Study on community structure and nitrogen metabolism mechanism in A^2O process under different hydraulic retention time conditions at high altitude region

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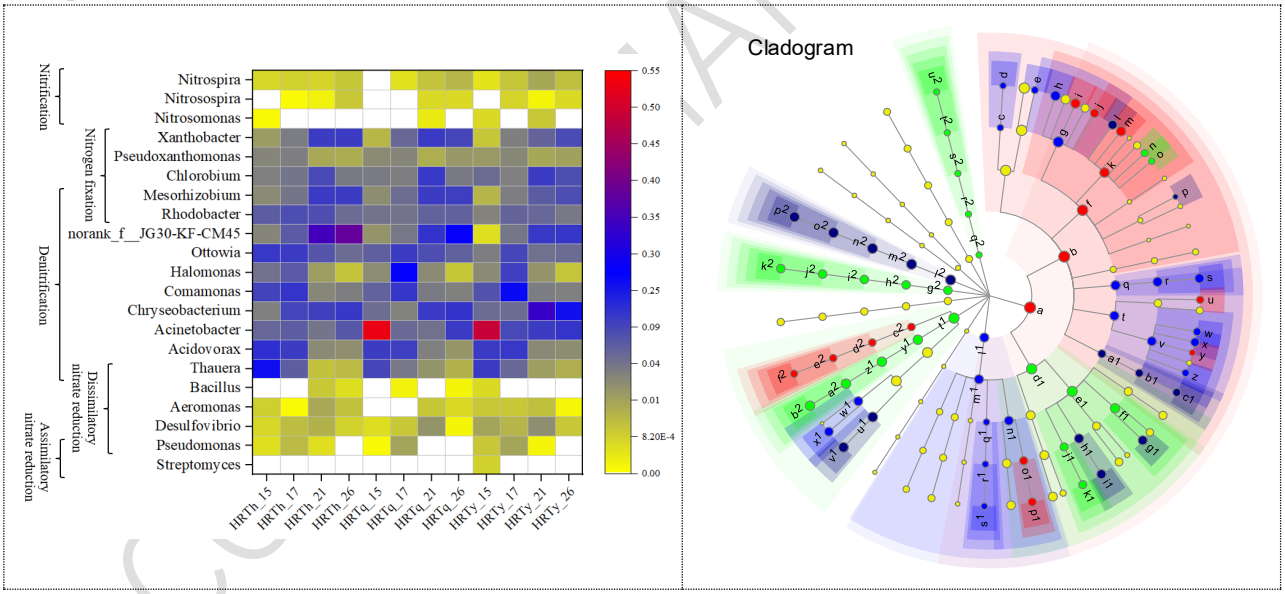
Graphical abstract



Abstract

The unique high-altitude environmental factors in the Linzhi region of Tibet have not been fully investigated in terms of their impact on the microbial mechanisms of water ecology, for which we explored the response of activated sludge microorganisms to different hydraulic retention times (HRT) based on 16S rRNA gene sequencing and PICRUST2 functional prediction. The results showed that different HRT had significant effects on Phylum, Class, Genus, Species and OTU levels, as well as on diversity, richness and evenness ($P < 0.05$). The relative abundance of *Bacteroidetes, Chloroflexi, Firmicutes* and *AAP99* in the dominant bacterial phylum and genus was significantly influenced by different HRT. In addition, we identified the denitrification reaction

as the major pathway in nitrogen metabolism in this study, as well as most of the significantly enriched genera were denitrifying genera as determined by LEfSe analysis. The overall low relative abundance of the nitrifying bacteria genera AOB and NOB may also be an important reason for the poor nitrogen removal. The effect of HRT on the expression of genes of the five functional modules of nitrogen metabolism was inconsistent. In conclusion, the above studies further complement the studies on the effects of plateau-specific environments on the microbial mechanisms of water ecology under different HRT conditions.

Keywords

High altitude region, HRT, Functional dominant bacterial genera, Nitrogen metabolism functional module

1. Introduction

Nowadays, due to accelerated urbanization, increasing population and rapid industrial development, water consumption has increased substantially and the threat of wastewater to the water ecosystem is becoming more and more serious (Priya et al., 2022). Many water pollutants originate from nitrogen and phosphorus in agriculture, as well as from harmful heavy metals and chemicals in industry (Priya et al., 2022). At present, the urbanization and industrial development in Tibetan region of China are accelerating and the population is increasing, coupled with the fact that the region is at high altitude and the water temperature and dissolved oxygen are low in the water ecosystem, the situation of urban wastewater treatment is therefore more severe. The Linzhi region of Tibet, with an average altitude of about 3000 m, has unique high-altitude environmental factors. High altitude leads to low temperature, low dissolved oxygen and high UV intensity etc. climatic environmental factors (Fang et al., 2018), and wastewater microbial characteristics are in turn influenced by temperature, dissolved oxygen and other natural environments. Therefore, the unique high-altitude climatic environmental factors indirectly affect the structural composition, diversity, energy metabolic pathways and genes abundance of microbial communities. Then the nitrogen metabolism pathway in energy metabolism must be affected by the high-altitude climate environment, including the relevant dominant genera and related genes involved in the six functional modules of nitrification, denitrification and nitrate reduction in nitrogen metabolism. It has been shown that altitude is an important factor affecting the community structure of nitrogen removal bacteria in municipal wastewater treatment when the altitude exceeds 1500 m (Niu et al., 2016), and microbial abundance and diversity are negatively correlated with altitude (Fang et al., 2018). And the abundance of denitrifying bacteria was found to be significantly reduced compared to lower altitudes, but there was no significant difference in microorganisms specifically related to degradation of refractory organic matter (Fang et al., 2018).

The hydraulic retention time (HRT) in wastewater treatment process parameters is also one of the important parameters affecting the microbial community structure and genes abundance. HRT directly determines the contact time between pollutants and microorganisms (Marais et al., 2020), which further affects the degradation rate of pollutants. Too long HRT increases the reactor volume, thus increasing the economic cost, as well as causing biological dead zone. Too short HRT leads to short contact time between pollutants and microorganisms and may increase hydraulic dead zone (Li et al., 2016). The homogeneity, diversity (Moreno-Andrade et al., 2015) and abundance (Wang et al., 2022) of microbial communities have been reported to decrease with lower HRT. However, Wang et al. (2022) showed that *paracoccus* is one of the very few denitrifying bacterial genera that can adapt to extreme HRT, which is negatively correlated with reduced HRT. And it was indicated that the denitrification genes *norBC* and *nosZ* increased significantly with the decrease of HRT, while the abundance of dissimilatory / assimilatory nitrate reduction genes *nirBD*, *nrfAH* and *nirA* first increased and then decreased significantly with the decrease of HRT. In addition, HRT had a

significant effect on the nitrifying genera in the nitrification reaction phase, and ammonia-oxidizing bacteria (AOB) had a higher effect with decreasing HRT (Boonnorat et al., 2019). In conclusion, the selection of a suitable HRT is important for improving the abundance of dominant genera of nitrogen removal related microorganisms, the abundance of related genes, the metabolic rate of nitrogen metabolic pathways, and the removal rate of nitrogen pollutants.

At present, there are few studies on the structure and related metabolic mechanisms of wastewater microorganisms under high altitude environment, and even the studies on the related functional genera and genes under the nitrogen metabolism pathway are still in a blank stage. Therefore, this paper investigates the effects of different HRT conditions on colony structure and nitrogen metabolism-related functional dominant genera and genes in high-altitude areas. Using A^2O process system as the wastewater treatment method, not only analyses the microbial community composition structure in the A^2O process at the Phylum and Genus levels affected by HRT changes, but also analyses the response of the dominant genera of nitrogen metabolism-related functional microorganisms and functional genes in the nitrogen metabolism functional module under different HRT conditions in detail. It provides a theoretical basis for the wastewater treatment system under the unique high-altitude environmental factors and reveals the wastewater microbial mechanism.

2. Materials and Methods

2.1 Test setup and operation

The pilot-scale A^2O process (Fig. 1) is divided into anaerobic, anoxic and aerobic tanks with volumes of 6.5L, 6.5L and 16.25L, respectively, with a ratio of 1:1:2.5, followed by a sedimentation tank. The anaerobic and anoxic tanks were equipped with stirring devices at the top, and the aerobic tank was equipped with an oxygen supply device. A thermostatic circulator was used for constant temperature control of the test. The experiment inlet water, sludge and mixed liquid return flow were controlled by peristaltic pumps. A sampling outlet was provided at the end of each tank.

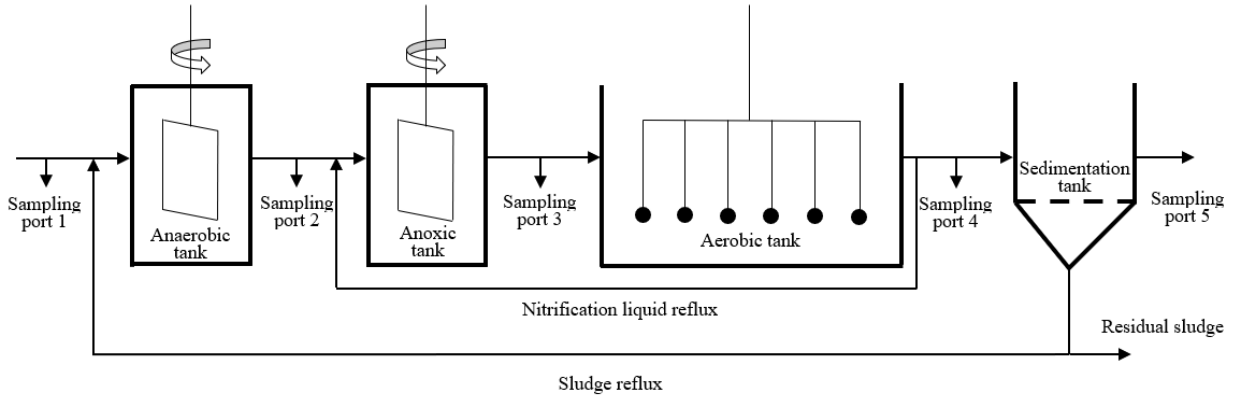


Fig. 1. Schematic diagram of A^2O process.

The activated sludge was incubated at a temperature of 20° C for 42 days, sludge settling ratio (SV_{30}) was (21±3%), and mixed liquor suspended solids (MLSS) was 2148～2864 mg/L. The test water was septic tank effluent from Tibet Agricultural and Animal Husbandry University, Linzhi, Tibet, and the influent water quality conditions are shown in Table 1.

Table 1. Wastewater quality indicators

pH	COD/(mg·L^{-1})	TN/(mg·L^{-1})	TP/(mg·L^{-1})	NH_3-N/(mg·L^{-1})
7.21～8.72	111.14～522.65	34.54～51.68	2.22～4.96	18.84～35.14

The process was carried out by controlling the influent flow rate to achieve four different HRT (15h, 17h,

21h, 26h), each operating for 9 days, with an interval of 72h for each operating condition. Sludge and mixed liquor were continuously refluxed by peristaltic pumps with reflux ratios of sludge reflux ratio (R) was 50% and mixed liquor reflux ratio (Ri) was 200%, respectively. The sludge age (SRT) was 4 days. The dissolved oxygen (DO) content of its aerobic pool was 2.0～3.0 mg/L, and the temperature of each pool was controlled at (23±0.5) °C.

2.2 16S rRNA gene sequencing

Samples for this study were commissioned to Majorbio Biotechnology Co., Ltd., Shanghai, China for sequencing (htpp://www.majorbio.com). The 16S rRNA gene sequencing was first run by putting the genomic DNA from the extracted samples into 1% agarose gel electrophoresis, followed by PCR amplification using PCR primers 338F (ACTCCTACGGGAGGCAGCAG) and 806R (GGACTACHVGGGTWTCTAAT) in the variable region (V3-V4) of the 16S rRNA gene to synthesize specific primers with barcode. PCR products from three replicate samples were mixed into 2% agarose gel electrophoresis for detection, and the PCR products were recovered by cutting the gel using the AxyPreDNA gel kit. Subsequently, the PCR products were detected and quantified using the QuantiFluor™-ST Blue Fluorescence Quantification System with reference to the electrophoretic quantification results. Finally, species annotation was performed on the QIIME2 cloud platform based on the Silva database (http://www.arbsilva.de) (Quast et al. 2012).

2.3 Data processing methods

All statistical analyses in this study were performed using the software SPSS 20.0. Data for each indicator of microorganisms were expressed using mean standard deviation (SD), as well as one-way analysis of variance (ANOVA) to test for significant differences between data, namely: when Pvalue<0.05, significant differences existed between data from different samples. Circos plots of sample-species relationships were drawn using the software Circos-0.67-7 (http://circos.ca/), and heat maps and histograms were drawn using the software Origin 2021. LEfSe analysis plots were drawn using the software LEfSe (http://huttenhower.sph.harvard.edu/galaxy/root?tool_id=lefse_upload), which was used to detect the non-parametric Kruskal-Wallis (KW) sum-rank test for different species abundance differences between groups to obtain significantly different species; then the Wilcoxon rank-sum test was used to test the consistency of the differences between species in the previous step in different subgroups between groups, and finally LDA linear discriminant analysis was applied to estimate the magnitude of the effect of these different species on the difference between groups. Based on the KEGG database, the abundance of nitrogen metabolism functional genera was normalized by 16Sr RNA function prediction software (PICRUSt2), and nitrogen metabolism pathways were mapped using the LianChuan Biological Platform (https://www.omicstudio.cn/tool/81).

3. Results and Discussion

3.1 Basic information

The statistical analysis of Domain, Kingdom, Phylum, Class, Order, Family, Genus, Species, and OTU (operational taxonomic unit) was performed using taxonomic level analysis, and the OTU of each sample was determined to use the Silva database, and the specific results of the statistical analysis are presented in Table 2.

Table 2. Sample information

Sample	Domain	Kingdom	Phylum	Class	Order	Family	Genus	Species	OTU
HRTh_15	1	1	27	61	134	234	424	633	1134
HRTh_17	1	1	26	59	129	234	447	679	1159

HRTh_21	1	1	29	62	136	224	387	580	964
HRTh_26	1	1	28	64	135	217	367	537	865
HRTq_15	1	1	28	62	138	238	441	654	1032
HRTq_17	1	1	26	59	129	226	416	613	1103
HRTq_21	1	1	27	62	133	223	392	588	999
HRTq_26	1	1	26	60	130	206	335	495	785
HRTy_15	1	1	28	62	127	206	371	525	734
HRTy_17	1	1	26	60	131	229	460	691	1260
HRTy_21	1	1	29	68	146	251	444	665	985
HRTy_26	1	1	25	61	130	208	345	504	827

As shown by sequencing number statistics from Table 2, both Domain and Kingdom levels are only 1, indicating that they are not affected by reactor and HRT. Phylum ∈ [25,29], Class ∈ [59,68], Order ∈ [127,146], Family ∈ [206,251], Genus ∈ [335,460], Species ∈ [495,691], and OUT ∈ [734,1260]. The above results are in accordance with Sun et al. (2020) in exploring the effect of different C/N ratios on the structure of microbial communities during aerobic nitrification at the Phylum and Class levels, but higher than that literature at the Order, Family, Genus and Species levels. At the OTU level, it is consistent with the number of OTU 1063 obtained in the literature (Shchegolkova et al., 2016) and OTU ∈ [1035,1168] in the literature (Zhang et al., 2019).

At the Phylum level, there was a significant difference between HRT_17 and HRT_21, HRT_21 and HRT_26 samples (Pvalue < 0.05). At the Class level, there was a significant difference between HRT_17 and HRT_21 samples (Pvalue < 0.05). It is worth noting that there is no significantly different sample group at the Order and Family levels. At both Genus and Species levels, there were significant differences between HRT_15 and HRT_26, HRT_17 and HRT_26, HRT_21 and HRT_26 samples (Pvalue < 0.05). At the OTU level, there was a significant difference between HRT_17 and HRT_26 samples (Pvalue < 0.05). In conclusion, different HRT had significant effects on Phylum, Class, Genus, Species and OTU levels, while they had less effect on Order and Family levels.

3.2 Alpha diversity analysis of microbial communities in the A^2O process system

Alpha diversity analysis can reflect the diversity and richness of microorganisms. Alpha diversity analysis reflects the colony richness index: ace; reflects the colony coverage index: coverage; reflects the colony diversity index: shannon; reflects the colony uniformity index: shannoneven; The specific values of the Alpha diversity index for each sample are shown in Table 3.

Table 3. Alpha diversity statistics

Sample	shannon	ace	coverage	shannoneven
HRTh_15	4.480764	1295.807	0.994155	0.63706
HRTh_17	5.066885	1309.725	0.994271	0.718166
HRTh_21	4.152191	1137.035	0.995187	0.604299
HRTh_26	3.827701	997.7249	0.995415	0.565999
HRTq_15	4.794359	1164.284	0.995251	0.690904

HRTq_17	3.966695	1253.691	0.994317	0.566202
HRTq_21	3.73019	1161.785	0.994726	0.540079
HRTq_26	3.415972	914.8477	0.995964	0.512471
HRTy_15	4.206137	773.1918	0.997826	0.637437
HRTy_17	4.771013	1402.091	0.994699	0.668315
HRTy_21	4.152946	1145.642	0.99462	0.602519
HRTy_26	3.049556	1015.683	0.995358	0.453951

As shown in Table 3, the colony coverage index coverage $\in$ [0.994155,0.997826], which indicates that the samples were sequenced with excellent coverage and the probability of undetected sequences in the samples was extremely low. The auxiliary software SPSS 20.0 was used to analyze whether there was a significant difference between them under the four different HRT conditions by ANOVA. The colony diversity index shannon showed in the significance analysis that there was a significant difference between HRT_15 and HRT_26, HRT_17 and HRT_26. Colony richness index ace in the significance analysis showed that there was a significant difference between HRT_17 and HRT_26. Colony uniformity index shannoneven in the significance analysis showed that there was a significant difference between HRT_15 and HRT_26, HRT_17 and HRT_26. There were 1 or 2 significant differences in colony richness, evenness and diversity in four groups of different HRT conditions.

The colony diversity index shannon $\in$ [3.049556,5.066885], the range of values is consistent with the literature (Chen et al., 2018) shannon value of 4.493, but lower than shannon $\in$ [5.905,7.148] in the literature (Zhang et al., 2019). The colony richness index ace $\in$ [773.1918,1402.091], the range of values is much higher than ace $\in$ [145.05,394.66] mentioned in the study of Zhang et al. (2020), but consistent with the study of Sun et al. (2020). The colony uniformity index shannoneven $\in$ [0.453951,0.718166]. The above Alpha diversity analysis index values were significantly different compared to other scholars' studies, possibly due to different treatment processes or unique high-altitude environmental factors, the exact reasons for which need to be further investigated.

3.3 Effect of different HRT on microbial community composition

To explore the abundance of dominant species in each sample and the percentage distribution of dominant species in different samples, the software RDP classifier was used to analyze the dominant species of the samples. Under the four HRT conditions, only two taxonomic levels, Phylum and Genus, were counted for classification and only species with microbial abundance greater than 1% were shown.

3.3.1 Analysis of colony composition structure at the Phylum level

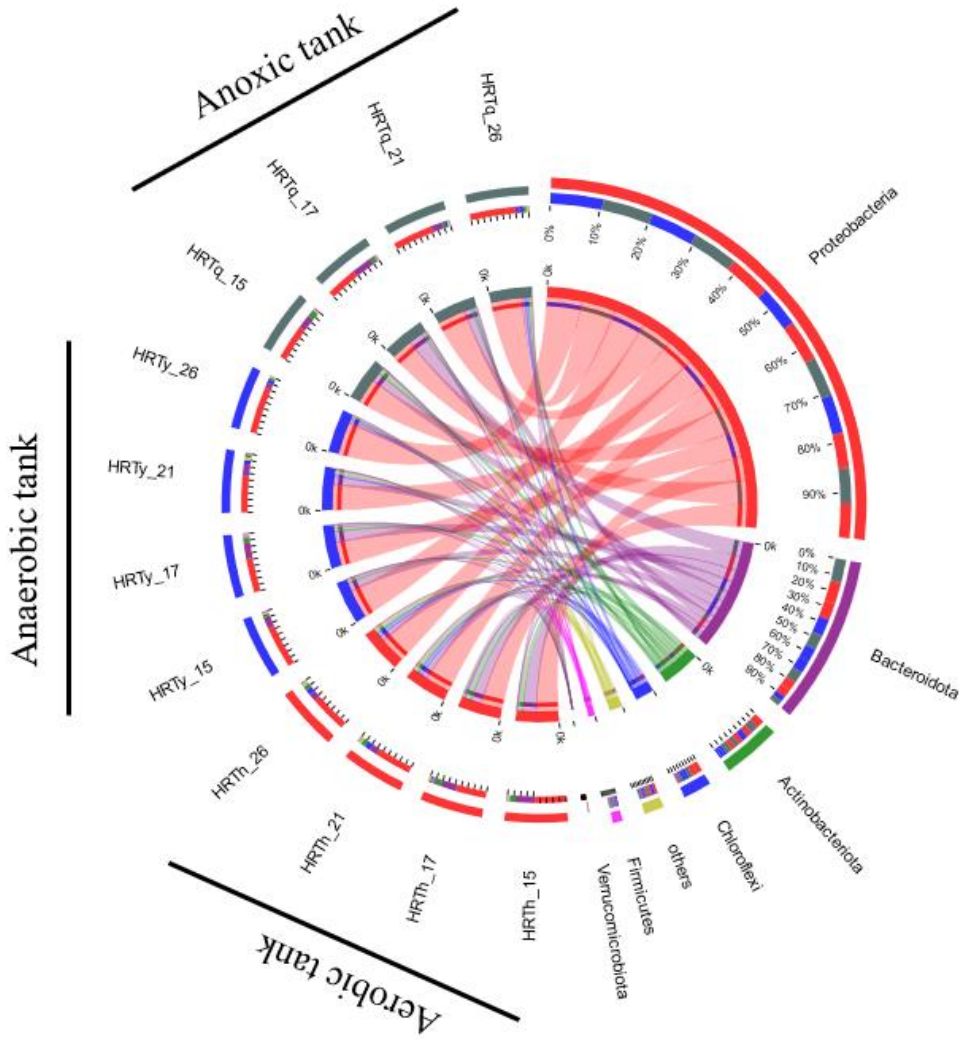


Fig. 2. Structural analysis of the colonies at the Phylum level.

As shown in Fig. 2, a total of 34 phyla were obtained in this study, where the top five dominant colonies at the Phylum level were *Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi and Firmicutes*, which are the dominant phyla at high altitude region, consistent with the results of Zong et al. (2022) who studied the effect of UV on community structural characteristics in the A^2O process under highland habitats. Tian and Wang (2021) found that at the Phylum level *Cyanobacteria* and *Acidobacteria* were also the dominant phyla but not *Chloroflexi*, while Yang et al. (2018) showed that *Proteobacteria, Bacteroidetes, Chloroflexi, Ignavibacteriae,* and *Acidobacteria* were the dominant phyla. It can be found that *Proteobacteria* and *Bacteroidetes* are the common dominant phyla, indicating that these two phyla are more stable, while several other dominant phyla may be affected differently by environmental factors at high altitude. In the anaerobic pool, the total abundance of the five dominant colonies ranged from 93.9% to 97.3%. In the anoxic and aerobic pools, the sum of abundance of the five dominant colonies decreased with the increase of HRT, and the sum of abundance ranged from 95.98% to 97.82% and 92.4% to 97.14%, respectively.

Proteobacteria, the main phylum in the wastewater treatment system, whose main function is responsible for organic matter removal (Huang et al., 2019), had the highest abundance under all four HRT conditions, in

agreement with the studies of Cydzik-Kwiatkowska and Zielińska (2016) and Yang et al. (2018). In the anaerobic pool, *Proteobacteria* abundance decreased and then increased with increasing HRT, from 70% (HRTy_15) to 78% (HRTy_26). In the anoxic pool, *Proteobacteria* abundance decreased and then increased with increasing HRT, from 60% (HRTq_15) to 75% (HRTq_26). In the aerobic pool, *Proteobacteria* abundance decreased and then increased with increasing HRT, from 53% (HRTh_15) to 63% (HRTh_26). It can be found that *Proteobacteria* abundance was lowest in anaerobic, anoxic and aerobic pools all at HRT_17. This is in accordance with the results of Alpha diversity analysis of colony abundance. *Proteobacteria* abundance in all three pools was higher than in the literature (36±2%) (Muszyński et al., 2015), but not significantly different from the literature [26.74%,63.22%] (Yang et al., 2018). However, *Proteobacteria* abundance was found to be insignificantly affected by HRT using the Kruskal-Wallis rank sum test (Pvalue > 0.05).

Bacteroidetes are specialized anaerobic bacilli that can degrade carbohydrates (Meng and He 2015) and also degrade organic matter into acetic acid, lactose and formic acid (Hill et al., 2007). It belongs to the second most dominant phylum in the present study, which is the same as the findings of Yang et al. (2018). In the anaerobic pool, *Bacteroidetes* abundance increased and then decreased with increasing HRT, from 19% (HRTy_15) to 8.2% (HRTy_26). In the anoxic pool, *Bacteroidetes* abundance increased and then decreased with increasing HRT, decreasing from 23% (HRTq_15) to 8% (HRTq_26). Both pools reached the highest at HRT_17. In the aerobic pool, *Bacteroidetes* abundance decreased with increasing HRT, from 32% (HRTh_15) to 12% (HRTh_26). *Bacteroidetes* abundance was significantly affected by HRT (Pvalue < 0.05). The above analysis was higher than the literature (3±0.3%) (Muszyński et al., 2015), but in accordance with the results of Nielsen et al. (2010) with 12% abundance of *Bacteroidetes*.

Actinobacteria is a phylum of heterotrophic aerobic bacteria suitable for survival in effluents containing high concentrations of ammonia nitrogen (You et al., 2021) and contribute to the nitrogen cycle (Li et al., 2013). The range of abundance in aerobic pool was from 7% to 13%, with the highest at HRT_17 and the lowest at HRT_26. The abundance range in the anoxic pool was 5% to 12%, highest at HRT_15 and lowest at HRT_26. In the anaerobic pool the abundance ranged from 3.9% to 9.4%, was highest at HRT_17 and lowest at HRT_26. The above results are slightly lower compared to the 15±1% abundance of *Actinobacteria* obtained by Muszyński et al. (2015). However, *Actinobacteria* abundance was not significantly affected by HRT (Pvalue > 0.05).

Chloroflexi plays an important role in the degradation of carbohydrates and cellular materials (Choi et al., 2017). Its abundance increased in anaerobic, anoxic and aerobic pools all with increasing HRT, but in contrast to the results of Su et al. (2019), *Chloroflexi* abundance increased when HRT was reduced from 24 to 12 h. The range of abundance in this study was 0.24% to 9.4%, which was significantly influenced by HRT (Pvalue < 0.05). It was slightly lower compared to the literature (12±1%) (Muszyński et al., 2015), but consistent with the literature (Yang et al., 2018).

Firmicutes are Gram-positive bacteria that degrade lactic acid and COD (Jing et al., 2019) and can also denitrify under anaerobic conditions using nitrate (Li et al., 2017). Its abundance increased and then decreased with increasing HRT in anaerobic, anoxic and aerobic pools, reaching a maximum at HRT_17 in all pools and a minimum at HRT_26 in all pools, with an abundance range of 0.88% to 3.2%, significantly influenced by HRT (Pvalue < 0.05), and its abundance was higher than that of the literature (0.4±0.2%) (Muszyński et al., 2015).

The above HRT had a significant effect on the abundance of the dominant phyla *Bacteroidetes,*

Chloroflexi, and *Firmicutes*. The first five dominant phyla showed significant differences in abundance and species compared to other regional studies.

3.3.2 Analysis of colony composition structure at the Genus level

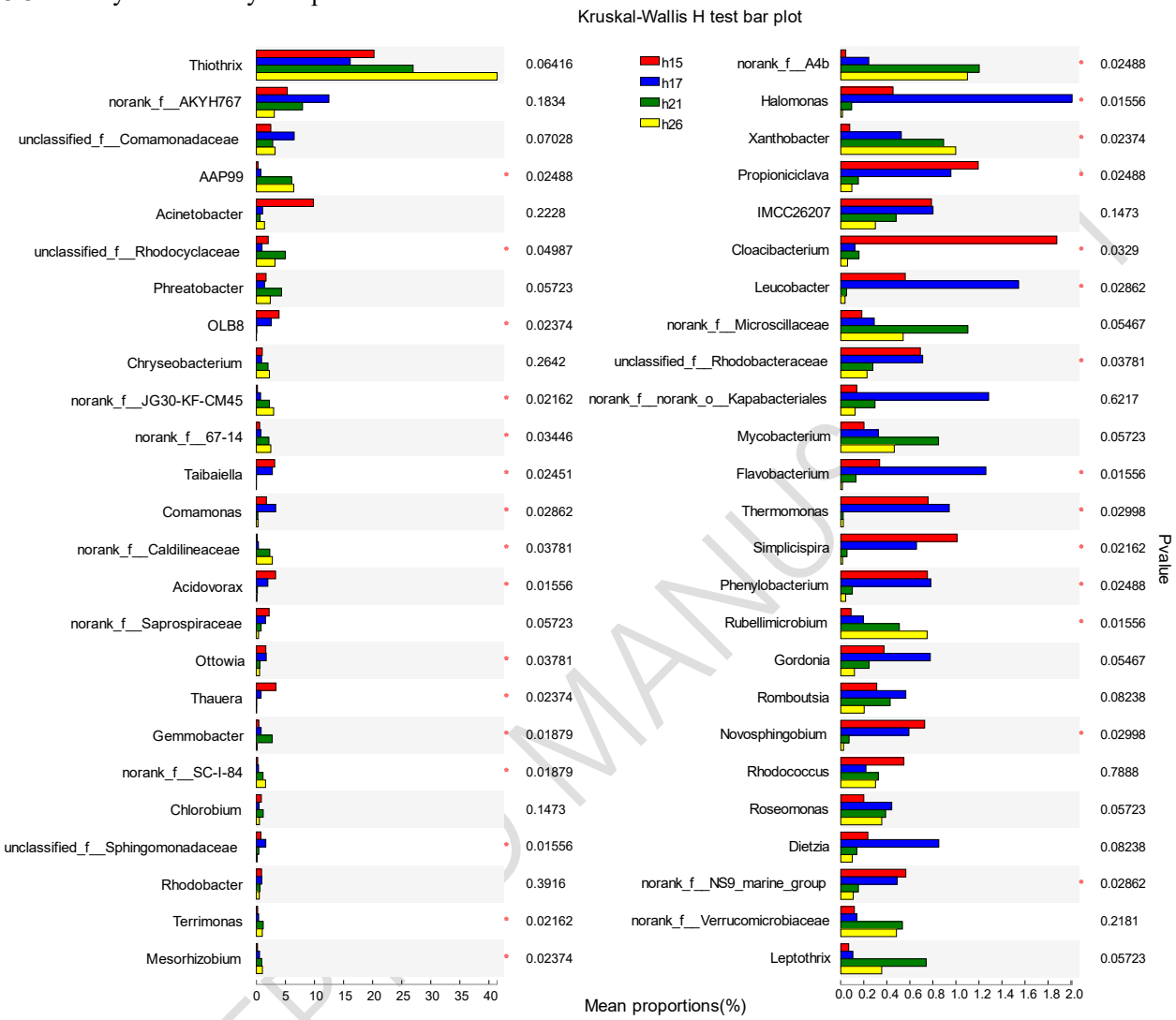


Fig. 3. Mean relative abundance of colonies in different subgroups at the Genus level. (Three reactors as a group, Y-axis indicates species name at the Genus level, X-axis indicates mean relative abundance in different subgroups, color bars indicate different subgroups, when Pvalue < 0.05, denoted by *)

As shown in Fig. 3, a total of 688 genera were obtained in this study, and the top 50 genera were screened for abundance ranking. The top 5 dominant genera at the Genus level were *Thiothrix, norank_f__AKYH767, unclassified_f__Comamonadaceae, AAP99,* and *Acinetobacter*. The average total relative abundance of the five dominant genera ranged from 36.997% to 55.537%.

Among the top 50 genera in terms of abundance, 30 genera showed significant differences in mean relative abundance under different HRT conditions (Pvalue < 0.05), with a mean total relative abundance range of 26.237% to 31.389%. Among the 30 genera with significant differences, the average relative abundance of eight genera, *AAP99, norank_f__JG30-KF-CM45, norank_f__67-14, norank_f__Caldilineaceae, norank_f__SC-I-84, Mesorthizobium, Xanthobacter* and *Rubellimicrobium*, showed a positive correlation with HRT, increasing with HRT. The mean relative abundance of nine genera, *OLB8, Taibaiella, Acidovorax, Thauera, Propioniciclava, Simplicispira, Novosphingobium, and norank_f__NS9_marine_group,* was

negatively correlated with HRT and decreased with HRT. Notably, *norank_f__Saprospiraceae* did not show significant differences, but it was negatively correlated with HRT. Another 20 genera did not show significant differences in mean relative abundance (Pvalue > 0.05), indicating that the abundance changes under the four HRT conditions showed relative stability and were not significantly influenced by HRT, with a mean total relative abundance range of 47.347% to 58.482%. The above study showed that 3/5 of the dominant genera showed significant variability to the changes of HRT, 2/5 of the dominant genera showed relative stability, and the sum of abundance of genera showing relative stability was higher than the sum of abundance of genera showing significant variability.

3.4 Functional microorganisms of nitrogen metabolism in activated sludge at four different HRT

3.4.1 Dominant genera of functional microorganisms related to nitrogen metabolism

Municipal wastewater treatment for nitrogen and phosphorus removal cannot be achieved without the role of functional microbial genera, and the changes in the abundance of dominant functional genera involved in nitrogen metabolism related to different HRT conditions in the three reactors were discussed in this study, as shown in Figure 4. A total of 21 dominant genera involved in five functional modules of nitrogen metabolism were screened. It is noteworthy that this test did not find anaerobic ammonia oxidizing genera, so the anaerobic ammonia oxidizing functional module was missing. 10 genera of denitrifying bacteria, 3 genera of nitrifying bacteria, 5 genera of nitrogen-fixing bacteria, 5 genera of dissimilatory nitrate-reducing bacteria genera and 2 genera of assimilatory nitrate-reducing bacteria genera were screened, among which 4 dominant genera were jointly involved in the reaction of two functional modules of nitrogen metabolism.

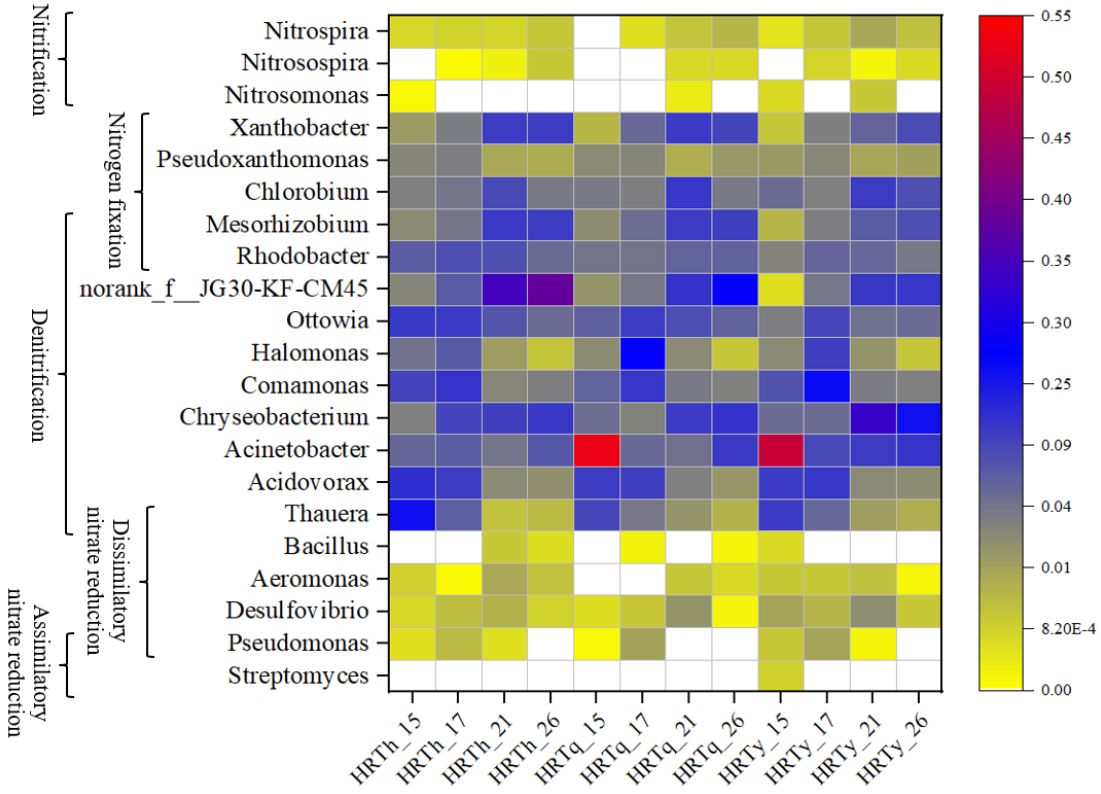


Fig. 4. Dominant genera of functional microorganisms related to nitrogen metabolism.

As shown in Fig. 4, it is not difficult to find that the dominant genera with greater relative abundance are all denitrifying genera, indicating that denitrification reaction is a major pathway in nitrogen metabolism, and

the five most abundant denitrifying genera are *Acinetobacter, Chryseobacterium, Acidovorax, Comamonas,* and *norank_f__JG30-KF- CM45*, with the highest total relative abundance among the reactors HRT_15. It is noteworthy that the relative abundance of *Acinetobacter* was highest at HRT_15 in the anaerobic and anoxic pools, and was much higher than the other three HRT. The relative abundance of *Acidovorax* was highest at HRT_15 in all three reactors, and then decreased with increasing HRT, which is consistent with the previous analysis of species composition. The relative abundance of *Comamonas* was the highest at HRT_17, which increased and then decreased with increasing HRT. *norank_f__JG30-KF-CM45* was different, which increased with increasing HRT and the highest abundance was at HRT_26. However, *Chryseobacterium* did not show regular fluctuations in relative abundance with HRT in the three reactors, and its average relative abundance was highest in the anaerobic pool. Jiang et al. (2020) found that *Acidovorax* was the predominant denitrifying genus in a vertical folded-flow solid-phase nitrifying biofilm reactor. Chen et al. (2016) and Wen et al. (2016) showed that *Thauera* and *Comamonas* were the key functional genera involved in denitrification reaction in wastewater treatment plants. Li et al. (2022) found that *norank_f__JG30-KF-CM45* was among the top 10 functionally dominant genera of known metabolic types screened for denitrification.

Notably, the denitrifying genus *Thauera* was also involved in the dissimilatory nitrate reduction reaction, and abundant denitrification and dissimilatory nitrate reduction genes were detected in this genus: *napB, narH,* and *norC* (Sun et al., 2019), and *Thauera* was also the dominant genus for dissimilatory nitrate reduction, while the abundance of the other four dissimilatory nitrate-reducing bacteria genera was much lower than that of *Thauera*, indicating a smaller contribution to the dissimilatory nitrate reduction reaction, which, as mentioned in the introduction, was the desired result of this experiment. And the total relative abundance of the five dominant genera was highest at HRT_15. *Pseudomonas* is a genus of dominant dissimilatory nitrate-reducing bacteria as well as assimilatory nitrate-reducing bacteria. It has been shown that the combination of *Pseudomonas* and *Bacillus* showed better efficiency in bioaccumulation and degradation of heavy metals. (Priya et al., 2015). It was also found that *Streptomyces* was detected only at HRTy_15 with small relative abundance. The total relative abundance of the dominant genera of assimilatory nitrate-reducing bacteria was highest at HRT_17. The denitrifying genera *Mesorhizobium* and *Rhodobacter* were also involved in the nitrogen fixation reaction, in which *Mesorhizobium, Rhodobacter, Chlorobium* and *Xanthobacter* genera were the most abundant, with the highest sum of relative abundance at HRT_21. A detailed analysis of the genera related to the nitrification reaction is given in the next subsection.

3.4.2 Effect of different HRT on AOB and NOB of nitrifying bacteria genera

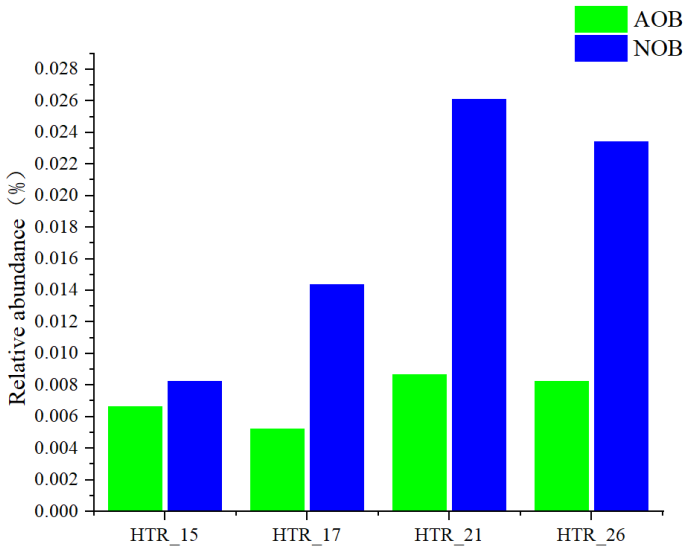


Fig. 5. Average relative abundance of AOB and NOB as affected by changes in HRT.

Ammonia-oxidizing bacteria (AOB) in this pilot study detected only *Nitrosomonas* and *Nitrosospira* at the Genus level, in agreement with the literature (Jiang et al., 2020) study, and nitrite-oxidizing bacteria (NOB) detected only *Nitrospira* at the Genus level and not *Nitrobacter*, in agreement with the results of the literature (Xiao et al., 2021). Fig. 5 demonstrates the trend of the average relative abundance of nitrifying bacteria genera AOB and NOB as influenced by the variation of HRT. In general, it can be observed that the abundance of nitrifying bacteria genera AOB and NOB was lower and significantly lower than that of such studies in the literature (Cui et al., 2021.; Feng et al., 2021.; Xiao et al., 2021). Therefore, the lower abundance of nitrifying bacterial genera may be influenced by environmental factors at high altitude, which is an important reason for the poor nitrogen removal effect. The average relative abundance of AOB was lower than the average relative abundance of NOB under all four HRT conditions.

The relative abundance of AOB showed fluctuating changes with increasing HRT, and was highest at HRT_21, followed by HRT_26, and lowest at HRT_17. The relative abundance of NOB increased and then decreased with increasing HRT, and was highest at HRT_21, followed by HRT_26, and lowest at HRT_15. In summary, the abundance of AOB and NOB was highest at HRT_21, indicating that the nitrification reaction was optimal.

3.4.3 Discriminant analysis of significant differences in LEfSe multilevel species

In addition to Alpha diversity analysis, another main objective of comparing microbial communities was to identify microbial taxa that had a significant effect on intergroup differences. Therefore, based on nitrogen metabolism-related microbial communities, taxa with significant differences in abundance were found using LEfSe multilevel species difference discriminant analysis software, and linear discriminant analysis (LDA) was used to estimate the magnitude of the effect of species abundance on intergroup differences. Since the number of OTU detected in this study was too large, the analysis was performed from the Phylum to Genus level, as shown in Fig. 6A.

LDA discriminant bar charts counted microbial taxa with significant effects in multiple groups and showed only microbial taxa with significant effects on intergroup differences for LDA scores (≥3.5), as shown in Fig. 6 B.

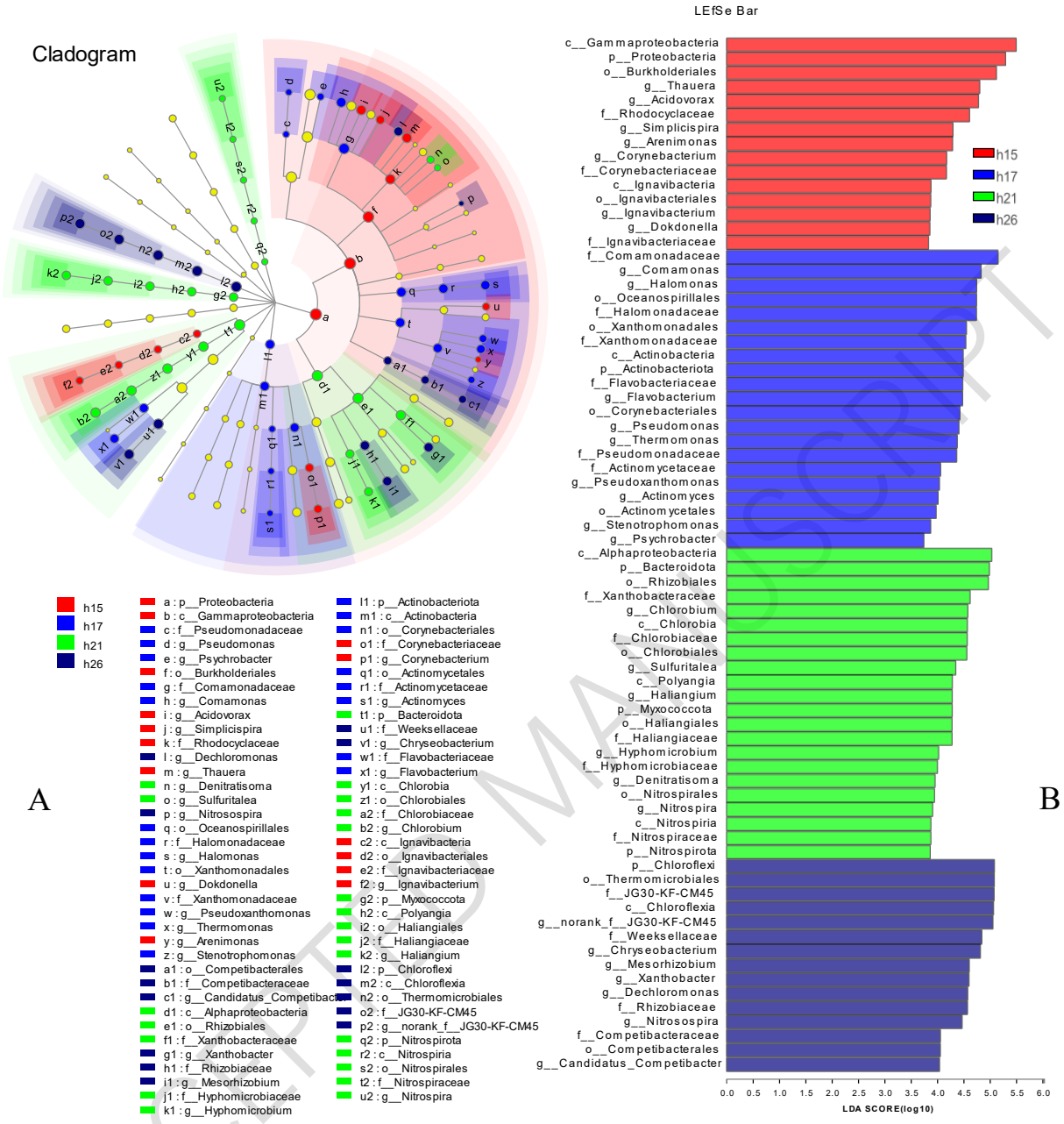


Fig. 6. Comparative LEfSe analysis of nitrogen metabolism county-related microbial abundance in four different groups of HRT samples. A: Five-level microbial community branching diagram from Phylum to Genus level (Different color nodes indicate microbial taxa that are significantly enriched in the corresponding groups and have a significant effect on the difference between groups, and light-yellow nodes indicate microbial taxa that have no significant effect on the difference between groups in different subgroups). B: LDA discriminant bar chart counting microbial taxa with significant effects in the grouping (The LDA threshold is 3.5, and a larger LDA value represents a greater effect of species abundance on the differential effect).

As shown in Fig. 6A, 3 groups of colonies were significantly enriched in the HRT_15 samples, namely: *Proteobacteria* (from Phylum to Class *Gammaproteobacteria* to Order *Burkholderiales* to Family *Rhodocyclaceae* to Genus *Thauera* and Family *Rhodanobacteraceae* to Genus *Dokdonella*), *Ignavibacteria* (from Class to Genus), *Corynebacteriaceae* (from Family to Genus). In HRT_17 samples, 6 groups of colonies

were significantly enriched, namely: *Actinobacteriota* (from Phylum to Class *Actinobacteria* to Order *Corynebacteriales* and Actinomycetaceae (from Order to Genus)), *Flavobacteriaceae* (from Family to Genus), *Xanthomonadales* (from Order to Family *Xanthomonadaceae* to Genus *Pseudoxanthomonas*, *Thermomonas* and *Stenotrophomonas*), *Pseudomonadales* (from Family to Genus), *Comamonadaceae* (from Family to Genus *Comamonas* and *Psychrobacter*), *Oceanospirillales* (from Order to Genus). In HRT_21 samples, 4 groups of colonies were significantly enriched, namely: *Myxococcota* (from Phylum to Genus), *Alphaproteobacteria* (from Class to Order *Rhizobiales* to Family *Hyphomicrobiaceae* to Genus and Family *Xanthobacteraceae*), *Bacteroidota* (from Phylum to Class *Chlorobia* (from Class to Genus)), and *Nitrospirota* (from Phylum to Genus). In HRT_26 samples, 4 groups of colonies were significantly enriched, namely: *Chloroflexi* (from Phylum to Genus), *Weeksellaceae* (from Family to Genus), *Rhizobiaceae* (from Family to Genus), and *Competibacterales* (from Order to Genus).

A total of 29 bacterial species involved in nitrogen metabolism-related dominant genera were significantly enriched at the Genus level under four different HRT of five nitrogen metabolism functional modules. In HRT_15, *Acidovorax, Simplicispira, Thauera, Dokdonella, Arenimonas, Ignavibacterium* and *Corynebacterium* were significantly enriched in denitrification reaction, but there was no dominant genus significantly enriched in nitrogen fixation reaction, assimilatory nitrate reduction reaction and nitrification reaction. In HRT_17, *Pseudomonas, Comamonas, Thermomonas, Flavobacterium, Psychrobacter, Halomonas*, and *Stenotrophomonas* were significantly enriched in denitrification reaction, among which *Pseudomonas* was also significantly enriched in the nitrogen fixation reaction, assimilatory/ dissimilatory nitrate reduction reaction. *Pseudoxanthomonas* and *Actinomyces* were significantly enriched in the nitrogen fixation reaction, while there was no significant enrichment of the dominant genus in the nitrification reaction. In HRT_21, only four dominant genera were found to be significantly enriched in denitrification reaction, namely: *Denitratisoma, Hyphomicrobium, Haliangium,* and *Sulfuritalea*. *Chlorobium* was significantly enriched in nitrogen fixation reaction, and *Nitrospirota* was significantly enriched in nitrification reaction. In HRT_26, *Dechloromonas, Candidatus_Competibacter, Mesorhizobium, Chryseobacterium* and *norank_f__JG30-KF-CM45* were significantly enriched in denitrification reaction, where *Mesorhizobium* was also a significantly enriched genus in the nitrogen fixation reaction, and *Xanthobacter* was another significantly enriched genus in the nitrogen fixation reaction. *Nitrosospira* was a significantly enriched genus of nitrifying bacteria. No dominant genus was found to be significantly enriched in the other two functional modules. In the above, it is easy to find that most of the dominant genera related to nitrogen metabolism were significantly enriched in denitrification reaction, which is consistent with the previous analysis that the dominant genera of nitrogen metabolism related functional microorganisms was denitrifying genera in greater relative abundance.

As shown in Fig. 6B, there were 73 microbial taxa with significantly different abundance with LDA score higher than 3.5, and 15, 21, 22, and 15 bacteria were significantly enriched in HRT_15, HRT_17, HRT_21, and HRT_26, respectively. The differences among these categories were statistically analyzed for significance (Pvalue<0.05). As an example, HRT_15 included 3 Phyla (*Proteobacteria, Actinobacteriota, Bacteroidota*), 3 classes (*Ignavibacteria, Actinobacteria, Gammaproteobacteria*), 4 orders (*Ignavibacteriales, Corynebacteriales, Burkholderiales, Xanthomonadales*), 6 families (*Ignavibacteriaceae, Corynebacteriaceae, Comamonadaceae, Xanthomonadaceae, Rhodocyclaceae, Rhodanobacteraceae*), 6 genera (*Simplicispira, Arenimonas, Acidovorax, Dokdonella, Thauera, Corynebacterium*).

3.5 Functional genes in activated sludge at different HRT

Based on the KEGG database, PICRUST2 function prediction was used to understand microbial community function and nitrogen metabolism. A total of 7132 genes and 380 ko pathways were obtained, among which a total of 49 genes related to nitrogen metabolism were obtained.

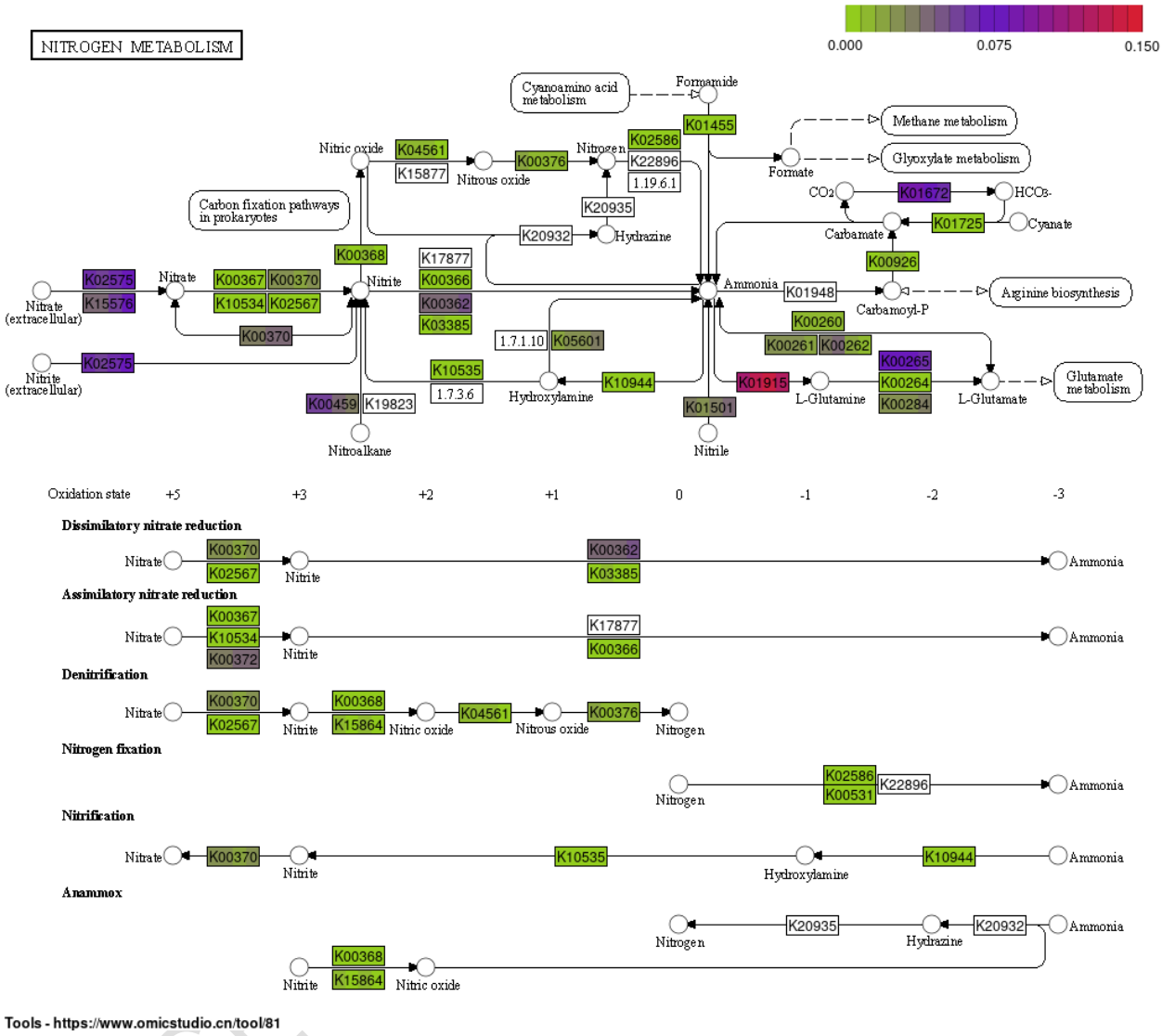


Fig. 7. Nitrogen metabolic pathways. (Gene codes involved in nitrogen metabolism are indicated in each metabolic pathway, but each gene code may contain the sum of the relative abundance of several gene codes, shown as one of the genes. Each gene code box is divided into four parts, and representing the average relative abundance of genes in the three reactors under four different HRT conditions. Different colors represent the relative abundance size, and no color marked means the gene is not detected)

As shown in Fig. 7, the average relative abundance of functional genes related to the nitrogen metabolic pathway was analyzed in detail under four different HRT conditions. The gene codes involved in the nitrification reaction module: K10944 (including K10944, K10945, K10946), K10535 and K00370 (including K00370, K00371). It was noteworthy that the relative abundance of K10944 and K10535 genes were the highest at HRT_15, and the relative abundance of K00370 gene was the highest at HRT_26. But the relative abundance of K10944 and K10535 genes was much smaller than the relative abundance of K00370 gene, and the expression of K00370 gene was mainly in the nitrification reaction module. The overall indicated that the

best expression was HRT_26, but this was inconsistent with the previous analysis that the relative abundance of nitrifying bacteria genera AOB and NOB was highest at HRT_21, and the reason might be that AOA (ammonia oxidizing archaea) or other bacteria containing nitrification genes were best expressed at HRT_26.

The gene codes involved in the denitrification reaction module: K00370 (including K00370, K00371, K00374), K02567 (including K02567, K02568), K00368, K15864, K04561 (including K04561, K02305) and K00376, all with high relative abundance. The total relative abundance was the highest at HRT_15 and the lowest at HRT_17, indicating that the best genes expression in denitrification reaction was at HRT_15. This is consistent with the fact that the total relative abundance of the dominant genera of denitrifying bacteria was highest at HRT_15.

The gene codes involved in the nitrogen fixation reaction module: K02586 (including K02586, K02591, K02588) and K00531. The relative abundance of K02586 gene was much greater than that of K00531 gene, indicating that the expression of K02586 gene was mainly in the nitrogen fixation reaction, and the best expression was at HRT_21 and the worst at HRTh_17, which was consistent with the result that the total relative abundance of the dominant genera involved in the nitrogen fixation reaction was highest in HRT_21 when analyzed.

The gene codes involved in the assimilatory nitrate reduction reaction module: K00367, K10534, K00372 (including K00372, K00360) and K00366. The expression of the genes K00372 and K00367 was dominant during the conversion of NO_3^- to NO_2^-. The contribution of K10534 gene expression was small, and the total relative abundance of the other four genes was greatest at HRT_26 and lowest at HRT_17, indicating that the best expression of the assimilatory nitrate-reducing genes was found at HRT_26, but this was inconsistent with the previous total relative abundance of the dominant assimilatory nitrate-reducing genera being highest at HRT_17. The reason might be that the lower abundance of unlisted assimilatory nitrate-reducing bacteria genera contained a greater sum of related genes than the sum of related genes of the dominant genera.

The gene codes involved in the dissimilatory nitrate reduction reaction module: K00370 (including K00370, K00371, K00374), K02567 (including K02567, K02568), K00362 (including K00362, K00363) and K03385 (including K03385, K15867). In the process of NO_3^- to NO_2^- conversion, it was mainly the expression of K00370 gene, and the contribution of K02567 gene expression was smaller. In the process of NO_2^- to NH_4^+ conversion, it was mainly the expression of K00362 gene, and the contribution of K03385 gene expression was smaller. The relative abundance of K00362 and K00370 was the largest at HRT_26 and the lowest at HRT_17, indicating that the best expression of the dissimilatory nitrate reduction genes was at HRT_26. However, the sum of the abundance of the dominant genera of dissimilatory nitrate-reducing bacteria in the previous analysis was highest at HRT_15, and the inconsistency between these two results might also be that the lower abundance of unlisted dissimilatory nitrate-reducing bacteria genera contained a greater sum of related genes than the sum of related genes of the dominant genera.

Finally, it could be found that the anaerobic ammonia oxidation reaction module pathway was incomplete, and no relevant functional gene was detected, which was due to the fact that no anaerobic ammonia oxidizing bacteria genus was detected in this study as mentioned in the previous analysis of the dominant functional genera related to nitrogen metabolism.

4. Conclusions

In this paper, different HRT had significant effects on the structural composition of microbial communities and the dominant genera and genes for functions related to nitrogen metabolism. Different HRT had significant effects on Phylum, Class, Genus, Species and OTU levels ($P < 0.05$), while they had less effect

on Order and Family levels ($P > 0.05$). The main dominant bacteria phyla at high altitude region included *Proteobacteria, Bacteroidetes, Actinobacteria, Chloroflexi, Firmicutes*, etc. and have significant differences compared with other regions. Denitrification reaction was the main pathway of nitrogen metabolism in this study. The overall low relative abundance of the nitrifying bacteria genera AOB and NOB may also be an important reason for the poor nitrogen removal. A total of 73 taxonomic branches of nitrogen metabolism-related microbial markers with LDA thresholds greater than 3.5 were found to have a significant effect on the differences between groups by LEfSe analysis, and most of the significantly enriched genera were denitrifying bacteria. In addition, the effect of HRT on the expression of genes of the five functional modules of nitrogen metabolism was inconsistent. Notably, the anaerobic ammonia oxidation reaction module reaction module pathway was incomplete because anaerobic ammonia-oxidizing bacteria genus and functional genes were not detected.

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Conflicts of Interest

The authors declare no conflict of interest.

References

Boonnorat, J., Kanyatrakul, A., Prakhongsak, A., Honda, R., Panichnumsin, P., and Boonapatcharoen, N. **(**2019) Effect of hydraulic retention time on micropollutant biodegradation in activated sludge system augmented with acclimatized sludge treating low-micropollutants wastewater. *Chemosphere* **230**: 606-615.

Chen, C., Ouyang, W., Huang, S., and Peng, X. (2018) Microbial community composition in a simultaneous nitrification and denitrification bioreactor for domestic wastewater treatment. IOP Conf Ser: Earth Environ Sci **112**: 012007.

Chen, Y., Zhao, Z., Peng, Y., Li, J., Xiao, L., and Yang, L. (2016) Performance of a full-scale modified anaerobic/anoxic/oxic process: high-throughput sequence analysis of its microbial structures and their community functions. *Bioresour Technol* **220**: 225-232.

Choi, J., Kim, E.S., and Ahn, Y. (2017) Microbial community analysis of bulk sludge/cake layers and biofouling-causing microbial consortia in a full-scale aerobic membrane bioreactor. *Bioresour Technol* **227**: 133-141.

Cui, H., Zhang, L., Zhang, Q., Li, X., Huang, Y., and Peng, Y. (2021) Advanced nitrogen removal from low C/N municipal wastewater by combining partial nitrification-anammox and endogenous partial denitrification-anammox (PN/A-EPD/A) process in a single-stage reactor. *Bioresour Technol* **339**: 125501.

Cydzik-Kwiatkowska, A., and Zielińska, M. (2016) Bacterial communities in full-scale wastewater treatment systems. *World J Microb Biot* **32**: 1-8.

Fang, D., Zhao, G., Xu, X., Zhang, Q., Shen, Q., Fang, Z., et al. (2018) Microbial community structures and functions of wastewater treatment systems in plateau and cold regions. *Bioresour Technol* **249**: 684-693.

Feng, Y., Peng, Y., Wang, B., Liu, B., and Li, X. (2021) A continuous plug-flow anaerobic/aerobic/anoxic/aerobic (AOAO) process treating low COD/TIN domestic sewage: Realization of partial nitrification and extremely advanced nitrogen removal. *Sci Total Environ* **771**: 145387.

Hill, V.R., Kahler, A.M., Jothikumar, N., Johnson, T.B., Hahn, D., and Cromeans, T.L. (2007) Multistate evaluation of an ultrafiltration-based procedure for simultaneous recovery of enteric microbes in 100-liter

tap water samples. *Appl Environ Microb* **73**: 4218-4225.

Huang, W., Liu, L.Y., Wu, M.H., Chen, L.H., Lü, X., Ye, M.F., et al. (2019) Microbial community structure and dynamics in swine wastewater treatment system, *China Environ Sci* **39**: 839-848. (in Chinese)

Jiang, L., Wu, A., Fang, D., Zhang, Y., Shen, Q., Xu, X., et al (2020) Denitrification performance and microbial diversity using starch-polycaprolactone blends as external solid carbon source and biofilm carriers for advanced treatment. *Chemosphere* **255**: 126901.

Jing, S., Li, Y., Yu, L., Yang, W., Zhu, H., and Li, W. (2019) Denitrification efficiency of the special moving-bed biofilm reactor (SMBBR) process for domestic sewage treatment and its microbial diversity. *Chin J Appl Environ Biol* **25**: 206-214. (in Chinese)

Li, Q., You, X., Zhang, Y., Mao, Y., and Jiao, H. (2013) Characteristics of bacterial community structure in the central aquaculture area of Xiangshan Port and its changes during habitat restoration. *J Fish Sci China* **20**: 1234-1246. (in Chinese)

Li, S., Nan, J., and Gao, F. (2016) Hydraulic characteristics and performance modeling of a modified anaerobic baffled reactor (MABR). *Chem Eng J* **284**: 85-92.

Li, T., Yu, L., and Liu, P., (2017) Cheng, L. Ammonia removal and microbial community structure in wave-flowing ecological filtering bed. *Huanjing Kexue Xuebao* **37**: 3664-3671. (in Chinese)

Li, Y., Hao, K., Zong, Y., You, J., and Guo, M. (2022) Study on the microbial metabolic mechanism of A^2O process activated sludge with different hydraulic retention time in plateau environment. *Environ Pollut Control* **44**: 20-26. (in Chinese)

Marais, T.S., Huddy, R.J., Harrison, S.T.L. and van Hille, R.P. (2020) Effect of hydraulic residence time on biological sulphate reduction and elemental sulphur recovery in a single-stage hybrid linear flow channel reactor. *Biochem Eng J* **162**: 107717.

Meng, F., and He, X. (2015) Effects of naturally occurring grit on the reactor performance and microbial community structure of membrane bioreactors. *J Membrane Sci* **496**: 284-292.

Moreno-Andrade, I., Carrillo-Reyes, J., Santiago, S.G., and Bujanos-Adame, M.C. (2015) Biohydrogen from food waste in a discontinuous process: effect of HRT and microbial community analysis. *Int J Hydrogen Energ* **40(48)**: 17246-17252.

Muszyński, A., Tabernacka, A., and Miłobędzka, A. (2015) Long-term dynamics of the microbial community in a full-scale wastewater treatment plant. *Int Biodeter Biodegr* **100**: 44-51.

Nielsen, P.H., Mielczarek, A.T., Kragelund, C., Nielsen, J.L., Saunders, A.M., Kong, Y., et al. (2010) A conceptual ecosystem model of microbial communities in enhanced biological phosphorus removal plants. *Water Res* **44**: 5070-5088.

Niu, L., Li, Y., Wang, P., Zhang, W., Wang, C., Cai, W., et al. (2016) Altitude-scale variation in nitrogen-removal bacterial communities from municipal wastewater treatment plants distributed along a 3600-m altitudinal gradient in China. *Sci Total Environ* **559**: 38-44.

Priya, A. K., & Nagan, S. (2015). Bioremediation of Dye Effluent and Metal Contaminated Soil: Low-Cost Method for Environmental Clean up by Microbes. *J Environ Sci Eng*, *57*(2), 109-119.

Priya, A. K., Gnanasekaran, L., Dutta, K., Rajendran, S., Balakrishnan, D., & Soto-Moscoso, M. (2022). Biosorption of heavy metals by microorganisms: Evaluation of different underlying mechanisms. *Chemosphere*, *307*, 135957.

Priya, A. K., Jalil, A. A., Vadivel, S., Dutta, K., Rajendran, S., Fujii, M., & Soto-Moscoso, M. (2022). Heavy metal remediation from wastewater using microalgae: Recent advances and future

trends. *Chemosphere*, *305*, 135375.

Quast, C., Pruesse, E., Yilmaz, P., Gerken, J., Schweer, T., Yarza, P., ... & Glöckner, F. O. (2012). The SILVA ribosomal RNA gene database project: improved data processing and web-based tools. *Nucleic acids research*, *41*(D1), D590-D596. https://doi.org/10.1093/nar/gks1219

Shchegolkova, N.M., Krasnov, G.S., Belova, A.A., Dmitriev, A.A., Kharitonov, S.L., Klimina, K.M., et al. (2016) Microbial community structure of activated sludge in treatment plants with different wastewater compositions. *Front Microbiol* **7**: 90.

Su, C., Deng, Q., Lu, Y., Qin, R., Chen, S., Wei, J., et al. (2019) Effects of hydraulic retention time on the performance and microbial community of an anaerobic baffled reactor-bioelectricity Fenton coupling reactor for treatment of traditional Chinese medicine wastewater. *Bioresour Technol* **288**: 121508.

Sun, F.L., Fan, L.L., Wang, Y.S., and Huang, L.Y. (2019) Metagenomic analysis of the inhibitory effect of chromium on microbial communities and removal efficiency in A^2O sludge. *J Hazard Mater* **368**: 523-529.

Sun, H. Shi, W. Cai, C. Ge, S. Ma, B. Li, X. et al. (2020) Responses of microbial structures, functions, metabolic pathways and community interactions to different C/N ratios in aerobic nitrification. *Bioresour Technol* **311**: 123422.

Tian, L., and Wang, L. (2021) Multi-omics analysis reveals structure and function of biofilm microbial communities in a pre-denitrification biofilter. *Sci Total Environ* **757**: 143908.

Wang, K. Qaisar, M. Chen, B. Xiao, J. and Cai, J. (2022) Metagenomic analysis of microbial community and metabolic pathway of simultaneous sulfide and nitrite removal process exposed to divergent hydraulic retention times. *Bioresour Technol* **354**: 127186.

Wen, X., Zhou, J., Li, Y., Qing, X., and He, Q. (2016) A novel process combining simultaneous partial nitrification, anammox and denitrification (SNAD) with denitrifying phosphorus removal (DPR) to treat sewage. *Bioresour Technol* **222**: 309-316.

Xiao, J., Huang, J., Wang, M., Huang, M., and Wang, Y. (2021) The fate and long-term toxic effects of NiO nanoparticles at environmental concentration in constructed wetland: Enzyme activity, microbial property, metabolic pathway and functional genes. *J Hazard Mater* **413**: 125295.

Yang, N., Zhan, G., Li, D., He, X., Zhang, Y., Jiang, Q., et al. (2018) Performance and microbial community of a novel non-aeration-based up-flow bioelectrochemical filter (UBEF) treating real domestic wastewater. *Chem Eng J* **348**: 271-280.

You, X., Wang, S., and Du, L. (2021) Characteristics of an endogenous compound microbial inoculant and its immobilization effect on wastewater treatment from pig feedlots. *J Zhejiang Univ* **47**: 98-106. (in Chinese)

Zhang, L., Zhang, M., Guo, J., Zheng, J., Chen, Z., and Zhang, H. (2019) Effects of K+ salinity on the sludge activity and the microbial community structure of an A^2O process. *Chemosphere* **235**: 805-813.

Zhang, L., Zhong, M., Li, X., Lu, W., and Li, J. (2020) River bacterial community structure and co-occurrence patterns under the influence of different domestic sewage types. *J Environ Manage* **266**: 110590.

Zong, Y., Hao, K., Lu, G., Li, Y., and Huang, D. (2022) Characteristics of the colony structure of A^2O processes under different ultraviolet conditions in plateau areas. *Environ Sci Pollut R* 1-12.