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Nitrogen Metabolism Pathway in Dry Wet Alternating Constructed Wetlands

Jing Zhu[1,3], Xinyong Chen[1,3]*, Jianjian Lu[2], Yuguo Zhou[1,3], Zhongxu Wang[1], Yingying Chen[4]

[1]Hebei University of Environmental Engineering, Qinhuangdao 066102, China
[2]School of Ecological and Environmental Sciences, East China Normal University, Shanghai 200062, China
[3]Hebei Engineering Research Center for ecological rehabilitation of rivers and coastal waters, Qinhuangdao City, Hebei 066102, China
[4]Middling coal Technology and Industry Group Hangzhou Research Institute Co., Ltd, Hangzhou City, Zhejiang 311200, China

Research Article

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***For Correspondence:**
Xinyong Chen, Hebei University of Environmental Engineering, Qinhuangdao 066102, China
E-mail: chenxinyong2007@163.com

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ABSTRACT

This article uses metagenomic sequencing technology, using the nitrogen metabolism pathways of COG and KEGG databases as tools, and groups functional genes under different dry wet alternation conditions (DAWT4h, DAWT8h, DAWT12h) as the research object to analyse the main microbial species and functional genes that affect the nitrogen metabolism pathway in the dry wet alternation artificial wetland ecosystem. Based on the KEGG metabolic pathway hierarchy and the abundance analysis of nitrogen metabolism related functional genes, a nitrogen metabolism pathway in constructed wetlands was constructed. The gene annotation results show that the abundance of various functional genes increases with the increase of DWAT, indicating that increasing the dry wet alternation time can increase microbial diversity and functional gene abundance, which is beneficial for pollutant removal. According to metagenomic data analysis, under dry wet alternation conditions, the abundance of nosZ is close to 2%, the abundance of nirK is close to 3%, and the abundance of norB is over 4%. This indicates that the abundance of denitrification functional gene enzymes is relatively high (over 65000 hits), indicating that dry wet alternation is beneficial for nitrogen removal. The analysis of inter group differences in metabolic pathways showed significant differences ($P<0.01$) among the denitrification functional gene enzymes in each group. The abundance of functional gene enzymes in each group showed a "V" shape change with the change of dry wet alternation time. The dry wet alternation in artificial wetlands significantly affected the metabolic pathway of nitrogen.

Keywords: Dry wet alternation; Artificial wetlands; Nitrogen metabolism; COG; KEGG; Metagenomic

INTRODUCTION

Various microorganisms in artificial wetland ecosystems utilize biochemical reactions to consume nutrients in water bodies, and the amount of nitrogen removed through microbial action can account for 60% to 90% of the total nitrogen removal amount [1-5]. The role of microorganisms is very important [6]. Research has found that the alternation of dry and wet conditions can promote microbial interactions in soil, which is beneficial for the increase of mineralized nitrogen, and its activity tends to stabilize, forming its unique adaptation mechanism [7,8]. Huang, et al. research pointed out that microbial communities undergo succession under alternating dry and wet conditions, with the dominant microbial community gradually transitioning from *Proteobacteria* to *Firmicutes* [6]. Research by Lv, et al. shows that the diversity index of microbial population in subsurface flow constructed wetlands is higher than that in tidal flow constructed wetlands [9]. Through the combination of tidal flow and subsurface flow constructed wetlands, the TN removal rate of wetlands is increased by 20% to 30% compared to general wetlands. Juan simulated the changes in gene abundance of denitrifying bacteria (nirS) during the dry wet alternation process under tidal action in the laboratory, and the results showed a decreasing trend from low tidal flats to high tidal flats [10].

Therefore, the alternation of dry and wet conditions is crucial for the diversity of microorganisms, abundance of functional microbial communities, and denitrification efficiency in constructed wetlands, which directly affects the final denitrification effect. However, the research on the microbial ecological characteristics of dry wet alternation in artificial wetlands is not clear enough, and further research is needed on nitrogen metabolism pathways in wetlands [11-13]. This article is based on the basic characteristics of dry wet alternation in artificial wetlands, and adopts metagenomic sequencing technology to study the functional genes and main metabolic pathways of substrate denitrification microorganisms under dry wet alternation conditions in artificial wetlands. The aim is to reveal the adaptation mechanisms of denitrification microorganisms to different environmental factors during dry wet alternation.

MATERIALS AND METHODS

Experimental design

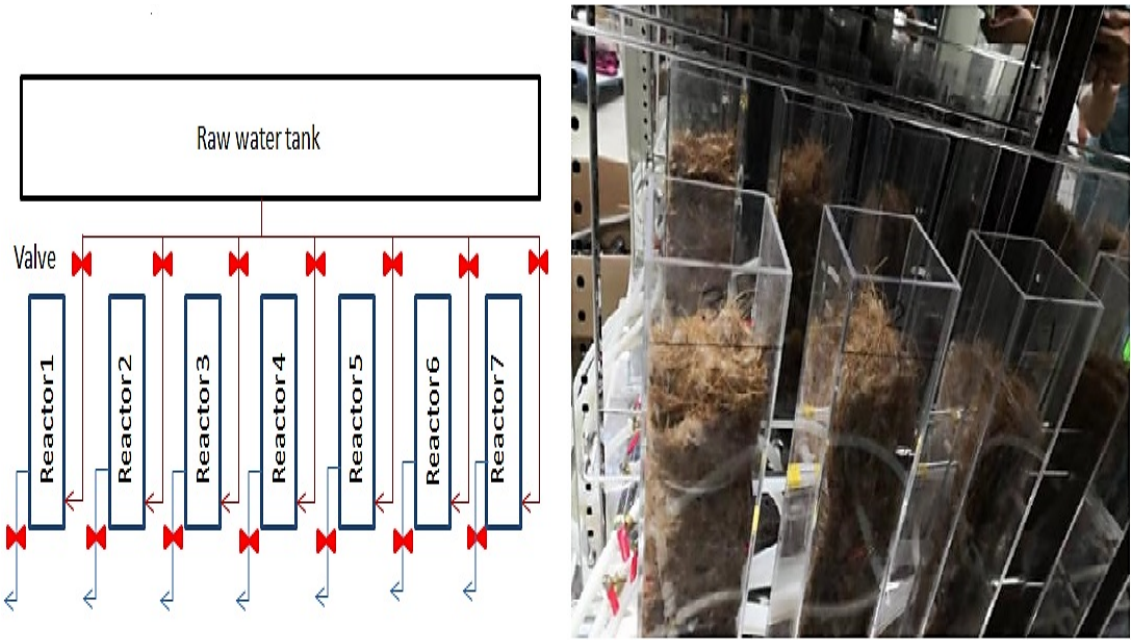
The constructed wetland experimental device is mainly composed of raw water tank and substrate reactor, and the experimental device is placed in a constant temperature box for control (Water Environment Laboratory, Hebei Provincial Academy of Ecological and Environmental Sciences, China) (Figure 1) [12-13].

Matrix: Biocarbon matrix (R1-3, organic fiber material), size 100 mm × 100 mm × 50 mm; gravel (R4), particle size 10 mm-20 mm; zeolite (R5), particle size 20 mm-40 mm; volcanic rock (R6), particle size 20 mm-30 mm.

Raw water: The raw water comes from the tail water of a sewage treatment plant in Shijiazhuang, Hebei Province, China. The tail water is regularly transported back to the laboratory through a transport vehicle in a bucket for standby.

Dry and wet alternation time (DAWT): 4 h, 8 h and 12 h, respectively. The environment condition is 10°C constant temperature control.

Figure 1. Experimental apparatus composed of raw water tank and substrate reactor.



Analysis index and methods

Sample: The overall run time of the experiment was from July 2019 to December 2019. Three parallel samples were collected at each time after the experimental device was running steadily (3 weeks-4 weeks). At the end of each operation, the substrate microbial samples were collected and pre-treated as follows: At each operation, 30 cm^3 to 50 cm^3 of the existing filled substrate in the experimental device was taken out, put into a self-sealing bag and immediately brought back to the laboratory for cold storage at 4°C; The sample is then rapidly oscillated in a cone-shaped bottle until the attachment on the surface of the substrate is shaken off. The suspension is centrifuged by a centrifugal pump, the solids on the top of the filter paper are removed and frozen at -10°C. The samples were sterilized before use and sent to Beijing Berry and Kang Biotechnology Company for metagenome sequencing.

Metagenome sequencing: Library building

DNA extraction: After taking samples in the laboratory, DNA is extracted from the samples by Kit (Omega), and the extracted DNA is transported at low temperature (below 0°C) and sent to the samples for testing (completed by Beijing Berry and Kang Biotechnology Company).

DNA testing: Quantifying DNA concentration using the Qubit method.

Library preparation and library examination: The qualified DNA samples were first cut into fragments (about 350 bp in length), then the library was constructed by purification, PCR amplification, and then quantitative analysis and library dilution were carried out.

On-machine detection: The library carries on illumina PE150 sequencing, obtains the raw data.

Bioinformatics: The data analysis is combined with the results of Shanghai Meiji bio-pharmaceutical Technology Company.

1. Convert the raw data file into the original sequenced reads and store it in the Fastq file format (fq for short).

2. Clean Data was obtained after quality control, analyzed by the megahit assembly software, and ORF was predicted by MetaGene.
3. Gene species annotation and classification, including COG, KEGG, etc.
4. Similar clustering, difference comparison and so on.

Data analysis

1. Using Excel, SPSS and other software to analyse data and mapping with Origin 9.0.
2. SPSS20.0 software was used to analyse the correlation between dry-wet alternation and microbial community.

RESULTS

Results of functional gene annotation of COG

From the annotation results of COG in Figure 2 (Table 1 for the main functional gene symbols), it can be seen that: Among the 24 functional classifications annotated, except for the largest abundance of unknown functional genes (S), amino acid transport and metabolism (E) are the most abundant metabolic types, followed by energy production and transformation (C). In addition, the abundance of biogenesis (M), signal transduction mechanisms (T), coenzyme transport, and metabolism (H) in cell walls/membranes/envelopes is also high, which is conducive to the formation of biofilms within the matrix and the increase of microbial activity. Furthermore, metabolic types such as transcription (K), ribosome translation and structural formation (J), lipid transport, and metabolism (I) are relatively high [14]. Therefore, in the artificial wetland system of this study, the metabolic pathways of functional genes are consistent with the characteristics of microbial biodiversity.

Figure 2. Results of functional gene annotation of COG. **Note:** 4 h; 8 h; 12 h.

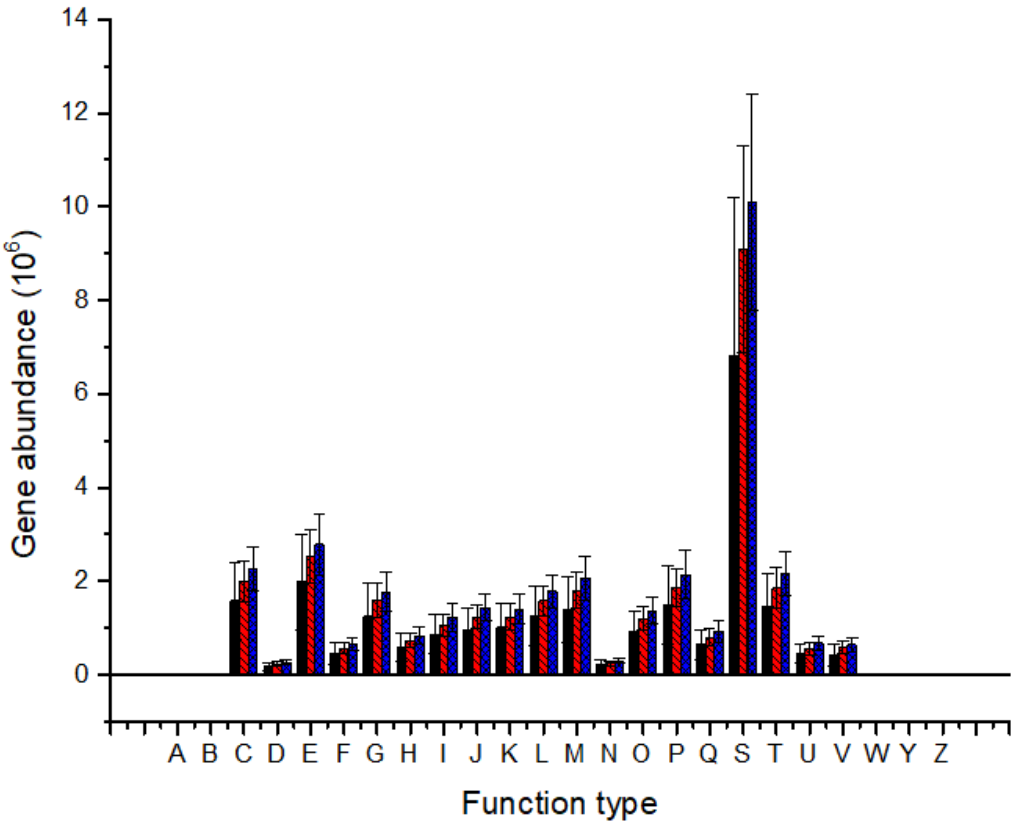


Table 1. The functional gene symbol.

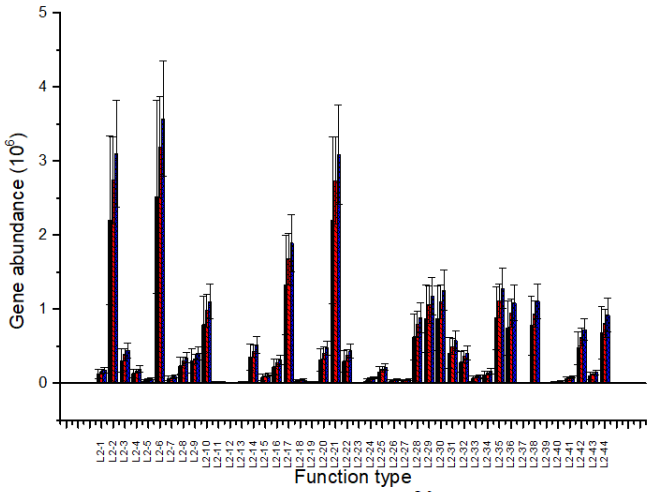
Gene annotation	Description	References
COG Function		
A	RNA processing and modification	-
B	Chromatin structure and dynamics	-
C	Energy production and conversion	-
D	Cell cycle control, cell division, chromosome partitioning	-
E	Amino acid transport and metabolism	-
F	Nucleotide transport and metabolism	[14]
G	Carbohydrate transport and metabolism	-
H	Coenzyme transport and metabolism	[15]
I	Lipid transport and metabolism	-
J	Translation, ribosomal structure and biogenesis	-
K	Transcription	-
L	Replication, recombination and repair	-
M	Cell wall/membrane/envelope biogenesis	-
N	Cell motility	-
O	Post-translational modification, protein turnover, chaperones	-
P	Inorganic ion transport and metabolism	-
Q	Secondary metabolites biosynthesis, transport and catabolism	-
S	Function unknown	-
T	Signal transduction mechanisms	-
U	Intracellular trafficking, secretion, and vesicular transport	-
V	Defense mechanisms	-
W	Extracellular structures	-
Y	Nuclear structure	-
Z	Cytoskeleton	-
KEGG Level 2		
L2-1	Digestive system	-
L2-2	Drug resistance: Antimicrobial	-
L2-3	Endocrine and metabolic diseases	-
L2-4	Endocrine system	-
L2-5	Energy metabolism	[14]
L2-6	Environmental adaptation	-
L2-7	Excretory system	[16]
L2-8	Folding, sorting and degradation	-
L2-9	Global and overview maps	-
L2-10	Glycan biosynthesis and metabolism	-
L2-11	Immune diseases	-
L2-12	Immune system	-
L2-13	Infectious diseases: Bacterial	-
L2-14	Infectious diseases: Parasitic	-
L2-15	Infectious diseases: Viral	-
L2-16	Lipid metabolism	-
L2-17	Membrane transport	-
L2-18	Metabolism of cofactors and vitamins	-
L2-19	Metabolism of other amino acids	-
L2-20	Metabolism of terpenoids and polyketides	-
L2-21	Nervous system	-
L2-22	Neurodegenerative diseases	-
L2-23	Nucleotide metabolism	-
L2-24	Replication and repair	-
L2-25	Sensory system	-
L2-26	Signal transduction	-
L2-27	Signaling molecules and interaction	-
L2-28	Substance dependence	-
L2-29	Transcription	-

Gene annotation	Description	References
L2-30	Translation	-
L2-31	Transport and catabolism	-
L2-32	Xenobiotics biodegradation and metabolism	-

Results of functional gene annotation of KEGG

From the annotation results of the second level of KEGG metabolic pathway in Figure 3 (the main functional gene symbols are shown in Table 1), it can be seen that the functional classification abundance of glucose metabolism (L2-6) is the highest, followed by amino acid metabolism (L2-2), followed by energy metabolism (L2-17, mainly nitrogen and methane metabolism), membrane transport (L2-29), cofactor and vitamin metabolism (L2-30), nucleotide metabolism (L2-35), and signal transduction (L2-38) copy and repair (L2-36), etc [17].

Figure 3. Results of functional gene annotation of KEGG. **Note:** 4 h; 8 h; 12 h.



According to COG analysis, the transport and metabolism (E) abundance of amino acids is the highest, while coenzyme transport and metabolism (H) are higher. KEGG analysis shows that sugar metabolism and amino acid metabolism are the highest, indicating that the amino acid nutrients available for microbial utilization in wetlands are abundant, which is conducive to the growth and activity enhancement of microorganisms; Free amino acids can also serve as intermediate transporters for metabolizing ammonia, playing a positive role in nitrogen metabolism; At the same time, the energy production and conversion (C) abundance of COG and the energy metabolism of KEGG are also high (mainly N and C energy metabolism), indicating that microorganisms have strong nitrogen metabolism characteristics [18,19].

The research results show that the abundance of each functional classification shows an increasing trend with the increase of DWAT, indicating that dry wet alternation can improve the diversity and functional gene abundance of denitrification microorganisms. Microorganisms play a decontamination role while adjusting their own bacterial structure to adapt to changes in different dry wet alternation environments, thereby promoting N metabolism and removal.

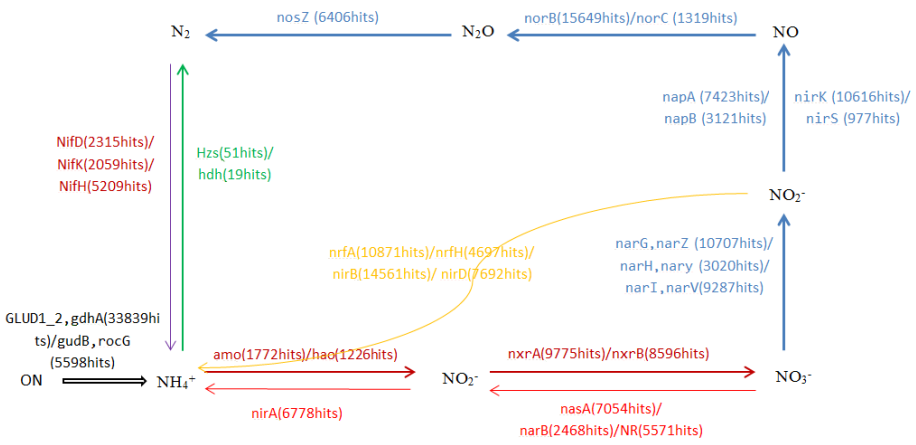
The metabolic pathway of nitrogen

Based on the KEGG metabolic pathway hierarchy and the abundance of nitrogen metabolism related functional genes, nitrogen cycling pathways were analysed, and a nitrogen metabolism pathway map of the constructed

wetland system was constructed in this paper, as shown in Figure 4 [14,17,20,21]. It can be seen that the functional gene abundance of nitrogen reductase is the highest.

1. Ammonia nitrogen oxidation process: The relative abundance of functional genes for aerobic ammonia oxidizing bacteria enzymes is amo 1772 hits and hao 1226 hits.
2. Nitrification process: The abundance of functional genes for nitrite oxidase is nxrA 9775 hits and nxrB 8596 hits, indicating that the system has good nitrification performance.
3. Denitrification process: Abundance of nitrate reductase narG/narZ (10707 hits), narH/nary (3020 hits), narI/narV (9287 hits); The abundance of functional genes for nitrite nitrogen reductase, nirK (10616 hits), nirS (977 hits), napA (7423 hits), napB (3121 hits); The abundance of NO reductase functional genes norB (15649 hits), norC (1319 hits); the functional gene abundance of N_2O reductase nosZ (6406 hits). This indicates that the system has good denitrification performance.
4. The abundance of functional genes Hzs (51 hits) and HDh (19 hits) in anaerobic ammonia oxidizing bacteria indicates the presence of anaerobic ammonia oxidation process in this system.
5. In addition, it was found that the abundance of nitrogen fixing enzyme genes was NifD (2315 hits), NifK (2059 hits), and NifH (5209 hits); Assimilation reduction of nitrate to the abundance of functional genes for ammonia nitrogenase, nasA (7054 hits), narB (2468 hits), NR (5571 hits); Alienation reduction of nitrate to the abundance of ammonia nitrogenase functional genes nirA (6778 hits); The abundance of phosphate dissimilatory reductase functional genes nrfA (10871 hits), nrfH (4697 hits), nirB (14561 hits), and nirD (7692 hits). The above indicates that there are abundant types of nitrogen functional enzyme genes.

Figure 4. Nitrogen transformation pathways. **Note**: $N_2 \rightarrow NH_4^+$ in presence of nitrogenase: nifD, nifK, nifH, ferritin genes; $NH_4^+ \rightarrow NO_2^-$ in presence of ammonia monooxygenase gene: amo (A/B/C); $NO_2^- \rightarrow NO_3^-$ in presence of nitrite oxidoreductase gene: nxrA, nxrB; $NO_3^- \rightarrow NO_2^-$ in presence of membrane-bound nitrate reductase: narG, narZ, narH, nary, narI, narV; $NO_3^- \rightarrow NO_2^-$ in presence of periplasmic-bound nitrate reductase: napA, napB; $NO_2^- \rightarrow NO$ in presence of copper-containing nitrite reductase: nirK; $NO_2^- \rightarrow NO$ in presence of cytochromecd1-containing nitrite reductase: nirS; $NO \rightarrow N_2O$ in presence of nitric oxide reductase: norB, norC; $N_2O \rightarrow N_2$ in presence of nitrous oxide reductase: nosZ; $NO_2^- \rightarrow NH_4^+$ in presence of dissimilatory nitrate reductase: nrfA, nrfH, nirB, nirD; $NO_3^- \rightarrow NO_2^-$ in presence of assimilatory nitrate reductase: nasA, narB, NR; $NO_2^- \rightarrow NH_4^+$ in presence of nirA; $NH_4^+ \rightarrow N_2$ in presence of anaerobic ammonium oxidase: Has, hdh[9,14].



DISCUSSION

Microbial annotation of nitrogen metabolism pathway function

This study found that under the condition of dry wet alternation, the abundance of functional gene enzymes in each group showed a "V" shape change with the change of dry wet alternation time, which is consistent with the nitrogen removal efficiency [13]. At 8 hours of DAWT, there is a significant difference in microbial abundance, with the highest abundance of denitrifying functional bacteria such as *Sphingomonas* and *Sphingobium*; The abundance of denitrification functional gene enzymes is relatively high (over 65000 hits), including narG/narZ (10707 hits), narH/nary (3020 hits), narI/narV (9287 hits), nirK (10616 hits), nirS (977 hits), napA (7423 hits), napB (3121 hits), norB (15649 hits), norC (1319 hits), nosZ (6406 hits); The above indicates that microorganisms adjust their own bacterial structure to adapt to changes in different dry wet alternating environments, thereby exerting better nitrogen removal effects [22].

The dry wet alternation time significantly changes the species and functional gene abundance of nitrogen metabolism pathways in artificial wetlands. Bacteria in the dry wet alternation artificial wetlands exhibit greater denitrification potential, which is speculated to be related to the impact of dry wet alternation on anaerobic/aerobic processes in wetlands [14,23]. At the same time, the abundance of microbial nitrogen metabolism functional genes is relatively high at DAWT8h compared to DAWT4h and DAWT12h, indicating that bacteria in the dry wet alternate artificial wetland have the best nitrogen removal potential. Therefore, the detected species can be used as marker species for the study of nitrogen metabolism microbial diversity in dry wet alternation constructed wetlands, and functional genes can be used as markers to detect microbial gene abundance and its response to environmental factors in constructed wetlands, providing convenient conditions for further research on the nitrogen metabolism microbiome of this wetland system [23].

Analysis of differences in nitrogen metabolism pathways

Based on the KEGG annotation results, functional genes were grouped under different dry wet alternation conditions (DAWT4h, DAWT8h, DAWT12h), and differential enzymes in the nitrogen metabolism pathway were tested and visualized to identify the impact of dry wet alternation on functional genes.

From Figure 5 of the nitrogen metabolism pathway and Figure 6 of the differential detection of metabolic pathway composition, it can be seen that there is a significant difference ($P<0.01$) between the denitrification functional gene enzymes 1.7.2.2 (narB, iron oxidoreductase) and 1.7.2.4 (nosZ, N_2O reductase), indicating that dry wet alternation significantly affects the abundance and changes of denitrification functional gene enzymes. However, it was also found that the abundance of functional gene enzymes in the DAWT8h and DAWT12h groups was relatively close, with significant differences compared to DAWT4h, and consistent with the peak ("V"/inverted "V") trend of nitrogen concentration or removal efficiency at DAWT8h, which is consistent with the RDA analysis that external conditions NH_3-N (AN) and TN are the main factors affecting the distribution of bacterial communities [13]. Similarly, the alternation of dry and wet conditions affects the abundance of nitrogen removal gene enzymes in constructed wetlands, thereby affecting nitrogen concentration and removal efficiency.

Meanwhile, as shown in Figure 6, the abundance of nosZ is close to 2%, the abundance of 1.7.2.1 (nirK, nitrite reductase gene, catalyzing $NO_2^- \rightarrow NO$) is close to 3%, and the abundance of 1.7.2.5 (norB, NO reductase functional gene, catalyzing $NO \rightarrow N_2O$) is over 4%, indicating that the abundance of denitrification functional gene enzymes is relatively high, indicating that dry wet alternation is beneficial for nitrogen removal.

In summary, the alternation of dry and wet conditions in constructed wetlands significantly affects the abundance and changes of nitrogen removal functional gene enzymes, affecting the metabolic pathway of nitrogen. The abundance of functional gene enzymes in the DAWT8h and DAWT12h groups is relatively close, with a significant difference compared to the DAWT4h group.

Figure 5. Nitrogen metabolism pathway in dry wet alternating constructed wetlands.

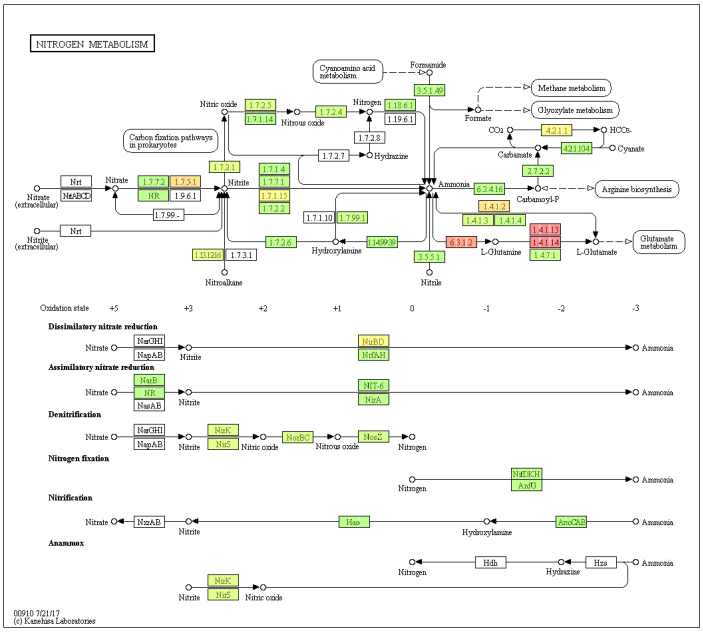
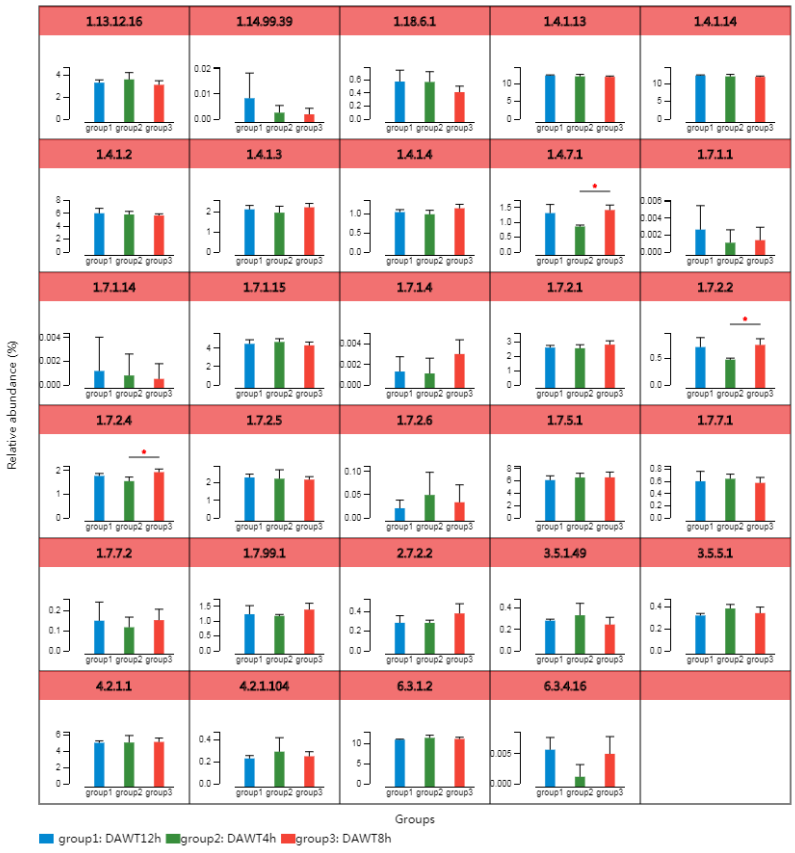


Figure 6. Differential detection map of Nitrogen metabolism pathway between groups. **Note:** Group 1: DAWT12h; Group 2: DAWT4h; Group 3: DAWT8h.



The effect of dry wet alternation on microbial nitrogen metabolism

The changes in nitrogen concentration and form in the constructed wetland with dry wet alternation can lead to changes in the nitrogen metabolism pathway of the systems microorganisms. The absorption and utilization mechanisms of nitrogen nutrients by microorganisms and their molecular responses exhibit different physiological activities and growth conditions to better adapt to changes in different dry wet alternation environments [21,24,25].

There are complete nitrogen metabolism pathways such as ammonia oxidation, nitrification, denitrification, and anaerobic ammonia oxidation in the constructed wetland with alternating wet and dry conditions. *Flavobacterium*, *Hydrogenophaga*, *Pseudomonas*, and other denitrifying bacteria are the most commonly distributed genera in wetlands (with a total abundance of 26.76%), and narG/narZ (10707 hits), nirK (10616 hits), norB (15649 hits), norC (1319 hits) are the main functional genes for nitrogen metabolism. As the dry wet alternation time changes, the concentration or removal efficiency of functional genes and nitrogen change are consistent with the peak ("V"/inverted "V") trend of DAWT at 8 h, indicating a certain degree of synergy between nitrogen removal and wetland microbial nitrogen metabolism functional genes [13,21]. The analysis of inter group differences in metabolic pathways shows that the alternation of dry and wet conditions in artificial wetlands significantly affects the metabolic pathway of nitrogen, which is beneficial for maintaining the nitrogen metabolism activity and greenhouse gas emissions balance of artificial wetlands. It has certain guiding significance for the sustainable use and fertilizer conservation and emission reduction of artificial wetlands.

CONCLUSIONS

1. Based on the KEGG metabolic pathway hierarchy, this article establishes a nitrogen metabolism pathway in a dry wet alternative constructed wetland.
2. The gene annotation results show that the abundance of various functional genes increases with the increase of DWAT, indicating that increasing the dry wet alternation time can increase microbial diversity and functional gene abundance, which is beneficial for pollutant removal.
3. According to the analysis of metagenomic data, under dry wet alternation conditions, the abundance of nosZ is close to 2%, the abundance of nirK is close to 3%, and the abundance of norB is over 4%. This indicates that the abundance of denitrification functional gene enzymes is relatively high (over 65000 hits), indicating that dry wet alternation is beneficial for nitrogen removal.
4. The analysis of inter group differences in metabolic pathways showed significant differences in denitrification functional gene enzymes among different groups ($P<0.01$). The abundance of functional gene enzymes in each group showed a "V" pattern with changes in dry wet alternation time. The dry wet alternation in artificial wetlands significantly affected the metabolic pathway of nitrogen.

DECLARATIONS

Funding

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Competing interests

The authors have no relevant financial or non-financial interests to disclose.

Ethics approval

This is an observational study. The China Research Ethics Committee has confirmed that no ethical approval is required.

Consent to participate

This is an observational study, the research does not involving human subjects.

Consent to publish

The authors affirm that the research does not involving human research participants.

Data and material availability

The datasets generated and/or analyzed and material and software used during the current study are available from the corresponding author on reasonable request.

Author contributions

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jing Zhu, Xinyong Chen, Jianjian Lu, Yuguo Zhuo, Zhongxu Wang, Yingying Chen. The first draft of the manuscript was written by Xinyong Chen and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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