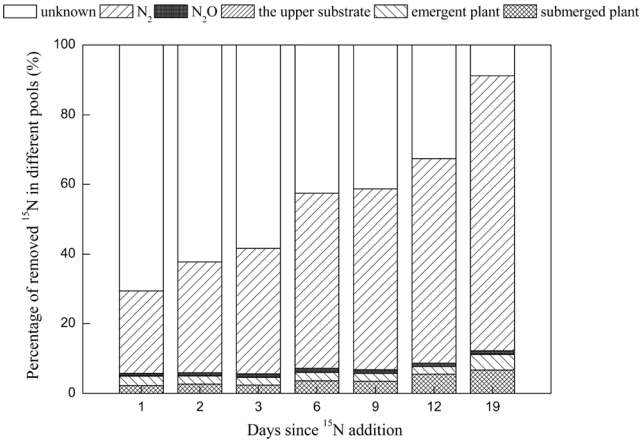

Fig. 5 Proportion of ^{15}N removed through different processes during the 19-day experimental period

from the system. Emission of N_2O contributed merely around 1 %, which was small enough to be ignored, whereas N_2 emission became the main pathway to remove N over time. In most previous studies, nitrogen denitrified to N_2 was assumed as the unknown part of ^{15}N loss and could only be calculated by subtraction method (Harrison et al. 2012; Liu et al. 2014; Matheson and Sukias 2010). However, in this study, the statistics of N_2-^{15}N flux emitted from constructed wetlands was more reliable because they were directly calculated based on $^{15}N_2$ isotope abundance and N_2 concentration which were actually measured in the experiment.

Among the substrate, zeolite played a much more significant role in nitrogen removal than the mixed soil at the upper layer which contributed less than 0.1 %. In the beginning 1–2 days of the experiment, substrate made a dominant contribution to nitrogen removal owing to the rapidity of zeolite adsorption. During the following days, plant and microbial assimilation as well as nitrification and denitrification kept consuming N in the water, resulting in the release of nitrogen retained in substrate before for the balance of N concentration. Therefore, wetland substrate was considered to be a relatively labile nitrogen sink.

Conclusions

With few studies available on contribution of individual N removal process in constructed wetlands, this study not only revealed the quantitative contribution of each nitrogen removal pathway in constructed wetlands treating slightly polluted water but also demonstrated the temporal variation of nitrogen balance making full use of stable nitrogen isotope technique. Microbial conversion and substrate retention were the dominant pathways to remove nitrogen contributing 24.4–79.9 and 8.9–70.7 %, respectively, while plant contributed only 4.6–11.1 % through direct assimilation but promoted the efficiency of other pathways. As time passed by, microbial conversion took the main role in nitrogen removal whereas N retained in substrate at first was released to be utilized by microbes and plants. Nitrogen emission is not only the major but also the permanent nitrogen removal process, thus keeping a high efficiency of microbial conversion is important for stable and efficient nitrogen removal in constructed wetlands, which can be achieved by cultivation of high-efficiency nitrogen-cycle bacteria and addition of external carbon source. This study elucidated the efficacy of stable isotope labeling technique in laboratory-constructed wetlands, illustrating the temporal variation of nitrogen balance within constructed wetlands treating slightly polluted water.

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Compliance with ethical standards We confirm that all the data are original and this manuscript has not been published elsewhere and is not under consideration for publication anywhere else. All authors have read the manuscript and accept responsibility for this manuscript. Its publication has been approved by all authors.

We understand that the corresponding author is the sole contact for the editorial process (including editorial manager and direct communications with the office). He is responsible for communicating with the other authors about progress, submissions of revisions, and final approval of proofs. We confirm that we have provided a current, correct email address which is accessible by the corresponding author and which has been configured to accept an email from zhkuili@nju.edu.cn.

Conflict of interest The authors declare that they have no competing interests.

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