Dynamic changes of bacterial community in activated sludge with pressurized aeration in a sequencing batch reactor

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ABSTRACT

This study aimed to investigate the organic removal efficiency and microbial population dynamics in activated sludge with pressurized aeration. The activated sludge was fed with synthetic wastewater composed of simple carbon source to avoid the effect of complex components on microbial communities. The pressurized acclimation process was conducted in a bench-scale sequencing batch reactor (SBR) under 0.3 MPa gage pressure. Another SBR was running in atmospheric environment as a control reactor, with the same operation parameters except for the pressure. Bacterial diversity was investigated by Illumina sequencing technology. The results showed that the total organic carbon removal efficiency of the pressurized reactor was significantly higher, while the mixed liquor suspended solids concentrations were much lower than those of the control reactor. Moderate pressure of 0.3 MPa had little effect on Alpha-diversity of bacterial communities due to the similar running conditions, e.g., feed water, solids retention time (SRT) and the cyclic change of dissolved oxygen (DO) concentrations. Although the relative percentage of the bacterial community changed among samples, there was no major change of predominant bacterial populations between the pressurized group and the control group. Pressurized aeration would have a far-reaching impact on microbial community in activated sludge when treating wastewaters being unfavorable to the dissolution of oxygen.

Key words | activated sludge, bacterial community diversity, population dynamics, pressurized aeration, sequencing batch reactor

INTRODUCTION

The activated sludge process is commonly used in wastewater treatment as a biological method. Aerobic bacteria involved in activated sludge play an important role in organic degradation through aerobic activated sludge process. Oxygen supply is a major problem due to limited oxygen solubility of wastewater under atmospheric pressure, especially when high concentration organic wastewater is treated by the process. Several methods have been taken to improve oxygen transfer impetus, e.g. pure-oxygen aeration, deep-shaft aeration and pressurized aeration (Calderón et al. 2012; Niu et al. 2013). The pressurized activated sludge process enhances the solubility of oxygen by increasing total air pressure, with a result of promoted oxygen transfer rate (Jin et al. 2010).

Pressurized biological method has been applied for treatments of industrial wastewater, such as high concentration pesticide wastewater, wastewater from canning of sour vegetables, pickled mustard tuber wastewater, tannery wastewater, etc. (Krauth & Staab 1993; Krauth 1996; Jin et al. 2010). Activated sludge and biofilm with pressurized aeration technology are both confirmed to be more effective than those in traditional aeration system. Although pressurized aeration system would require more aeration energy, the degradation rate of organic matters could be dramatically increased especially when activated sludge process was running under high organic load (Xu et al. 2016), which meant that volume of aeration tank and hydraulic detention time could be reduced effectively by the
application of pressurized aeration. Besides, pressurized treatment could significantly decrease the sludge production. This advantage would bring about an appreciably lower sludge disposal cost. An economic evaluation performed by Berktay & Ellis (1997) showed that, compared with conventional activated sludge process, pressurized unit could obtain a substantial saving, especially when the treatment process was for larger populations.

There is a general tendency of microbial growth inhibition under high pressure of several hundred bars. High pressures could inactivate and eliminate microorganisms, and consequently provide a longer storage time for various materials and food (Santos et al. 2015). However, the effects of high pressure are not of relevance to industrial aerobic bioreactors, where the moderate pressure is often controlled to less than 1.0 MPa. Moderate pressures have been demonstrated to cause no damage to several culture processes, such as pressurized culture of Aureobasidium pullulans (Dufresne et al. 1990). However, several factors have changed under moderate pressure condition, including oxygen transfer rate from air to water, dissolution rate of carbon dioxide and elimination speed of other metabolites, etc. Little work has been conducted to investigate the change of microbial community structure when activated sludge is cultivated in moderate pressure.

A better understanding of bacterial composition could provide useful information for elucidating the microbial effects on the formation, development, and function of activated sludge. Traditional molecular techniques such as clone library and denaturing gradient gel electrophoresis combined with other molecular tools have been successfully used to study microbial community composition and dominant microbial population in activated sludge, granular sludge or biofilm (Qin et al. 2016). However, it is difficult to achieve complete information about microbial community due to the high microbial diversity in activated sludge. In recent years, high-throughput sequencing method, which could provide enough sequencing depth to cover the complex microbial communities, has been applied to investigate change of the microbial community composition induced by environmental factors in activated sludge (Pala-Ozkok et al. 2013; Al-Kindi & Abed 2016).

The objective of this study was to evaluate the organic degradation efficiency and the response of bacterial community in activated sludge with pressurized aeration. The activated sludge was cultivated in a sequencing batch reactor (SBR) under 0.3 MPa gage pressure. Another SBR was running in atmospheric environment as a control reactor, with the same operation parameters except for the pressure. Samples were collected routinely and bacterial diversity was investigated by Illumina sequencing technology. Since pressurized aeration was more effective and economical for organic degradation under high organic load, this study is intended to reveal the possible dynamic changes of microbial population in activated sludge when exposed from atmospheric environment to the gage pressure of 0.3 MPa.

MATERIALS AND METHODS

Wastewater and seed sludge

Activated sludge was obtained from a municipal wastewater treatment plant in Nanjing as seed sludge and pre-cultured in a 150 L plastic container under normal environment with synthetic wastewater whose composition was as follows: CH3COONa, 500 mg · L\(^{-1}\); glucose: 250 mg · L\(^{-1}\); NH4Cl: 170 mg · L\(^{-1}\); KH2PO4: 20 mg · L\(^{-1}\); NaHCO3: 40 mg · L\(^{-1}\); CaCl2: 40 mg · L\(^{-1}\); MgSO4·7H2O: 164 mg · L\(^{-1}\). The characteristics of the synthetic wastewater were total organic carbon (TOC) 240–260 mg · L\(^{-1}\), chemical oxygen demand (COD) 550–610 mg · L\(^{-1}\), ammonia nitrogen (NH3-N) 40–50 mg · L\(^{-1}\), total phosphorus (TP) 4–5 mg · L\(^{-1}\) and pH 6.5–7.5. The pre-culture process was in sequencing mode and had four cycles a day. The exchange volume ratio was 75 L/cycle. Each cycle (6 h) consisted of the following steps: feeding (0.5 h), aeration (3 h), settling (1 h), and decanting (0.5 h) and idling (1 h). Aeration rate and solids retention time (SRT) were controlled at 300 L · h\(^{-1}\) and 10 days, respectively. The pre-culture process ran over 2 months until the activated sludge had completely adapted to the synthetic wastewater. Then, the experimental reactors were inoculated by the pre-cultured sludge with initial mixed liquor suspended solids (MLSS) concentration of 2,500 mg · L\(^{-1}\).

Experimental design and operating conditions

The experiments were conducted with sequencing batch operation mode in two parallel bench-scale bioreactors as described by Zhang et al. (2016). The synthetic wastewater was directly used as influent without diluting to keep high organic load. Pressurized bioreactor was operated under gage pressure of 0.3 MPa by an air compressor. The exchange volume ratio of both reactors was 25 L/cycle. The SBR had four cycles a day and each cycle (5 h) consisted of feeding (30 min), aeration (3 h), settling (1 h) and decanting (30 min). Aeration rate was controlled at
160 L·h\(^{-1}\) for each reactor. SRT was sustained at 7.5–10 days. The two SBRs were operated under the same conditions except for the pressure. All experiments were performed at ambient temperature (25 ± 2 °C).

**Activated sludge samples collection**

The sludge samples were collected at the end of the aeration stage from each SBR at regular intervals to investigate the response of microbial community structure of activated sludge under the gage pressure of 0.3 MPa. Seven sludge samples were collected: A0 was sampled from inoculated sludge on the first day of the experiment (representing the initial status of the activated sludge of both reactors); A1 and P1 were sampled from the control reactor and the pressurized reactor on the 5th day, respectively; A2 and P2 were sampled from the two reactors on the 15th day; and A3 and P3 were sampled from the two reactors on the 30th day. The process of pre-culture and pressurized acclimation is shown in Figure 1.

**Analytical methods**

Water sampling and on-site measurements were conducted daily. TOC concentration was measured by a TOC analyzer (TOC-VCPH, SHIMADZU, Japan) and dissolved oxygen (DO) by a dissolved oxygen meter (HI 9146, HANNA, Italy). COD and MLSS were assayed according to the standard method (SEPAC1999). Microscopic morphology of sludge was observed by a scanning electron microscope (JSM-7600F, Japan).

**DNA extraction, PCR amplification and MiSeq sequencing**

Microbial DNA was extracted from sludge samples with the E.Z.N.A.® soil DNA Kit (Omega Bio-tek, Norcross, GA, USA). The V4-V5 region of the bacteria 16S ribosomal RNA gene was amplified by polymerase chain reaction (PCR), with an initial 2-min denaturation at 95 °C, followed by 25 cycles at 95 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s and completed with a final extension at 72 °C for 5 min. The primers were 338F (5’-ACTCCTACG GAGGCAGCA-3’) and 806R (5’-GGACTACHVGGTWTCTAAT-3’) where barcode is an eight-base sequence unique to each sample. PCR reactions were performed in triplicate 20 μL mixture composed of 4 μL 5× FastPfu Buffer, 2 μL 2.5 mM dNTPs, 0.8 μL of each primer (5 μM), 0.4 μL FastPfu Polymerase, and 10 ng template DNA. Amplicons were then sequenced on an Illumina MiSeq platform described by Zhang et al. (2016).

The raw reads were submitted to the NCBI Sequence Read Archive (SRA) database and the Accession Number was SRP054752.

**Statistical analysis**

ACE and Chao indices were used to evaluate the community richness of activated sludge samples. Shannon index values were also calculated to illustrate the community diversity of the samples. The Venn diagram with shared and unique operational taxonomic units (OTUs) was employed to compare the similarity and difference of samples during pressuring. The significance of results was tested by t-test, and \( p < 0.05 \) was considered to be statistically significant.

**RESULTS AND DISCUSSION**

**TOC removal efficiency**

The daily TOC removal rates are shown in Figure 2. The removal efficiency of the pressurized reactor was significantly higher than that of the control reactor throughout
the process \( p < 0.05 \), illustrating that the degradation activity of activated sludge would quickly increase with pressure. The result was consistent with previous studies (Sharma and Huang 2004; Jin et al. 2010). The improvement of treating efficiency of the pressurized reactor also related to the organic concentration of influent, designed as high as 240–260 mg · L\(^{-1}\) (TOC). The corresponding COD value is approximately 550–610 mg · L\(^{-1}\). A previous study has demonstrated that the degradation rate of unacclimated activated sludge with pressurized aeration increases obviously when the biological process runs under higher organic load with much more oxygen demand (Xu et al. 2016).

An increasing trend of TOC removal rates was shown at the beginning of the process in both reactors before the 5th day. Afterwards, the removal rates tended towards stability. This trend was associated with the increasing MLSS concentration, particularly in the initial stage. The MLSS concentrations were determined daily (three replicates) to investigate the sludge growth tendency during cultivating. Figure 3(a) shows the changes of the MLSS concentration of the two reactors during the whole process. The initial MLSS concentrations of both reactors were approximately 2,500 mg · L\(^{-1}\). There was a rapid growth of activated sludge in both reactors at the start due to the high influent organic load. MLSS concentrations on the 5th day reached 3,364 mg · L\(^{-1}\) in the control reactor and 2,900 mg · L\(^{-1}\) in the pressurized reactor, much higher than the initial MLSS concentration (2,500 mg · L\(^{-1}\)). Since then MLSS remained stable in the later period of the process. At the end of the acclimation process, MLSS concentrations of the pressurized reactor and the control reactor were approximately 4,070 mg · L\(^{-1}\) and 3,368 mg · L\(^{-1}\), respectively. Increasing MLSS improved the treatment efficiency as well as decreasing the sludge loading. The variation regularity of MLSS was in line with the TOC removal rules shown in Figure 2.

![Figure 3](https://iwaponline.com/wst/article-pdf/75/11/2639/452891/wst075112639.pdf)

**Figure 3** | Increase of MLSS concentrations in the pressurized reactor and the control reactor (a) and the periodic variations of DO concentrations on the first day (b) and the 30th day (c).
In this study the synthetic wastewater with simple carbon sources was used in the whole process. All substrates in the wastewater were biodegradable and soluble. In the circumstances, increasing MLSS concentrations were mainly due to the microbial growth. Increasing biomass would consume more oxygen, while the aeration rate was constant (160 L · h⁻¹). Cyclic variation of DO concentration was also detected during the experiment, as was shown in Figure 3(b) and 3(c). In the early days of the pressurized experiment (Figure 3(b)), DO concentration in the pressurized reactor reached 7.5 mg · L⁻¹ after 3 hours’ aeration, much higher than that in the control reactor (2.6 mg · L⁻¹). After a rapid increase of MLSS concentration in the early stage of the acclimation process, DO concentrations were 6.2 mg · L⁻¹ and 2.0 mg · L⁻¹ in the pressurized reactor and the control reactor on the 30th day, respectively (Figure 3(c)). The results showed that the increasing MLSS concentrations in both reactors consumed more oxygen and brought about a decrease of DO concentration in the two reactors, especially in the control reactor.

The MLSS concentrations of pressurized activated sludge were lower than those in the control reactor, which demonstrated the sludge reduction ability of pressurized bioreactor (Berklay & Ellis 1979). The reason was due to the higher DO concentration obtained by higher oxygen transfer impetus under pressurized condition. Xu et al. (2016) also reported that more oxygen expenditure leads to more extracellular polymeric substances (EPS) consumption as well as promoting the biodegradation of organics, which may be an important way of sludge reduction by pressurized technology.

**Alpha-diversity of bacterial communities**

The sequence information of samples and calculated microbial diversity index including community richness (ACE, Chao) and community diversity (Shannon) are listed in Table 1. The coverage per each sample was higher than 99%, indicating adequate sequence depth. The OTUs (from 1,105 to 1,163) did not differ significantly (p > 0.05) between the pressurized group (A0, P1, P2 and P3) and the control one (A0, A1, A2 and A3), which showed a very similar richness between the two groups. Neither community richness indicated by ACE estimator and Chao estimator nor community diversity indicated by Shannon index differed significantly between the pressurized group and the control one (p > 0.05).

Venn analysis was also conducted to evaluate the distribution of OTUs of activated sludge in the pressurized reactor (Figure 4). Shared OTUs among samples A0, P1, P2, P3 were calculated at phylum level. It showed that 825 OTUs, embracing 60.0% of the sequences, were common to all four samples. The number of special OTUs of the four samples was 21, 15, 14, and 36, respectively.

The effect of moderate pressure in the relevant range (in general, up to 2 or 3 bars, approximate maximum 10 bars) on microbial growth has been generalized to be negligibly small, including activated sludge process (Onken & Liefke 1979). The similar Alpha-diversity between the two groups may be due to the similar running conditions (e.g., feed water, DO, SRT), which played an important role in changing microbial community structure of activated sludge (Pala-Ozkok et al. 2013; Yadav et al. 2014).

**Bacterial community composition and similarity analysis**

The classifier test results of the taxon assignments at phylum level for each bacterial community composition are
depicted in Figure 5(a). *Proteobacteria*, *Bacteroidetes* and *Chloroflexi* were the dominant phyla in all samples, accounting for 60.35–71.51%, 9.31–15.22% and 5.95–13.71% of total effective bacterial sequences, respectively. These phyla have been reported to be abundant in activated sludge or biomembrane samples in previous studies (Palo-Ozkok et al. 2015). The relative abundances of *Proteobacteria* in pressurized samples P2 and P3 were 71.5% and 71.0%, respectively, much higher than that in initial sample A0 (61.6%). *Proteobacteria* was the dominant phylum in many wastewater treatment bioreactors and municipal wastewater treatment plants (Ma et al. 2015). *Bacteroidetes* obviously grew in the control samples (A2 and A3). Especially with regard to sample A3, the percentage of *Bacteroidetes* was up from 9.31% of sample A0 to 15.22%. Increasing percentage of *bacteroidetes* could also be confirmed by scanning electron microscopy (SEM) (×20,000) (Figure 5(b) and 5(c)). In addition, several phyla always accounted for more than 1% in the samples, such as *Acidobacteria*, *Chlorobi*, *Planctomycetes*, *Firmicutes* and *Spirochaetae*. These phyla have also been demonstrated to be always abundant in wastewater biological treatment systems (Yadav et al. 2014; Ma et al. 2015).

Further analysis was made at class level (Figure 6(a)) and genus level (Figure 6(b)). At the class level, abundant classes (above 1.0%) in all samples were *Betaproteobacteria* (43.9–63.2%), *Sphingobacteriia* (7.0–11.6%), *Anaerolineae* (4.5–9.9%), *Gammaproteobacteria* (3.4–10.5%), *Alphaproteobacteria* (2.1–5.5%), *Deltaproteobacteria* (1.4–3.1%) and *Ignavibacteria* (1.1–1.9%). Increase of the abundance of *Proteobacteria* in samples P2 and P3 was mainly due to the increase of percentage of *Betaproteobacteria*, which was the most abundant bacterial group in all samples. *Betaproteobacteria* are highly versatile in pollutant degradation capacities (Wang et al. 2012). Yadav et al. (2014) reported a higher percentage of *Betaproteobacteria* in activated sludge under circumstances of lower DO concentrations. Figure 3 shows that the DO concentration of the pressurized reactor

![Figure 5](https://iwaponline.com/wst/article-pdf/75/11/2639/452891/wst075112639.pdf)

**Figure 5** | Microbial community bar plot of the seven activated sludge samples (phylum level) (a) and corresponding SEM of sludge sample P3 (b) and A3 (c) on Day 30, respectively.
was lower than that of the control one in the early stage of the batch reaction, even though pressure multiplies transfer impetus of oxygen from air to water. The phenomenon has also been elaborated by Xu et al. (2016). Gammaproteobacteria showed a downward trend in pressurized samples from initial 7.06% in sample A0 to 3.43% in sample P3.

At the genus level, there were 381 genera classified in the 7 activated sludge samples; 265 genera were shared by all samples. Figure 6(b) shows the top 26 genera in each sample. The percentage of uncl. Rhodocyclaceae maintained population dominance in both reactors. Rhodocyclaceae were the core family in many wastewater treatment plants, responsible for denitrifying and aromatic degrading processes (Loy et al. 2005). Thauera, also belonging to the family Rhodocyclaceae, remained relatively stable in all samples, accounting for 8.8–15.8%. The genus was identified as heterotrophic bacteria capable of aromatic biodegradation and denitrification (Ma et al. 2015). In this study the addition of NH4Cl to the synthetic wastewater was to provide enough nutrition for microorganisms to grow and perform normal metabolism. The intermittent aeration of SBR mode provided an alternating aerobic-anaerobic environment, which may lead to the abundance of these bacteria. Besides, Thauera are also capable of internal polyhydroxyalkanoates (PHA) storage by using acetate as a substrate (Jena et al. 2016). Thus, the abundance of the genus may be associated with the composition of the synthetic wastewater, in which concentration of CH3COONa was as high as 500 mg L⁻¹. Other genera whose percentages were above 1.0% in all samples were Zoogloea, Dechloromonas, Brachymonas, Anaerolineaceae, Saprospiraceae, and uncl. Comamonadaceae. These heterotrophic bacteria possess wastewatertreatment capabilities of removing COD through carbon metabolism (Tian et al. 2015). For example, Dechloromonas has been reported in activated sludge to be capable of reducing perchlorate and phosphorus from wastewater (Achenbach et al. 2001). Anaerolineae are known as semi-synthetic and fatty acids-oxidizing bacteria (Narihiro et al. 2012). The genus Saprospiraceae has also been detected in activated sludge, playing an important role in protein degradation (Xia et al. 2008).

The abundant OTUs (relative abundance > 1.0%) of the pressurized group and the control group at phylum, class and genus level are shown in Table 2. Although the relative percentage of the bacterial community changed among samples, there was no major change of predominant bacterial populations between the two groups on each taxonomic level. The result was accordance with the stable TOC removal rate during most of the process (Figure 2) and the similar Alpha-diversity (Table 1) above mentioned.

Many researches focused on the bacterial population dynamics in activated sludge cultivated in a specific industrial wastewater (Liu et al. 2015; Stalder et al. 2013). Effects of some important parameters (e.g. influent quality, DO, SRT) on activated sludge bacterial population have also been studied (Pala-Ozkok et al. 2015; Yadav et al. 2014). Yadav et al. (2014) reported a greater diversity of bacteria in reactor maintained at 2 mg · L⁻¹ DO than that at 4 mg · L⁻¹ DO. Ma et al. (2015) also reported that microbial diversity decreases with the increase of aeration intensity. This study concluded a similar Alpha-diversity and predominant bacterial populations between activated sludge with/without pressurized aeration, even though the peak DO
concentration of pressurized aeration was pretty higher than that of the control one (Figure 3(b) and 3(c)). It may be due to the similar periodic change of DO concentrations of both reactors in SBR mode, which deserves further discussion in future studies. Microbial diversity and community structure of activated sludge play an important role in pollutant degradation process. Even though the Alpha-diversity of activated sludge in the pressurized reactor and the control reactor was very similar, and there was even no major change of predominant bacterial populations between both reactors, the pressurized activated sludge could achieve significantly higher TOC removal rate during the whole acclimation process. The promoted biodegradation of organics may be due to the increasing utilization efficiency of oxygen achieved by high oxygen transfer impetus with pressure (Xu et al., 2016).

In this study, the reactors were fed with the synthetic wastewater composed of simple and stable carbon source to avoid the effect of complex components on microbial communities. Besides, the activated sludge had been pre-cultured with the synthetic wastewater for 2 months to adapt to the wastewater before the pressurized experiments. The pressurized reactor and the control reactor were running under same conditions except for the pressure. Thus, the difference of community structure between both reactors was related to the aeration methods. The results differed from a previous study concluding that pressurized aeration would improve bacterial richness and community diversity of activated sludge in high-salinity wastewater (Zhang et al., 2016), in which oxygen solubility would decrease sharply with the increase of salinity. It indicated that pressurized aeration would have a far-reaching impact on microbial community in activated sludge when treating complicated industrial wastewaters, especially wastewaters being unfavorable to the dissolution of oxygen.

**CONCLUSIONS**

TOC removal efficiency and response of microbial population dynamics in activated sludge with pressurized aeration (0.3 MPa gage pressure) were studied using comparative methods. The TOC removal efficiency of the pressurized reactor was significantly higher than that of the control reactor throughout the whole process. MLSS concentrations of pressurized activated sludge were much lower, which led to sludge reduction. Moderate pressure of 0.3 MPa had little effect on Alpha-diversity of bacterial communities, including community richness indicated by ACE and Chao estimator and community diversity indicated by Shannon index. Increase of the abundance of *Proteobacteria* in pressurized samples P3 was mainly due to the increase of percentage of *Betaproteobacteria*. The percentage of *Rhodocyclaceae* maintained population dominance in the pressurized reactor while decreasing in the control reactor. Although the relative percentage of the bacterial community changed among samples, there was no major change of predominant bacterial populations between the pressurized group and the control group. Pressurized aeration would have a far-reaching impact on microbial community in activated sludge when treating complicated industrial wastewaters, especially wastewaters being unfavorable to the dissolution of oxygen.

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