# Effect of dry-wet alternation on denitrogen bacteria in constructed wetland

**Jing Zhu**
Hebei Provincial Academy of Ecological and Environmental Sciences
**Xinyong Chen** ( chenxinyong2007@163.com )
Hebei Provincial Academy of Ecological and Environmental Sciences
**Jianjian Lu**
East China Normal University
**Zaifeng Tian**
Hebei Provincial Academy of Ecological and Environmental Sciences
**Yihong Wu**
Hebei Provincial Academy of Ecological and Environmental Sciences
**Qi Zhao**
Hebei Provincial Academy of Ecological and Environmental Sciences

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## Abstract

The metagenome sequencing technique was used to study the effect of dry-wet alternation on denitrogen bacteria in constructed wetland. The results showed that the dry-wet alternate condition had a great effect on the abundance of denitrogen bacteria in the system. With the increase of dry-wet alternation time (DWAT4h-DWAT8h-DWAT12h), the number of nitrifying bacteria showed an inverted "V" pattern, the distribution of denitrifying bacteria showed an inverted "V" pattern at 10°C, a "V" pattern at 20°C, and a stable pattern at 30°C (both appeared peak at DWAT8h). The DWAT was important factors affecting the distribution of denitrogen bacteria ($P < 0.05$). RDA Analysis showed that $NH_3$-N (AN) and TN were significantly correlated with denitrogen bacteria ($P < 0.05$) and were the main factors affecting the distribution of denitrogen bacteria. Reasonable control of dry-wet alternation time was crucial to increase the number of denitrogen bacteria and denitrogen efficiency.

## Introduction

Dry and Wet Alternate is one of the most common water ecological processes in nature. Natural factors such as storm runoff, tidal process, continuous rainfall or drought evaporation, and man-made factors such as artificial water transfer, it is easy to cause the wetland hydrology situation change, causes the wetland to present the dry-wet alternation phenomenon. As a basic feature of wetland, the alternation of moisture is coupled with the cycle of nutrient salt[1–2]. The effect of dry-wet alternation on nitrogen transport and transformation is significant [3–5], to achieve the purification capacity of reclaimed wetlands. In the constructed wetland system, oxygen in the air can be inhaled into the wetland substrate to improve the dissolved oxygen in the wetland through periodic submergence/drainage, further improvements in denitrogen (nitrogen removal) efficiency [6–9].

Various microorganisms in constructed wetland systems use biochemical reactions to deplete nutrients in water bodies [10–11], the amount of denitrogen through microbial interaction can account for 60% ~ 90% of the total denitrogen [12–14], the role of microorganisms is very important [15]. Dry-wet alternate conditions were found to promote microbial activity in the soil, increase its mineralized nitrogen[16], and stabilize its activity [17], forming its own adaptive mechanism [18]. Zhuang Linjie pointed out that the microbial community succession occurred under the condition of dry-wet alternation, and the dominant microbial community changed from Proteobacteria to Firmicutes. The results show that the microbial diversity index in the subsurface flow constructed wetland is higher than that in the tidal flow constructed wetland. Through the combination of the tidal flow and the subsurface flow constructed wetland, the removal rate of TN in the subsurface flow constructed wetland is increased by 20%  30% than that in the general constructed wetland [19]. Therefore, dry-wet alternation is very important for the diversity of microorganism, the abundance of functional bacteria and the efficiency of denitrogen in constructed wetland, which directly affects the final denitrogen efficiency.

In recent years, with the development and application of molecular biology techniques such as high-throughput DNA sequencing technology and metagenome sequencing technology[13,20–21], the important progress has been made in the functional microbial abundance, community structure, and spatial and temporal distribution of constructed wetlands, which has greatly improved people's understanding of the diversity of denitrogen bacteria in constructed wetlands. However, the study on microbial ecological characteristics of dry-wet alternation in constructed wetlands is not clear enough, and there is no report on the effect of dry-wet alternation on denitrogen bacteria in constructed wetland.

In this paper, based on the basic characteristics of dry-wet alternation of constructed wetland, the constructed wetland ecosystem was constructed by using degradable, supplementary carbon source and large specific surface area materials made of coconut fiber and Straw, to explore the ecological effect of substrate microorganism in wetland under the dynamic process of dry-wet alternation. A Dry and Wet Alternate (DWA) experiment was designed to simulate the aerobic/anaerobic environment of natural wetlands, in order to reveal the adaptation mechanism of denitrogen bacteria to different environmental factors, the community structure of denitrogen bacteria under dry-wet alternate condition in constructed wetland was studied.

## Methods And Materials

# Experimental design

(1) Experimental device of constructed wetland

The experimental device is mainly composed of raw water tank and substrate reactor, and the experimental device is placed in a constant temperature chamber for control, as shown in Fig. 1.

1) Structure Size: single device 100mm×100mm×700mm, altogether 6 groups, placed in the constant temperature chamber, unified water supply by the raw water tank, each device alone, through the control valve to control the inflow and outflow of water.

2) substrate: bio-carbon substrate (organic fiber material), dimensions 100mm×100mm×50mm.

3) monomer structure: The bio-carbon substrate was composed of three types of Straw and Coir, which were respectively placed in the 1–6 unit.

4) water distribution: The inlet water is controlled by the valve from the bottom to the experimental device, and the outlet water is controlled by the outlet valve. The raw water comes from the tail water of the Sewage Treatment in a town in north China and is regularly transported back to the laboratory in a bucket by a truck.

(2) Experimental mode of operation

1) Operating Load: DAWT is 4h, 8h, 12h, and HRT is 4h, 8h, 12h.

2) The operating conditions of the dry-wet alternate experiment are detailed in Table 1.

Table 1
Operation Mode and control condition of laboratory

| No. | DAWT | inlet/outlet mode | Control condition |
|---|---|---|---|
| 1#-6# | 4h | The dry/wet interval is 4h, i.e. the first hour is full of water, the fourth hour is 1/2V, the eighth hour is 1/ 2V, the 12th hour is 1/2V, the 16th hour is 1/2V,... | The samples were collected at 10°C, 20°C and 30°C for 3 weeks |
| | 8h | The dry/wet interval is 8h, i.e. the first hour is full of water, the eighth hour is 1/2V, the 16th hour is 1/ 2V, the 24th hour is 1/2V, the 32th hour is 1/2V,... | |
| | 12h | The dry/wet interval is 12h, i.e. the first hour is full of water, the 12th hour is 1/2V, the 24th hour is 1/ 2V, the 36th hour is 1/2V, the 48th hour is 1/2V,... | |
| 7# | Ditto | Continuous flow in and out of water, not dry or wet | Ditto |

# 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 (3–4 weeks). At the end of each operation, the substrate microbial samples were collected and pretreated 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 Co. for metagenome sequencing.

# Metagenome sequencing

(1) Library building

1)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 Co.).

2)DNA testing: Quantifying DNA concentration using the Qubit method.

3)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.

4)On-machine detection: The library carries on Illumina PE150 sequencing, obtains the Raw Data.

(2) Bioinformatics

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

# Determination of water sample

Hydrographic $NH_3$-N, $NO_2$-N, $NO_3$-N, and TN were all determined by the Laboratory Continuous Flow Chemical Analyzer (Auto Analyzer 3).

# Data analysis

(1) Using Excel, SPSS and other software to analyze data and mapping with Origin9.0. (2) SPSS20.0 software was used to analyze the correlation between dry-wet alternation and microbial community. (3) Redundancy analysis between environmental factors and Microbial Flora (RDA). Firstly, we used COG/KEGG/gene or species abundance table to analyze DCA, then got the environmental factor subset, analyzed the species/function distribution table with environmental factor or environmental factor, and judged the significance of RDA by permutest analysis similar to ANOVA.

## Results

# Diversity of microbial community structure

By using the Megahit (version 1.12) assembly software, a total of 14254368 contigs were obtained, and the total sequence length predicted by metaGene was 9282883724bp. This study was conducted at the level of phylum and genus according to the experimental conditions.

At phylum level, the abundance of Proteobacteria was more than 59.87%, Bacteroidetes was more than 5.77%, Actinobacteria was more than 4.88%, Acidobacteria was more than 3.39%, and planktomycetes was more than 3.25%, see Fig. 2(a). With the dry-wet alternation time (DAWT 4h/8h/12h, see Fig. 2(b)), Proteobacteria showed a "V" pattern, while other bacteria, include Nitrospirae showed an inverted "V" pattern.

At genus level, the abundance of Mesorhizobium, Hyphomicrobium, Bradyrhizobium, Hydrogenophaga and Pseudoxanthomonas were more than 3.08%, 2.70%, 1.92%, 1.70% and 1.15%, respectively, see Fig. 3(a). With the dry-wet alternation time (DAWT 4h/8h/12h,Fig. 3(b)), the main microbial species showed a decreasing trend at genus level: Mesorhizobium, Hyphomicrobium, Hydrogenophaga, Bradyrhizobium and Genophaga showed an inverted "V" decreasing

trend, the number of denitrifying bacteria such as Sphingomonas and Sphingobium increased in a "V" pattern, while the number of ammonia-oxidizing bacteria such as Nitrotomonas and Nitrosospira increased in a "V" pattern. It can be seen that the abundance of microorganisms varied greatly at DAWT 8h, which indicated that the microbes were adjusting the structure of their own genus to adapt to the change of dry-wet alternate environment.

## Community distribution characteristics of Denitrogen bacteria

The genus level distribution of denitrogen bacteria is shown in Table 2 and Fig. 4, the total abundance of denitrifying bacteria is more than 10.0%. Among them, Nitrosomonas, Nitrosospira and Nitrosococcus are the main ammonia-oxidizing bacteria, the total abundance is 0.18%, the proportion of denitrogen bacteria is about 1.8%. Nitrospira, Nitrospira and Nitrococcus are the main nitrifying bacteria, the total abundance is 0.72%, the proportion of denitrogen bacteria is about 7.1%. The main denitrifying bacteria, such as Pseudomonas, Pseudomonas, chloomonas, Flavobacterium, Rubrivivax, Bacillus, Pedobacter, Thauera, Hyphomicrobium, Hydrogenophaga, Rhizobium, Azoira and Acidovorax, the total abundance was 9.04%, accounting for about 90.4% of the total denitrogen bacteria. The abundance of Candidatus, Candidatus, Candidatus and Candidatus was 0.07%, which accounted for about 0.7% of the total Anammox. Among all the denitrogen bacteria, denitrifying bacteria were the most abundant, nitrifying bacteria the second, and Anammox the least.

Table 2
The relative abundance of nitrifying bacteria in Genus cell(%)

| Taxonomy | H-121814 | H-121815 | H-121816 | H120521 | H120522 | H120523 | H-112331 | H-112335 | H-112336 |
|---|---|---|---|---|---|---|---|---|---|
| Nitrosomonas | 0.003571 | 0.004048 | 0.006582 | 0.004355 | 0.004333 | 0.006356 | 0.002755 | 0.007518 | 0.007767 |
| Nitrosococcus | 0.004818 | 0.003665 | 0.006676 | 0.004224 | 0.004126 | 0.005666 | 0.002613 | 0.006286 | 0.006902 |
| Nitrosospira | 0.004216 | 0.004019 | 0.005335 | 0.003475 | 0.004108 | 0.00517 | 0.001665 | 0.005384 | 0.005637 |
| Nitrospira | 0.036161 | 0.154328 | 0.07075 | 0.033127 | 0.070847 | 0.060423 | 0.042152 | 0.060783 | 0.025703 |
| Nitrospina | 0.001368 | 0.001577 | 0.002308 | 0.00126 | 0.001281 | 0.001649 | 0.000671 | 0.002243 | 0.00215 |
| Nitrococcus | 0.00154 | 0.001276 | 0.002043 | 0.001223 | 0.001207 | 0.00156 | 0.000495 | 0.001904 | 0.00178 |
| Bacillus | 0.008502 | 0.01072 | 0.012414 | 0.006831 | 0.008149 | 0.009092 | 0.003667 | 0.013039 | 0.011989 |
| Terrimonas | 0.031969 | 0.04859 | 0.04786 | 0.029913 | 0.050711 | 0.030891 | 0.020916 | 0.11891 | 0.068656 |
| Flavobacterium | 0.030308 | 0.022656 | 0.048869 | 0.02005 | 0.023516 | 0.017929 | 0.011496 | 0.040032 | 0.036726 |
| Pedobacter | 0.01716 | 0.015249 | 0.017477 | 0.010259 | 0.014051 | 0.009979 | 0.007036 | 0.026693 | 0.024509 |
| Pseudomonas | 0.066751 | 0.042267 | 0.065602 | 0.038607 | 0.04459 | 0.052721 | 0.021127 | 0.063141 | 0.067214 |
| Rubrivivax | 0.021594 | 0.026745 | 0.029076 | 0.017428 | 0.014441 | 0.017231 | 0.010591 | 0.026823 | 0.027149 |
| Dechloromonas | 0.009804 | 0.007698 | 0.014551 | 0.013937 | 0.019105 | 0.019209 | 0.007687 | 0.013236 | 0.024752 |
| Thauera | 0.013404 | 0.01308 | 0.016417 | 0.011293 | 0.012353 | 0.017332 | 0.007325 | 0.014217 | 0.015694 |
| Hyphomicrobium | 0.255264 | 0.221496 | 0.235097 | 0.262102 | 0.23177 | 0.238969 | 0.085411 | 0.230893 | 0.243098 |
| Hydrogenophaga | 0.194108 | 0.067588 | 0.164097 | 0.113839 | 0.136871 | 0.10198 | 0.116131 | 0.213241 | 0.146844 |
| Rhizobium | 0.085387 | 0.072496 | 0.05181 | 0.047517 | 0.035739 | 0.044031 | 0.01544 | 0.059142 | 0.086671 |
| Azospira | 0.005949 | 0.005127 | 0.008731 | 0.005874 | 0.007195 | 0.013896 | 0.003384 | 0.006305 | 0.01382 |
| Acidovorax | 0.214717 | 0.102938 | 0.131994 | 0.071753 | 0.069273 | 0.075746 | 0.109424 | 0.182268 | 0.120183 |
| Candidatus_Brocadia | 0.002418 | 0.002779 | 0.003385 | 0.001546 | 0.00213 | 0.002533 | 0.000781 | 0.003412 | 0.003058 |
| Candidatus_Jettenia | 0.0009 | 0.001128 | 0.00145 | 0.000578 | 0.000938 | 0.001272 | 0.00044 | 0.001554 | 0.001138 |
| Candidatus_Kuenenia | 0.000877 | 0.001065 | 0.001572 | 0.000752 | 0.000917 | 0.00115 | 0.000412 | 0.001434 | 0.001158 |
| Candidatus_Scalindua | 0.000701 | 0.000911 | 0.001137 | 0.000539 | 0.000665 | 0.000873 | 0.000303 | 0.001156 | 0.000877 |

The composition of denitrogen bacteria in constructed wetland reactor was different under different dry-wet alternate conditions:

(1) At 10°C, compared the abundance of ammonia-oxidizing bacteria, DWAT12h (H-121816) > DWAT4h (H-121814) > DWAT8h (H-121815); Compared with nitrifying bacteria, DWAT8h(H-121815) > DWAT4h(H-121814) > DWAT12h(H-121816); Compared with denitrifying bacteria, DWAT4h(H-121814) > DWAT12h (H-121816) > DWAT8h(H-121815); Compared with Anammox, DWAT12h (H-121816) > DWAT8h (H-121815) > DWAT4h (H-121814).

(2) At 20°C, compared the abundance of ammonia-oxidizing bacteria, DWAT12h (H-121816) > DWAT8h (H-121815) > DWAT4h (H-121814); Compared with nitrifying bacteria, DWAT4h(H-121814) > DWAT12h(H-121816) > DWAT8h(H-121815); Compared with denitrifying bacteria, DWAT8h(H-121815) > DWAT4h(H-121814) > DWAT12h(H-121816); Compared with Anammox, DWAT12h (H-121816) > DWAT8h (H-121815) > DWAT4h (H-121814).

(3) At 30°C, compared the abundance of ammonia-oxidizing bacteria, DWAT12h (H-121816) > DWAT8h (H-121815) > DWAT4h (H-121814);; Compared with nitrifying bacteria, DWAT8h (H-121815) > DWAT4h(H-121814) > DWAT12h(H-121816); Compared with denitrifying bacteria, DWAT8h(H-121815) > DWAT12h

(H-121816) > DWAT4h (H-121814); Compared with Anammox, DWAT12h (H-121816) > DWAT8h (H-121815) > DWAT4h (H-121814).

The results showed that the abundance of DWAT4h and DWAT8h of ammonia-oxidizing bacteria was higher at 12h than at 8h of nitrifying bacteria, and higher at 20 °c than at 4h of DWAT12h of nitrifying bacteria Compared with DWAT4h and DWAT12h, the abundance of DWAT4h and DWAT12h of denitrifying bacteria was highest at 10°C, and that of Anammox was highest at 12h.

# Response Analysis of denitrogen bacteria and dry-wet alternation

(1) Response Analysis at 10°C

Table 3 shows that the main ammonia-oxidizing bacteria (Nitrosomonas, Nitrospira and Nitrosococcus) and Nitrospira (Nitrospira, Nitrospina and Nitrococcus) are significantly correlated with $NO_2$-N, DO and DWAT at 10°C($P < 0.05$), the results showed that the concentration of $NO_2$-N and DWAT affected ammonia oxidation and nitrification to some extent. The main denitrifying bacteria such as Terrimonas, Pseudomonas, Dechloromonas and Flavobacterium were significantly correlated with $NO_3$-N, ON and TOC ($P < 0.05$), which indicated that the concentration of $NO_3$-N affected the growth of Denitrification cells to some extent. The main Anammox bacteria such as Candidatus_Brocadia, Candidatus_Jettenia, Candidatus_kuenenia and Candidatus_Scalindua were significantly correlated with $NH_3$-N, $NO_2$-N, DO and DWAT ($P < 0.05$). The results showed that $NO_2$-N, $NO_3$-N, DO and DWAT were important factors affecting the distribution of denitrogen bacteria in the substrate at 10°C.

Table 3
Analysis of correlation between denitrogen bacteria and environmental factors (10°C)

| Taxonomy | TN | $NH_3$-N | $NO_3$-N | $NO_2$-N | ON | DO | TO |
|---|---|---|---|---|---|---|---|
| Nitrosomonas/Nitrosospira/Nitrosococcus | -0.346 | -0.512 | -0.03 | -0.912* | -0.173 | -0.870* | 0.4 |
| Nitrospira/Nitrospinav/ Nitrococcus | 0.419 | 0.578 | -0.049 | 0.877* | 0.215 | 0.828* | -0.3 |
| Terrimonas/Pseudomonas/Dechloromonas/Flavobacterium /… | -0.698 | -0.555 | 0.915* | -0.763 | -0.835* | 0.820* | -1.0 |
| Candidatus_Brocadia/Candidatus_Jettenia/Candidatus_kuenenia/Candidatus_Scalindua | 0.527 | 0.674* | -0.171 | -0.811* | 0.333 | 0.753* | -0.2 |
| Note: N = 3;*, P < 0.05 **, P < 0.01 | | | | | | | |

(2) Response Analysis at 20°C

Table 4 shows that the main ammonia-oxidizing bacteria (Nitrosomonas, Nitrospira and Nitrosococcus) is significantly correlated with TN, $NO_3$-N, DO and DWAT at 20°C($P < 0.05$). The Nitrospira (Nitrospira, Nitrospina and Nitrococcus) is significantly correlated with $NO_3$-N, $NO_2$-N, DO and DWAT ($P < 0.05$). The main denitrifying bacteria such as Terrimonas, Pseudomonas, Dechloromonas and Flavobacterium were significantly correlated with $NO_3$-N, $NO_2$-N, DO and DWAT ($P < 0.05$). The main Anammox bacteria such as Candidatus_Brocadia, Candidatus_Jettenia, Candidatus_kuenenia and Candidatus_Scalindua were significantly correlated with $NH_3$-N, $NO_2$-N and TOC ($P < 0.05$). The results showed that $NO_2$-N, $NO_3$-N, DO and DWAT were important factors affecting the distribution of denitrogen bacteria in the substrate at 20°C.

Table 4
Correlation between denitrogen bacteria and environmental factors (20°C)

| Taxonomy | TN | $NH_3$-N | $NO_3$-N | $NO_2$-N | ON | DO | |
|---|---|---|---|---|---|---|---|
| **Nitrosomonas/Nitrosospira/Nitrosococcus** | -0.772* | -0.460 | -0.979* | 0.608 | -0.410 | -1.000** | |
| **Nitrospira/Nitrospinav/ Nitrococcus** | -0.608 | -0.248 | -0.908* | 0.999* | -0.193 | -0.974* | |
| **Terrimonas/Pseudomonas/Dechloromonas/Flavobacterium /…** | 0.333 | -0.064 | 0.734* | -0.931* | -0.120 | 0.857* | |
| **Candidatus_Brocadia/Candidatus_Jettenia/Candidatus_kuenenia/Candidatus_Scalindua** | 0.524 | 0.816* | 0.067 | 0.870* | 0.847* | -0.137 | |
| Note: N = 3;*, P < 0.05 **, P < 0.01 | | | | | | | |

(3) Response Analysis at 30°C

Table 5 shows that the main ammonia-oxidizing bacteria (Nitrosomonas, Nitrospira and Nitrosococcus) is significantly correlated with TN, $NO_3$-N, $NO_2$-N, ON, DO and DWAT at 30°C($P < 0.05$). The Nitrospira (Nitrospira, Nitrospina and Nitrococcus) is significantly correlated with $NO_3$-N, ON and TOC ($P < 0.05$). The main denitrifying bacteria such as Terrimonas, Pseudomonas, Dechloromonas and Flavobacterium were significantly correlated with $NO_3$-N and TOC ($P < 0.05$). The main Anammox bacteria such as Candidatus_Brocadia, Candidatus_Jettenia, Candidatus_kuenenia and Candidatus_Scalindua were significantly correlated with TN, $NO_3$-N, $NO_2$-N ON, DO and TOC ($P < 0.05$). The results showed that $NO_2$-N, $NO_3$-N, DO and DWAT were important factors affecting the distribution of denitrogen bacteria in the substrate at 30°C.

Table 5
Correlation between denitrogen bacteria and environmental factors (30°C)

| Taxonomy | TN | $NH_3$-N | $NO_3$-N | $NO_2$-N | ON | DO | TC |
|---|---|---|---|---|---|---|---|
| Nitrosomonas/Nitrosospira/Nitrosococcus | 0.953* | -0.482 | 0.871* | -0.984* | 0.999* | 0.700* | -0. |
| Nitrospira/Nitrospinav/ Nitrococcus | 0.293 | 0.447 | -0.899* | -0.410 | 0.609* | -0.191 | -0. |
| Terrimonas/Pseudomonas/Dechloromonas/Flavobacterium /… | 0.026 | -0.709 | -0.713* | 0.098 | -0.325 | 0.494 | 0.9 |
| Candidatus_Brocadia/Candidatus_Jettenia/Candidatus_kuenenia/Candidatus_Scalindua | 0.823* | -0.204 | 0.977* | -0.887* | 0.970* | 0.459 | -0. |

Note: N = 3;*, P < 0.05 **, P < 0.01

## Characteristics of denitrogen bacteria and environmental factors

The results of Redundancy Analysis (RDA) between denitrogen bacteria and environmental factors are shown in Fig. 5. The main environmental factors, $NH_3$-N (VIF value 2.32597), $NO_3$-N (VIF value 1.90636), TN (VIF value 1.931025), were screened by analysis of Variance inflation factor (VIF). Then, the main environmental variables were analyzed, and the results showed that $NH_3$-N (AN) and TN (TN) had a significant effect (Table 6, $P_{AN}0.019 < 0.05$, $P_{TN}0.001 < 0.05$), the correlation coefficients between denitrogen bacteria and environmental factors were 0.63 and 0.84, respectively. At the same time, different DAWT4h, 8h and 12h groups were distributed in four quadrants, and the distances between the points were larger, which indicated that the functional composition of the samples were different. The analysis shows that $NH_3$-N (AN) and TN are the main factors that influence the difference of denitrogen microbial community, that is, the difference of environmental factors caused by dry-wet alternation affects the structure and distribution of denitrogen microbial community [8, 21–22].

Table 6
Redundancy analysis (RDA) of denitrogen bacteria

| | RDA1 | RDA2 | r2 | P_values |
|---|---|---|---|---|
| A_N | 0.996577 | 0.082664 | 0.632249 | 0.019 |
| X_N | 0.956327 | -0.2923 | 0.476429 | 0.061 |
| T_N | -0.56045 | -0.82819 | 0.837563 | 0.001 |

## Discussion

## Effect of denitrogen bacteria

In this paper, the effect of dry-wet alternation on the abundance of denitrogen bacteria was analyzed from the point of view of nitrogen transformation.

(1) Ammonia oxidation process

The results showed that the abundance of ammonia-oxidizing bacteria in DWAT4h was higher than that in DWAT8h and 12h, and that in low temperature 10°C was higher than that in DWAT8h. In addition, the major ammonia-oxidizing bacteria Genera (Nitrosomonas, Nitrosospira and Nitrosococcus) were negatively correlated with $NO_2$-N, DO and DWAT as a whole. This paper studies showed that increasing the dry-wet alternate time (reducing frequency) and the ratio of dry-wet time were beneficial to increasing the nitrite nitrogen content in wetland, that is, the abundance of ammonia-oxidizing bacteria was consistent with the concentration of $NO_2$-N and DO, and was negatively correlated with DWAT. It is further suggested that DWAT can increase the abundance of ammonia-oxidizing bacteria and affect the ammonia-oxidizing process from ON to $NO_2$-N to some extent.

Therefore, it can be concluded that the abundance of ammonia-oxidizing bacteria decreases with the increase of dry-wet alternate time, and the potential ammonia-oxidizing rate may also decrease. The reason for this may be that higher DO levels play an important role in maintaining the stability of the ammonia-oxidizing bacteria [23], the dry-wet alternation promoted the substrate to be exposed to air, and the increase of oxygen concentration promoted the ammonia oxidation reaction, and the abundance of ammonia-oxidizing bacteria increased[24–25]. However, the accumulation of $NO_2$-N gradually decreased the activity of ammonia-oxidizing bacteria and the abundance of ammonia-oxidizing bacteria [26].

(2) Nitrification process

The abundance of nitrifying bacteria at DWAT4h was higher than that of DWAT8h and DWAT12h, while the main nitrifying bacteria (Nitrospira, Nitrospina, Nitrococcus) were positively correlated with ON and $NO_2$-N, negatively correlated with $NO_3$-N and TOC. The results showed that increasing the dry-wet alternate time (reducing frequency) and the dry-wet time ratio were beneficial to the reduction of nitrate and organic nitrogen contents in wetland, the results showed that the abundance of nitrifying bacteria was consistent with the concentration change of $NO_2$-N and ON, but opposite to $NO_3$-N and TOC, which indicated that the dry-wet alternate (DWAT) affected the nitrification process from $NO_2$-N to $NO_3$-N to some extent.

Therefore, we hypothesized that the nitrification rate might increase with the increase of the abundance of nitrifying bacteria. The reason for this may be that the substrate is exposed to air in a wet-dry cycle, and nitrification is facilitated by an increase in oxygen concentration, increasing the abundance of nitrifying bacteria [24]. At the same time, with the increase of dry-wet alternation time, the concentration of nitrite nitrogen increased in accordance with the increasing trend of nitrifying bacteria.

(3) Denitrification process

The abundance of denitrifying bacteria at DWAT8h and 12h was higher than that of DWAT4h. At this time, the main denitrifying bacteria (Territonas, Pseudomonas, Dechloromonas, Flavobacterium, etc.) showed significant positive correlation with $NO_3$-N and TOC, and negative correlation with $NO_2$-N and ON, indicating that the concentration of $NO_3$-N and TOC affected Denitrification. The results of this study showed that the denitrogen efficiency could be improved by increasing dry-wet alternate time, and the abundance of denitrifying bacteria was consistent with the concentration change of $NO_3$-N and Toc, but opposite to that of $NO_2$-N and ON. This indicated that the dry-wet alternation affected the denitrification process to a certain extent.

Therefore, the abundance of denitrifying bacteria increased with the increase of dry-wet alternate time, and we can infer that the potential denitrification rate also increased. The reason may be that the dry-wet alternation promoted the diversity and abundance of denitrifying bacteria, and the nitrate concentration increased gradually with the dry-wet alternation time, the abundance of denitrifying bacteria was increased.

(4) Anammox process

The abundance of Anammox bacteria was relatively high at DWAT4h and DWAT12h, and was positively correlated with $NH_3$-N and $NO_2$-N. The results of this study showed that the increase of dry-wet alternate time and dry-wet time increased the concentration of ammonia nitrogen and nitrite nitrogen, that is, the abundance of Anammox bacteria was corresponding to the change of $NH_3$-N and $NO_2$-N concentration, that suggests that DWAT interacts with the Anammmox. The reason for this analysis may be that $NH_3$-N is the main factor affecting ammonia-oxidizing microorganisms [27–28], as the dry-wet alternation time increased, the concentration of ammonia nitrogen and nitrite nitrogen decreased, which affected the abundance of Anammox bacteria to some extent.

# Analysis of denitrogen efficiency

It was found that the removal efficiency of TN (13.0 mg/l →8.0 mg/l) and ON (10.0 mg/l →1.5 mg/l) was better at 10°C and 30°C with the increase of dry-wet alternate time, but at 20°C, the reverse is true (probably due to the initial concentration of nitrogen in the water and the microbial activity of the water), while $NH_3$-N and $NO_2$-N are generally effective, as shown in Fig. 6.

Distribution of microbial community: Firstly, under different temperature conditions, the number of AOB increased slightly with the increase of dry-wet alternation time, the concentration of $NO_2$-N showed a "V" pattern at 10°C, a "V" pattern at 20°C and an upward pattern at 30°C, indicating that dry-wet alternation could promote the conversion of $NH_3$-N to $NO_2$-N, that is, the ammonia oxidation process was gradually enhanced. Secondly, the number of nitrobacteria (NOB) showed an inverted "V" pattern with the increase of dry-wet alternate time (both peaks appeared at DAWT8h), and the concentration of $NO_3$-N increased at 10°C, 20°C and 30°C, the results showed that dry-wet alternation could promote the conversion of $NO_2$-N to $NO_3$-N, and the nitrification was strong. Furthermore, the distribution of denitrifying bacteria (DNOB) showed a "V" pattern at 10°C, a stable pattern at 20°C and an inverted "V" pattern at 30°C (all peaking at DAWT8h) with the increase of dry-wet alternate time, the results showed that dry-wet alternation had a great effect on denitrifying bacteria. Finally, the presence of Anammox bacteria (Candidatus_Brocadia and so on) was found, but the number of distribution did not change much.

On the whole, the dry-wet alternate constructed wetland could increase the number of nitrifying and denitrifying bacteria, which played a positive role in denitrogen.

## Conclusion

The main conclusions of this study are:

At the phylum level, the dominant microbial species are Proteobacteria, Bacteroidetes, Actinobacteria, etc. At genus level, the abundance of ammonia oxidizing bacteria, nitrifying bacteria, denitrifying bacteria and Anammox bacteria was 10% of the total bacterial abundance. The diversity and relative abundance of denitrogen bacteria were the largest, nitrifying bacteria the second, and Anammox the least.

The dry-wet alternate condition has a great influence on the abundance of denitrogen bacteria in the system. The results showed that $NH_3$-N, $NO_2$-N, $NO_3$-N, DO and DWAT were important factors affecting the distribution of denitrogen bacteria in constructed wetlands ($P < 0.05$). RDA Analysis showed that $NH_3$-N (AN) and TN were significantly correlated with denitrogen bacteria ($P < 0.05$) and were the main factors affecting the distribution of denitrogen bacteria. The abundance of Ammonia oxidizing bacteria at DWAT12h was higher than that of DWAT4h and DWAT8h, the abundance of nitrifying bacteria at DWAT8h was higher than that of DWAT4h and Dwat12h, but was higher at DWAT4h at 20°C; the abundance of denitrifying bacteria at DWAT8h was higher than that of DWAT4h and DWAT12h, but was higher at DWAT4h at 10°C; The abundance of Anammox at DWAT12h was relatively high.

Although the denitrogen effect of wetland system was better under dry-wet alternate condition, the accumulation of nitrite nitrogen was found at the same time, which indicated that the nitrate nitrogen reduction process might be hindered, the relative abundance and absolute abundance of denitrifying bacteria were also decreased. On the other hand, it is very important to control dry-wet alternate time reasonably for increasing the number of denitrifying bacteria and denitrogen efficiency.

## Declarations

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### Competing Interests

Financial interests Author Jing Zhu Xinyong Chen Zaifeng Tian Yihong Wu, Qi Zhao declare they have no financial interests. Author Jianjian Lu has received speaker and consultant honoraria from Hebei Provincial Academy of Ecological and Environmental Sciences.

Non-financial interests: Author Jianjian Lu has served on advisory boards for Hebei Provincial Academy of Ecological and Environmental Sciences.

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 analysed during the current study are available in the [MJ20200805033] repository, [https://cloud.majorbio.com/page/project/task.html?project_id=311_5f2cbdaea04a6].

All authors contributed to the study conception and design. Material preparation, data collection and analysis were performed by Jing Zhu Xinyong Chen Zaifeng Tian Jianjian Lu Yihong Wu Qi Zhao. The first draft of the manuscript was written by Jing Zhu and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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## Figures

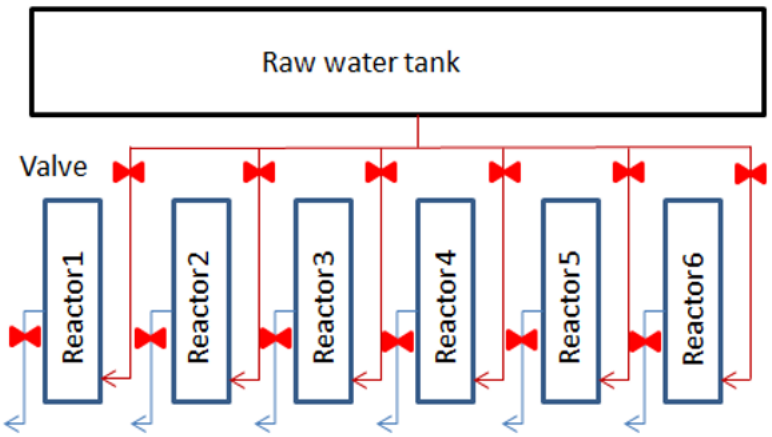


**Figure 1**

Experimental apparatus

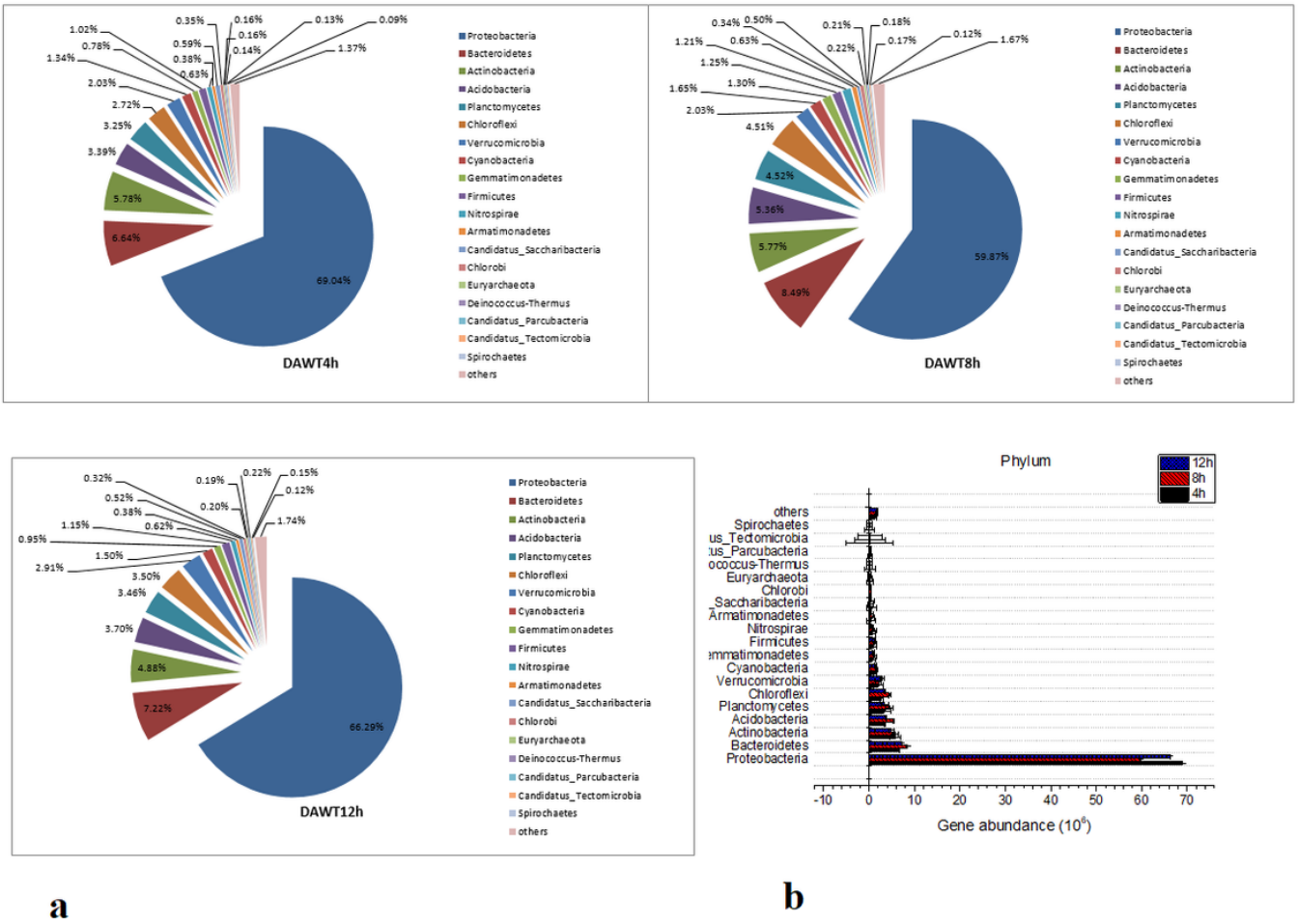


Figure 2

(a) Microbial community composition at phylum levels.

(b) Microbial community composition at phylum levels

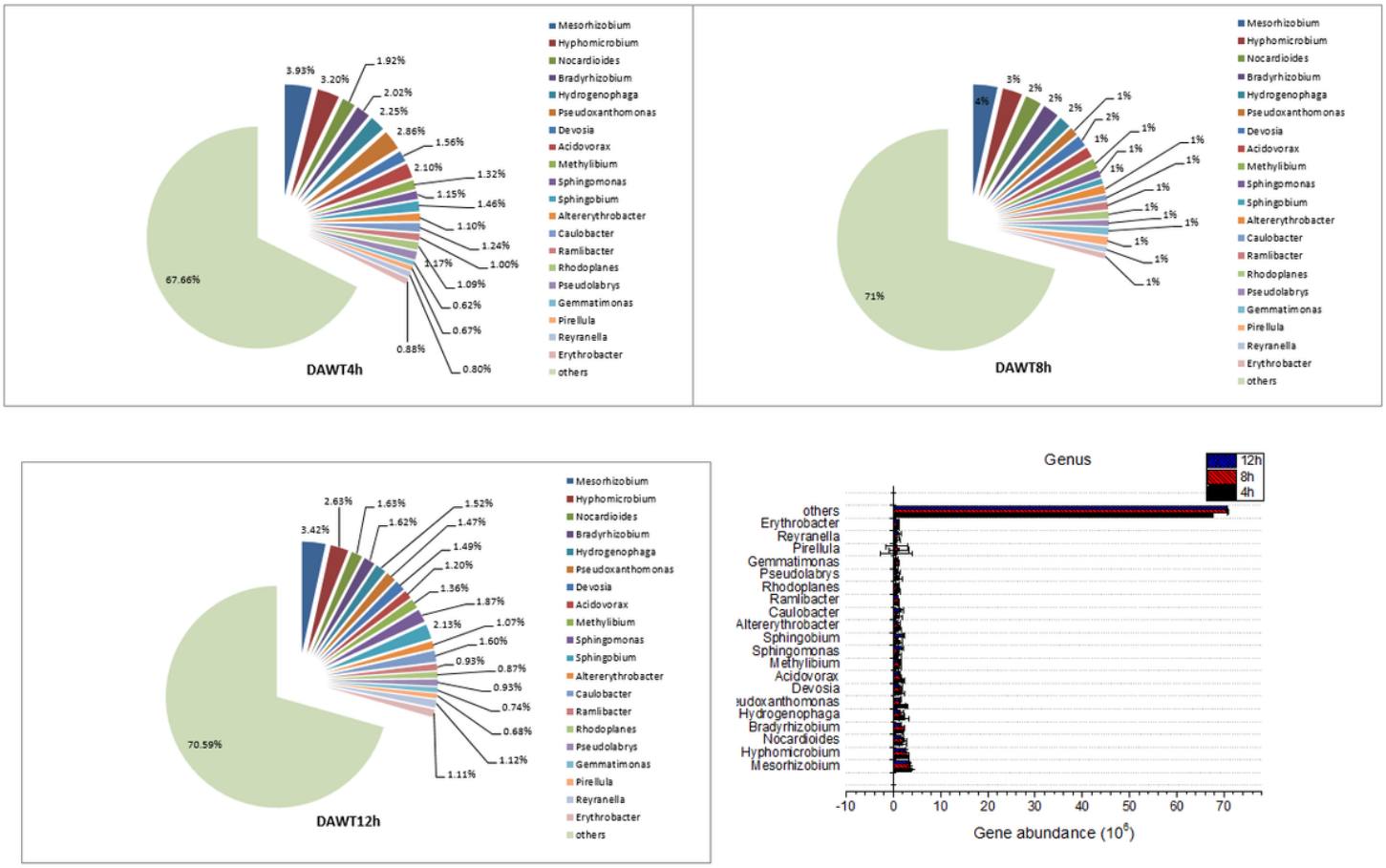


Figure 3

(a) Microbial community composition at genus levels.

(b) Microbial community composition at genus levels

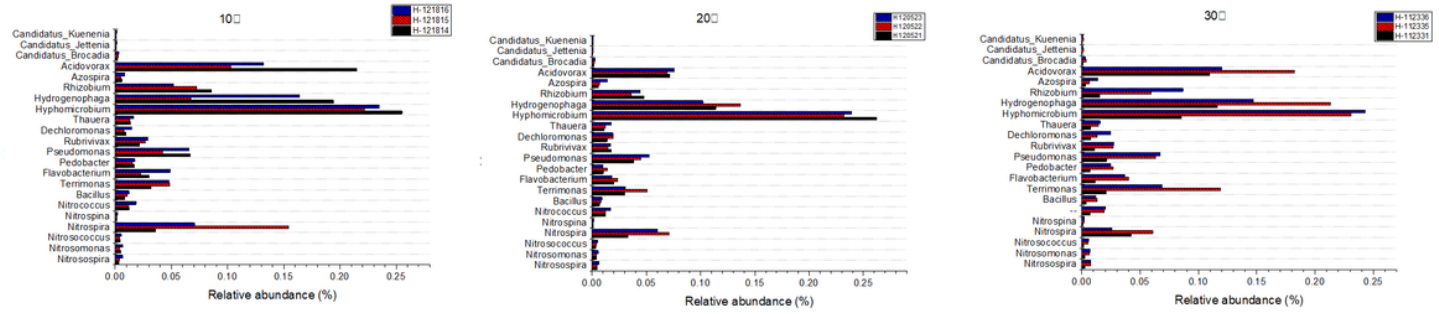


Figure 4

Relative abundance of denitrifying bacteria at genus level

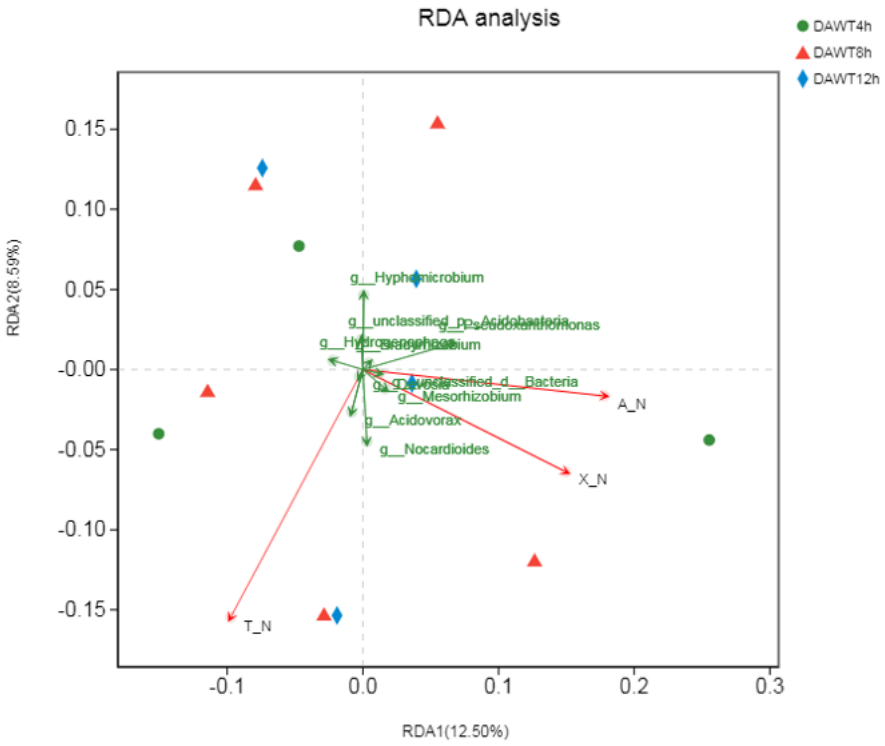


Figure 5

RDA Analysis of denitrifying bacteria and environmental factors

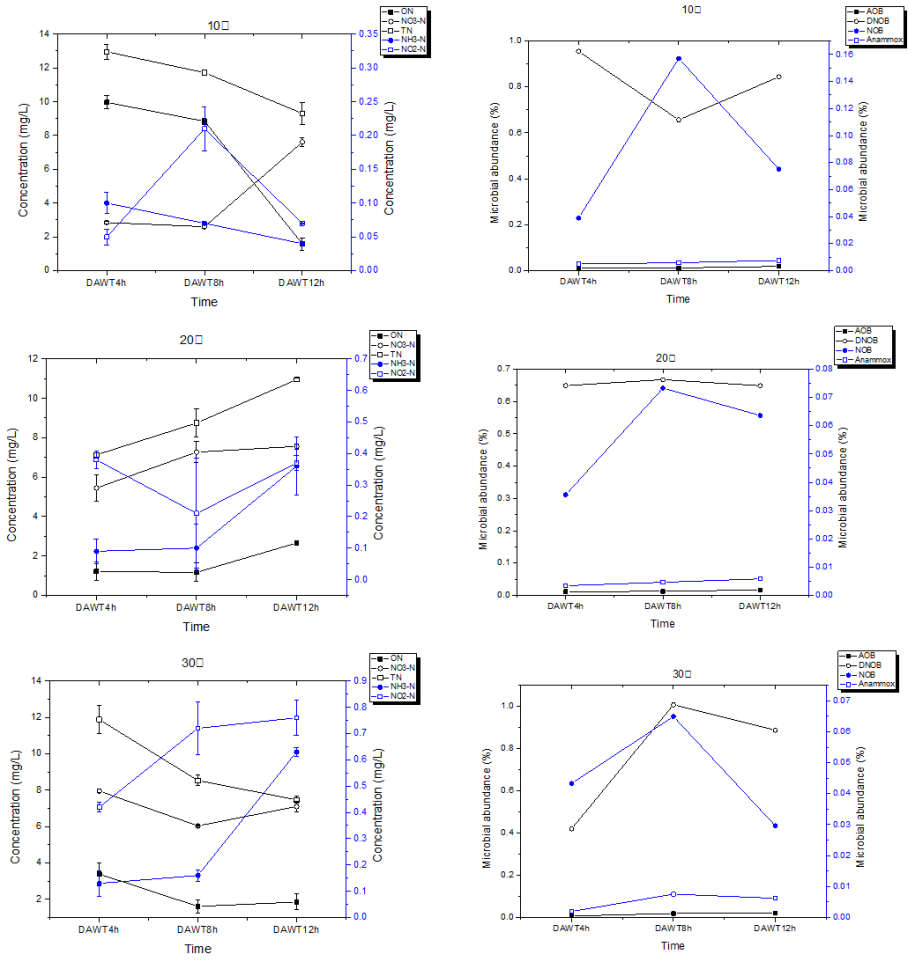


Figure 6

Relationship Between denitrogenation bacteria and denitrogenation effect under the influence of dry-wet alternation