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The Changes of Pollutants and Microbial Community Structures of the Activated Sludge in Response to Different Temperature Levels in Tibet of China

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Abstract. This study evaluated the efficiency of wastewater treatment in constantly aerated CASS under different temperature levels, 4±1, 10±1, 15±1 and 20±1°C at Tibet Nyingtri. The change of microbial community structure of activated sludge reference to temperature levels was also analyzed. Results demonstrated that the treatment performance was the largest at the 20±1°C by comparing the 4±1, 10±1, 15±1 and 20±1°C four conditions. Bacteria belonging to the phylum proteobacteria, firmicutes, bacteroidetes, acidobacteria, nitrospira and chloroflexi have only been detected in this reactor. Ammonia and nitrate levels in all four reactors indicated efficient nitrification process. Results of this study offer new insights into understanding the performance of activated biomass vis-à-vis microbial community structures and degradative efficiency with reference to temperature levels on plateaus. This information would be useful in improving the efficiency of any wastewater treatment plant at high altitudes.

1. Introduction
With rapid social and economic development at high altitudes, sewage discharge increased gradually in big cities of plateau region. Sewage treatment is urgent for protecting water environment at high-altitude regions, which may decrease the organic and inorganic pollutants, especially nutrients such as nitrogen and phosphorus. The activated sludge process, which uses activated sludge for the transformation of organic and inorganic pollutants, has contributed greatly to the improvement of the aquatic environment worldwide, and is still the most widely used process for the treatment of municipal wastewater because of its low operation cost and high performance[1].

The pollution level of organic matter in water is often evaluated in terms of chemical oxygen demand (COD) removal efficiency. Similarly, COD removal efficiency is often used for reflecting the level of wastewater treatment[2]. To date, many treatment processes have high COD removal rates at room or high temperature[3-4]. The performance of most treatment processes however, is negatively affected by low temperature conditions which often result in a deterioration of process performance[5]. Alternating seasons leads to the majority of these studies of COD removal by biological wastewater treatment being conducted at 15°C conditions[6-7].
It has been reported that some researchers have tried to change operating conditions like hydraulic retention time (HRT) or adding some measure of pre-treatment or post-treatment to improve COD removal efficiency at low temperature\[6,7,8\]. It is well known that microbial properties such as microbial biomass and activities during the process of biological wastewater treatment are closely related to COD removal efficiency\[9\]. Therefore, in order to improve the COD removal efficiency at low temperatures, it is more practical to investigate microbial characteristics.

Most previous research has focused on either the enzyme activity or diversity of microbial communities to reflect microbial characteristics[10-11]. Currently, there have been some investigations into the relationship between removing pollution and microorganism characteristics at room temperature\[12\], but few at low temperature such as 4°C. Most of the knowledge is directed at the low altitude wastewater treatment plants (WWTPs). More information on the changes of pollutants and the microbial community structures of activated sludge at different temperature levels, especially at low temperature is needed to better understand the stable operation of WWTPs at high altitude.

In order to provide some practical guidance to the operation of WWTPs in treating domestic wastewater in plateaus, this study was aimed at investigating the impact of different temperature levels, 4±1, 10±1, 15±1 and 20±1°C in Tibet Nyingtri. The study covers reactor performance evaluation and microbial community structures analysis of activated sludge at different different temperature levels. These results are expected to provide practical guidance to the operation of WWTPs in treating domestic wastewater in plateau.

2. Material and Methods

2.1. Reactor and operation
The Cyclic Activated Sludge System (CASS) process unit was placed in the environmental science and engineering laboratory of the Tibet Agricultural and Animal Husbandry College in Nyingtri of Tibet. The experiment was carried out in the same four CASS process units. The CASS process unit was shown in Fig.1. The main body of the reactor is made of organic glass. The reactor volume was 0.045m³. The activated sludge of Lhasa sewage treatment plant was used for the use of sludge. Initial sludge concentration was 2.5g/L. The reactors run continuously. The Influent sewage and aeration system of the device was adjusted by the creep pump. The influent sewage pH was maintained between 7.2 and 8.4. The stable dissolved oxygen in water (2-2.5mg/L) was maintained during the different test stages with different temperature levels. The Influent sewage was made by artificial water. The composition of synthetic sewage was shown in Table 1 at different temperature levels. Reactors designated was maintained with a temperature of 4±1, 10±1, 15±1 and 20±1°C, respectively. Each stage lasted for about 25 days, including 10 days of adjustment and 15 days of stabilization.

The CASS reactor ran for four cycles per day, with each cycle for 6h, divided into two stages. The water aeration phase of reactor took 4h. The precipitation drainage stage after the aeration completion of natural precipitation took 2h. And then, the reactor went above the clear liquid through the outlet, and ran back to the next cycle.

Fig.1. The CASS process unit
Table 1. The composition of synthetic sewage

| Species                        | Composition | Concentration (mg/L) | Confect          |
|-------------------------------|-------------|----------------------|------------------|
| Organics                      | COD         | 210                  | glucose          |
| Nitrogen compounds            | NH₄⁺-N      | 30                   | NH₄Cl            |
| Phosphorus compounds          | TP          | 5                    | K₂HPO₄           |
| Other additions and trace elements | MgSO₄·7H₂O(12);FeSO₄·7H₂O(10);CaCl₂(30);NaHCO₃(50);pH(7.2-8.4);H₂BO₃(0.15);CoCl₂·6H₂O(0.15);CuSO₄·6H₂O(0.03);FeCl₃·6H₂O(1.5);KI(0.03);MnCl₂·2H₂O(0.12);(NH₄)₂Mo₇O₂₄·2H₂O(0.06);ZnSO₄·7H₂O(0.12) |

2.2. Collection of samples and effluent quality analysis

The effluent quality parameters were measured per day during the stable stage. The effluent quality parameters such as COD, NH₄-N, NO₂-N, NO₃-N and TP were measured according to Chinese standard methods. The water temperature and DO were measured by the portable dissolved oxygen meter (JPBJ -608). PH was measured by digital display (PHS-25).

The 10ml MLSS reaction mixture was extracted from the reactor during the stable stage for microbiological analysis. The 10ml MLSS reaction mixture was kept in a dry ice box immediately after the collection for express and stored at -80°C in a lab for DNA extraction. After melting at room temperature, the sample was centrifuged at 14000g for 8 min. The supernatant was decanted, and 5g of the pellets were weighted out for the next step of DNA extraction.

2.3. DNA extraction and PCR amplification

The microbial DNA was extracted from AS samples with the E.Z.N.A.® Soil DNA Kit (Omega Bio-tek, Norcross, GA, U.S.) according to the manufacturer’s protocol. The V4-V5 regions of the bacteria 16S ribosomal RNA gene were amplified by PCR (at 95 °C for 5 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min) by use of primers 341F 5’- CCTAYGGGRBGCASCAG-3’ and 806R 5’- GGACTACNNGGGTATCTAAT-3’, where the barcode was an eight-base sequence unique to each sample. The archaeal gene was amplified by PCR (at 95 °C for 5 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min, 10°C until being stopped by user) using primers Arch519F 5’-CAGCCGCCGCGGTAA-3’ and Arch915R 5’-GTGCTCCCCCGCCAATTCCT-3’, where the barcode was an eight-base sequence unique to each sample. The fungal gene was amplified by PCR (at 95 °C for 5 min, followed by 27 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 45 s and a final extension at 72 °C for 10 min, 10°C until being stopped by user) using primers ITS1F 5’-CTTGGTCAATTTAGAGGAATCAA-3’ and ITS2R 5’- GCTGCGTTCATTCCATCGATG-3’, where the barcode was an eight-base sequence unique to each sample. The PCR reactions were performed in triplicate 20 μL mixture containing 4 μL of 5 × FastPfu Buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL of FastPfu Polymerase, and 10 ng of template DNA. The amplicons were extracted from 2% agarose gel, and purified by use of the AxyPrep DNA Gel Extraction Kit (Axygen Biosciences, Union City, CA, U.S.) according to the manufacturer’s instructions and quantified by use of Quantifluor™-ST (Promega, U.S.).

2.4. Library Construction and Sequencing

The purified PCR products were quantified by Qubit®3.0 (Life Invitrogen), and every 24 amplicons with different barcodes were different were mixed equally. The pooled DNA product was used to construct an Illumina pair-end library following the preparation procedure of Illumina’s genomic DNA library. Then the amplicon library was sequenced in pairs (2×250) on an Illumina MiSeq platform (Shanghai BIOZERON Co., Ltd) according to the standard protocol.
2.5. Processing of sequencing data
Raw FASTQ files were demultiplexed, and the filtered based on the quality by use of QIIME (version 1.17) with the following criteria: (i) The 250 bp reading should be truncated at any site, and an average quality score <20 over a 10 bp sliding window should be gotten by discarding the truncated reading which was shorter than 50bp. (ii) Exact barcode matching. 2 nucleotide mismatches in prime matching and the reading containing ambiguous characters should be removed. (iii) Only the sequences which overlap longer than 10 bp could be assembled according to their overlapped sequences. The reading which could not be assembled should be discarded.

Operational Units (OTUs) were clustered with 97% similarity and cut off by use of UPARSE (Version 7.1 http://drive5.com/uparse/), and the chimeric sequences were identified and removed by use of UCHIME. The phylogenetic affiliation of each 16S rRNA gene sequence was analyzed by RDP Classifier (http://rdp.cme.msu.edu/) against the silva 16S rRNA database by use of 70% of confidence threshold[13].

2.6. Microbial community structure Analyses
The microbial community structure of each sample was detected by T-RFLP method. The similarity analysis of the OTU compositions of samples was performed by clustering analysis based on the Bray-Curtis similarity by PAST 3.0[14]. Use AutoCAD2012, Origin8.0 and R language statistical analysis software to complete the chart.

3. Results and discussions

3.1. Process performance
The removal result of carbon, nitrogen and phosphorus was shown in Fig.2 under different temperature levels. The mean effluent COD concentrations of the 4±1, 10±1, 15±1 and 20±1℃ reactors were 100.49, 66.32, 41.79 and 26.61 mg/L, respectively, and the mean COD removal efficiencies were 52.14%, 68.41%, 80.1% and 87.33%, respectively. It shows that the average removal rate of COD was the largest when the temperature was at 20±1℃.

Fig.2. The effluent parameter NH4-N+, NO3--N, NO2--N, TP values and COD removal efficiency under different temperature levels
As shown in Fig. 2, the Effluent NH₄-N and TP concentration of the temperature 20±1°C were lower than the 4±1, 10±1 and 15±1°C. The treatment performance was the largest when the temperature was at 20±1°C by comparing the 4±1, 10±1, 15±1 and 20±1°C four conditions. The nitrification concentration in effluent increased from 8.24mg/L (20±1°C) to 9.58mg/L (4±1°C), indicating a strong relationship between the temperature and nitrogen removal activity.

3.2. Microbial community structures

Fig. 3 summarized the relative abundances of the bacterial community on the phylum and genus level of activated sludge under different temperature levels. At phylum level, proteobacteria were predominant while bacteroidetes were also a major group in activated sludge when the temperature were 4±1, 15±1 and 20±1°C. Bacteroidetes (39.09%) were predominant while proteobacteria (34.98%) were also a major group when the temperature was 10±1°C. The relative abundances of acidobacteria of the temperature were 10±1, 15±1 and 20±1°C (0.22%, 0.36% and 0.72%) were lower than the temperature was 4±1°C (2.34%) from Fig 3. In addition, a large portion of the sequences was grouped into unclassified sequences in this study. The most abundant phylum observed in this study across all reactors and previously reported in activated biomass are proteobacteria, firmicutes, bacteroidetes, acidobacteria, nitrospira and chloroflexi[15-16].

Fig. 3. Relative abundance of bacterial phyla and genus of activated sludge

As shown in Fig. 3, at genus level, uliginosibacterium were predominant while zoogloea was also a major group in activated sludge when the temperature was 4±1°C. Porphyromonadaceae uncultured were predominant while Acinetobacter were also a major group in activated sludge when the temperature was 10±1°C. Acinetobacter were predominant while Porphyromonadaceae uncultured were also a major group in activated sludge when the temperature was 15±1°C. Arcobacter were predominant while pseudomonas were also a major group in activated sludge when the temperature was 20±1°C. The relative abundances of ferribacterium of 10±1, 15±1 and 20±1°C (0.15%, 0.14% and 0.54%) were lower than the temperature was 4±1°C (7.48%) from Fig 3. In addition, a large portion of the sequences was grouped into unclassified sequences in this study.

Fig. 4 summarized the relative abundances of the fungal on the phylum and genus level of activated sludge under different temperature levels. At phylum level, ascomycota (>80%) were predominant while basidiomycota (<10%) were also a major group in activated sludge when the temperature were 10±1 and 20±1°C. Ascomycota (50.79%) were predominant while basidiomycota (47.38%) were also a major group when the temperature was 4±1°C. Basidiomycota (54.95%) were predominant while ascomycota (43.76%) were also a major group when temperature was 15±1°C. In addition, a large portion of the sequences was grouped into unclassified sequences in this study.
As shown in Fig. 4, at genus level, *nectria* (>80%) were predominant while *tausonia* (<10%) were also a major group in activated sludge when the temperature were 10±1 and 20±1°C. *Nectria* (46.5%) were predominant while *tausonia* (41.41%) were also a major group when the temperature was 4±1°C. *Tausonia* (53.29%) were predominant while *nectria* (40.54%) were also a major group when the temperature was 15±1°C. In addition, a large portion of the sequences was grouped into unclassified sequences in this study.

Fig. 5 summarized the relative abundances of the archaeal on the phylum and genus level of activated sludge under different temperature levels. At phylum level, *Euryarchaeota* were predominant while *bacteroidetes* were also a major group in activated sludge when the temperature were 4±1, 15±1 and 20±1°C. *Bacteroidetes* (43.62%) were predominant while *firmicutes* (42.56%) were also a major group when the temperature was 10±1°C. *Euryarchaeota* (99.21%) were significant increase in activated sludge when the temperature was 20±1°C. In addition, a large portion of the sequences was grouped into unclassified sequences in this study.

As shown in Fig. 5, at genus level, *methanobacterium* were predominant in activated sludge when the temperature was 15±1 and 20±1°C. *Methanobacterium* (85.59%) were significant increase in activated sludge when the temperature was 20±1°C. *Woesearchaeota (DHVEG-6)_norank* (30.77%) were predominant while *methanobacterium* (10.46%) were also a major group when the temperature was 4±1°C. *Clostridium sensu stricto 13* (10.48%) were predominant while *methanobacterium* (3.27%) were also a major group when the temperature was 10±1°C. In addition, a large portion of the sequences was grouped into unclassified sequences in this study.
4. Conclusions
This study demonstrated the changes of pollutants during different temperature levels using CASS sludge reactor on plateau. Microbial community structure was also analyzed. The analysis provides a better understanding sewage treatment under different temperature levels in plateau areas. In addition, the data generated could be used to select the optimum parameters required to run a WWTP efficiently at high altitudes, although pilot scale trials would be further required to take lab-scale results to the field.

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