Characteristics of the community-structure of A2O processes under different dissolved oxygen conditions in plateau areas

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Abstract. This study conducted a Pilot-Scale Anaerobic-Anoxic-Aerobic Process (A2O) experiment in a highland city of Linzhi. Four Dissolved oxygen (DO) working conditions of 2.0, 1.5, 1.0, and 0.5 mg/L was designed in this experiment. The 16S rRNA gene sequencing was performed on sludge from the anaerobic, anoxic and aerobic tanks. Through the composition analysis of sludge on Phylum, Class and Genus level, it can be found that the number of bacterial community at each level of the bacterial community was relatively low. Indicators of community richness, community evenness and community diversity were relatively low compared to other regions. The bacterial communities at different levels are significantly different from the reported dominant community and abundance. Correlation analysis between environmental factors and bacterial community structure proved that DO had significant correlation with bacterial community structure (P<0.05). The removal rates of the total phosphorous (TP), Total nitrogen (TN), Ammonia nitrogen (NH3-N) and Chemical Oxygen Demand (COD) were all affected by the sample communities structure. The composition of the bacterial community structure included nitrifying bacteria, denitrifying bacteria and polyphosphate-accumulating organisms, but the abundance was relatively low. The results also showed that the dominant community in different DO conditions and different reactors has large differences.

1. Introduction

Tibet has an average elevation of 4,000 meters and is known as the third pole of the world. It has unique environmental factors, among which the change of atmospheric pressure and oxygen partial pressure caused by high altitude has been universally recognized [1]. According to the measurement and calculation [2], the atmospheric pressure and oxygen partial pressure in Linzhi with an altitude of 3,000 meters are about 67.24% of the standard atmospheric pressure. In addition, higher altitude is negatively correlated with temperature [3], which may affect the water temperature negatively, thus affecting the reaction speed and removal effect.

Fick's Law [4] holds that oxygen is transferred from gas to liquid phase through the gas-liquid interface in the aeration process of sewage treatment, and there are gas and liquid films on both sides of the interface, while the mass transfer theory of gas molecules passing through gas and liquid films is called the two-film theory, which was established by Lewis and Whitman in 1923 [5]. According to the two-film theory, oxygen transfer depends on oxygen partial pressure, which is about 21kPa under normal
circumstances and only about 14 kPa at the research site [6]. The lower oxygen partial pressure will prolong the time required for the transfer of DO, and reduce the efficiency of oxygen transfer.

DO affect the sludge sedimentation rate, sludge nitrification activity, anaerobic phosphorus release rate, and nitrogen removal rate by denitrification, etc, and its final impact is reflected in the efficiency of nitrogen and phosphorus removal. At present, DO in the aeration tank are controlled within 1.5-2mg/L [7]. A pilot study of A²O process was conducted on domestic sewage of Yushu prefecture with an altitude of 4000m, and was found that the removal effect of NH3-N and TP was positively correlated with DO concentration by controlling DO concentration in the aerobic tank, while the removal effect of TN showed no significant correlation with DO concentration [8]. This conclusion is being supported by increasing research results [9-11]. Prolonged aeration time can promote nitrification, while prolonged anaerobic condition can stimulate denitrification [12]. Nitrogen and phosphorus removal effect in A²O system could be significantly reduced by low aeration [13]. The level of DO affected the conversion of polyhydroxyalkanoate (PHA) and glycogen, and the activity of desulfurization phosphatase and polyphonically, which further affected the level of phosphorus removal [14]. In an analysis of sludge performed, the ratio of anaerobic phosphorus absorption rate to aerobic phosphorus absorption rate reached 69% in such a system, while the introduction of different organic substrates in the anaerobic stage had a significant influence in the phosphorus absorption content of anoxic phase[8]. Meanwhile, it was found that DO concentration exhibited a primary effect on the ammonia-oxidizing activity of ammonia-oxidizing cells [15]. After Wen et al.[16] examined the bacterial communities of activated sludge from nine wastewater treatment plants located in three cities in China by using high-throughput MiSeq sequencing, and canonical correspondence analysis (CCA) results indicated that the bacterial community variance correlated most strongly with water temperature, DO, concentrations of COD, and solids retention time (SRT).Chen et al. [17] examined the sequences of activated sludge from two municipal wastewater treatment plants (WWTPs) located in a high altitude Plateau in Tibet, China (3650 m above the sea level), and got the core microbial communities in the WWTPs at the taxonomic level of phylum, class, order, family, genus and species.

At present, the research area mainly adopts A²O process. Therefore, A²O was selected as a typical sewage treatment process to conduct experimental research under DO working conditions. Using SPSS20.00 software, starting from the analysis of bacterial community structure characteristics, the experimental rule of DO change was discussed the colony structure in in anaerobic stage, anoxic stage, aerobic stage to explore the microbial response mechanism under the influence of plateau environmental factors.

2. Materials and Methods

A²O system is selected as a typical sewage treatment process in the study, the microbial characteristics were used as the research content to explore the influence of colonies structure under DO working conditions on plateau environmental factors, and to explore the microbial characteristics of A²O process under the Pilot Scale of DO working conditions under plateau environmental factors.

2.1. Experimental setup

A Pilot-Scale A²O was designed and produced in this research, with an effective volume of 210L, which could be divided into 3 grids, namely, anaerobic tank, anoxic tank, and aerobic tank (volume ratio of 35:58:117), and the effective volume of sedimentation tank was 39L. There were stirring devices at the bottom of both anaerobic tank and anoxic tank, with the stirring speed of 50rpm, and the aerator was installed at the bottom of aerobic tank for oxygen supply. The peristaltic pump was used to control water inflow, sludge and nitrated liquid reflux. In order to ensure the constant temperature in the experiment, constant temperature circulators was used to control the water temperature. Additionally, the sampling place was set on each tank wall. Schematic of A²O process is shown in Figure 1.
Before the experiment, the activated sludge was cultured for 37 days, with the temperature controlled at 20.0. Sludge settling ratio (SV30) is 35% and mixed liquor suspended solids (MLSS) are 4557mg/L. The test water was directly urban sewage of Linzhi City. The main water quality conditions of urban sewage of Linzhi City are shown in Table 1.

### Table 1. The main water quality conditions of influent

|          | COD (mg/L) | TN (mg/L) | TP (mg/L) |
|----------|------------|-----------|-----------|
| Influent | 93.102~521.78 | 69.875~175.45 | 1.975~4.55 |

2.2. Operation of the A2O

The basic control conditions were as follows: mixed liquid reflux ratio (Ri)=200%, sludge reflux ratio (R)=100%; continuous reflux was used in influent and mixed sludge; there are two aeration heads at the bottom of the aeration tank, and the change of DO was achieved by changing the air blowing aeration quantity, and the water quality sampling would be made 72h after the DO reached the design value, and the online DO detector is used to read the data. Designed influent flow is 10.0±0.1L/h, and hydraulic retention time (HRT) is 21.0±0.2h, and temperature is controlled at 20℃ in the aerobic tank, and DO is controlled at 2.0, 1.5, 1.0, and 0.5mg/L in the aerobic tank.

3. Results and discussion

According to the purpose of the experiment and operating parameters, research indicators in the article were microorganisms. All statistical analyses were performed using SPSS 20.0 analyses, and the data for microbial indicators were expressed as mean standard deviation (SD). The single factor analysis of variance (ANOVA) was adopted to test the different significance of data. When P<0.05, there are significant differences between different samples. Significance test using single factor analysis of variance (P <0.05) for biological indicators meets the requirements.

According to the working conditions set to 2.0, 1.5, 1.0, and 0.5mg/L, the sludge of the anaerobic, anoxic and aerobic tanks under each working condition were sampled and 16SrRNA. The relevant results obtained are analysed as following.

3.1. General Information

The statistical content of taxonomic analysis was the Domain, Kingdom, Phylum, Class, Genus, Operational Taxonomic Unit (OTU), and the silva database was used to analyze each sample. The statistical results are shown in Table 2.

### Table 2. Sample information

| Sample  | Domain | Kingdom | Phylum | Class | Genus | OTU |
|---------|--------|---------|--------|-------|-------|-----|
| do_ana_20 | 1      | 1       | 21     | 34    | 308   | 580 |
| do_ana_15 | 1      | 1       | 24     | 42    | 406   | 828 |
As showed in Table 2, An RDP classifier was used to assign these sequence tags into different OTUs with 3% of nucleotide cutoff. There was only one Domain and one Kingdom in the sequence sample, while the Phylum $\in (20, 27)$, Class $\in (34, 51)$, Genus $\in (308, 460)$ and Operational Taxonomic Unit (OTU) $\in (580, 957)$. The corresponding genealogical value is significantly lower than the number reported in existing literature. This is much lower than Tian et al. [18] who measured 51 on Phylum level and over 800 on Genus level in the A2O process. Wen et al. [19] measured the number of OTU in the Sequencing Batch Reactor Activated Sludge Process (SBR) from 924-1363, which was higher than that in this experiment. Jing Wang et al. [20] measured that the number of OTU in the anaerobic, anoxic, and aerobic tanks of the A2O process exceeds 1,800, and Zhang et al. [21] on the structure of activated sludge populations have found that the number of OTU ranges from 1,000-1,600, and Fan et al. [22] found that the OTU ranged from 800-1,900. Comparing the data obtained in this study with the above-mentioned existing literature, the difference in microbial species is large, that is, plateau environmental factors have a greater impact on microbial species.

### 3.2. Alpha diversity analysis

Alpha diversity includes the diversity indexes and the test of the difference between the indices. The commonly used measurement standards of Alpha diversity indexes include Shannon (community diversity), Ace (community richness), Chao (community richness), Coverage (community coverage), Shannoneven (community uniformity). Alpha diversity indexes of every sample are shown in Table 3.

| Sample     | Shannon | Ace   | Chao   | Coverage | Shannoneven |
|------------|---------|-------|--------|----------|-------------|
| do_Oxic_5  | 4.103   | 524.468 | 523.800 | 0.998   | 0.669       |
| do_Oxic_20 | 3.845   | 442.516 | 438.326 | 0.998   | 0.648       |
| do_ana_20  | 2.785   | 456.948 | 437.111 | 0.998   | 0.486       |
| do_Oxic_10 | 4.018   | 541.266 | 540.122 | 0.998   | 0.655       |
| do_ana_20  | 3.446   | 437.486 | 438.098 | 0.998   | 0.582       |
| do_ana_15  | 4.164   | 493.662 | 503.022 | 0.998   | 0.691       |
| do_ana_5   | 4.289   | 562.870 | 593.900 | 0.997   | 0.700       |
| do_ana_5   | 3.737   | 521.285 | 525.200 | 0.997   | 0.615       |
| do_Oxic_15 | 3.937   | 479.287 | 478.878 | 0.997   | 0.653       |
| do_ana_10  | 4.006   | 488.262 | 504.023 | 0.997   | 0.665       |
| do_ana_10  | 3.631   | 514.108 | 534.349 | 0.998   | 0.597       |
| do_Oxic_5  | 4.168   | 470.779 | 471.283 | 0.998   | 0.694       |

According to Table 3, the Ace index of community richness was 654.7-998.8. The current Ace value was 1,300-2,200, and the Ace index in the literature [23] was 2,700-3,400. The Shannoneven index of community evenness was 0.0117-0.0691, and no correlation coefficient is reported. The Shannon index of community diversity is 2.785-4.289. A literature study [22] has obtained a Shannon index of 0.016-0.049. Another research [24] indicates that the value is 0.835. The community Coverage [25] has a coverage coefficient of 0.994025 to 0.997255, all of which exceed 0.994. The sample sequencing coverage is good, indicating that the possibility of not detecting the sequence in the sample is extremely low, and it can fully reflect the situation of the sample.
3.3. **Bacterial community structure analysis**

The research uses Ribosomal Database Project (RDP) Classifier to sequentially perform the Domain, Kingdom, Phylum, Class and Genus in three reactors under four operating conditions. Classification statistics on Genus level focus on the analysis of strain function, and count the results of the number of sequences at different classification levels greater than 1%. The analysis found that there were only **Bacteria** on Domain level and only **norank_d__Bacteria** on Kingdom level.

### 3.3.1. Analysis of bacterial community structure on Phylum level

![Figure 2. Bacterial community structure characteristics on Phylum level](image)

Figure 2 shows that there is 31 types at the Phylum level. The dominant communities are **Proteus**, Bacteroides, Actinomyces, Sclerotium and Campylobacter. The abundance of 5 grass was 93.86-98.66%. The DO in the anaerobic, anoxic and aerobic pools declined with the decrease of DO.

**Proteobacteria** are organic degradation bacteria with a relatively high abundance. The abundance was 29.21-61.98% and in the anaerobic tank, anoxic tank, and aerobic tank showed a decreasing trend as the DO decreased. When DO were 2.0 mg/L, the abundance in the anaerobic and aerobic tanks reached more than 50%. Meanwhile, the abundance of Proteobacteria in the anaerobic, anoxic and aerobic tanks decreased in turn. The abundance of Proteobacteria decreased with the decrease of DO in the anaerobic tank, while it decreased firstly and then increased as DO dropped in the anoxic and aerobic tanks. When DO were 0.5 mg/L, the abundance in the aerobic and anoxic tanks increased, which may be caused by the anaerobic reaction when DO were low.

**Bacteroidetes** are a Phylum of obligate anaerobes, the abundance of which increased with the decrease of DO in anaerobic, anoxic and aerobic tanks. The abundance was 13.61-21.63%. When DO was 2.0 mg/L and 1.5 mg/L, their abundance in the anaerobic tank was higher, while they were more dispersed at other DO levels. This phenomenon may be related to certain denitrification in anaerobic and aerobic tanks at low DO levels.

**Actinobacteria** are a heterotrophic aerobic Phylum. The abundance was 6.26-27.80%. When DO was 2.0 mg/L, it showed that the abundance of aerobic tank was higher than other tanks. In aerobic tank, the abundance decreased with the decrease of DO.

**Firmicutes** are denitrifying bacteria and are mainly existing in anoxic conditions. The abundance was 6.26-27.80%. When DO was 2.0 mg/L and 1.5 mg/L, their abundance in the anaerobic tank was higher, while they were more dispersed at other DO levels. This phenomenon may be related to certain denitrification in anaerobic and aerobic tanks at low DO levels.

**Chloroflexi** are thiobacillus aerobic bacteria, and their abundance decreased in anoxic and aerobic...
tanks with decreasing DO. The abundance was 4.99-10.21%.

The above analysis showed that the sum of the dominant communities (Proteobacteria, Bacteroidetes, Actinobacteria, Firmicutes, and Chloroflexi) was more than 93%, which are the dominant communities on Phylum level in the plateau environment. Proteobacteria were a Phylum with the highest abundance, which was consistent with the result reported in the literature [26]. However, corresponding DO were 1.0 and 0.50 mg/L which were lower than that in the literature [27]. And there was one extra Phylum of Firmicutes with higher abundance, which was not identified in the literature [26]. The abundance of Bacteroidetes was lower than that reported by Juretschko et al. [28] and Kong et al. [29] when DO was 2.0 and 1.5mg/L. The abundance of Firmicutes was lower than that in the studies by Beer et al. [30] and Wong et al. [31].

3.3.2. Analysis of bacterial community structure on class level

Figure 3 shows that the shared dominant community on Class level were Bacteroidia(3.02-38.54%), Gammaproteobacteria(18.31-56.89%), Actinobacteria(13.61-21.63%), Alphaproteobacteria(5.06-14.32%), Clostridia(3.60-18.22%), and Chloroflexia(3.35-8.42%). And a total of 63 Classes were obtained. The abundance of 6 Classes was 83.35-92.91%. The abundance in anoxic tank was higher than other tanks.

Gammaproteobacteria and Alphaproteobacteria belong to the Phylum of Proteobacteria, which are denitrifying bacteria. The abundance of Gammaproteobacteria decreased with the decline of DO in anoxic tank, while it firstly decreased and then increased with the decrease of DO in anaerobic and aerobic tanks, and this may be related to the denitrification in both anaerobic and aerobic tanks at low DO levels. Its abundance in anoxic tank was the highest. The abundance of Alphaproteobacteria in anoxic tank decreased as DO dropped. Bacteroidaceae a class of obligate anaerobes, which demonstrated an increasing trend with the decrease of DO in anaerobic, anoxic and aerobic tanks. Clostridia belong to the Chloroflexi Phylum, which changed greatly in different reactors under different working conditions, but its regularity was not obvious. Chloroflexia belong to the Chloroflexi Phylum, which tended to decrease with the decrease of DO.

The above analysis shows that the dominant community on the Class level was Bacteroidia, Gammaproteobacteria, Actinobacteria, Alphaproteobacteria, Clostridia, and Chloroflexia. The sum of the abundance of the six colonies was above 83%, which are the dominant community on the Class level in plateau environment. The above composition shows that bacteria in the samples were quite different from the Alphaproteobacteria reported in the literature [26], and the Class and abundance of dominant microorganisms were significantly different from those in the study [32]. The abundance of
Alphaproteobacteria and Gammaproteobacteria was higher than the literature [27], while the abundance of Betaproteobacteria was obviously lower than Nielsen et al. [33], Wong et al. [31] and Schmid et al. [34]. The abundance of Chloroflexia was substantially lower than Schmid et al. [34] and Nielsen et al. [33].

3.3.3. Analysis of bacterial community structure on Genus level

Figure 4. Bacterial community structure characteristics on Genus level

Figure 4 shows that the shared dominant communities were JG30-KF-CM45(2.58-6.75%), IMCC26207(1.87-4.72%), Romboutsia(1.26-5.15%), 67-14(1.91-3.76%), Acinetobacter(1.93-47.76%), Mesorhizobium(1.00-2.52%) and Mycobacterium(1.17-2.21%). In addition, we need to pay attention to the large bacterial Genera variations of AKYH767(0.59-30.83%), Ottowia(0.60-6.13%), Simplicispira(0.27-5.98%), Enterobacteriaceae(0.09-10.44%), and Trichococcus(0.27-5.50%) were subject to significant changes. The above analysis shows the abundance varies widely, which means that the abundance and Genus of dominant communities change dramatically in the plateau environment with the change of DO. On Genus level, the differences between the dominant Genera identified in this study and other relevant literature [35] were large, and only the Pseudomonas was also identified in existing studies. The dominant Genera was significantly different from those in the existing research [18][31].

When the DO are 2.0 mg/l, the top five dominant community on Genus level is AKYH767, JG30-KF-CM45, 67-14, Acinetobacter, IMCC26207, and the abundance of 5 Genus were 45.41%. When the DO are 1.5mg/l, the top five dominant community on Genus level is AKYH767, norank_f__JG30-KF-CM45, Romboutsia, Acinetobacter, IMCC26207, and the abundance of 5 Genus were 30.93%. When the DO are 1.0mg/l, the top five dominant community on Genus level is AKYH767, JG30-KF-CM45, Ottowia, Acinetobacter, IMCC26207, and the abundance of 5 Genus were 39.76%. When the DO are 0.5mg/l, the top five dominant community on Genus level is AKYH767, JG30-KF-CM45, Ottowia, Acinetobacter, IMCC26207, and the abundance of 5 Genus were 37.30%. AKYH767, JG30-KF-CM45, Acinetobacter, IMCC26207 are the common Genus of the top five dominant community under four working conditions under.

3.4 Relationship between A2O process colony structure and environmental factors

The correlation analysis of environmental factors and bacterial community structure uses RDA/CCA analysis, which mainly performs regression analysis on the community structure and environmental factors under DO conditions to reflect the relationship between the community structure and
environmental factors. The classification level of microorganisms is Species. The factors correspond to DO, TN, TP, and COD. CCA analysis was performed for anaerobic, anoxic, and aerobic tanks.

Figure 5 shows that the correlation coefficients R of DO, TN, TP, and COD was 0.6542, 0.3078, 0.4631 and 0.048, respectively. According to the significance analysis results, there was significant correlation between DO and samples (P<0.05). The relationship between environmental factors and bacterial community structure of the sample was described above. The removal rates of TP, TN, and COD were affected by the sample bacterial community structure, and the degree of influence was weakened in turn. Explanation degrees of RDA1 and RDA2 were 58.15% and 5.62%, respectively.

CCA analysis using the abundance extraction function, the highest in anaerobic tank dominant Genera in the top ten were Acinetobacter, Ottowia, Bacteroidetes_bacterium_OLB10, Chryseobacterium, Acinetobacter_celticus, metagenome_g__Gordonia, Ferruginibacter, Novosporangiobium, Pseudomonas, Proteocatella; the advantages of anoxic bacteria were Bacteroidetes_bacterium_OLB10, Acinetobacter, Trichococcus, Acinetobacter_celticus, Romboutsia, Psychrobacter_faecalis, Simplicispira, Enterococcus, JG30-KF-CM45, OLBI7. Aerobic tank dominant Genera were acteroideetes_bacterium_OLB10, Acinetobacter, Enterobacteriaceae, Chryseobacterium, Trichococcus, JG30-KF-CM45. Proteocatella, Acinetobacter_celticus, Saprospiraceae, Ottowia. The dominant Genera corresponding to 2.0 mg/L was Acinetobacter, Enterobacteriaceae, Bacteroidetes_bacterium_OLB10, Trichococcus, Gordonia, Acinetobacter_celticus, JG30-KF-, M45, Dietzia_maris, JG30-KF-CM45, Romboutsia successively. The dominant Genera corresponding to 1.5 mg/L was Bacteroidetes_bacterium_OLB10, Simplicispira, Ottowia, Romboutsia, IMCC26207, Proteocatella, JG30-KF-CM45, Pseudomonas, Ottowia, SC-I-84 successively. The dominant Genera corresponding to 1.0 mg/L were Bacteroidetes_bacterium_OLB10, Acinetobacter, Romboutsia, Proteocatella, unclassified_g__Ottowia, Saprospiraceae, OLBI7, Ottowia, Acinetobacter_celticus, SC-I-84 successively. The dominant Genera corresponding to 0.5 mg/L was Bacteroidetes_bacterium_OLB10, Acinetobacter, Acinetobacter_celticus, Proteiniclasticum, IMCC26207, OLB17, Romboutsia, Ottowia, Solirubrobacterales_bacterium_67-14, Novosporangiobium successively.

The above analysis shows that the dominant Genera corresponding to different reactors was quite different, and also there were huge differences in the dominant Genera corresponding to different DO. Nitrifying Genera only had one Phylum of Nitrospirae, the abundance of which was low at 0-0.65%. The reported denitrifying Genera included Thiobacillus denitrificans, Micrococcus denitrificans,
Paracoccus denitrificans, Alkaligenes, Bacillus and Pseudomonas [38], There were only two Genera of Bacillus and Pseudomonas. The abundance of Bacillus in anoxic tank was 0.00-0.04%, while the abundance of Pseudomonas was 0.02-1.62%. The abundance of Pseudomonas was higher than that reported in existing research [38].

4. Conclusion
The bacterial community of anaerobic, anoxic and aerobic tanks under the DO working conditions of 2.0, 1.5, 1.0 and 0.5 mg/L was analyzed by the 16SrRNA gene sequencing. The result is as following:

(1) The statistical information of the 12 gene sequencing samples showed that the number of species in the samples at different levels is lower than that in other regions, and diversity indexes such as richness, evenness and diversity are lower. Which indicates that the species richness in the plateau region is lower than that reported in previous studies, but the higher coverage indicates that there was a good sample coverage.

(2) The analysis of bacterial community composition showed that the bacterial community composition includes nitrifying bacteria, denitrifying bacteria, and polyphosphate-accumulating organisms. DO are the influencing factor of bacterial community structure, and the dominant community in different reactors is different. The abundance varies widely on Order, Family, Genus, and Species level in the plateau environment with the change of DO. In other words, there are large differences in the community composition and quantity composition of bacteria in different reactors at different levels.

(3) The CCA diagram further verified that DO are an important factor affecting the bacterial community structure and composition. The dominant community in the same reactor is quite different under different conditions, indicating that DO is an important factor affecting the bacterial community structure.

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