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Original Research

Enhancing nitrogen removal in constructed wetlands: The role of influent substrate concentrations in integrated vertical-flow systems

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ABSTRACT

Recent advancements in constructed wetlands (CWs) have highlighted the imperative of enhancing nitrogen (N) removal efficiency. However, the variability in influent substrate concentrations presents a challenge in optimizing N removal strategies due to its impact on removal efficiency and mechanisms. Here we show the interplay between influent substrate concentration and N removal processes within integrated vertical-flow constructed wetlands (IVFCWs), using wastewaters enriched with NO_3^--N and NH_4^+-N at varying carbon to nitrogen (C/N) ratios (1, 3, and 6). In the NO_3^--N enriched systems, a positive correlation was observed between the C/N ratio and total nitrogen (TN) removal efficiency, which markedly increased from 13.46 ± 2.23% to 87.00 ± 2.37% as the C/N ratio escalated from 1 to 6. Conversely, in NH_4^+-N enriched systems, TN removal efficiencies in the A-6 setup (33.69 ± 4.83%) were marginally 1.25 to 1.29 times higher than those in A-3 and A-1 systems, attributed to constraints in dissolved oxygen (DO) levels and alkalinity. Microbial community analysis and metabolic pathway assessment revealed that anaerobic denitrification, microbial N assimilation, and dissimilatory nitrate reduction to ammonium (DNRA) predominated in NO_3^--N systems with higher C/N ratios (C/N ≥ 3). In contrast, aerobic denitrification and microbial N assimilation were the primary pathways in NH_4^+-N systems and low C/N NO_3^--N systems. A mass balance approach indicated denitrification and microbial N assimilation contributed 4.12–47.12% and 8.51–38.96% in NO_3^--N systems, respectively, and 0.55–17.35% and 7.83–33.55% in NH_4^+-N systems to TN removal. To enhance N removal, strategies for NO_3^--N dominated systems should address carbon source limitations and electron competition between denitrification and DNRA processes, while NH_4^+-N dominated systems require optimization of carbon utilization pathways, and ensuring adequate DO and alkalinity supply.

1. Introduction

Constructed wetland (CW) was eco-friendly, cost-effective, and promising technology for removing organic matter and nutrients from various wastewater sources, including industrial, agricultural, and domestic wastewater and the tailwater of sewage treatment plants [1]. Under the synergistic effects of vegetation, microorganisms, and substrates [2], CWs displayed a high organic pollutants removal capability [3]. However, due to the insufficient dissolved oxygen (DO) and the lack of electron donors, the N removal efficiencies in CWs were usually unsatisfactory [4]. Since the N discharges were ever-increasing, an urgent need was to enhance the N removal performance in CWs.

In recent years, numerous intensified N-removal strategies have been studied and implemented in various scenarios [5–8]. These strategies included increasing of C/N ratios [9], employing artificial aeration (AA) [9,10], utilizing various emerged substrates [11], adopting hybrid CWs (HCWs) [12,13], etc. Nevertheless, the effectiveness of these enhanced strategies under different scenarios was usually unstable due to the variations of influent substrate concentrations and N transformation pathways in CWs [4,14]. For

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Abbreviations

N	Nitrogen
C	Carbon
CW	Constructed Wetland
IVFCWs	Integrated vertical-flow constructed wetlands
HCWs	Hybrid constructed wetlands
NO_3^--N	Nitrate nitrogen
NH_4^+-N	Ammonia nitrogen
AA	Artificial aeration
TN	Total nitrogen
C/N ratio	Carbon to nitrogen ratio
DO	Dissolved oxygen
DNRA	Dissimilatory nitrate reduction to ammonium
COD	Chemical oxygen demand
TP	Total phosphorus
PAOs	Phosphate accumulating organisms
GAOs	Glycogen accumulating organisms
Anammox	Anaerobic ammonium oxidation
HNAD	Heterotrophic nitrification and aerobic denitrification
SND	Simultaneous nitrification and denitrification
ANRA	Assimilatory nitrogen reduction
NR	Nitrate reductases
Nar	Respiratory nitrate reductase
Nap	Periplasmic nitrate reductase
Nas	Assimilatory nitrate reductase
Nir	Nitrite reductase
LDH	Lactate dehydrogenase
GS	Glutamine synthetase
GOGAT	Glutamate synthase
Amo	Ammonia monooxygenase
Hao	Hydroxylamine oxidoreductase

instance, Fu et al. [15] reported that sufficient electron donors were important for N removal. A higher TN removal efficiency was obtained when the influent C/N ratio was higher than four, and the partial nitrification-denitrification process was recognized as the main N removal pathway. However, another study showed that the high denitrification rate occurred at the C/N ratio of 2.5–5, and the highest TN removal efficiency was achieved at the C/N ratio of 2.5 compared with the C/N ratios of 5 and 10 [16]. Li et al. [17] used AA to improve the N removal with simulated domestic wastewater and achieved a 68.57% increase in TN removal in AA-CWs compared with the control set. However, a low TN removal efficiency in AA-CWs was obtained in the treatment of domestic sewage by Pan et al. [12]. These studies indicated a substantial effect of influent substrate concentrations on the effectiveness of strategies for enhanced N removal. The increasing C/N ratio did not indicate a better N removal performance. When insufficient electron donors were responsible for the low N removal efficiency, increasing the C/N ratio was an effective strategy. However, when the concentration of organic matter was detrimental to N transformation, the increase in the C/N ratio was unreasonable. Similar results were also found in the applications of other enhanced strategies. For emerged substrates, taking iron-carbon fillers as an example, the effectiveness of this material for enhanced N removal was also closely related to the influent C/N ratios, N source types, and N concentrations [18,19]. Sometimes, it might promote the dissimilatory nitrate reduction to ammonium (DNRA) process, which is a competing pathway to denitrification [20]. Integrated vertical-flow constructed wetlands (IVFCWs) had the advantages of a small footprint, high oxygen transmission capacity, and long hydraulic retention time [21–23]. Benefiting from the structure of "down-flow" followed by "up-flow", IVFCWs offered an alternate "aerobic–anaerobic–aerobic" condition which created a suitable environment for the colonization of nitrifying and denitrifying bacteria. Previous studies on IVFCW and IVFCWs coupled with other enhanced strategies have mainly focused on the N removal performance [24–26], but the exploration of N removal pathways with different influent substrate concentrations and guidelines for enhanced strategy selections were rarely reported.

Considering that NO_3^--N and NH_4^+-N were the two most common N types in CWs [4,15,27,28], this study was carried out in two stages with two different N source types (stage I: NO_3^--N; stage II: NH_4^+-N) to investigate the N removal pathways in IVFCWs, and three C/N ratios (6, 3, and 1) were controlled for each stage. The following three specific objectives were pursued: (1) to evaluate the pollutants removal performance and characteristics of the microbial community under different influent substrate concentrations; (2) to reveal the microbial degradation pathways of pollutants by analyzing the changes in microbial ecological functions, metabolic intensity and related functional gene abundance with different influent substrate concentrations; and (3) to calculate the contribution of the corresponding N removal pathways using the mass balance approach and propose a selection method for enhanced N removal strategies in IVFCWs.

2. Materials and methods

2.1. IVFCWs set up and operation

Three lab-scale IVFCWs made of plexiglass were established. As shown in Fig. 1, every system consisted of a down-flow VFCW followed by an up-flow VFCW (L × B × H = 250 × 250 × 500 mm) with a total working volume of 24 L. Each VFCW was filled with 350 mm gravel (10–30 mm in diameter) and 50 mm sand (3–5 mm in diameter) from the bottom to the surface and was wrapped with black plastic to prevent algal growth. Five sampling spots were set along the bed following the flow of water. *Acorus calamus* were

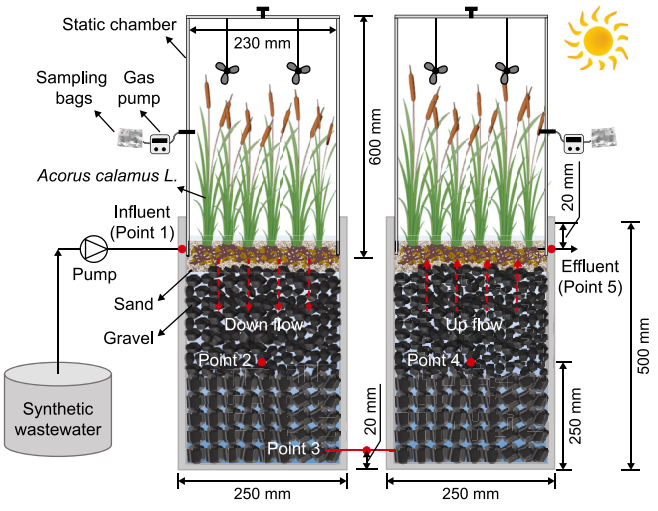

Fig. 1. Schematic description of the IVFCW configuration.

planted at a density of 14 rhizomes per system.

The experiments were conducted in two stages. In the first stage, NO_3^--N was used as the nitrogen source ("N-" system), and then NH_4^+-N was used as the nitrogen source ("A-" system) in the second stage. Three C/N ratios (6, 3, and 1) were set for each stage. Synthetic wastewater was used in the experiment (Table 1). The composition of the synthetic wastewater was shown in Table S1. Before each stage started, the systems were fed synthetic wastewater for two weeks to cultivate the microorganisms. The influent was supplied by a peristaltic pump with a continuous flow, and each stage lasted for one month with a 0.10 $m^3\ m^{-2}\ d^{-1}$ hydraulic load.

2.2. Sampling and analysis

2.2.1. Water sampling and analysis

Water samples were collected daily from five sampling points. The COD was tested using the HACH standard method (HJ/T399-2007). TN was tested by a TOC/TN analyzer (multi N/C 3100, analytikjena, Germany). Total phosphorus (TP), NH_4^+-N, NO_3^--N, and NO_2^--N were determined according to standard methods (GB3838-2002). DO, pH, and water temperature were measured immediately after sampling by a portable DO meter (AZ 8403, AZ Instrument Corp, China), a pH meter (Five Easy Plus, METTLERTOLEDO, Switzerland), and an electronic thermometer. The pollutant concentrations were measured in triplicate.

2.2.2. Gas collection and analysis

Plexiglass chambers (L × B × H = 230 × 230 × 600 mm) were installed on the IVFCWs. Gas emissions were monitored through the static chamber–gas chromatography method [29]. With a gas pump (DC-125, HOPAR), gas samples were pumped into 0.1 L sampling bags (Dalian Haide Technology Co., Ltd.). The concentrations of the gases were then determined using gas chromatography (7890B, Agilent Technologies, USA). The gas emission flux was calculated by the following equation:

$$J = \frac{dc}{dt} \times \frac{M}{V_0} \times \frac{P}{P_0} \times \frac{T_0}{T} \times H \quad (1)$$

where J was gas emission flux (mg $m^{-2}\ h^{-1}$); dc/dt was the slope of the straight line obtained by fitting the GHG concentration with time (mg h^{-1}); M was the mole mass of CH_4, CO_2, and N_2O (g mol^{-1}), with the values of 16, 44, and 44 g mol^{-1}, respectively; P was the atmospheric pressure (kPa); T was the namely temperature (K); H was the height of static chamber above the water level (m), with a value of 0.55 m; V_0, P_0, T_0 represented the gas volume (L mol^{-1}), pressure (kPa), temperature (K) under standard conditions, with the values of 22.4 L mol^{-1}, 101.325 kPa and 273.15 K, respectively.

2.2.3. Plants sampling and analysis

Plants were collected from every system before and after the operation. The fresh weight and the maximum length of the roots and leaves were recorded to calculate the amount of plant growth during the experiment using equation (2). Plant samples were oven-dried at 60 °C and then were ground with the mortar. The P content of plants was measured by digestion methods (NY/T2017-2011) by an inductively coupled plasma optical emission spectrometer (ICP-OES, PerkinElmer Optima 2000 DV). The C and N contents of the plants were analyzed by an elemental analyzer (Elementar vario EL cube, Germany).

$$M_{plant} = M_{final} - M_{initial} \quad (2)$$

In equation (2), M_{plant} was the amount of plant growth during the experiment (mg); $M_{initial}$ and M_{final} were the fresh weight of plants before and after the operation (mg), respectively.

2.2.4. Microbial sampling and analysis

Samples were collected from the aera of the inlet (from point 1 to 2), middle (from point 2 to 4), and outlet (from point 4 to 5) with a five-point sampling method, which was named "-I", "-M", and "-O", and then stored at −20 °C before the high-throughput sequencing analysis.

Total genomic DNA was extracted from the samples using an E.Z.N.A™ Mag-Bind Soil DNA Kit (Omega, USA). The extracts were quantified by using a Qubit 3.0 DNA Kit (Life, USA). Universal primers 341F and 805R were used for polymerase chain reaction amplification of the bacterial 16S rRNA gene V3–V4 regions. Amplicon sequencing was performed using an Illumina MiSeq platform (Shanghai Sangon Biotech Co., Ltd.). Sequences of each sample were quality-filtered and then clustered into operational taxonomic units (OTUs) with a 97% similarity by the Usearch program. The predictions of bacterial metabolic and ecologically relevant functions were conducted by the Functional Annotation of Prokaryotic Taxa (FAPROTAX). Functional genes were predicted using the PICRUSt package and the Kyoto Encyclopedia of Genes and Genomes (KEGG) databases.

2.3. Mass balance calculations

In IVFCWs, N removal efficiency was determined by plant uptake, substrate adsorption, NH_3 volatilization, and microbial N transformation. N removal and mass balance analyses were based on the following assumptions: (1) N removal by substrate adsorption could be ignored because of the weak adsorption capacity of gravel and sand [30]; (2) NH_3 volatilization was negligible due to the low concentration of NH_4^+-N and neutral pH in the influent; and (3) Biological N fixation and N loss caused by litter release were negligible [31].

N removal in IVFCWs could be attributed to plant uptake, microbial assimilation, and microbial conversion (the generation of N-containing gases). With the data of N contents in plants and the amount of plant growth during the operation period, the amount of N removal by plant uptake was calculated via the following

Table 1
Concentration of influent pollutants in different systems.

Stage (Period) (day)	System	Parameters (mg L^{-1}) COD	NO_3^--N	NH_4^+-N	TP	pH	C/N
I (1–30)	N-6	182.47 ± 9.30	32.88 ± 2.12	0	2.10 ± 0.18	7.04	6
	N-3	92.77 ± 5.66	32.85 ± 1.47	0	2.08 ± 0.20	7.12	3
	N-1	35.63 ± 5.79	32.61 ± 2.05	0	2.11 ± 0.18	7.03	1
II (46–75)	A-6	173.35 ± 10.94	0	33.96 ± 2.55	2.10 ± 0.12	6.93	6
	A-3	91.87 ± 6.57	0	34.04 ± 2.42	2.08 ± 0.11	6.92	3
	A-1	34.60 ± 4.09	0	33.65 ± 2.45	2.11 ± 0.13	6.94	1

equation:

$$N_{plant} = m_{final} \times N_{final} - m_{initial} \times N_{initial} \quad (3)$$

where N_{plant} was the amount of N removed by plant uptake (mg); $m_{initial}$ and m_{final} were the dry weights of plants before and after the operation (mg), respectively; $N_{initial}$ and N_{final} were the proportions of N content in plants before and after the operation (%).

N removal through microbial assimilation was evaluated by carbon mass balance based on the chemical formula $C_5H_7NO_2P_{0.074}$. The amount of N removed by microbial assimilation was calculated using equation (4):

$$N_{assimilation} = (C_{in} - C_{out} - C_{gas}) \times \frac{M_N}{5 \times M_C} \quad (4)$$

where $N_{assimilation}$ was the amount of N removed by microbial assimilation (mg); C_{in} and C_{out} were the contents of organic matters in the influent and effluent (mg), respectively; C_{gas} was the carbon content of CH_4 and CO_2 (mg); M_N and M_C were the relative atomic masses of N and C, with the values of 14 and 12, respectively.

The amount of N removal by denitrification was calculated by equation (5):

$$N_{denitrification} = N_{in} - N_{out} - N_{plant} - N_{assimilation} \quad (5)$$

where $N_{denitrification}$ was the amount of N removed by denitrification (mg); N_{in} and N_{out} were the TN contents in the influent and effluent (mg), respectively.

The contributions of plant uptake, microbial assimilation, and denitrification to the TN removal were calculated using equation (6):

$$E_{assimilation/denitrification/\ plant} = \frac{N_{assimilation/denitrification/plant}}{TN_{removal}} \quad (6)$$

where $E_{assimilation/denitrification/plant}$ was the contribution of microbial assimilation/denitrification/plant uptake to TN removal (%); $TN_{removal}$ was the TN removal amount (mg).

3. Results and discussion

3.1. Pollutant removal performance in IVFCWs

The pollutant removal efficiencies during the operation were analyzed and shown in Fig. 2. The effluent COD concentrations of all systems were lower than 15 mg L^{-1}, as shown in Fig. S1. The COD degradation efficiency was positively correlated with the C/N ratio, as it showed an increasing trend with an increasing C/N ratio at both stages (Fig. 2a). The highest COD removal efficiencies were obtained in the N-6 and A-6 systems, which were 91.79 ± 2.03% and 92.25 ± 1.43%, respectively. Compared with the N-3 (87.47 ± 2.60%) and N-1 (76.96 ± 4.38%) systems, the COD removal efficiency of the N-6 system was increased by 4.32% and 14.83%, respectively. Compared to A-3 (88.08 ± 2.09%) and A-1 (72.22 ± 5.46%) systems, the COD removal efficiency of the A-6 system was increased by 4.17% and 20.03%, respectively.

The TP degradation efficiency was related to the C/N ratio and N source types (Fig. 2b). At stage I (NO_3^--N system), average TP removal efficiency was first increased from 49.50 ± 6.04% in the N-1 system to 61.02 ± 4.33% in the N-3 system and then decreased to 54.03 ± 6.42% in the N-6 system. The same tendency was observed at stage II (NH_4^+-N system), where the highest TP removal efficiency was 76.23 ± 12.38% in the A-3 system. TP removal efficiencies in A-6 and A-1 systems were 54.18 ± 6.33% and 39.36 ± 7.28%, respectively.

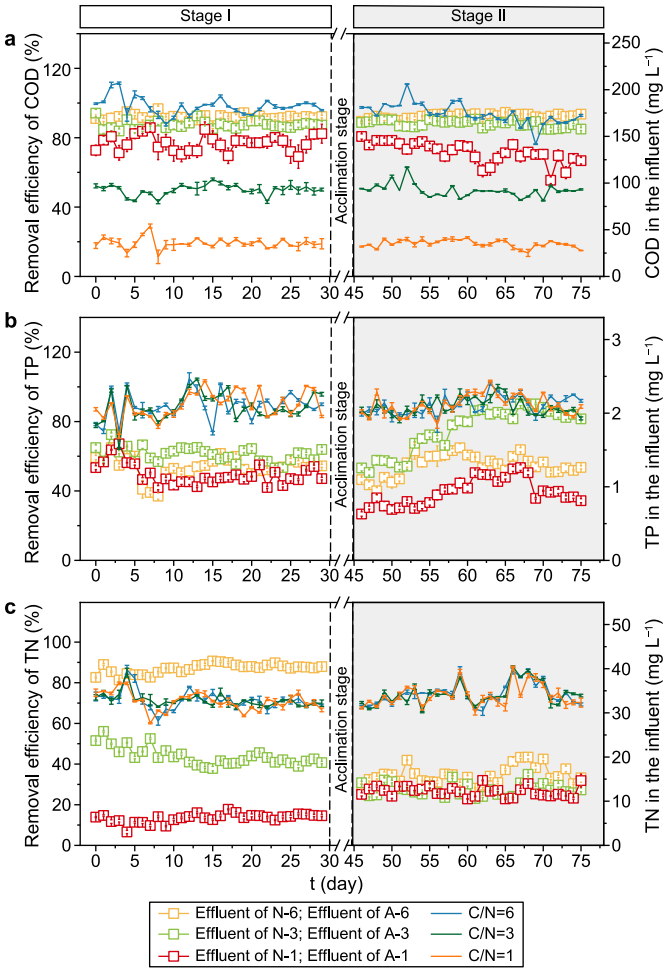

Fig. 2. Concentrations in the influent and removal efficiencies of COD (**a**), TP (**b**), and TN (**c**).

The discrepancies of NO_2^--N accumulation (Fig. S1) [32], the main N types (NO_3^--N, NO_2^--N, or NH_4^+-N), and the competition between phosphate accumulating organisms (PAOs) and glycogen accumulating organisms (GAOs) [33] were responsible for the variations of TP removal. Although NO_3^--N and NO_2^--N could be the elector acceptors in the denitrifying phosphorus removal process, the presence of excess NO_2^--N would affect the absorption of organic matters by phosphate accumulating organisms (PAOs), and inhibit the growth and metabolism of PAOs. It was reported that 2 mg NO_2^--N L^{-1} was the threshold for anoxic phosphate uptake [34]. The NO_2^--N concentrations in the effluent of different systems were shown in Fig. S1f. Except for the N-3 system (2.62 mg L^{-1}), the NO_2^--N concentrations of other systems were similar, which were lower than 0.5 mg L^{-1}. Therefore, the A-3 system showed a better TP removal efficiency than the N-3 system. When the C/N ratio was 1, the existence of NO_3^--N in the N-1 system promoted the denitrifying phosphorus removal process and achieved a higher TP removal compared with the A-1 system. When the C/N ratio was 6, NO_3^--N was completely converted in the N-6 system, which led to a similar TP removal efficiency to the A-6 system. Except for that, the C/N ratio also was the key factor for TP removal. Insufficient organic matter affected the synthesis of poly-β-hydroxyalkanoates (PHAs) and the growth of PAOs, while excess organic matter increased the biomass of glycogen accumulating organisms (GAOs), which would compete organic matters with PAOs and then decreased the TP removal. Therefore, the TP removal was first increased with the

increasing C/N ratio from 1 to 3 and then decreased at the C/N ratio of 6.

For N removal, despite the increase in TN removal with the increasing C/N ratio (Fig. 2c), the linear relationship between TN removal and the C/N ratio under the two different N sources was quite different (Fig. S2). At the stage I (NO_3^--N system), the TN removal efficiencies of N-1, N-3, and N-6 systems were 13.46 ± 2.23%, 43.83 ± 4.42%, and 87.00 ± 2.37%, respectively. A linear increase was observed as the C/N ratio increased. At stage II (NH_4^+-N system), the increase in TN removal was not obvious with the increasing C/N ratio. Average TN removal efficiencies were 26.02 ± 2.78%, 26.95 ± 3.32%, and 33.69 ± 4.83% in the A-1, A-3, and A-6 systems. This result indicated that TN removal was not always significantly improved with the increasing C/N ratios because it was also closely related to the N source types. Therefore, enhanced strategies, such as the addition of external carbon sources, might be unsuitable for increasing TN removal towards NH_4^+-N-dominated wastewaters in IVFCWs.

3.2. Nitrogen transformation process in IVFCWs

To investigate the impacts of the C/N ratio and N types on TN removal, different N species in the effluent were tested, as shown in Fig. 3. At stage I (NO_3^--N system), NO_3^--N was transformed entirely in the N-6 system. The predominant N form in the effluent was NH_4^+-N (Fig. 3a), with a concentration of 3.65 ± 0.78 mg L^{-1}. NH_4^+-N had also been detected in N-3 and N-1 systems, but NO_3^--N was still the dominant N form in the two systems, accounting for 75.71 ± 2.07% and 92.86 ± 1.88% of the effluent TN concentration, respectively. Because NO_3^--N was the only N source in the influent during stage I, the presence of NH_4^+-N in the effluent probably originated from the DNRA process [4]. Relevant studies have shown that the DNRA positively correlated with the C/N ratio [35,36], and was not conducive to denitrification [28]. Except for NO_3^--N and NH_4^+-N, some NO_2^--N was accumulated in the N-3 system, accounting for 13.19 ± 0.54% of the effluent TN concentration. NO_2^--N accumulation was attributed to the incomplete denitrification

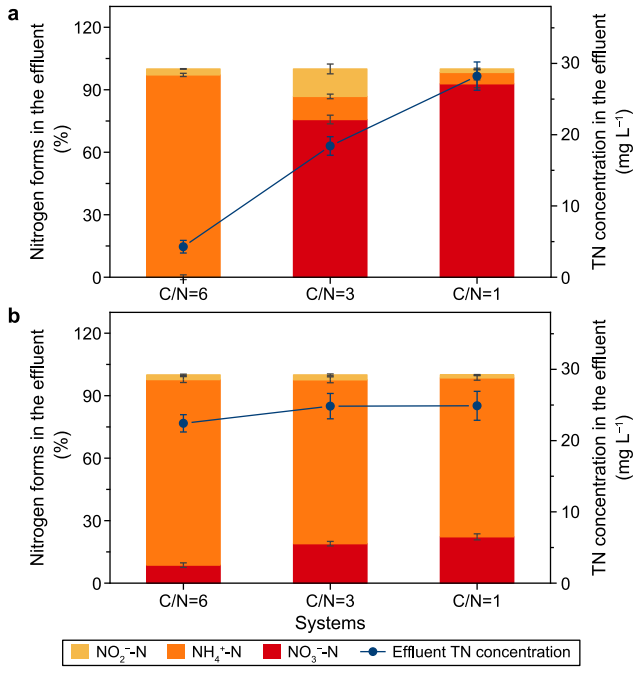

Fig. 3. The N forms and TN concentrations in the effluent of stage I (**a**) and stage II (**b**).

caused by insufficient carbon sources, and nitrite reductase activity was more sensitive than nitrate reductase at low C/N ratios [37]. Therefore, the factors of limited TN removal in the NO_3^--N system were carbon source availability and the electron competition between DNRA and the denitrification process. External carbon source supplements, emerged substrates [11], and effluent recirculation were effective strategies for addressing the problem of carbon source deficiency. Some studies have reported that a relatively low C/N ratio [38], low dissolved oxygen (DO) [39], and neutral or subacidic conditions [40] were more favorable for denitrification than DNRA.

At stage II (NH_4^+-N system), NH_4^+-N was the primary N form in A-6, A-3, and A-1 systems, accounting for 89.09 ± 1.40%, 78.66 ± 1.41%, and 76.30 ± 1.11% of the effluent TN concentration, respectively (Fig. 3b). In the NH_4^+-N system, the experimental conditions were consistent except for the different influent substrate concentrations. Therefore, DO, C/N ratios, pH, and alkalinity were recognized as the crucial factors for nitrification. The variations of DO concentrations in the aerobic aera of IVFCWs were shown in Fig. S3, and the average DO concentrations in the aerobic aera were 0.72 mg L^{-1} (down-flow) and 1.31 mg L^{-1} (up-flow) in the A-6 system, 2.54 mg L^{-1} (down-flow) and 2.07 mg L^{-1} (up-flow) in the A-3 system, and 3.25 mg L^{-1} (down-flow) and 2.50 mg L^{-1} (up-flow) in the A-1 system. Because the DO concentration should be maintained at more than 1 mg L^{-1} for nitrification [41], it could be inferred that the lower DO concentration was the limited factor for NH_4^+-N transformation in the A-6 system. However, the DO concentrations in the A-3 and A-1 systems exceeded 2 mg L^{-1}, indicating that DO was not the limited factor in the two systems. A relevant study reported that the degradation of every 1 g of BOD could produce 0.3 g alkalinity, since there was no extra alkalinity addition and a low carbon source concentration in the influent, it could be inferred that alkalinity deficiency was the limiting factor of the A-3 and A-1 systems [42,43]. The proportions of effluent NO_3^--N to effluent TN were 8.64 ± 1.11% in the A-6 system, 18.95 ± 1.08% in the A-3 system, and 22.23 ± 1.46% in the A-1 system. The effluent NO_3^--N concentration showed a negative linear correlation with the C/N ratio, and this trend was consistent with that of stage I (Fig. S4). However, as mentioned in Section 3.1, the variation of TN removal at stage II differed from that in stage I. TN removal in the A-1 system was nearly two times higher than that of the N-1 system, which meant that autotrophic N removal processes took place in the A-1 system, for instance, anaerobic ammonium oxidation (anammox).

3.3. Microbial community and metabolic pathways analysis

3.3.1. Microbial community composition

To understand the effects of influent substrate concentration on N removal pathways, the microbial community and abundance in different systems were evaluated using high-throughput sequencing analysis. All microbial samples' coverage values exceeded 99% (Table S2), demonstrating that the sequencing depth was suitable and the data were credible. The alpha diversity indices showed that the closer the samples were to the inlet, the higher the microbial community richness and diversity were, which might be attributed to a higher influent substrate concentration in the inlet aera (Figs. S5 and S6).

The microbial community structures of stage I (NO_3^--N system) at the phylum, class, and genus levels are shown in Fig. 4a and b. The main phylum was Firmicutes, and the dominant class was Clostridia, followed by Bacilli in the N-6 and N-3 systems. Most Firmicutes bacteria were associated with anaerobic denitrification [44], and Clostridia and Bacilli performed admirably in NO_3^--N reduction [45]. The dominant bacterial groups at the genus level in

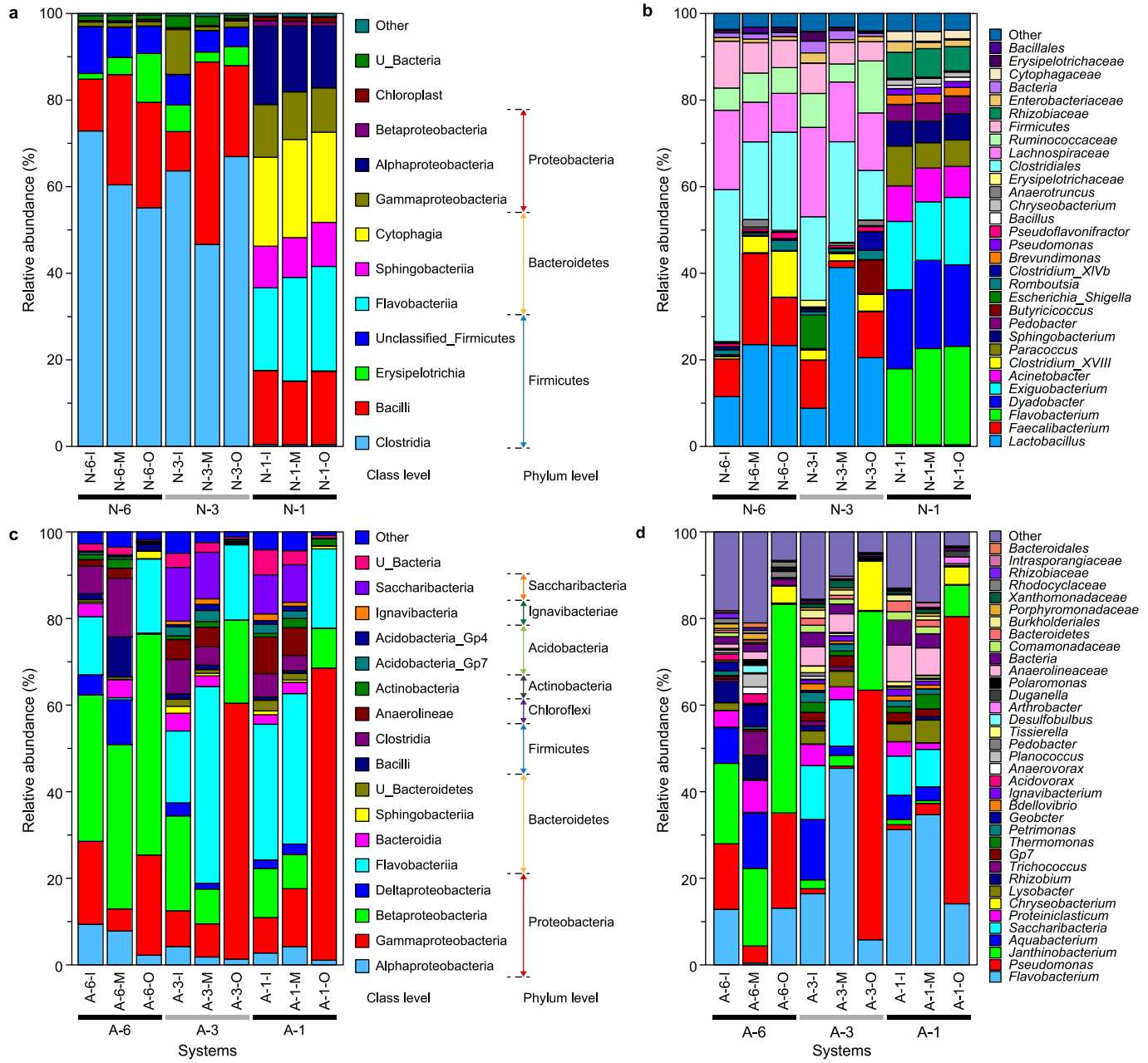

Fig. 4. Relative abundances of microbial communities. **a**–**b**, Stage I: Phylum and class level (**a**) and genus level (**b**). **c**–**d**, Stage II: Phylum and class level (**c**) and genus level (**d**).

both systems were unclassified_*Clostridiales*, *Lactobacillus*, unclassified_*Lachnospiraceae*, and *Faecalibacterium*. At the genus level, most of these genera were heterotrophic bacteria able to utilize C and N compounds through fermentation [46], demonstrating that anaerobic denitrification was the primary denitrification process in the N-6 and N-3 systems. Because of its sensitivity to organic C concentration (Fig. S7), the microbial community composition of the N-1 system was significantly different from those of the N-6 and N-3 systems, suggesting that N removal pathways were altered. The dominant phyla were Bacteroidetes and Proteobacteria in the N-1 system, while the primary classes were Flavobacteriia, Cytophagia, Bacilli, and Alphaproteobacteria. The principal genera of the system were *Flavobacterium*, *Dyadobacter*, and *Exiguobacterium*, which were related to the heterotrophic nitrification and aerobic denitrification (HNAD) process [47–49].

At stage II (NH_4^+-N system), the microbial compositions at the phylum and class levels were similar in the A-6, A-3, and A-1 systems (Fig. 4c). The dominant phyla in the three systems were Proteobacteria and Bacteroidetes, whereas the primary classes were Betaproteobacteria, Gammaproteobacteria, and Flavobacteriia. At the genus level (Fig. 4d), *Janthinobacterium* and *Pseudomonas* were the dominant genera in the A-6 system, whereas *Flavobacterium* and *Pseudomonas* were the main genera in the A-3 and A-1 systems. Previous research reported that *Janthinobacterium* was associated with the HNAD process [50], and *Pseudomonas* was a type of mixotrophic denitrifying bacteria [51], indicating that anaerobic denitrification and aerobic denitrification existed simultaneously in the NH_4^+-N systems.

3.3.2. Microbial ecological functions

To gain insights into the effect of influent substrate concentration on the ecological role of bacteria, FAPROTAX was used to predict the microbial functional characteristics from the 16S rRNA gene data. The ecological functions of IVFCWs included chemoheterotrophy, cellulolysis, chitinolysis, denitrification, nitrification, anammox, and nitrogen fixation. Chemoheterotrophy was the most abundant functional group in all systems, including aerobic chemoheterotrophy and fermentation [52].

Microbial functions related to carbon metabolism were shown in Fig. 5a. Most of the chemoheterotrophs in the N-6 and N-3 systems were fermentation, whereas many inferred functions were aerobic chemoheterotrophs in the N-1, A-6, A-3, and A-1 systems. Both processes were closely related to the C/N ratio and N forms. Fermentation was positively correlated with the C/N ratio and resulted in a higher relative abundance in the NO_3^--N systems. Aerobic chemoheterotrophy was negatively correlated with the C/N ratio, resulting in a higher relative abundance in the NH_4^+-N systems. Cellulolysis provided an organic carbon source for biological denitrification through the degradation of plant biomass [53]. The highest relative abundance of cellulolysis was observed in the N-1 system, indicating that wetland plants were a kind of potential carbon source under low C/N ratio conditions in the NO_3^--N system. Chitinolysis in the A-6, A-3, and A-1 systems indicated that NH_4^+-N-dominated conditions were more suitable for algal growth because chitin was an abundant renewable natural resource that could be extracted from algae [54].

Fig. 5b showed the N metabolism-related microbial functions. The N-1 and N-6 systems had the highest and the lowest relative abundances of denitrification, respectively, contrary to the TN removal trend in stage I. The high TN removal efficiency and low relative abundance of denitrification in the N-6 system indicated that many N sources might be utilized for microbial growth; microbial N assimilation was crucial for N removal in IVFCWs. Because microbial N assimilation has usually been neglected in previous studies [30,31], the contribution of this process to TN removal should be reassessed. Furthermore, the N-6 system showed the highest relative abundance of nitrification with no NO_3^--N content in the effluent, indicating that a simultaneous nitrification and denitrification (SND) process occurred in the N-6 system. In the NH_4^+-N system, a higher relative abundance of denitrification was observed in the A-6 system, and the relationship between nitrification intensity and the C/N ratio was not obvious in the three NH_4^+-N systems. This result was consistent with the variation of TN removal in stage II, and it could be inferred that limited nitrification was responsible for the low TN removal in stage II. Because the anammox process was only found in the A-3 and A-1 systems, it could be inferred that the NH_4^+-N dominated condition with the low C/N ratio was a desirable enrichment condition for anammox bacteria in IVFCWs.

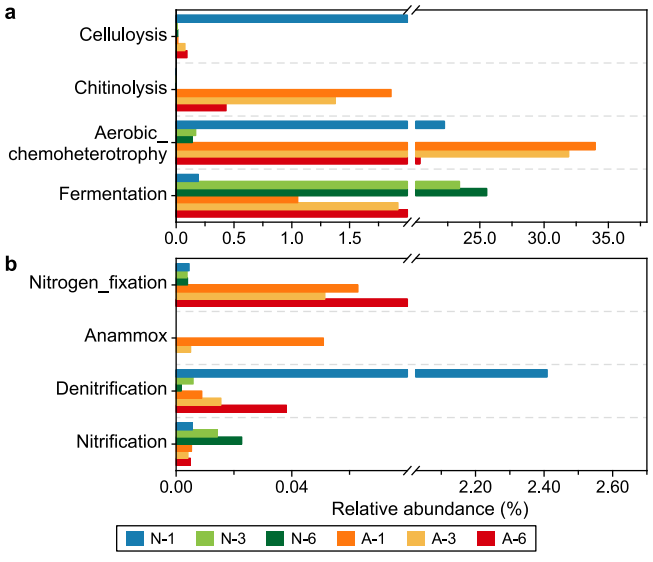

Fig. 5. C- (**a**) and N- (**b**) related ecological functions prediction.

3.3.3. Microbial metabolic pathway

To further confirm N removal pathways in IVFCWs at different influent substrate concentrations, PICRUSt software and the KEGG database were used to analyze the microbial metabolic processes. The main processes involved in the C-cycle and N-cycle included N metabolism, glutamate metabolism, sucrose metabolism, pyruvate metabolism, and methane metabolism. Biotransformation and biodegradation pathways of the pollutants, microbial metabolic intensity, and related metabolic genes are shown in Fig. 6.

At stage I (NO_3^--N system), the NO_3^--N degradation pathways included denitrification, DNRA, and assimilatory nitrogen reduction (ANRA). Firstly, NO_3^--N was reduced to NO_2^--N by nitrate reductases (*NR*). *NR* had three distinct types: respiratory nitrate reductase (*Nar*), periplasmic nitrate reductase (*Nap*), and assimilatory nitrate reductase (*Nas*), respectively [55]. *Nar* was severely restricted by oxygen associated with anaerobic denitrification [56]. *Nap* could be expressed under aerobic and anaerobic conditions related to aerobic denitrification [57]. Comparing the relative abundances of *Nar* and *Nap*, it was confirmed that anaerobic denitrification was the predominant denitrification process in the N-6 and N-3 systems, while aerobic denitrification was the primary denitrification process in the N-1 system. Secondly, nitrite reductase (*Nir*) was essential for the NO_2^--N degradation, including *nirK*, *nirS*, *nirB*, *nirD*, *nrfA*, and *nirA* genes. Among these genes, *NirK* and *nirS* were involved in the production of nitric oxide; *nirB*, *nirD*, and *nrfA* played essential roles in DNRA, and *nirA* was one of the essential ANRA genes. The two processes could both convert NO_3^--N to NH_4^+-N, but there was some difference between them. NH_4^+-N produced by DNRA could be used to grow DNRA bacteria or released into the extracellular environment to grow other bacteria under anoxic or hypoxic conditions [58]. NH_4^+-N produced by ANRA was involved in glutamate metabolism under oxic and anoxic conditions [59]. Both processes occurred in the NO_3^--N dominated system, but the abundance of DNRA-related genes was higher than ANRA-related genes in the N-1 system, whereas the opposite result was observed in the N-6 and N-3 systems. In the C cycle, sucrose (the C source in this study) was first degraded to pyruvate through sucrose and pyruvate metabolism. Subsequently, lactate dehydrogenase (LDH) transformed pyruvate into L-lactate in the N-6 and N-3 systems. In the N-1 system, pyruvate was metabolized into acetyl-CoA and acetate before entering the tricarboxylic acid cycle (TCA cycle), methane metabolism, or other pathways.

At stage II (NH_4^+-N system), the NH_4^+-N transformation pathways included microbial N assimilation, nitrification, and anammox. Microbial N assimilation was a process in which NO_3^--N and NH_4^+-N were utilized by microorganisms to synthesize organic N compounds, such as L-glutamate and L-glutamine [60,61]. NH_4^+-N was the preferred inorganic N source for microorganisms, which could be obtained directly from the environment or produced by amino acid deamination. The relevant genes were *glnA*, *gudB*, *GLUD1_2*, *gdhA*, *gltB*, and *gltD*. The relative abundance of these genes indicated that the glutamine synthetase—glutamate synthase pathway (GS-GOGAT) was the primary microbial N assimilation process [62]. Ammonia monooxygenase (*Amo*) and hydroxylamine oxidoreductase (*Hao*) were the critical genes involved in nitrification and

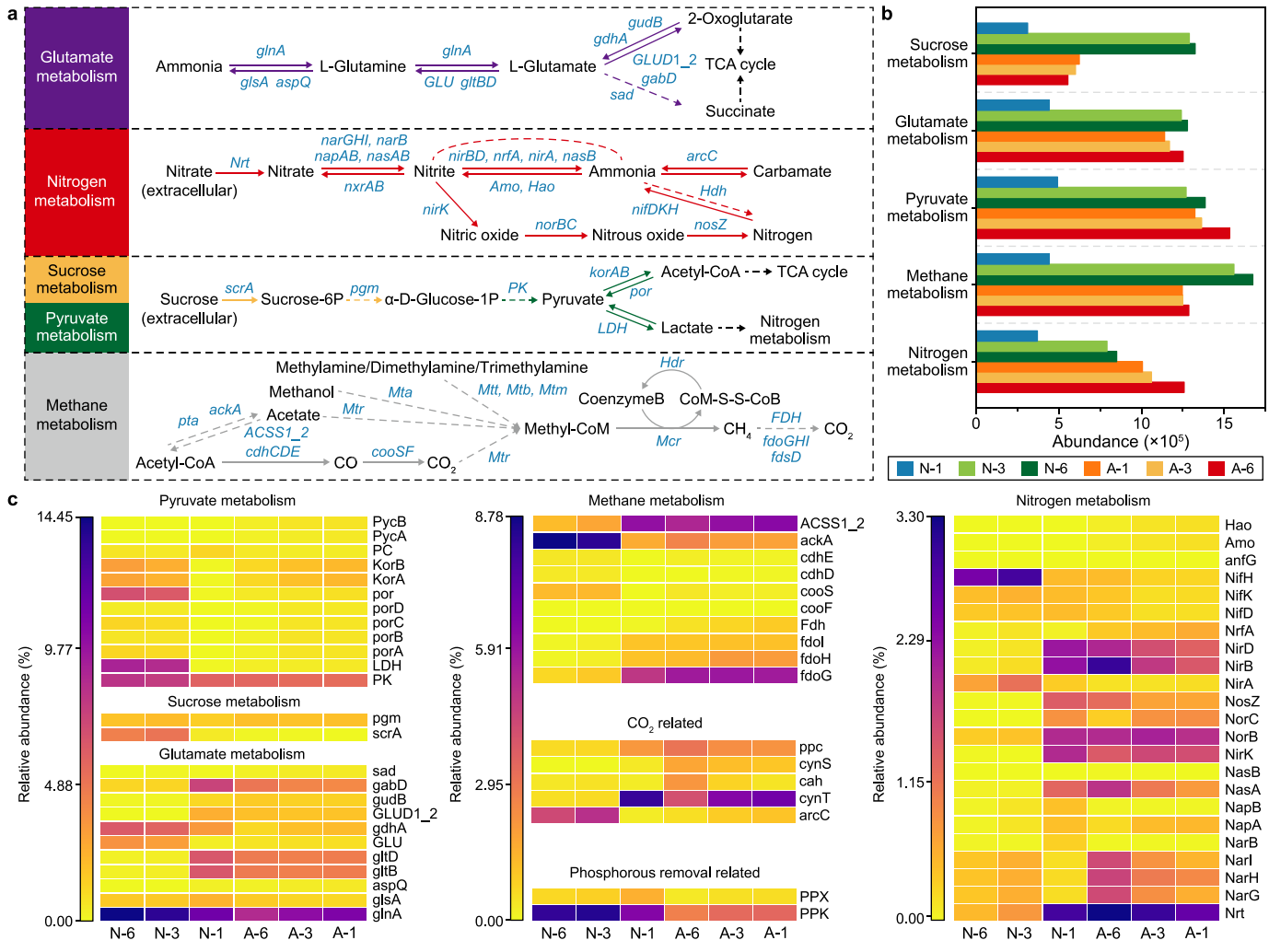

Fig. 6. **a**, Microbial metabolic pathways-flow chart. **b**, Metabolic intensity. **c**, Relative abundance of functional genes.

showed a positive relationship with the decreasing C/N ratios. Abundant denitrification genes and insufficient nitrification genes verified that nitrification was the limited process in the NH_4^+-N systems. For C degradation, C source pathways in the NH_4^+-N systems differed from those in the NO_3^--N systems. Genes related to CO_2 synthesis, including *FDH*, *fdoD*, *fdo*G, *fdo*H, and *fdo*I, were highly expressed in the NH_4^+-N systems. Combined with the COD removal efficiencies of the two systems, it concluded that more C sources were used to produce carbon-containing gases in the NH_4^+-N systems, whereas most C sources were utilized in denitrification and microbial reproduction in the NO_3^--N systems. This explained why adding external carbon sources was unsuitable for improving TN removal with the NH_4^+-N-dominated wastewater in IVFCWs. For the treatment of NH_4^+-N-dominated wastewater, more attention should be paid to the pathways of carbon utilization and adequate supply of DO and alkalinity. Methods, such as carbon source separation, CWs coupled with bioelectrochemical systems, and the aggregation of anammox bacteria in CWs, could be considered.

3.4. Contribution of different nitrogen removal pathways

Based on the results of the above analysis, the main microbial N transformation pathways in six IVFCWs are shown in Table 2. N mass balance calculations in this study included plant assimilation, microbial assimilation, and microbial conversion. N removal through plant assimilation was calculated using equations (3) and (6) in Section 2.3, and the results were shown in Table S3 and Fig. S8. N uptake by plants accounted for 0.92–4.90% of the TN removal.

Table 2
Main nitrogen removal pathways in different IVFCWs.

Systems	Main nitrogen transformation pathways
N-6	Anaerobic denitrification, Microbial assimilation, DNRA, SND
N-3	Anaerobic denitrification, Microbial assimilation, DNRA
N-1	Microbial assimilation, HNAD, DNRA
A-6	Microbial assimilation, HNAD
A-3	Microbial assimilation, HNAD
A-1	Microbial assimilation, HNAD, anammox

Owing to the abundant N_2 in the atmosphere, it was difficult to calculate the contribution of denitrification to TN removal directly through the emission of nitrogen-containing gases from IVFCWs. Therefore, the contribution of microbial assimilation to TN was first calculated with the carbon content of microbial proliferation based on calculation equations (4) and (6). Then, the contribution of denitrification was evaluated by an indirect calculation based on equations (5) and (6). The amounts of gases generated during the microbial conversion process are shown in Table S4 and Fig. S9. The

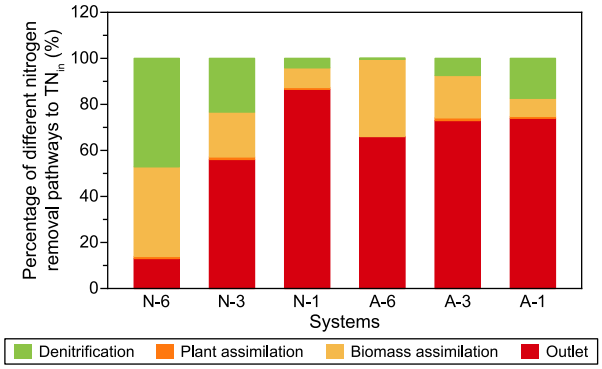

Fig. 7. Contributions of different nitrogen pathways.

emission of C-containing gas was increased with the increasing C/N ratios. The N_2O emission flux was first increased and then decreased with the increasing C/N ratios in the NO_3^--N systems, while it was increased with the increasing C/N ratios in the NH_4^+-N systems.

The contribution of denitrification was affected by both the C/N ratio and the N source forms (Fig. 7). In the NO_3^--N system, the contribution of denitrification increased with the increment of the C/N ratio, and the contributions of denitrification in the N-6, N-3, and N-1 systems were 47.12%, 23.33%, and 4.12%, respectively. In the NH_4^+-N systems, the contribution of denitrification to TN removal decreased with the increasing C/N ratio. The highest contribution of denitrification to TN removal was 17.35% in the A-1 system. The contribution of microbial N assimilation was primarily affected by the C/N ratio, and it was slightly higher in the NO_3^--N systems than in the NH_4^+-N systems. When the C/N ratios were 6, 3, and 1, the contributions of N assimilation to TN removal were 33.55–38.96%, 18.40–19.50%, and 7.83–8.51%, respectively. Since microbial N assimilation would lead to biological clogging [63], and was not conducive to the long-term stable operation of IVFCWs, enhancing the TN removal capacity by increasing the influent C/N ratio was not a good strategy.

4. Conclusions

The N removal pathways were significantly affected by influent substrate concentration. Denitrification and microbial N assimilation were the two main N removal pathways in IVFCWs. The contribution of denitrification to TN removal was affected by the C/N ratios and N source types, which accounted for 4.12–47.12% in the NO_3^--N systems and 0.55–17.35% in the NH_4^+-N systems. Microbial N assimilation was mainly affected by the C/N ratio. When the C/N ratios were 6, 3, and 1, the contributions of microbial N assimilation to TN removal were 33.55–38.96%, 18.40–19.50%, and 7.83–8.51%, respectively. Under the same C/N ratio conditions, better N removal efficiencies could be obtained with a high C/N ratio ($\geq$3) in the NO_3^--N systems and a low C/N ratio (=1) in the NH_4^+-N systems. Enhanced N removal strategies of IVFCWs should focus on the problem of carbon source deficiency and the electron competition between DNRA and denitrification in the NO_3^--N dominated systems. At the same time, the main concerns should be the carbon utilization pathways and the adequate supply of DO and alkalinity in the NH_4^+-N-dominated systems.

CRediT authorship contribution statement

Tongtong Liu: Writing - Original Draft, Investigation, Methodology, Visualization. **Da Li:** Writing - Review & Editing, Funding Acquisition. **Yan Tian:** Project Administration, Investigation. **Jiajie Zhou:** Writing - Review & Editing. **Ye Qiu:** Investigation. **Dongyi Li:** Investigation. **Guohong Liu:** Writing - Review & Editing, Project Administration, Funding Acquisition. **Yujie Feng:** Supervision, Project Administration, Funding Acquisition.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

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