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Effect of hydraulic retention time on microbial community structure and nitrogen metabolism in anaerobic-anoxic-oxic process

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ABSTRACT

Microorganisms in wastewater treatment play a critical role in the degradation and removal of organic wastes and foreign contaminants, but they are vulnerable to the effects of operational working conditions. We investigated the changes in microbial community structure and abundance while the A^2/O process was operated at four different hydraulic retention durations using 16S rRNA gene sequencing. The results showed that microbial abundance and diversity increased with hydraulic retention time (HRT), and the genus level was more significantly affected by HRT than the phylum level. The dominant phylum and genus were Proteobacteria, Bacteroidetes with norank_f__AKYH767, Saprospiraceae, etc. The bacteria under the level of dominant phylum and dominant genus affected by HRT each had certain antagonistic effects, and the anaerobic pool microorganisms had the highest abundance and diversity. In nitrogen metabolism, HRT significantly affects the abundance of enzymes and functional genes. Enzymes involved in denitrification (44%) and denitrification functional genes (30.68%) have the highest abundance; HRT affects nitrogen removal efficiency by influencing the abundance of denitrification functional genes. The abundance of denitrification functional genes is the highest at 17 h; the increase of HRT is beneficial to denitrification, but has little effect on nitrification. The results provide a reference for understanding microbial and nitrogen metabolism influenced by HRT.

Keywords: 16S rRNA; Hydraulic retention time; A^2/O process; Microorganism; Nitrogen metabolism

1. Introduction

In recent years, with the continuous improvement of living standards, the production of domestic and industrial sewage has continued to increase, and research on sewage treatment has attracted more and more attention. Wastewater treatment methods are mainly divided into chemical, physical and biological methods, with biological methods being the most mainstream wastewater treatment methods today. The A^2/O process in the biological method is widely used in various wastewater treatment, and processes based on the A^2/O process coupled with biofilm have also been developed, such as the modified Phoredox [1] and fixed bed activated sludge (IFAS) [2]. According to the survey, the wastewater treatment plants that have been built and put into operation in Tibet, China, mainly use the A^2/O process of the biological method [3]. We have found that previous studies of the influence of operational or environmental factors on the removal of pollutants from a macroscopic perspective are no longer sufficient, and as biological methods rely heavily on the degradation of pollutants by micro-organisms, it is essential to enhance understanding of the mechanisms by which operational conditions or environmental factors affect micro-organisms from a microscopic perspective.

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At present, high-throughput sequencing is widely used in microbial research. Through high-throughput sequencing, you can gain in-depth understanding of the classification and abundance of various bacteria, which will help improve the understanding of sewage biological treatment. Based on high-throughput sequencing technology, Hao et al. [4] studied the best temperature effects for pollutant removal and microbial communities to play a role. Huang et al. [5] explored the pollutant removal performance and the interaction between microbial species in constructed wetlands containing iron-carbon fillers based on high-throughput sequencing technology. Hu et al. [6] used high-throughput sequencing technology to interpret the microbial community structure and diversity in 12 municipal sewage treatment plants, and a total of 202,968 effective sequences were detected.

Biological treatment of wastewater has become the most widespread route in wastewater treatment due to its low operating cost and high efficiency in removing pollutants. Activated sludge consists of a complex microbial community of bacteria, archaea, eukaryotes and viruses, among which bacteria dominate and play a crucial role in the biological treatment process, and the microbial community is susceptible to the influence of external factors [7]. Therefore, studying the effects of external factors on microbial communities and their functional roles may provide new perspectives on the control of wastewater biological treatment processes and will help to improve pollutant removal rates. Process parameters as the main external influences in wastewater treatment process have a great impact on microorganisms, and hydraulic retention time (HRT) in process parameters is again considered as one of the important operational parameters affecting microbial communities [8]. For example, Jafari et al. [9] showed that a reduction in HRT leads to a reduction in the removal of chemical oxygen demand (COD). Mahdy et al. [10] studied the microbial response to the methanogenic performance of hydrolyzed sludge at different HRTs. Furthermore, Wang et al. [11] noted that increasing the HRT appeared to be a successful way to successfully compensate for the effect of low temperatures on the efficacy of the water treatment process in their study of the effect of low temperatures on the efficacy of the CANON-like MBR process for treating surface water. The background of this experiment is Tibet, which is located on the Qinghai-Tibet Plateau, known as the "roof of the world" and the last piece of pure land in the world. Its average altitude is over 4,000 m, and it has special environments such as low temperature and low dissolved oxygen (DO). At the same time, we found that there are few studies on the A^2/O process mainly used in sewage treatment plants in the Qinghai-Tibet Plateau. In the published papers, only Hao et al. [4] studied the effect of temperature on the A^2/O process and Li et al. [12] studied the effect of DO on the A^2/O process, but the effect of HRT on the A^2/O process has yet to be found.

The results of the above-mentioned studies have shown the importance and need to investigate the impact of HRT on the A^2/O process on the plateau, and the close correlation between process parameters and microbial structure characteristics highlights the importance of microbial community monitoring in wastewater treatment as a sign of innovation to ensure correct operational parameter control and process stability. Therefore, this study was conducted to run the A^2/O process under four different HRT conditions and obtain activated sludge samples from each stage. High-throughput sequencing technology was used to obtain information about bacteria, and through data processing and analysis, we aimed to reveal the effects of different HRT on microbial diversity, community structure, and nitrogen metabolism pathways.

2. Materials and methods

2.1. Experimental setup

Using a conventional A^2/O process municipal wastewater treatment simulator [9], a stirring device is provided in both the anaerobic and anoxic pools with a set stirring speed of 110 rpm/min and a diaphragm type microporous aeration head at the bottom of the aerobic pool to supply oxygen to it. Both the test water inlet and the sludge return are driven by a magnetic circulation pump, and the flow is controlled with the aid of a float flowmeter and a ball valve, and the nitrification liquid return is controlled by a peristaltic pump. A thermostatic circulator was used to control the temperature of the test water, and the nitrification liquid reflux ratio and sludge reflux ratio were controlled to be 150% and 100%, respectively. Use an aerobic pool for 35 d of sludge culture, control the temperature at 20.0°C ± 1.0, dissolved oxygen (DO) at 2~3 mg/L, and pH at 6~8. When the SV30 was measured at 28% and MLSS at 3,716 mg/L, different HRT operation tests were formally conducted. This experiment directly used domestic sewage in the office building area of Tibet Agriculture and Animal Husbandry College located in Linzhi City, Tibet Autonomous Region (3,000 m above sea level) as the test water [13]. The main characterization of influent wastewater to reactors are shown in Table 1.

2.2. HRT setup and sample collection

On the premise of keeping the control indicators in the culture stage unchanged, different HRT s were controlled by adjusting different influent flow rates. At an influent flow rate of 8 L/h, the HRT is 4.38 h for the anaerobic pool, 7.25 h for the anoxic pool and 14.62 h for the aerobic pool, giving a total HRT of 26.25 h. At an influent flow rate of 12 L/h, the HRT is 3.5 h for the anaerobic pool, 5.8 h for the anoxic pool and 11.7 h for the aerobic pool, giving a total HRT of 21 h. At an influent flow rate of 14 L/h, the HRT is 2.92 h for the anaerobic pool, 4.83 h for the anoxic pool and 9.75 h for the aerobic pool, giving a total HRT of 17.5 h. At an influent flow rate of 14 L/h, the HRT is 2.92 h for the anaerobic pool, 4.83 h for the anoxic pool and 9.75 h for the aerobic pool, giving a total HRT of 17.5 h. At an influent flow rate of 16 L/h, the HRT is 2.5 h for the anaerobic pool, 4.14 h for the anoxic pool and 8.36 h for the aerobic pool, giving a total HRT of 15 h. After 9 d of operation for each HRT condition, samples of activated sludge from the anaerobic, anoxic and aerobic pools were taken for testing. In order to eliminate the effect of working condition adjustment, after each set of HRT operation,

Table 1
Characterization of influent wastewater to reactors

pH	8.36 ± 0.06
COD (mg/L)	258.17 ± 86.39
TN (mg/L)	38.5 ± 7.14
TP (mg/L)	5.11 ± 2.34
NH_4^+–N (mg/L)	25.89 ± 8.21

the next set of HRT was carried out after an interval of 72 h. Meanwhile, in order to ensure the stable operation of the reactor, the sludge concentration (MLSS) and SV30 in the reactor were tested daily, and the MLSS was stable at 3,700~4,100 mg/L and the SV30 was stable at 27%~30%.

2.3. Sample detection and analysis methods

2.3.1. Sample testing

COD was measured by potassium dichromate method, total phosphorus (TP) was measured by spectrophotometry, total nitrogen (TN) was measured by potassium persulfate-ultraviolet spectrophotometry, and NH_4^+–N was measured by Nessler Reagent Spectrophotometry. Activated sludge samples was sent to Shanghai Meiji Biomedical Technology Co., Ltd. for testing (htpp://www.majorbio.com) for 16S rRNA gene sequence sequencing. 16S rRNA is a component of the 30S subunit in the ribosome of prokaryotes, with a length of about 1,542 nt, with a high degree of conservation and specificity. 16S rRNA gene sequencing generally uses MiSeq to sequence the V3-V4 variable regions of 16S rRNA genes to study microbial diversity [14].

2.3.2. Bioinformatics analysis

According to 97% sequence similarity, all sequences are clustered into OTUs (Operational Taxonomic Units) using RDP (Ribosomal Database Project) database classification method. Based on the number of OTUs, Mothur Software analyzes abundance and diversity indexes, including Chao1 index, ACE (abundance-based coverage estimator) index, Shannon index and Simpson index.

2.3.3. Community composition analysis and correlation Heatmap

Community composition analysis includes community column chart (Bar chart), community pie chart (Pie chart), multi-level species Sunburst chart, and community Heatmap chart. The community bar chart (Bar chart) is used this time. The community bar chart (Bar chart) is based on the results of taxonomic analysis. It can be known that different groups (or samples) are at each classification level (such as domain, boundary, door, class, Order, family, genus, species, OTU, etc.). According to the community Bar diagram, two aspects of information can be visualized: (1) Which dominant species are contained in each sample at a certain taxonomic level; (2) The relative abundance (proportion) of each dominant species in the sample. Use the data table based on the tax_summary_a folder and use the R language (version 3.3.1) tool to draw. Correlation Heatmap analysis is performed by calculating the correlation coefficients between the selected species (Spearman's rank correlation coefficient, Pearson's correlation coefficient, etc.) and visually displays the obtained numerical matrix through the Heatmap chart. The color change reflects the data information in the two-dimensional matrix or table. The color depth indicates the size of the data value, and it can intuitively express the size of the data value in a defined color depth. Use software R (version 3.3.1) (pheatmap package).

2.3.4. Sample hierarchical cluster analysis and PCoA analysis

In order to study the similarity or difference relationship between the structure of different sample communities, cluster analysis can be performed on the sample community distance matrix to construct a sample hierarchical clustering tree. UPGMA (unweighted pair-group method with arithmetic mean) is a cluster analysis method commonly used to solve classification problems. When used for sample-level clustering analysis, hierarchical clustering is performed according to the beta diversity distance matrix. Hierarchical clustering analysis uses the UPGMA algorithm to construct a tree structure to visualize the degree of similarity or difference in community composition in different environmental samples. Use the software Qiime to calculate the beta diversity distance matrix, and then use the R language (version 3.3.1) to make the picture tree. PCoA analysis (principal co-ordinates analysis), namely principal coordinate analysis, is a non-constrained data dimensionality reduction analysis method that can be used to study the similarity or difference of sample community composition. PCoA is based on the selected distance matrix through dimensionality reduction to find out the potential principal components that affect the difference in sample community composition, using python statistical analysis and R (version 3.3.1) drawing.

2.3.5. 16S rRNA gene function prediction

The OTU abundance table was standardized by PICRUSt (Phylogenetic Investigation of Communities by Reconstruction of Unobserved States) software, and the COG (Cluster of Orthologous Groups of Proteins) functional protein information corresponding to OTU and KEGG (Kyoto Encyclopedia of Genes and Genomes, http: //www.genome.jp/kegg/) Ortholog (KO) information; and calculate KO abundance. According to the information of KEGG database, KO and PATHWAY can be obtained. The KEGG PATHWAY database includes various metabolic pathways. At the same time, the types of enzymes and gene abundances involved in nitrogen-related metabolic pathways can be obtained, and the influence of HRT on them can be explored.

3. Results

3.1. Abundance and diversity of microbial communities

The Ace and Chao indices reflect the abundance of microflora, and higher values indicate higher microbial

abundance. In the three pools under four different HRT groups, the Ace and Chao indices fluctuated between 660 and 1,000 with large fluctuations. Table 2 shows that the Ace and Chao indices at HRT 26 and 21 h were greater than those at 17 and 15 h, and the highest values of Ace and Chao indices were reached at 26 h. By comparing the Ace and Chao indices of the three functional units, the results showed that the anaerobic pool was larger than the anoxic pool and larger than the aerobic pool. Shannon and Simpson indices reflected the diversity of microbial flora, and the high Shannon and low Simpson indices represented high diversity. By calculating from Table 2, it is concluded that the anaerobic pool has high diversity, followed by the anoxic and aerobic pool. From the HRT perspective, it was calculated that the diversity of species also showed an increasing trend with the increase of HRT, and the highest species diversity was found when the HRT was 26 h.

3.2. Microbial community composition

3.2.1. Composition of microbial community at phylum level

Fig. 1a shows that at the phylum level, there are 7 dominant bacteria phyla, namely Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, Chloroflexi, Patescibacteria, Acidobacteria. The highest percentage of Proteobacteria was average 35.1%, followed by Bacteroidetes (average 28.9%) and Actinobacteria (average 18.4%). the highest abundance of Proteobacteria and Bacteroidetes at HRT 21 and 26 h, and Actinobacteria at the highest abundance was found at 15 h. Among the different functional units, Proteobacteria had the highest percentage in the anaerobic pool, which is the same as the results of Zhang et al. [15], Proteobacteria are obligate or facultative anaerobic bacteria with nitrogen removal function [16]. Bacteroidetes and Actinobacteria have the highest proportions in aerobic and anoxic pools, which are related to the functions they belong to, with Bacteroidetes being mainly nitrification and nitrogen fixation and Actinobacteria being related to denitrification and nitrogen removal [17,18]. This will contribute to the nitrification denitrification efficiency and improve the nitrogen removal.

In Fig. 1b of the correlation network with HRT as a single factor, only the gates that satisfy the correlation coefficient $R > 0.50$ and the significance coefficient ≤ 0.05 are analyzed. There is 1 group that satisfies the significant positive correlation, and 10 groups that satisfies the significant negative correlation. Among them, Bacteroidetes and Proteobacteria are significantly positively correlated. To further explore the effect of HRT on microbial community structure, a joint analysis of hierarchical clustering (Fig. 1c) and principal component analysis (Fig. 1d) was performed to visualize differences and similarities. We found that both cluster analysis and PCoA appear to hrt_ano_15 this sample was independently split out. PCoA analysis showed that PC1 and PC2 explained 84.53% of the differences in phylum microbial composition. The closer the two samples were to each other, the more similar the species composition was. The four HRT groups are mainly concentrated on the left side of the figure, with 26 and 17 h on the upper left and 21 and 15 h on the lower left, which is very similar to the results of the cluster analysis. It indicates that HRT appears to have a differentiating effect on microbial communities, which is similar to the studies of Kim et al. [19] and Zhang et al. [15] who showed that microbial communities in bioreactors depend on HRT.

3.2.2. Genus level microbial community composition

From Fig. 2a we can see that the microorganisms at the genus level are diverse. norank_f__AKYH767 (average 13.15%), norank_f__Saprospiraceae (average 8.8%), and Ottowia (average 4.85%) were the dominant genera with the highest percentage. The abundance of norank_f__AKYH767 and Ottowia was highest at 21 h, norank_f__Saprospiraceae was highest at 17 h, norank_f__Saprospiraceae fluctuates greatly under the influence of HRT at 26, 17 h, and 21, 15 h. Among the different functional units, norank_f__AKYH767 and norank_f__Saprospiraceae had the highest percentage in the aerobic pool, norank_f__Saprospiraceae are denitrifying bacteria, which are strictly aerobic and can degrade organic matter [17]; Ottowia had the highest percentage in the anaerobic pool, it is a typical denitrifying bacterium that

Table 2
Abundance and diversity of microbial communities in different functional units under different HRT

Functional unit	Ace	Chao	Shannon	Simpson	Coverage
hrt_ana_26	906.317	930.5385	4.275531	0.046462	0.995227
hrt_ana_21	859.0467	886.4937	4.368855	0.042126	0.994775
hrt_ana_17	782.0825	820.5806	4.396031	0.034418	0.995205
hrt_ana_15	811.2419	838.1216	4.262753	0.043137	0.995582
hrt_ano_26	998.7863	866.2353	4.240109	0.048106	0.994803
hrt_ano_21	884.224	780.7051	4.353357	0.036729	0.994981
hrt_ano_17	733.4434	735.0395	4.112332	0.052290	0.994757
hrt_ano_15	756.5508	755.3750	3.905734	0.099564	0.994786
hrt_oxic_26	914.8647	916.8353	4.368186	0.041285	0.994613
hrt_oxic_21	761.2368	776.4085	4.150202	0.059924	0.995198
hrt_oxic_17	687.0719	665.6582	3.933853	0.060414	0.995973
hrt_oxic_15	702.2541	713.3788	3.953836	0.075543	0.995842

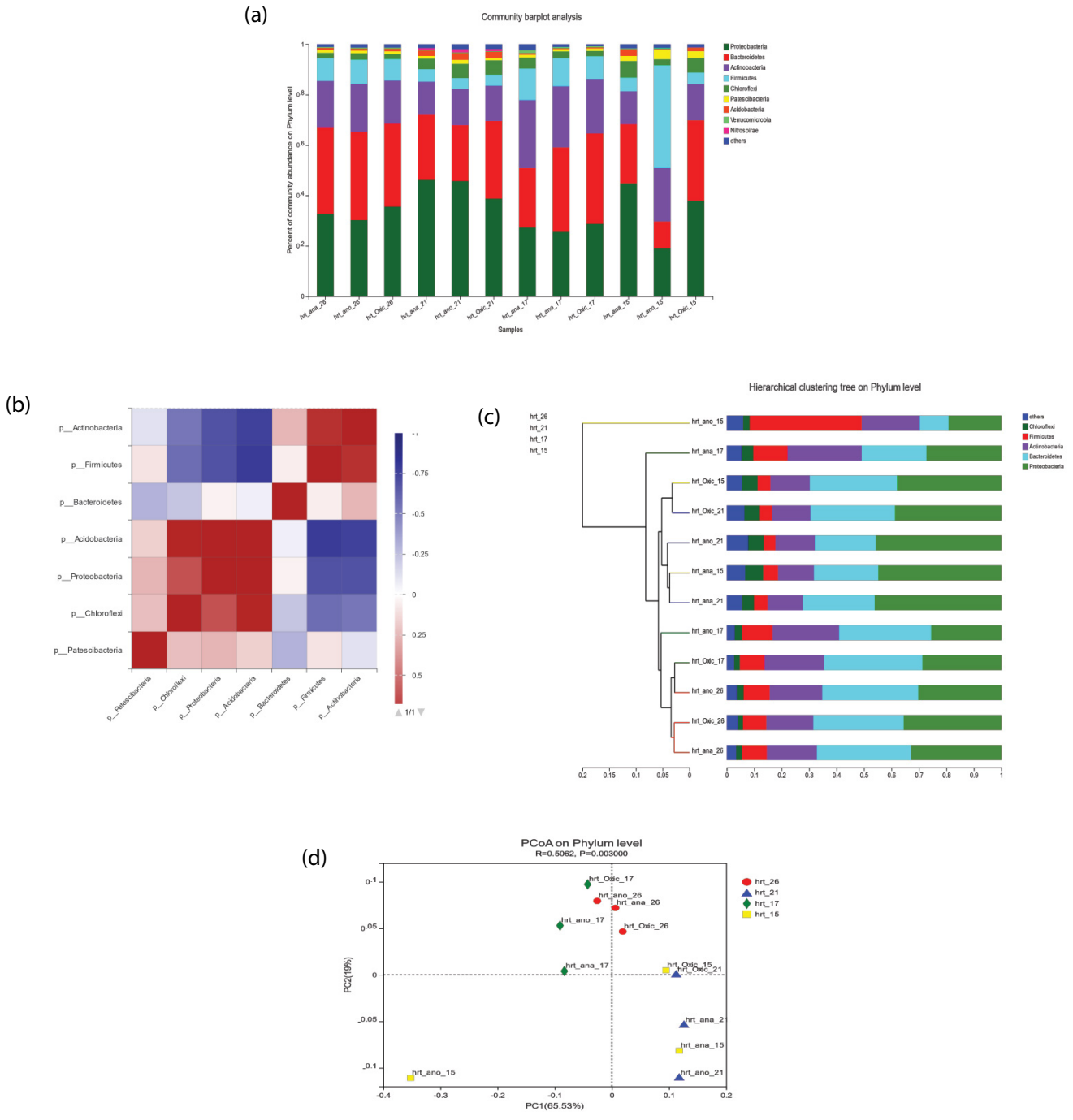


Fig. 1. (a) Species composition at the phylum level. (b) Heatmap diagram of species correlation at the phylum level. (c) Hierarchical clustering analysis at phylum level. (d) PCoA analysis at phylum level.

contains nitrate reductase with strong oxygen sensitivity and belongs to the anaerobic type [20]; it also laterally indicates a large percentage of denitrifying bacteria genus.

Fig. 2b shows that there are 21 groups with negative correlation and 4 groups with positive correlation. There is a 40%–60% correlation between each genus and other genera, which reflects the interaction between microorganisms. In the cluster analysis (Fig. 2c) and PCoA (Fig. 2d) analysis at the genus level, we likewise found the sample hrt_ano_15 to be independent. The PCoA analysis showed that PC1 and PC2 explained 87.17% of the variation in the microbial composition of the genus, with a high contribution. HRT 26 and 17 h were enriched in the lower right corner of the figure, while 21 and 15 h were in the lower left corner, and the samples of 21 and 15 h were closer together than those of 26

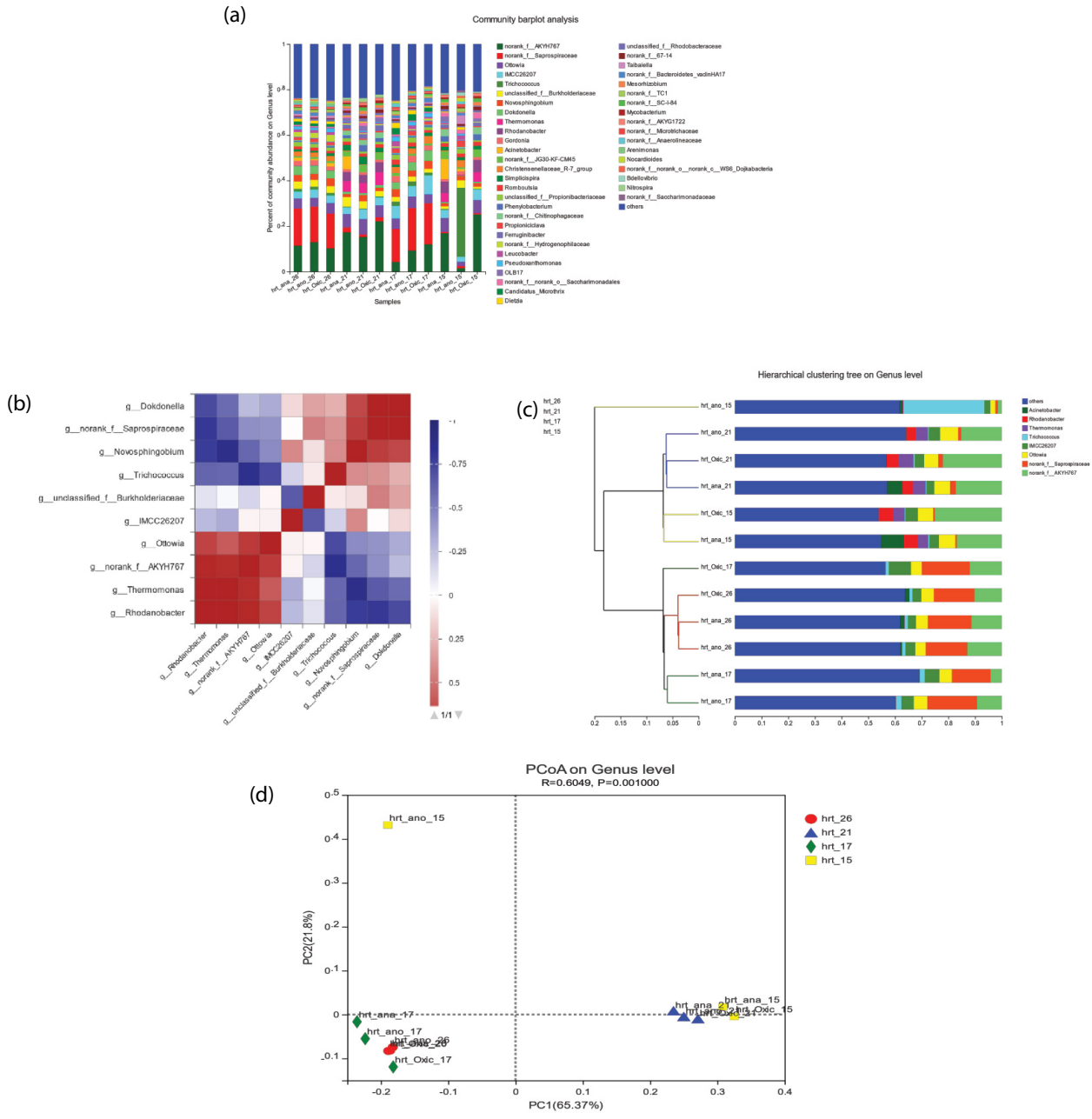


Fig. 2. (a) Species composition at the genus level. (b) Heatmap diagram of species correlation at the genus level. (c) Hierarchical cluster analysis at the genus level. (d) PCoA analysis at the genus level.

and 17 h. Compared with the cluster analysis results, it shows that the two results have a highly consistent relationship, and at the same time the phylum level results are also the same, but the degree of sample enrichment at the genus level is greater than the phylum level.

3.3. Nitrogen metabolism

3.3.1. Key enzymes in nitrogen metabolism pathway

As shown in Fig. 3, nitrogen metabolism mainly includes denitrification, nitrogen fixation, nitrification, and nitrate reduction, which constitute a closed metabolic pathway. Enzyme content is one of the main determinants of metabolic pathways, and the process of nitrogen metabolism is catalyzed by various enzymes. The numbers marked in red are the key enzymes involved in nitrogen metabolism in this sample, 12 in total, and all of them are oxidoreductases. Because nitrogen removal is carried out during denitrification and nitrification, oxidoreductases are required to catalyze the oxidation reaction (such as the oxidation of ammonia nitrogen to nitrite nitrogen). The most abundant enzyme was nitrite reductase (EC 1.7.1.15) accounting for

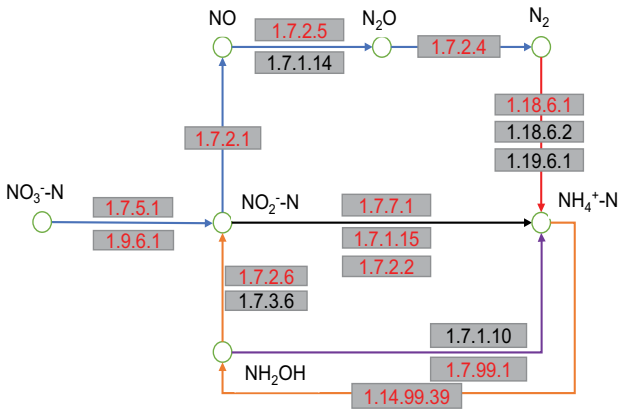


Fig. 3. Analysis of nitrogen metabolism pathway based on KEGG database A^2/O process.

30% of the total, which is mainly involved in assimilating and dissimilating nitrate reduction and catalyzing the reduction of nitrite nitrogen to ammonia nitrogen. As can be seen from the picture, the types of related enzymes involved in the denitrification process are complete, mainly NO_3^-–N is converted to NO_2^-–N under the action of nitrate reductase (EC 1.7.5.1, EC 1.9.6.1), nitrate reductase is an important rate-limiting factor in the reduction of nitrate nitrogen [21]; Then NO_2^-–N is reduced to NO by nitrite reductase, and then under the action of nitric oxide reductase (EC 1.7.2.5), NO is converted into N_2O, N_2O is converted to N_2 under the action of nitrous oxide reductase (EC 1.7.2.4). Of the 6 enzymes involved in denitrification, 5 enzymes have the highest abundance when HRT is 26 and 21 h, which indicates that longer HRT provides more complete and more abundant enzymes for denitrification, thereby increasing the rate of denitrification. Among them, nitrous oxide reductase (EC 1.7.2.4) was most significantly affected by HRT, and the relative abundance at 15 and 26 h was nearly twice that of 17 and 21 h. A trend of alternating size in the relative abundance share of nitrite reductase (EC 1.7.1.15, EC 1.7.2.1) and nitrate reductase (EC 1.7.5.1, EC 1.9.6.1) was also found under the variation of HRT. The reason may be due to the competition between nitrite reductase and nitrate reductase for electron donor [22].

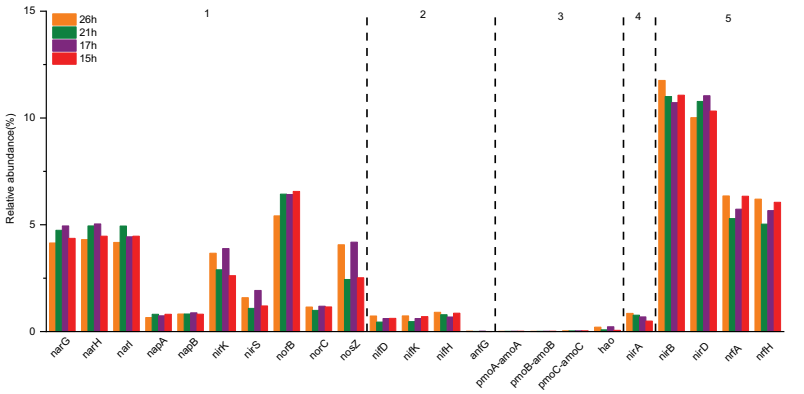
For nitrogen fixation, N_2 becomes NH_4^+–N under the redox of nitrogenase (EC 1.18.6.1). Nitrogenase (EC 1.18.6.1) requires strict anaerobic or micro-aerobic conditions [23]. As seen in Fig. 3 and Table 3, the only enzyme involved in nitrogen fixation was nitrogenase (EC 1.18.6.1), and HRT less than 17 h or greater than 26 h was not conducive to the growth of nitrogenase (EC 1.18.6.1). Nitrification is the oxidation of NH_4^+–N to NO_2^-–N by ammonia monooxygenase (EC 1.14.99.39), hydroxylamine dehydrogenase (EC 1.7.2.6). As seen in Table 3, the abundance of ammonia monooxygenase (EC 1.14.99.39) and hydroxylamine dehydrogenase (EC 1.7.2.6) increased with the increase in HRT, but the abundance remained low, indicating that the increase in HRT had a small effect on the increase in their abundance. In nitrate reduction, the relative abundance of the enzyme was second only to denitrification, and the relative abundance variation was less affected by HRT.

3.3.2. Genes in the pathway of nitrogen metabolism

Fig. 4 shows the relative abundance percentages of genes involved in nitrogen metabolism. As seen in the figure that the highest percentage of gene abundance is the dissimilated nitrate reduction *nirB*. Nitrification had the lowest percentage of gene abundance, while the largest number of genes involved was denitrification. For denitrification, *norB* genes were the most abundant, followed by *narH*, *narG*, *narI*, and *nosZ*, excluding the highest percentage of *norB* genes in abundance at 15 h, and the rest were in highest abundance at 21 and 17 h. Except for the *anfG* gene, all the other genes involved in nitrogen fixation were highest at 26 h. Nitration genes were highest at 17 and 15 h. In the assimilation and dissimilation of nitrate reduction, except for the *nirD* gene, the rest were highest at 26 h. The relative abundance of genes involved in nitrogen metabolism varied at different HRTs, indicating a significant effect of HRT on the abundance of functional genes.

Table 3
Information about enzymes related to nitrogen metabolism pathway

Enzyme code EC	Function	Enzymes	HRT 26 h	HRT 21 h	HRT 17 h	HRT 15 h
1.7.1.15	Nitrite reductase (NADH)	Oxidoreductase	30.026	28.320	30.014	32.809
1.7.5.1	Nitrate reductase (quinone)	Oxidoreductase	16.749	16.576	15.900	16.429
1.7.2.5	Nitric oxide reductase (Cytochrome c)	Oxidoreductase	7.206	7.656	6.754	5.872
1.7.7.1	Ferredoxin—nitrite reductase	Oxidoreductase	6.060	5.273	6.475	5.602
1.7.2.4	Nitrous oxide reductase	Oxidoreductase	7.187	3.764	3.809	7.538
1.18.6.1	Nitrogenase	Oxidoreductase	3.958	6.265	5.134	3.884
1.7.2.1	Nitrite reductase (NO-forming)	Oxidoreductase	3.011	5.974	5.220	2.716
1.7.2.2	Ammonia-forming	Oxidoreductase	0.800	1.106	0.809	0.884
1.9.6.1	Nitrate reductase (Cytochrome)	Oxidoreductase	0.853	0.405	0.407	0.763
1.7.99.1	Hydroxylamine reductase	Oxidoreductase	0.336	0.331	0.249	0.175
1.7.2.6	Hydroxylamine dehydrogenase	Oxidoreductase	0.351	0.240	0.216	0.126
1.14.99.39	Ammonia monooxygenase	Oxidoreductase	0.058	0.028	0.008	0.002



Note: 1 Denitrification 2 Nitrogen fixation 3 Nitrification 4 Assimilatory nitrate reduction 5 Dssimilatory nitrate reduction

Fig. 4. Relative abundance of nitrogen metabolism genes under different HRT.

4. Discussion

The results showed that HRT had significant effects on microbial diversity, community composition, and nitrogen metabolism. The abundance and diversity of microorganisms increase with the increase of HRT, and the abundance and diversity of the anaerobic pool are the highest among the three functional units. This is contrary to the result of Kim et al. [24] finding that the abundance of microorganisms in aerobic pools is higher than that in anaerobic pools and anoxic pools. The reason for this may be that the incoming organic matter is utilised in the anaerobic pool and more heterotrophic microorganisms multiply to increase the abundance of microbial community in the anaerobic pool, while the anoxic and aerobic pool form a decreasing nutrient-poor environment where the number of heterotrophic microorganisms becomes low.

The same results as previously reported [25,26], Proteobacteria, Bacteroidetes, Actinobacteria are still the dominant bacteria phyla, occupying an important position in the removal of pollutants. The dominant genus is different from previous reports [27,28]. The results of this experiment found that norank_f__AKYH767, norank_f__Saprospiraceae, and Ottowia as the dominant genera with the highest abundance. norank_f__Saprospiraceae, Ottowia are common denitrifying bacteria in wastewater treatment systems [17]. The highest abundance of norank_f__AKYH767 was a newly discovered genus with the highest abundance of aerobic pool among the three functional units, it is greatly affected by HRT, which also reflects its strong ability to adapt to the plateau, and its specific functions are expected to be studied later. In the species-related Heatmap, it was found that affected by HRT changes, bacteria at the phylum level had antagonistic effects with each other, and the same was true at the genus level. The reason may be caused by the competition between both for the same substrate [29,30], while it may result in unstable pollutant removal, but it may also provide a reference for subsequent addition of bacterial agents in the wastewater treatment system. Hierarchical clustering analysis and PCoA analysis show that different HRT has a significant separation effect on bacterial communities, which is the same as the results of Zhang et al. [15], but genus level is more significantly affected by HRT than the phylum level, and the degree of enrichment is higher. By comparing with other samples, it is found that the reason why the 15 h hypoxic pool samples at the phylum and genus levels are independent is that the relative abundance of Firmicutes at the phylum level and Trichococcus at the genus level is abnormally prominent. Because Trichococcus belongs to Firmicutes, studies have shown that the relative abundance of Firmicutes reaches the highest around HRT 15 h [31], at the same time, Zong et al. [32] and Kang [33] found that Firmicutes and Trichococcus had the highest relative abundance in anoxic pools in the A^2/O process. This explains the high abundance of Firmicutes and Trichococcus in the 15h anoxic pool, and also illustrates the stability of Firmicutes and Trichococcus in the A^2/O process. It was also found that the highest abundance of dominant bacteria in the phylum and genus was concentrated in HRT 17 and 21 h, and most of them belonged to denitrifying bacteria. This indicates that 17 and 21 h are conducive to the growth of dominant bacteria and the accumulation of denitrifying bacteria, which is the same as the results of previous studies [30].

Under the influence of HRT, the relative abundance of denitrification enzymes in nitrogen metabolism is higher than that of nitrogen fixation and nitrification enzymes, accounting for 44% of the total abundance, while the enzymes involved in nitrification are the least. This is because most of the dominant bacteria in the phylum and genera belong to denitrifying bacteria, and the activity of enzymes is related to the bacterial community to a certain extent, and bacteria contribute to the release of enzymes [34]. In nitrification, the abundance of enzymes involved is very low. Although it increases with the increase of HRT, it is still very low. Compared with denitrification, it shows that the increase of HRT is conducive to denitrification, but has little effect

on nitrification. Therefore, we should consider the carbon source and dissolved oxygen to increase the abundance of enzymes in nitrification [35,36].

Among the nitrogen metabolism genes, the total abundance of denitrification-related functional genes was also quite high (30.68%) and showed an increase followed by a decrease with increasing HRT, with the highest abundance at 17 h. This was consistent with the trend of TN removal rate in Fig. 5, which was highest at 17 h. The *norB*, *narH* and *narG* genes with the highest relative abundance percentages were all lowest at 26 h, which was the same as the lowest TN removal rate at 26 h, indicating that the abundance of the dominant genes had a certain degree of influence on the TN removal rate. At the same time, it was found in the denitrification functional genes that the *nosZ* gene was affected by HRT and its relative abundance showed a wave-like trend with the increase of HRT. The *nosZ* gene is considered to be the key factor in the final step of the denitrification process that converts N_2O to N_2, and the abundance of *nosZ* gene will affect the removal of TN [35]. The removal rate of TN in this test fluctuates between 50% and 90%, which can also be better confirmed. Therefore, it shows that HRT mainly affects the nitrogen removal performance by affecting the abundance of denitrification functional genes. This reveals that the increase in HRT is the pathway leading to the decrease in TN removal performance. The abundance of nitrogen fixation genes was less fluctuated by HRT changes. Stability of nitrogen fixation genes better maintained the nitrogen cycle and provided a stable nitrogen source for microorganisms.

The trend of the total abundance of dissimilative nitrate reduction genes *nirB*, *nirD*, *nirA*, and *nrfH* under the influence of HRT is the same as the trend of NH_4^+–N removal rate under the influence of HRT, as HRT increases, it first decreases and then increases. In this experiment, no enzymes and genes related to the oxidation-reduction of NH_4^+–N to N_2 were found in the nitrogen metabolism pathway, indicating that the removal of NH_4^+–N in this experiment mainly occurred on nitrification. The abundance of nitrification-related functional genes was very low (0.84%), the highest was *hao*, followed by *pmoC-amoC*, both of which reached the highest relative abundance at HRT 26h. The ammonia oxidation process (NH_4^+–N is converted to NH_2–OH) is the rate-limiting step in the entire nitrification process, *pmoC-amoC* is the gene of ammonia oxidation process, higher abundance of *pmoC-amoC* gene can accelerate the conversion of NH_4^+–N, and the *hao* gene accelerates the conversion of NH_2–OH to NO_2^-–N, and finally the average removal of NH_4^+–N The rate is as high as 91.11% at 26 h. However, it was found that the average removal of NH_4^+–N was the highest at 15 h (92.94%), while the abundance of nitrification genes at 15 h was the lowest. Analyze the reason may be related to dissolved oxygen, Studies have shown that dissolved oxygen as a process parameter has a significant impact on the catabolism of active substances in an aerobic environment [36]. The genes involved in nitrification this time are the *pmoA-amoA*, *pmoB-amoB*, *pmoC-amoC* and *hao* genes in the ammonia oxidation process. The highest dissolved oxygen (average 3.21 mg/L) was detected in the aerobic pool at 15 h. This indicates that although the abundance of nitrification functional genes was low at 15 h, the high dissolved oxygen

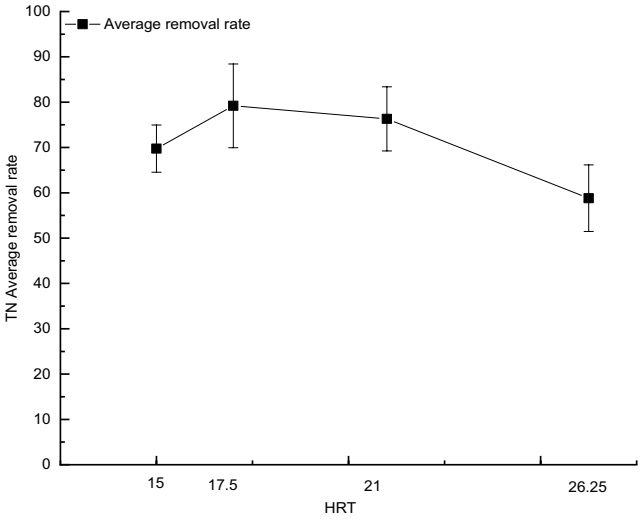


Fig. 5. Average removal rate of TN under different HRT.

created a better aerobic environment for their functional genes, thus improving the decomposition capacity of nitrification functional genes. It also shows that the A^2/O process has a strong impact resistance to NH_4^+–N removal.

5. Conclusion

The analysis showed that microbial abundance and diversity increased with increasing HRT. Proteobacteria, Bacteroidetes, Actinobacteria, norank_f__AKYH767, Saprospiraceae, Ottowia, etc. are the dominant bacteria phyla and genera in the A^2/O process. Under the influence of HRT, microorganisms under the dominant phylum have a mutually suppressive effect, as do microorganisms under the dominant genus. The impact of HRT changes on the composition of the genus microorganisms was more significant than that of the phylum, and it was found that the abundance, diversity and community composition of the anaerobic pool microorganisms were the highest among the three functional units. In nitrogen metabolism, HRT has a significant impact on the abundance of key enzymes and functional genes. The increase of HRT is beneficial to denitrification enzymes and related functional genes, but has little effect on nitrification enzymes and related functional genes. The removal of TN was consistent with the trend that the abundance of denitrification function genes was affected by HRT. The above conclusions provide us with insights for a better understanding of the effects of operating conditions on microorganisms and biological nitrogen removal in sewage treatment.

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