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# Cyanobacterial Nitrogen Fixation Influences the Nitrogen Removal Efficiency in a Constructed Wetland

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**Abstract:** Nitrogen removal efficiency in constructed wetlands (CW) is influenced by multiple environmental factors. However, little is known about the role of cyanobacterial nitrogen fixation in affecting nitrogen removal efficiency. This study investigated how cyanobacterial nitrogen fixation affects the efficiency, at which a CW removes nitrogen from an associated artificial lake (AL) in Beijing. For this purpose, we measured cell densities of N-fixing and non-N-fixing cyanobacteria, the aquatic nitrogen fixation rate ( $R_{Nfix}$ ), and the concentration of various nitrogen fractions over the growing season (April–November) of 2014 in both AL and CW. We found that the removal of particulate organic nitrogen (PON) contributed to >90% of the total nitrogen removal in the CW. The removal efficiency of PON was lower during August–October ( $55.45 \pm 27.49\%$ ) than during April–July ( $68.86 \pm 8.83\%$ ). Phytoplankton proliferation in summer, as one of the main sources of PON, may have exceeded the capacity of the CW and led to declines in PON removal efficiency.  $R_{Nfix}$  peaked in July–October ( $3\text{--}169 \text{ ng N}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ) and was positively correlated with both PON concentration and the cell density of N-fixing *Anabaena* sp. over the growing season, suggesting that aquatic nitrogen fixation (primarily in the AL) may increase PON and thereby reduce its removal efficiency in the CW.

**Keywords:** constructed wetland; nitrogen fixation; particulate organic nitrogen; *Anabaena* sp.

## 1. Introduction

Constructed wetlands ('CW', hereafter) are effective in reducing biological and chemical oxygen consumption, suspended matter, and pollution-related bacteria in waste waters. However, the removal efficiency of nitrogen is usually low (<50%) and variable in CW [1,2]. The nitrogen removal in CW involves complex processes and is influenced by many factors [3]. Total nitrogen (TN) in aquatic systems can be divided into particulate organic nitrogen (PON) and total dissolved nitrogen (TDN). PON mainly consists of bacteria, phytoplankton, sediments, and plant litter [4], while TDN is mainly comprised of dissolved organic nitrogen (DON), ammonia ( $\text{NH}_4^+\text{-N}$ ), and nitrate and nitrite ( $\text{NO}_x\text{-N}$ ) [5]. PON is an important nitrogen component in aquatic systems, accounting for more than 50% of TN in domestic wastewater, aquaculture wastewater, stormwater, and other sewage sources [5–7]. Most constructed wetlands have been designed for removing TDN from sewage through creating conditions for mineralization, nitrification, and denitrification in different segments [8]. Few studies have addressed PON, because it was usually classified into particulate organic matter which can be removed through sedimentation or biofilm adsorption [9]. However, organic nitrogen can transform between particulate and dissolved forms in the flow from sewage sources to CWs,

and factors influencing the transformation are still not clear. It is thus necessary to investigate the PON removal in CW for improving the efficiency at which TN is removed.

Some genera of filamentous cyanobacteria are capable of fixing nitrogen by transforming  $N_2$  into bioavailable forms, thereby adding new nitrogen into aquatic systems. Cyanobacterial nitrogen fixation is a high-energy-consuming process, and occurs only when bioavailable nitrogen cannot meet the demand by phytoplankton [10]. Most previous studies on cyanobacterial nitrogen fixation have focused on natural ecosystems, some of which were oligotrophic or mesotrophic [11]. The nitrogen fixation in CWs has long been overlooked due to previous findings that cyanobacterial nitrogen fixation is not active in eutrophic waters [1], excepting their symbiosis with *Azolla* in freshwater ponds and paddy fields [12]. However, abundant *Anabaena* sp. (a genus of N-fixing cyanobacteria) has been observed in an artificial lake ('AL', hereafter) constructed for waterfowl inhabitation and an associated CW in the Beijing Wildlife Rescue and Rehabilitation Center (40°06' N, 116°43' E). Therefore, we hypothesized that cyanobacterial nitrogen fixation may play a role in affecting nitrogen removal at this site.

The objective of this study was to investigate how cyanobacterial nitrogen fixation affects the efficiency at which the CW removes nitrogen from the associated AL at the study site. For this purpose, we measured the contents of multiple nitrogen fractions (i.e., PON, DON,  $NH_4^+$ -N, and  $NO_x$ -N) and their removal efficiencies during the growing season (April–November) of 2014. We also examined the dynamics of nitrogen fixation rate ( $R_{Nfix}$ ) and cell density of both N-fixing and non-N-fixing cyanobacteria. We analyzed the relationships among N-fixing cyanobacteria,  $R_{Nfix}$ , and the removal efficiency of various nitrogen fractions. This study will demonstrate the effects of nitrogen fixation on nitrogen removal in the CW, and will help improve our understanding of the mechanisms underlying nitrogen removal in wetlands.

## 2. Materials and Methods

### 2.1. Study Site

This study was carried out in the Beijing Wildlife Rescue and Rehabilitation Center, where an AL was constructed for waterfowl inhabitation and an associated CW was constructed for purifying the wastewater from the AL. The AL covers an area of about 1 ha. The water input to the AL is from the Chaobai River basin. The AL cannot itself maintain a good water quality due to the input of food detritus and feces of the waterfowl. Thus, a CW with a total area of 2400 m<sup>2</sup> was established adjacent to the AL in order to assist in water purification (Figure 1). Water from the AL was pumped into the CW at a flow rate of 300 m<sup>3</sup>·d<sup>-1</sup>, and the average hydraulic retention time was 24 h (see the design of the constructed wetland in Cui [13]). The inflow to the AL was characterized by a total phosphorous (TP) of 0.10–1.98 mg·L<sup>-1</sup>, a TN of 1.15–19.70 mg·L<sup>-1</sup>, a TDN of 0.04–0.62 mg·L<sup>-1</sup>, a DON of 0.72–16.23 mg·L<sup>-1</sup>, and a pH of 8.04–9.36. The CW has been running well since 2008 [14].

### 2.2. Sampling

From May to November 2014, four replicated water samples were collected monthly from each of the AL and CW at 0.1 m depth below the water surface for monitoring water quality. For each sample, a volume of 250 mL of water was filtered through a  $\phi$ 25 mm pre-combusted Whatman GF/F filter (450 °C, 5 h). The filters were stored at -20 °C until determination of the accumulated PON. The filtrates were stored at 4 °C, and then used for determining the  $NH_4^+$ -N and  $NO_x$ -N within a week of sampling. Another sample of 500 mL water was brought back to the laboratory on the same day for immediate determination of  $R_{Nfix}$  using the <sup>15</sup>N<sub>2</sub> stable isotope gas tracer method described below. Water samples were also taken biweekly for phytoplankton analysis. A volume of 2 L water was stained in situ with 20 mL Lugol's reagent and then settled for 48 h, before finally being concentrated into 40 mL solutions. The cell numbers of phytoplankton were counted with an optical microscope at 200× or 400× magnifications. The phytoplankton composition was identified to genus according to

Hu [15]. The in situ water temperature was determined using YSI 6820 Multiparameter Instrument (Xylem Inc., White Plains, NY, USA), and the air temperature data was obtained from the China Meteorological Data Network [16].

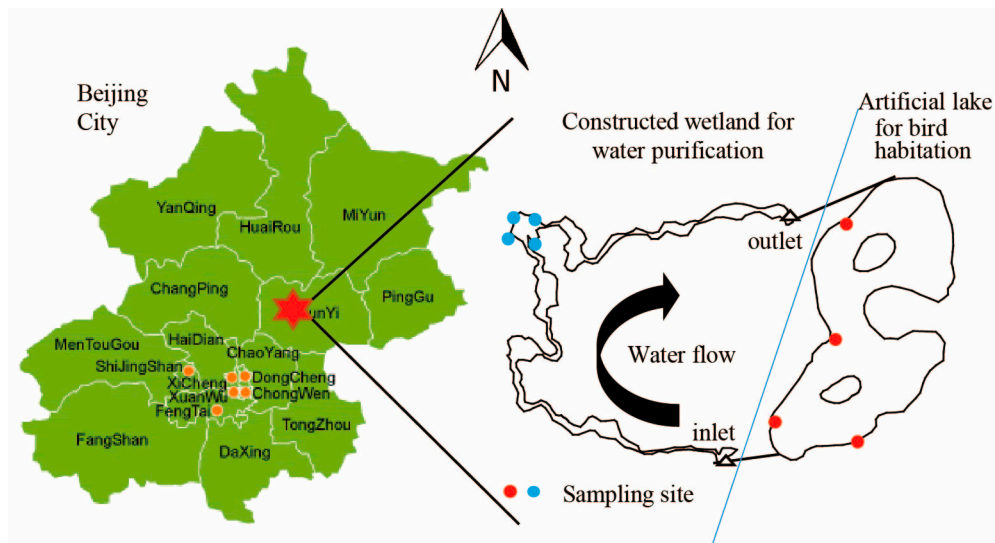


Figure 1. The location and design of the study site.

### 2.3. Concentration of Nitrogen Fractions

The aquatic PON concentration ( $\rho(\text{PON})$ ) was determined on the accumulated particulates on the filters. The refrigerant filters were dried at 50 °C for 48 h, and nitrogen content ( $W_N$ ) of the filter was determined using the Flash EA 1112 (Thermo Fisher Scientific Inc., Waltham, USA). The  $\rho(\text{PON})$  ( $\text{mg}\cdot\text{L}^{-1}$ ) was calculated as

$$\rho(\text{PON}) = W_1 \cdot \frac{W_N}{100} \cdot \frac{A_0}{A_1} \cdot \frac{1}{V_f} \cdot 1000 \quad (1)$$

where  $W_1$  is the weight (mg) of filter combusted in the Flash element analyzer,  $W_N$  the nitrogen content (%) of the combusted filter,  $A_0$  the total area of the accumulated particulates on the filters ( $\text{cm}^2$ ),  $A_1$  the tested area of filters ( $\text{cm}^2$ ) which was combusted, and  $V_f$  the water volume (mL) filtered. The coefficient 1000 transforms the unit  $\text{mg}\cdot\text{mL}^{-1}$  into  $\text{mg}\cdot\text{L}^{-1}$ .

The aquatic TDN concentration ( $\rho(\text{TDN})$ ) was determined on the filtrates using the Multi N/C 3100 Analyzer (Analytik Jena AG, Jena, Germany). The concentration of  $\text{NH}_4^+\text{-N}$  ( $\rho(\text{NH}_4^+\text{-N})$ ) and  $\text{NO}_x\text{-N}$  ( $\rho(\text{NO}_x\text{-N})$ ) were determined on the filtrates using the SmartChem® 200 Discrete Analyzer (WESTCO Scientific Instruments Inc., Brookfield, CT, USA). The aquatic DON concentration ( $\rho(\text{DON})$ ) was calculated as the difference between TDN and inorganic nitrogen (i.e.,  $\text{NH}_4^+\text{-N} + \text{NO}_x\text{-N}$ ). The removal efficiency of a nitrogen component  $r(\text{N})$  (%) was calculated as

$$r(\text{N}) = \frac{\overline{\rho(\text{N})}_{\text{AL}} - \overline{\rho(\text{N})}_{\text{CW}}}{\overline{\rho(\text{N})}_{\text{AL}}} \cdot 100\% \quad (2)$$

where  $\overline{\rho(\text{N})}_{\text{CW}}$  is the mean nitrogen content in CW and  $\overline{\rho(\text{N})}_{\text{AL}}$  the mean nitrogen content in AL.

### 2.4. Nitrogen Fixation Rate

The  $R_{\text{Nfix}}$  was determined using the  $^{15}\text{N}_2$  stable isotope gas tracer method [15]. For each sample, two 250 mL serum bottles were filled with water samples to overflow, and then were sealed with rubber caps. Afterwards, 0.5 mL of  $^{15}\text{N}_2$  gas (99.9%) was injected into one bottle, and the bottles were shaken for 1 h to make sure that  $^{15}\text{N}_2$  was well dissolved in water. The labeled bottles and control

bottles were incubated at 25 °C for 24 h with 12 h:12 h, light:dark. When the incubation was completed, water in the bottles were filtered through a  $\phi$ 25 mm pre-combusted Whatman GF/F filter (450 °C, 5 h), and the filters were stored at –20 °C for subsequent measurements. The refrigerant filters were dried at 50 °C for 48 h before  $^{15}\text{N}$  abundance was determined using Finnigan DELTAplus XP (Thermo Fisher Scientific Inc., Waltham, MA, USA). The  $R_{\text{Nfix}}$  ( $\text{ng N}\cdot\text{L}^{-1}\cdot\text{h}^{-1}$ ) was estimated according to Montoya [17]

$$R_{\text{Nfix}} = \left( \frac{1}{\Delta t} \right) \cdot \left( \frac{A_{\text{PNf}} - A_{\text{PN0}}}{A_{\text{N2}} - A_{\text{PN0}}} \right) \cdot \left( \frac{\rho(\text{PON})_0 + \rho(\text{PON})_f}{2} \right) \quad (3)$$

where  $\Delta t$  is the incubation time (h),  $A_{\text{PNf}}$  the  $^{15}\text{N}$  enrichment of particulate N at the end of incubation,  $A_{\text{PN0}}$  the  $^{15}\text{N}$  enrichment of particulate N at the start of incubation, and  $A_{\text{N2}}$  the  $^{15}\text{N}$  enrichment of the  $\text{N}_2$  available for fixation,  $\rho(\text{PON})_0$  and  $\rho(\text{PON})_f$  the initial and final of PON concentration of the solution ( $\text{ng N}\cdot\text{L}^{-1}$ ).

### 2.5. Statistical Analysis

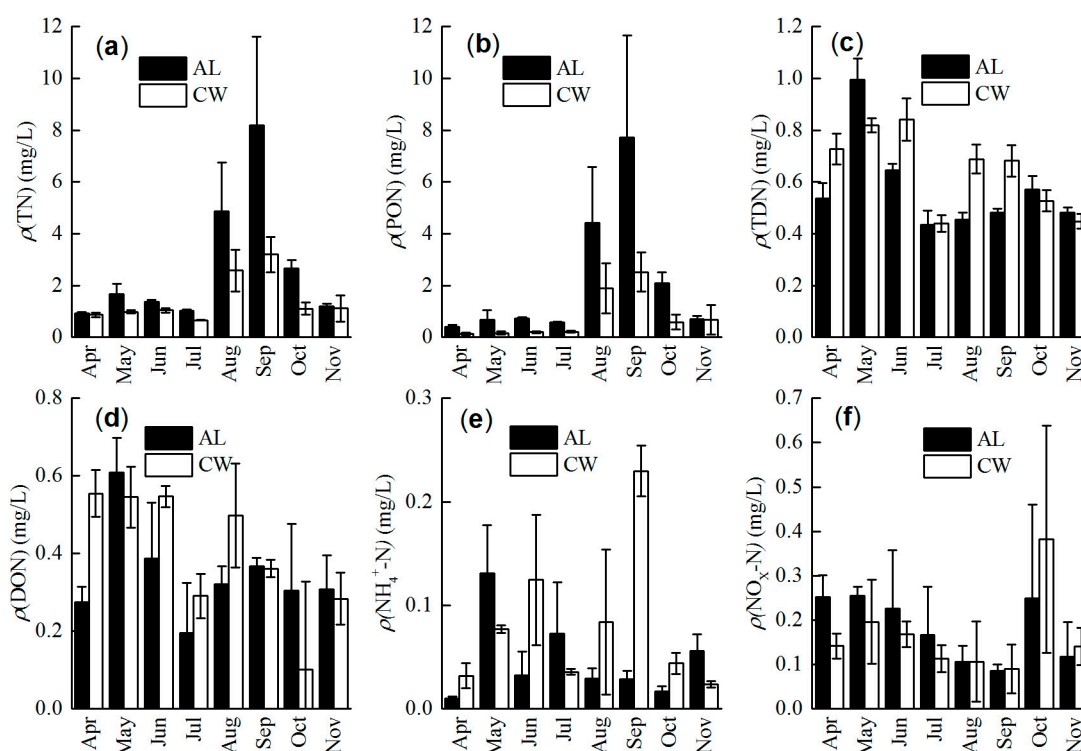
All data were presented as mean  $\pm$  standard deviation (mean  $\pm$  SD). Variables were compared between the AL and CW with paired *t*-test. The effects of months and sites (AL vs. CW) on the aquatic nitrogen fixation were tested using two-way analysis of variance (ANOVA). Pearson correlation coefficient was used to investigate the relationship between nitrogen fixation rates and water chemistry parameters. The concentration of nitrogen fractions and cell densities of phytoplankton were log transformed, and the  $R_{\text{Nfix}}$  were sine transformed to meet the requirements of these parametric tests. All data analysis was performed using R 3.2.2 [18].

## 3. Results

### 3.1. Concentration of Nitrogen Forms

The concentrations of TN ( $\rho(\text{TN})$ ) and PON in AL were significantly higher than those in the CW (TN:  $n = 32$ ,  $t = 6.19$ ,  $p < 0.001$ , Figure 2a; PON:  $n = 32$ ,  $t = 9.75$ ,  $p < 0.001$ , Figure 2b). During April–July, PON accounted for  $47.83 \pm 0.10\%$  and  $20.92 \pm 8.30\%$  of TN in the AL and CW, respectively (S1). During August–November,  $\rho(\text{PON})$  in the AL and CW increased and achieved the highest values in September (Figure 2b), accounting for  $86.93 \pm 7.71\%$  and  $65.82 \pm 15.92\%$  of TN in the AL and CW, respectively (S1). Conversely, the concentration of  $\rho(\text{TDN})$  was higher in the CW than that in the AL ( $n = 32$ ,  $t = -2.90$ ,  $p < 0.01$ , Figure 2c). The  $\rho(\text{TDN})$  was higher during April–June, and decreased during July–October in both AL and CW. The  $\rho(\text{TN})$  was positively correlated with  $\rho(\text{PON})$  ( $n = 64$ ,  $r = 0.94$ ,  $p < 0.001$ ), but was not correlated with  $\rho(\text{TDN})$  ( $n = 64$ ,  $r = -0.06$ ,  $p > 0.05$ ).

Among the fractions of TDN, the  $\rho(\text{NH}_4^+\text{-N})$  was higher in the AL than that in the CW ( $n = 32$ ,  $t = -3.00$ ,  $p < 0.01$ , Figure 2e), but there was no significant difference of  $\rho(\text{DON})$  and  $\rho(\text{NO}_x\text{-N})$  between the AL and CW (DON:  $n = 32$ ,  $t = -1.09$ ,  $p > 0.05$ , Figure 2d;  $\text{NO}_x\text{-N}$ :  $n = 32$ ,  $t = 0.56$ ,  $p > 0.05$ , Figure 2f). The  $\rho(\text{NO}_x\text{-N})$  was two to six times higher than  $\rho(\text{NH}_4^+\text{-N})$ . During April–July, DON accounted for  $28.46 \pm 11.19\%$  and  $54.47 \pm 10.74\%$  of TN in the AL and CW (S1). The proportions of DON in TN decreased during August–October, accounting for  $5.21 \pm 2.08\%$  and  $17.38 \pm 14.28\%$  of TN in the AL and CW, respectively (S1).

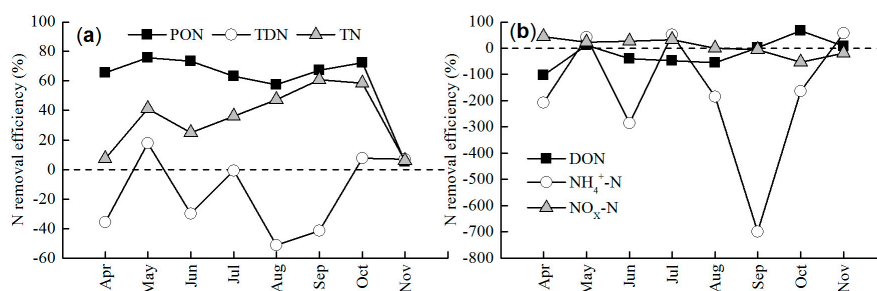


**Figure 2.** Concentration of nitrogen fractions in the artificial lake (AL) and the adjacent constructed wetland (CW). (a) Total nitrogen (TN); (b) particulate organic nitrogen (PON); (c) total dissolved nitrogen (TDN); (d) dissolved organic nitrogen (DON); (e)  $NH_4^+-N$ ; and (f)  $NO_x-N$ . All data were presented as mean  $\pm$  standard deviation (mean  $\pm$  SD).

### 3.2. Nitrogen Removal Efficiency by CW

The average removal efficiency of TN by the CW was  $35.22 \pm 21.18\%$  during the study period (Figure 3a). The removal efficiency of PON by the CW was  $60.22 \pm 97.97\%$  during the study period, being higher during April–July ( $68.86 \pm 8.83\%$ ) than during August–October ( $55.45 \pm 27.49\%$ ) (Figure 3a). The CW led to a reduction of  $\rho(PON)$  in the AL by  $0.04$ – $5.19$   $mg \cdot L^{-1}$ , accounting for more than 90% of the decrease in  $\rho(TN)$  ( $0.07$ – $4.19$   $mg \cdot L^{-1}$ ).

The removal efficiency of PON was significantly higher than that of TDN ( $n = 8$ ,  $t = 5.63$ ,  $p < 0.001$ , Figure 3a). The CW did not efficiently remove TDN, as indicated by the average change in TDN of  $-16.40 \pm 27.88\%$  from the AL to CW during the study period (Figure 3b). Among of the fractions of TDN, the removal efficiency of DON and  $NO_x-N$  fluctuated in the range of  $-102.40\%$ – $66.87\%$ , and did not significantly differ from 0 (DON:  $n = 8$ ,  $t = -1.38$ ,  $p = 0.209$ ;  $NO_x-N$ :  $n = 8$ ,  $t = 0.47$ ,  $p = 0.650$ ). Meanwhile, the removal of  $NH_4^+-N$  varied widely over a range of  $-699$ – $50\%$ , which was significantly negative ( $n = 8$ ,  $t = -1.96$ ,  $p < 0.05$ ), and dropped to the lowest value in September (Figure 3b). The water temperature and the daily maximum air temperature (S2) were negatively correlated with the PON removal efficiency, but not with the other nitrogen fractions (S4).



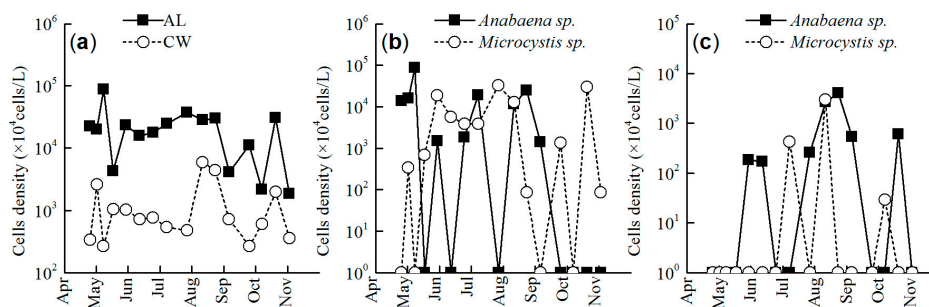
**Figure 3.** Removal efficiency of nitrogen fractions by the constructed wetland (CW). (a) Total nitrogen (TN), particulate organic nitrogen (PON), and total dissolved nitrogen (TDN); (b) dissolved organic nitrogen (DON), NH<sub>4</sub><sup>+</sup>-N, and NO<sub>x</sub>-N.

### 3.3. Cell Density of N-Fixing and Non-Nitrogen Fixing Cyanobacteria

The cell density of phytoplankton in the AL was 3–300 times higher than that in the CW (Figure 4a). Cyanophyta was the most dominant class, accounting for  $56.88 \pm 36.24\%$  and  $29.84 \pm 36.62\%$  of the total phytoplankton cells in the AL and CW, respectively. N-fixing *Anabaena* sp. and non-N-fixing *Microcystis* sp. were the dominant genera of Cyanophyta, accounting for  $52.3 \pm 46.7\%$  and  $42.3 \pm 45.5\%$  of Cyanophyta. Other observed classes of phytoplankton included Chlorophyta, Bacillariophyta, and Pyrrophyta (S3).

The average cell densities of *Anabaena* sp. and *Microcystis* sp. in the CW were lower than those in AL (*Anabaena* sp.:  $n = 32$ ,  $t = 4.09$ ,  $p < 0.01$ ; *Microcystis* sp.:  $n = 32$ ,  $t = 9.85$ ,  $p < 0.001$ , Figure 4b,c). The dynamics of *Anabaena* sp. and *Microcystis* sp. showed periodic fluctuations in both AL and CW (Figure 4b,c). The density of *Anabaena* sp. in the AL achieved peaks in May and September, fluctuating in the range  $1.41 \times 10^7$ – $86.76 \times 10^7$  cells·L<sup>-1</sup> during April–September, and accounting for 6.4–98.5% of total phytoplankton cells (S3). The density of *Microcystis* sp. during June–August ( $0.38 \times 10^8$ – $3.29 \times 10^8$  cells·L<sup>-1</sup>) was higher than that in other months in the AL, accounting for 31.8–89.9% of total phytoplankton cells.

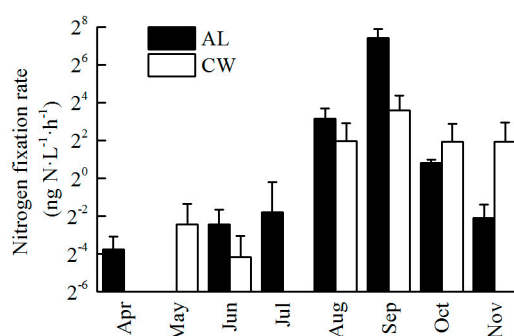
The  $\rho$ (PON) and  $\rho$ (DON) were positively correlated with the cell density of total phytoplankton and of *Anabaena* sp. in both AL and CW, but were not correlated with the cell density of *Microcystis* sp. (S5). The  $\rho$ (NH<sub>4</sub><sup>+</sup>-N) showed negative correlations with the cell density of total phytoplankton and of *Anabaena* sp. in the AL, but these relationships turned positive in the CW (S5). The daily maximum and minimum air temperature showed positively correlation with the cell density of *Microcystis* sp. in the CW, but neither with the *Anabaena* sp. nor total phytoplanktons (S4). Furthermore, no significant relationship was observed between the cell density of phytoplankton and the removal efficiency of nitrogen fractions, except for the negative correlation between the removal efficiency of PON and the cell density of *Microcystis* sp. in the AL (S6).



**Figure 4.** The cell density of (a) total phytoplankton, *Anabaena* sp. and *Microcystis* sp., in (b) the artificial lake (AL), and (c) the constructed wetland (CW).

### 3.4. Nitrogen Fixation Rate

The  $R_{N_{fix}}$  in water varied over the growing season and between sites (site:  $F_{1,48} = 8.62$ ,  $p < 0.01$ ; month:  $F_{7,48} = 4.41$ ,  $p < 0.001$ ; site  $\times$  month:  $F_{7,48} = 8.98$ ,  $p < 0.001$ ). The  $R_{N_{fix}}$  in the AL reached the highest value of  $169.00 \pm 65.98$  ng N·L<sup>-1</sup>·h<sup>-1</sup> in September, and maintained a high level ( $1.74$ – $8.92$  ng N·L<sup>-1</sup>·h<sup>-1</sup>) from August to October (Figure 5). However,  $R_{N_{fix}}$  in AL were lower than  $0.30$  ng N·L<sup>-1</sup>·h<sup>-1</sup> in other months.  $R_{N_{fix}}$  in the CW was lower than  $0.20$  ng N·L<sup>-1</sup>·h<sup>-1</sup> during April–July, and plateaued at a high average rate of  $5.93 \pm 4.89$  ng N·L<sup>-1</sup>·h<sup>-1</sup> during August–November (Figure 5). The highest rate of  $R_{N_{fix}}$  in CW ( $12.14 \pm 8.60$  ng N·L<sup>-1</sup>·h<sup>-1</sup>) occurred in September.  $R_{N_{fix}}$  was positively correlated with  $\rho(\text{PON})$ ,  $\rho(\text{DON})$ , and the cell density of *Anabaena* sp. in both AL and CW (Table 1). Moreover,  $R_{N_{fix}}$  in the CW was positively correlated with  $\rho(\text{NH}_4^+\text{-N})$  and total phytoplankton (Table 1).



**Figure 5.** Nitrogen fixation rate ( $R_{N_{fix}}$ ) in the artificial lake (AL) and the constructed wetland (CW). Statistical tests were performed using two-way analysis of variance.

**Table 1.** Pearson correlation coefficients between  $R_{N_{fix}}$  and water parameters (\*\* $p < 0.001$ ; \* $p < 0.01$ ; \* $p < 0.05$ ;  $n = 32$ ). AL represents the artificial lake, CW represents the constructed wetland.

	$\rho(\text{PON})$	$\rho(\text{DON})$	$\rho(\text{NH}_4^+\text{-N})$	$\rho(\text{NO}_x\text{-N})$	Total Phytoplankton	<i>Anabaena</i> sp.	<i>Microcystis</i> sp.
$R_{N_{fix}}$ in AL	0.648 ***	0.662 ***	-0.078	-0.244	0.309	0.467 **	-0.259
$R_{N_{fix}}$ in CW	0.739 ***	0.618 ***	0.417 *	-0.107	0.489 **	0.533 **	-0.223

## 4. Discussion

### 4.1. Dynamics of Nitrogen Removal Efficiency

In this study, the removal of TN by the CW was primarily due to the decreases in PON, which accounted for more than 90% of TN removal (Figure 3a). The PON in the AL was primarily composed of food debris and excreta produced by birds, plant litter, algae, and other microorganisms [4]. These sources of PON are usually higher in aquaculture wastewater, domestic wastewater, and storm runoff [5]. The removal efficiency of PON was lower during August–October than during April–July (Figure 3a). The key factor limiting the PON removal efficiency was the capacity of the CW during April–July, but turned into the cell density of phytoplankton in both the AL and CW during August–October. As one of the main sources of PON in wastewater, phytoplankton may have proliferated in the summer in the AL to a point beyond the capacity of the CW. This can lead to declines in PON removal efficiency. Particulate matter, whose concentration is generally reduced through sedimentation and adsorption, can be removed more efficiently with a longer hydraulic retention time (generally longer than 3–5 days [6]). However, the methods promoting sedimentation and adsorption may not be effective in removing PON in the studied system, especially in removing phytoplankton. A longer hydraulic retention time may even increase the phytoplankton cell densities, because the phytoplankton could actively grow in the CW during the summer.

The removal of TDN by CW was not as efficient as the removal of PON (Figure 3a). Among the fractions of TDN, DON and its proportion in TDN showed a decreasing trend from spring to summer (Figure 2d; S1). DON was probably rapidly decomposed under rising temperature and microbial activities [19], thereby leading to increases in  $\rho(\text{NH}_4^+\text{-N})$  and  $\rho(\text{NO}_x\text{-N})$  during the wastewater flowing through the CW (Figure 2e,f). Therefore, in the studied system the transformation from DON to inorganic nitrogen may cause declines in the removal efficiency of TDN (Figure 3b), as inorganic nitrogen was known as the most difficult form of nitrogen to remove from the CW [1].

#### 4.2. Dynamics of N-Fixing and Non-N-Fixing Cyanobacteria

The *Anabaena* sp. and *Microcystis* sp. bloomed alternately ( $>2 \times 10^7$  cells·L<sup>-1</sup>) in both AL and CW (Figure 4b,c). Similar dynamics were observed in natural lakes, such as the Iron Gate and Copco reservoirs in California [20] and the Mendota Lake in Wisconsin [21]. In those aquatic systems, *Microcystis* spp. broke out following the bloom of *Aphanizomenon* spp., which is an N-fixing genus of Cyanobacteria. Generally, N-fixing cyanobacteria usually proliferated in early spring and autumn [22,23]. The optimal temperature of N-fixing cyanobacteria (20–25 °C, e.g., *Anabaena* spp. and *Aphanizomenon* spp.) is generally lower than that of *Microcystis* spp. (25–30 °C) [24,25]. Such differences in optimal temperature may result in their alternate proliferation. For example, the alternately blooming of N-fixing and non-N-fixing cyanobacteria has been reported for Dianchi Lake [26] and Erhai Lake [27] in China, where suitable temperatures last long enough for the proliferation of N-fixing cyanobacteria. However, blooms of N-fixing cyanobacteria have barely been observed in East China, where temperatures usually increase rapidly in the spring and *Microcystis* populations develop in aquatic systems over a short time [28].

The site of this study was located in the north China, where so far no N-fixing cyanobacteria blooms had been reported. The daily average air temperature of the study site remained between 17.1 °C and 25.1 °C between April and June in 2014 [S2], which might be a suitable period for *Anabaena* sp. proliferation. The daily average air temperature increased to above 25 °C during July–August, when the cell density of *Microcystis* sp. increased (Figure 4b). When the air temperature decreased to ~20 °C in September, the cell density of *Anabaena* sp. increased again and reached the highest points in both AL and CW (Figure 4b,c). The cell density of phytoplankton in the AL was correlated with neither air nor water temperature (S4). Therefore, the growth of *Anabaena* sp. and *Microcystis* sp. may be activated when temperature rises above a threshold [29], but may not be so when continued warming occurs. However, the air temperature was positively correlated with the density of *Microcystis* sp. in the CW, and was negatively correlated with the PON removal efficiency (S4). This implied that high air temperature may facilitate the proliferation of *Microcystis* sp. in the CW, and thereby reduce the PON removal efficiency. Therefore, fluctuations in the cell densities of *Anabaena* sp. and *Microcystis* sp. were largely driven by temperature at the study site.

#### 4.3. Relationships among Nitrogen Fixation, N-Fixing Cyanobacteria, and PON Removal

Our finding is consistent with Patoine [30] showing that nitrogen fixation increased PON in aquatic ecosystems in the Canadian Great Plains.  $R_{\text{Nfix}}$  at our site averaged  $12.14 \pm 8.60$  ng N·L<sup>-1</sup>·h<sup>-1</sup> during August–November, and the highest  $R_{\text{Nfix}}$  in AL was  $169.00 \pm 65.98$  ng N·L<sup>-1</sup>·h<sup>-1</sup>. This value is close to the  $R_{\text{Nfix}}$  in the Baltic Sea and Pacific Ocean, where N<sub>2</sub> was the primary nitrogen source [31,32]. The observed positive  $R_{\text{Nfix}}-\rho(\text{PON})$ ,  $R_{\text{Nfix}}-\textit{Anabaena} sp., and  $R_{\text{Nfix}}-\text{phytoplankton}$  relationships (Table 1) suggested that the new nitrogen fixed by *Anabaena* sp. contributed a large proportion of PON in the study site.$

The nitrogen added by the *Anabaena* sp. could promote the proliferation of *Microcystis* sp. [22,33]. Since *Anabaena* sp. showed a positive correlation with  $\rho(\text{DON})$  (S3), the proliferated *Anabaena* sp. in the early-growing season was probably decomposed into dissolved nitrogen in the summer, and supplied available nitrogen for the subsequent bloom of *Microcystis* sp. in the AL. Furthermore, *Microcystis* sp. in the AL was negatively correlated with the removal efficiency of PON (S6), suggesting that



*Microcystis* sp. may have exceeded the capacity of the CW and led to a lower PON removal efficiency in summer (Figure 3a). Therefore, we speculated that the nitrogen fixation by N-fixing Cyanobacteria may reduce the PON removal in the CW in summer, through supplying available nitrogen for non-N-fixing phytoplankton.

## 5. Conclusions

In this study, the PON was a major fraction of the total nitrogen in the AL, and the cyanobacteria, which may be toxic for other aquatic organisms, was an important component of PON. Therefore, PON removal should be considered a priority. Our results showed that the PON contributed to more than 90% of the removal of TN in the study site. The removal efficiency of PON was lower in the summer than in the spring. Phytoplankton proliferation in summer, as one of the main sources of PON, may have exceeded the capacity of the CW and led to declines in PON removal efficiency. The nitrogen fixed by *Anabaena* sp. was likely used by *Microcystis* sp., promoting the summer *Microcystis* sp. blooms in AL. Such blooms increased  $\rho(\text{PON})$  in wastewater, and thus reduced the PON removal efficiency of the CW. Therefore, we concluded that the nitrogen fixation by cyanobacteria could reduce the nitrogen removal efficiency in the CW in the summer. Studies on phytoplankton management will greatly assist in the removal of TN in the CW, especially in the culture ponds and paddy fields with abundant N-fixing cyanobacteria.

**Supplementary Materials:** The following are available online at [www.mdpi.com/2073-4441/9/11/865/s1](http://www.mdpi.com/2073-4441/9/11/865/s1), Figure S1: Relative abundance of nitrogen forms in (a) the artificial lake (AL) and (b) the constructed wetland (CW), Figure S2: The (a) water temperature and (b) air temperature in the study site. Figure S3: Relative abundance of phytoplankton classes in (a) the artificial lake (AL) and (b) the constructed wetland (CW), Table S4: Pearson correlation coefficients between temperature and the cell densities and nitrogen removal efficiency, Table S5: Pearson correlation coefficients between cell densities of phytoplankton and nitrogen forms in the artificial lake (AL) and the constructed wetland (CW), Table S6: Pearson correlation coefficients between cell densities of phytoplankton and removal efficiency of nitrogen forms in the artificial lake (AL) and the constructed wetland (CW).

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**Conflicts of Interest:** The authors declare no conflict of interest. The founding sponsors had no role in the design of the study; in the collection, analyses, or interpretation of data; in the writing of the manuscript, or in the decision to publish the results.

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