# Analysis of the Differences in the Microbial Community and Structure of Calcified ONP Granular Sludge and Bagasse Granular Sludge

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Anaerobic biological technology has been widely used in the treatment of high-concentration organic wastewater such as old newspaper (ONP) pulping wastewater and bagasse spray washing wastewater before pulping. However, due to the high calcium content and complex chemical properties of the ONP pulping wastewater, granular sludge calcification occurs during the anaerobic treatment, which has negative effects. In contrast, calcification does not occur in the bagasse spray washing wastewater. Therefore, a comparative analysis of the biological flora and structure of the granular sludge for these two types of wastewater can provide theoretical and data support for revealing the cause of calcification of granular sludge. The results indicate a considerable difference between the anaerobic granular sludge for the treatment of bagasse spraying wastewater (B-GS) and the treatment of ONP pulping wastewater (P-GS). The microorganisms in the B-GS were mainly *Bactericides* (25.4%), *Proteobacteria* (20.2%), *Hyd24－12* (14.4%), *Chloroflexi* (10.6%), and *Firmicutes* (8.9%). The microorganisms in the P-GS were mainly *Bacteroidetes* (20.4%), *Chloroflexi* (19.5%), *Proteobacteria* (19.3%), *Firmicutes* (6.2%), *Spirochaetae* (4.8%), *Actinobacteria* (4.4%), and *Lentisphaerae* (4.3%). *Methanomassiliicoccus* were detected only in the P-GS. The methanogens had a higher relative abundance in the P-GS (50%), and only a small fraction (10%) of methanogens was detected in the B-GS.

*Keywords: High-throughput sequencing; Microbial community; Microbial diversity*

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## INTRODUCTION

An internal circulation reactor is used in the treatment of highly concentrated organic wastewater (Luo *et al.* 2016a). The efficiency of wastewater treatment is closely related to factors such as the microbial community structure and composition of the sludge. The composition of wastewater differs for different sources during the paper production process, which results in differences in the nature, structure, and formation mechanism of the granular sludge. Researchers investigating the community structure of sludge microbes have determined that the community structure of microbes in activated sludge depends on the substrate.

The microbial community structure obtained from an upflow anaerobic sludge blanket (UASB) of two breweries has been observed by scanning electron microscopy (SEM), transmission electron microscopy (TEM), and fluorescence *in situ* hybridization (FISH) (Batstone *et al.* 2004). The acetotrophic methanogens and syntrophic acetogenic clusters occupy the same region rather than forming a different layer. The use of

pyrophosphate sequencing has revealed methanogens in the activated sludge of sewage processing plants in mainland China, Hong Kong, Singapore, Canada, and the United States (Zhang *et al.* 2012). The most common phylum is *Proteobacteria*, followed by *Bacteroidetes*, *Firmicutes*, and *Actinobacteria*. When two kinds of microbes isolated from oil sands tailings ponds were investigated, the Anaerolineaceae sequences increased only in cultures amended with a four-alkane mixture and only during n-octane and n-decane biodegradation (Shahimin *et al.* 2016); the dominant methanogens were acetoclastic Methanosaetaceae.

There is still considerable debate on whether the community structure of activated sludge microbes depends on the substrate. There are few studies on the community structure in calcified granular sludge and general granular sludge. In this study, the microbial community diversity and relative abundance of the bacterial flora in activated sludge were analyzed. The heavy metals in the granular sludge were investigated using inductively coupled plasma mass spectrometry (ICP-MS), and the types of surface compounds in granular sludge were analyzed using Fourier-transform infrared (FTIR) spectroscopy. The elementary composition of the granular sludge surface was characterized by energy dispersive spectrometry (EDS), and high-throughput genetic sequencing was used to detect bacteria in the V3 and V4 regions.

## EXPERIMENTAL

### Anaerobic Granular Sludge

The organic matter content in the sludge for the treatment of bagasse wastewater (B-GS) was 75.4% and the average particle size was 0.24 cm; the particles were black. The chemical oxygen demand (COD) of the treated wastewater was 8997 mg/L. The 5-day biological oxygen demand ($BOD_5$) was 5047 mg/L, and the calcium content of the granular sludge was 67 mg/L. The pH was 4.2.

The organic matter content in the sludge for the treatment of ONP pulping wastewater (P-GS) was 54.0%. The average particle size was 0.38 cm; the particles were hollow. The main portions of the granular sludge were black, and some portions consisted of yellow soil. The particles of the sludge were spherical with a shiny surface. The COD of the treated wastewater was 2347 mg/L. The $BOD_5$ was 5047 mg/L, and the calcium content of the granular sludge was 334 mg/L. The granular sludge exhibited low biodegradability.

### Analysis Method

*Analysis of the heavy metal compositions*

The granular sludge was dehydrated, ground into a powder, and screened using a mesh size of 200. The samples were oven-dried until they weighed 0.50 g. The samples were placed into a polytetrafluoroethylene (PTFE) container, and 6 mL of concentrated hydrochloric acid, 2 mL of concentrated nitric acid, and 2 mL of hydrofluoric acid were added. Subsequently, the PTFE solution was digested in a microwave digestion instrument (MARS6, Power 1600 W). The temperature was increased for 3 min and the heat was maintained for 30 min; the final temperature was 190 °C. The samples were completely digested after treatment with bio-acid at 155 °C. The digestive solution was removed, filtered through a micropore filter, and diluted with deionized water to 50 mL. The elements Cu, Cr, Cd, Pb, Zn, Mn, As, and Ni were determined by ICP-MS.

*Analysis of anaerobic granular sludge by FTIR*

The granular sludge was dehydrated and ground into a powder. A total of 0.01 g of the sludge powder was mixed with 2.00 g of KBr, and the mixture was dried at 60 °C for 4 h and subjected to a vacuum at -101.3 KPa. After drying, the samples were poured into a compression mold and pressed at 10 to 80 MPa for 5 min, obtaining a completely transparent tablet. FTIR spectroscopy was conducted using a VERTEX 70 instrument (Siemens AG, Karlsruhe, Germany; resolution: 0.16 $cm^{-1}$; measurement range: 4000 to 400 $cm^{-1}$).

*Analysis of anaerobic granular sludge by SEM-EDS*

The granular sludge was flushed three times by using phosphate buffered saline (PBS) (pH 7.4) to remove the impurities and mucus on the granular sludge; natural sedimentation was allowed to occur. The cleaned granular sludge sample was fixed in 2.5% glutaraldehyde for 4 h and washed 3 times for 10 min with the PBS buffer. It was dehydrated by using 50%, 70%, 80%, and 90% alcohol for 30 min for each alcohol proportion (Tay *et al.* 2001; Lenz *et al.* 2008). After drying, the granular sludge sample was in the shape of a hollow sphere, which was fastened to a surface with an electrically conductive adhesive for the subsequent analysis by EDS.

*Analysis of the microbial diversity in the granular sludge*

The genomic DNA of the two granular sludge types was extracted and sequenced. The sample concentration was greater than 10 ng/μL. The sample size was 500 ng, and the purity of the samples was between 1.8 and 2.2. The samples were subjected to electrophoresis.

*Polymerase chain reaction (PCR) amplification purification and recycling in the target zone*

For PCR amplification, the samples were denatured for 5 min at 95 °C and went through 15 thermal cycles. Finally, the samples were incubated for 7 min at 72 °C and then stored at 4 °C. The process of PCR purification in the target zone was as follows. The magnetic particles were added to the pre-amplification product and blended well at room temperature for 5 min. Subsequently, the sample was placed on a magnetic rack for about 5 min, and the supernatant was discarded. The magnetic beads were cleaned by adding 80% alcohol, and the supernatant was discarded at room temperature for 30 s. The step was repeated. The sample was placed on the magnetic rack for 3 min, and the magnetic beads were resuspended with 37 μL of $ddH_2O$ at room temperature for 2 min, followed by 2 min on the magnetic rack, after which the supernatant of the flow-through was transferred to a new PCR tube. Solexa PCR was used with the following conditions: the samples were denatured for 30 s at 98 °C and went through 10 thermal cycles. Finally, the samples were incubated for 5 min at 72 °C. Subsequently, the samples were subjected to electrophoresis. Finally, the sample and the magnetic beads were well mixed at a ratio of 1:1.5. They were washed and dewatered by using 25 μL of 80% ethanol.

*Quantitative fluorescence and sequencing*

The purified product was quantified; it was mixed at a weight ratio of 1 to 1 and gelled by adding 1.8% agarose. The electrophoresis was performed at 120 V for 40 min, and the target fragment was cloned and recycled for computer sequencing. The sequencing

platform was an Illumina MIseq 2500 (San Diego, CA, USA). Each sample yielded no less than 20000 sequences.

*Analysis of biological information*

The paired-end (PE) reads were obtained by Miseq sequencing and the date of the original tags was obtained by stitching with overlap. The tags with lengths of less than 75% were filtered by using Trimmomatic (Aachen, Germany) to generate high-quality data. Finally, the UCHIME algorithm was used for the identification and removal of the chimera sequences. The data were categorized into operational taxonomic units (OTUs) using a cluster analysis. The OTUs were analyzed using biological information technology at a 97% similarity level. The samples for all communities were statistically analyzed at each level, and the composition and relative abundance of the species were obtained for the different taxonomic levels.

## RESULTS AND DISCUSSION

### Heavy Metal Elements in the Granular Sludge

The contents of heavy metals in the two sludge samples are shown in Table 1. The aerobic granular sludge was cultured at a low concentration of Pb (<10 mg/L). The dominant flora was Bacteroidetes. The aerobic granular sludge was cultured at a high concentration (>50 mg/L) of lead ion. The dominant flora was Beta-proteobacteria microorganisms (Liu 2012). When UASB was used to treat pig manure wastewater containing heavy metals, Methanomicrobiales were the dominant species (60%), Methanobacteriales, and Methanosarcinales (10%) were lower in the UASB reactor before zinc was added. When exogenous zinc exceeds 15 mg/L, the three methanogenic bacteria Methanomicrobiales, Methanobacteriales, Methanosarcinales are all significantly inhibited, and Methanomicrobiales are the most inhibited (Tuo 2014). If aged sludge is discharged directly without being treated, it causes serious pollution of the environment (Peraza *et al.* 1998; Duru *et al.* 2016). Pb is a common heavy metal and is harmful to humans. It enters the human body mainly through the skin, the respiratory system, and the digestive tract and may cause irreversible nerve injury (Zhang 2014). Aged sludge cannot be discharged unless the pollutants are treated based on appropriate standards.

**Table 1.** Heavy Metals in the Granular Sludge

| Element | Content of Heavy Metals in Anaerobic Granular Sludge (mg/kg) | |
|---|---|---|
| | B-GS | P-GS |
| Cu 327.393 | 30.70 | 90.99 |
| Pb 220.353 | 8.125 | 68.83 |
| Cd 228.802 | 0.000 | 0.000 |
| Cr 267.716 | 32.30 | 9.102 |
| Mn 257.610 | 1850.2 | 585.1 |
| As 193.696 | 95.12 | 32.06 |
| Zn 206.200 | 761.8 | 125.3 |
| Ni 231.604 | 25.09 | 31.96 |

Note: The unit is mg /kg

## SEM/EDS Analysis

The images shown in Fig. 1 are 5000x magnifications acquired by SEM. The Corynebacterium bacteria occurred mainly on the smooth granular sludge surface; because there are many indentations on the surface, the bacteria can easily contact the substrate. More indentations on the surface mean that nutrients and gas can easily enter and exit the granular sludge particles (B-GS). P-GS had a low porosity. The elements and their composition on the surface of the granular sludge were analyzed by EDS. The results showed that approximately the same elements and the same proportion of organic elements occurred in both types of sludge, although the proportion of the metallic elements exhibited some differences. The proportions of K, Fe, and Zn were higher in B-GS than in P-GS. The total mass of these three elements (C, N, and O) accounted for 86.6% of B-GS and 94.0% of P-GS.

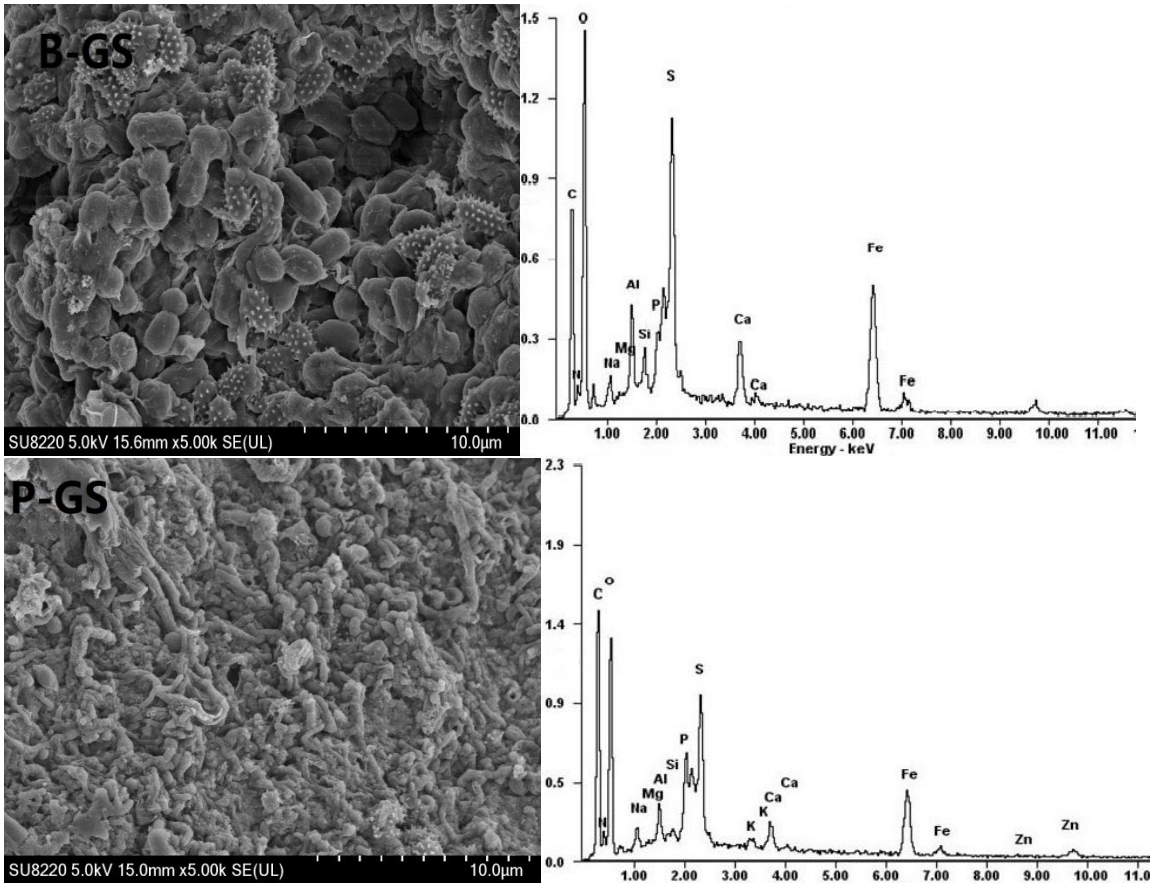


**Fig. 1.** Morphology of the granular sludge

Organic elements dominated in the surface of the sludge. The proportion of $Ca^{2+}$ was higher in P-GS than in B-GS. The wastewater with high concentrations of $Ca^{2+}$ did not result in $Ca^{2+}$-rich granular sludge, and this result should be explored further. It is speculated that when high calcium concentrations exist, the bacteria actively transport $Ca^{2+}$ to the outer section of the granular sludge. This increases the $Ca^{2+}$ concentration in this location and causes interactions between $CO_2$ and $Ca^{2+}$, resulting in calcification on the outer surfaces of the granular sludge particles.

## Infrared Spectra Analysis

The two types of sludge samples differed in their functional groups, but they exhibited the same absorption bands in specific areas. Weak absorption bands are due to the presence of acid compounds, and -OH stretching vibration is observed in the range of 3400 $cm^{-1}$ to 3100 $cm^{-1}$ (Sun *et al.* 2011; Liu *et al.* 2015). In the sugarcane bagasse, the sugar was anaerobically fermented, producing large amounts of acid substances that were absorbed by the granular sludge. Weak absorption bands occur due to the alkyl-CH stretching vibration in the range of 3000 $cm^{-1}$ to 2850 $cm^{-1}$ (Silva *et al.* 2012; Liu *et al.* 2015). The peak absorption in the range of 1170 $cm^{-1}$ to 1000 $cm^{-1}$ is attributed to the -OH stretching vibration of the mineral component (Francioso *et al.* 2010). In addition, the absorption peak at 1626.9 $cm^{-1}$ for the B-GS was caused by the deformation vibration of the N-H group of amines, and the absorption peak at 1234.7 $cm^{-1}$ was due to the $-CH_2$ vibration of the carboxylic acid. The absorption peak at 1408.9 $cm^{-1}$ for the P-GS was caused by the -OH stretching vibration of the mineral component (Smidt and Meissl 2007). The sharp absorption peaks at 872.8 $cm^{-1}$ and 712.2 $cm^{-1}$ represent an aromatic compound (Ma *et al.* 2013). These observations reflect that contaminants in the wastewater are relatively complex, and therefore, more aromatic compounds are absorbed, enriched, and degraded.

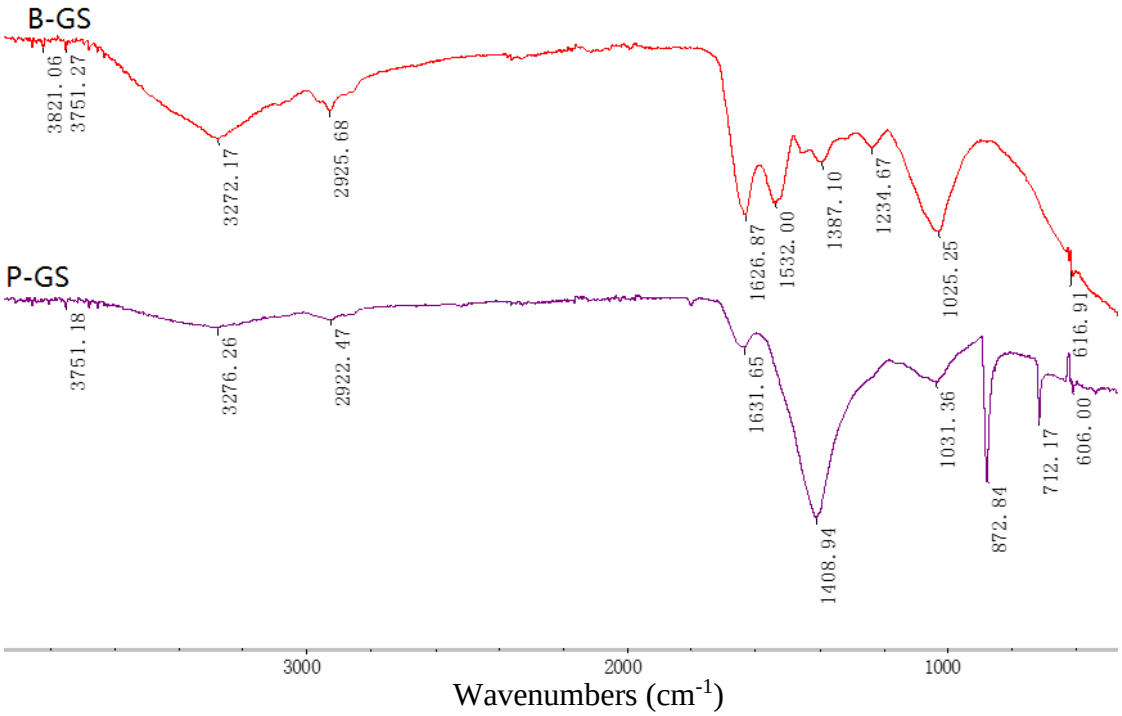


**Fig. 2.** Infrared spectrum analysis of two types of granular sludge

## Analysis of Microbial Diversity

The sequencing results for the two sludge samples are shown in Table 2. Chao1 and ACE are indices used for the estimation of the number of OTUs of the microbes; the rarefication (dilution) curves were smooth for the two samples (Fig. 3). This result indicated that the number of species determined during sequencing is progressive and as expected. The Simpson index and Shannon index represent the diversity of the flora. A

smaller Simpson index or a larger Shannon index indicates a greater diversity of flora (Wang *et al.* 2012). The Shannon index of B-GS is 4.71, and the Shannon index of P-GS is 5.19. The high values and the smooth curve indicated that the microbial diversity in the anaerobic granular sludge is high and that the number of samples was adequate (Boon *et al.* 2002).

**Table 2.** Alpha Diversity Statistics for Two Types of Granular Sludge

| Sample ID | Reads | | | 0.97 | | |
|---|---|---|---|---|---|---|
| | | OTU | ACE | Chao1 | Simpson | Shannon |
| B-GS | 44508 | 764 | 795.52 | 827.75 | 0.02679 | 4.71 |
| P-GS | 44875 | 874 | 890.33 | 931.19 | 0.01970 | 5.19 |

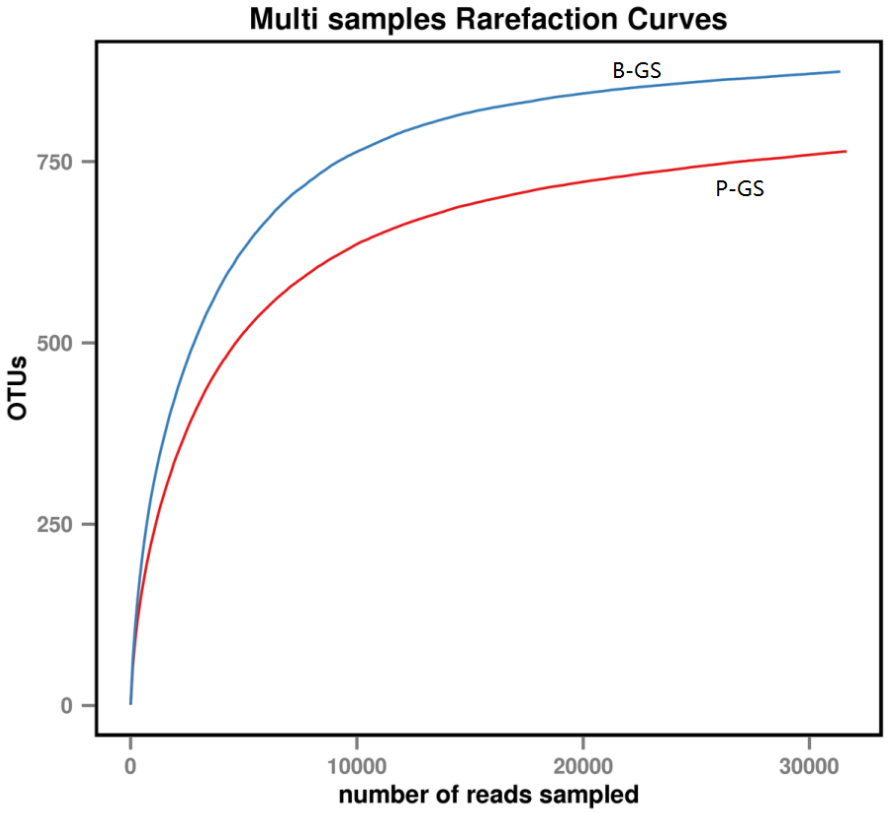


**Fig. 3.** Rarefaction curves of two kinds of granular sludge

As shown in Table 2, there were 89383 valid 16 s rRNA sequences in the two samples. There were 764 OTUs in the B-GS and 864 OTUs in the P-GS with a similarity of 97%. Alpha diversity is the analysis of species diversity in a single sample (Beazley *et al.* 2012): mainly including the Chao index, Ace index, Shannon index, Simpson index, and Coverage index. Chao or Ace is an index used to measure community abundance. The greater the index, the higher the degree of community richness. The bacterial community abundance in P-GS sludge is greater than B-GS. The Simpson index indicates the species diversity of the community. The smaller the Simpson index, the higher the species diversity. The bacterial diversity in P-GS sludge is greater than B-GS.

## Analysis of Bacterial Community

The sequence of the microbial abundance in the B-GS was as follows: Bacteroidetes (25.4%), Proteobacteria (20.2%), Hyd24-12 (14.4%), Chloroflexi (10.6%), Firmicutes (8.9%), and Nitrospirae (4.3%). The sum of the relative abundance of the six classes of flora was 83.8%. The sequence of the microbial abundance in the P-GS was as follows: Bacteroidetes (20.4%), Chloroflexi (19.5%), Proteobacteria (19.3%), Firmicutes (6.2%), Spirochaetae (4.8%), Actinobacteria (4.4%), and Lentisphaerae (4.3%). The sum of the relative abundance of these seven classes of flora was 78.9%. Furthermore, there was a high abundance of Bacteroidetes that were stable in the different sources of the sludge. Bacteroidetes and Firmicutes degrade complex organic microbes during anaerobic acidification processes (Wen 2015).

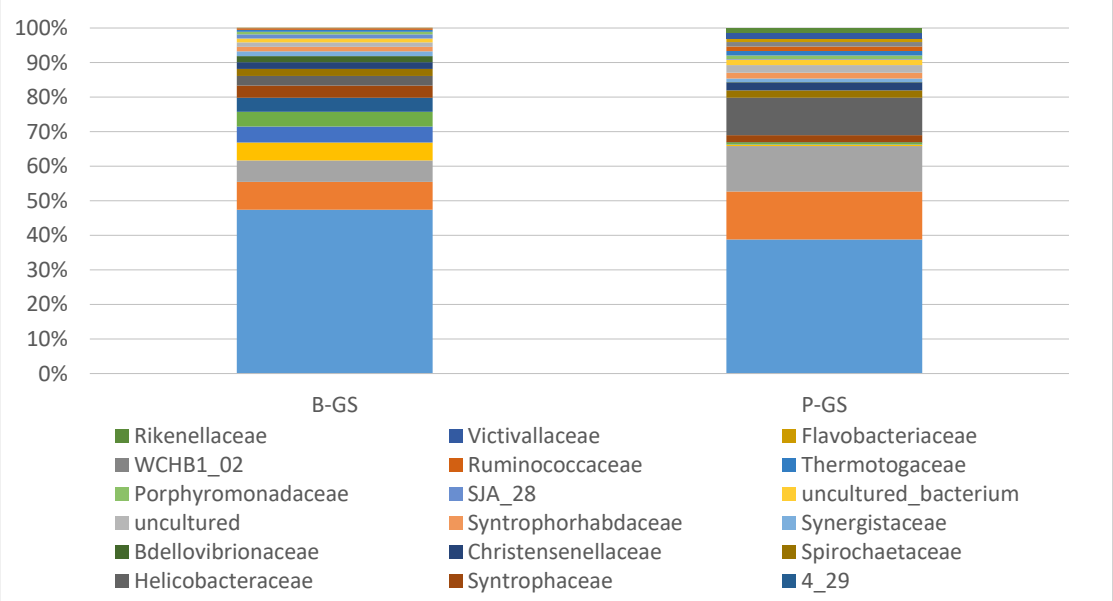


**Fig. 4.** Bacterial distribution at the family level in two types of granular sludge

In addition, the abundance of Nitrospirales in the B-GS was 0.58%; it performs nitrite oxidation in the microbial anaerobic reactor. The abundance of Nitrospirales in P-GS was 1.30%. At the order level, Tag sequences were clearly assigned at 53.4% (B-GS) and 62.0% (P-GS). The allocation rate was similar at the family level with 51.3% (B-GS) and 59.0% (P-GS). At the genus level, the tag sequences allocated proportions of only 29.4% (B-GS) and 36.0% (P-GS). For a better comparison of the flora in the two types of anaerobic granular sludge, the microbial diversity was analyzed at the family level.

In B-GS, the relative abundance of the 13 species was greater than 1%, and none of the bacteria had more than 10% abundance. In P-GS, two species of bacteria had a relative abundance of more than 10%, and there were 13 families of bacteria with a relative abundance of more than 1%. The relative abundance of the major bacteria was very different for the two samples. The major differences between the bacteria were selected for analysis and comparison. The relative abundance of the Anaerolineaceae in the B-GS and P-GS was 8.1% and 13.9%, respectively. This family belongs to the group of strictly anaerobic bacteria and is fairly common in anaerobic reactors. It is adapted to wastewater with high concentrations of phenol and participates in the degradation of phenol (Rosenkranz *et al.* 2013).

The genus *Anaerolinea* in the Anaerolineaceae family was relatively abundant in the B-GS and P-GS at 1.3% and 5.5%, respectively. The literature indicates that the genus *Anaerolinea* is positively correlated with salinity and can be used as an indicator of the changes in the salinity of the flora (Zhang *et al.* 2016). The relative abundance of the Syntrophomonadaceae in the B-GS and P-GS was 5.3% and 0.38%, respectively. The genus of *Syntrophomonas* in the Syntrophobacteraceae family had a relative abundance in the B-GS and P-GS of 5.27% and 0.38%, respectively. The genus *Syntrophomonas* as a co-cultured bacterium plays an important role in the oxidation of butyrate and long chain fatty acids. *Syntrophomonas* interacts with *Methanosarcina*, Hydrogenotrophic, Methanoculleus, and Methanobacterium in the treatment of organic wastewater.

*Syntrophomonas* is highly abundant and can interact with the amount of Methanosarcinaceae and *Methanobacterium*, which can strengthens the butyrate metabolic process (Narihiro *et al.* 2015). The relative abundance of Geobacteraceae in the B-GS and P-GS was 4.6% and 0.09%, respectively; these are important bacteria involved in the process of extracellular electron transfer under hypoxia conditions and also drive the metal cycle (Ishii *et al.* 2013). The Syntrophobacteraceae and Syntrophaceae belong to the order Syntrophobacterales; the relative abundance of the Syntrophobacteraceae in the B-GS and P-GS was 4.3% and 0.68%, respectively, and the relative abundance of the Syntrophacea in the B-GS and P-GS was 3.5% and 1.96%, respectively. Both families are involved in the oxidation of long chain fatty acids and produce hydrogen and acetate and they act in synergy with the methanogens (Gray *et al.* 2011). The relative abundance of the Helicobacteraceae in the B-GS and P-GS was 2.8% and 10.8 %, respectively. The samples of Helicobacteraceae mainly included *Sulfuricurum* and *Sulfurovum*. The relative abundance of the *Sulfurovum* in the B-GS and P-GS was 2.6% and 9.9%, respectively. This genus belongs to the facultative anaerobic bacteria. In neutral chemical conditions, it can be used as an electron donor with elemental sulfur or sulfate. It is an electron acceptor with oxygen and nitrate and uses carbon dioxide as a carbon source and is a self-supporting bacterium (Inagaki *et al.* 2004).

The relative abundance of the Spirochaetaceae was 2.0% and 2.2% in the B-GS and P-GS, respectively. Members of the genus *Spirochaeta* are chemical heterotrophic bacteria that make use of glucose, maltose, and cellobiose as single carbon and energy sources. It cannot use cellulose or other alcohol sugars as single carbon or energy sources (Dubinina *et al.* 2011). Therefore, the cellulose in the substrate needs to be hydrolyzed before it can be used by the Spirochaeta. The relative abundance of the Ruminococcaceae was 0.45% and 1.32% in the B-GS and P-GS, respectively. Their abundance was relatively low and they can degrade starch, cellulose, protein, organic acids, and short-chain polysaccharides. It is one of the important bacteria during the hydrolysis stage of the anaerobic fermentation (Shen *et al.* 2013). The hydrolysis stage is relatively slow during cellulose fermentation and determines the speed of the anaerobic fermentation process. The present analysis indicated that acids-degrading bacteria were more abundant in the B-GS, while bacteria that degrade fiber and more complex compounds were more abundant in the P-GS; this corresponds with the contaminants present in the wastewater.

## Analysis of the *Archaea* Community

The relative abundance of the Archaea in the microbial system was only 0.52% (B-GS) and 0.51% (P-GS) although they play a very important role in the methane-producing phase of anaerobic digestion. The following details of these ancient bacteria are described at the genus level.

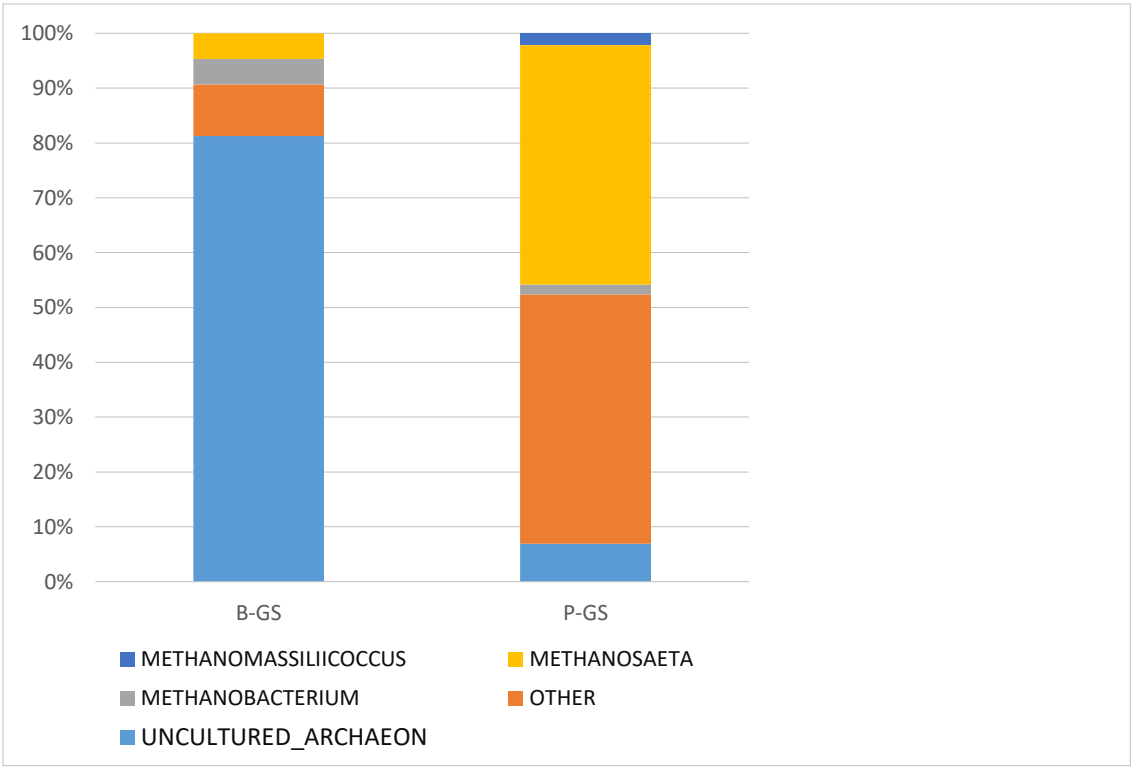


**Fig. 5.** Archaea distribution at the genus level in two types of granular sludge

The abundances of the methanogens *Methanobacterium* (methane bacteria genus) and *Methanosaeta* (methane bacteria genus) were determined in the two types of sludge. These two methanogens accounted for 10% of the total B-GS *Archaea*. The non-acclimated bacteria accounted for 10% and the unknown bacteria accounted for 80% (Fig. 5).

In the P-GS, the methanogens accounted for 50% and the *Methanomassiliicoccus* accounted for 2.2% of the Archaea in the P-GS. These three methanogens belong to the Euryarchaeota. The relative abundance of the *Methanobacterium* in the B-GS and P-GS was 4.7% and 1.8%, respectively. *Methanobacterium* utilizes $H_2$ as an electron donor and $CO_2$ as an electron acceptor, and three metabolic pathways are used, using $H_2$, $CO_2$, and acetate to produce methane carbon compounds (Luo *et al.* 2016b). The relative abundance of the *Methanosaeta* was 4.68% and 42.12% for B-GS and P-GS, respectively, exhibiting a large difference. The *Methanosaeta* belongs to the methanogenic bacteria responsible for acetic acid decomposition. As a strictly anaerobic bacterium, *Methanobacterium* utilizes $H_2$, formate, dibasic alcohol, and CO as its electron donor. It can reduce $CO_2$ to $CH_4$. In addition, electrons can be exchanged by an interspecific electron transfer between *Geobacter* and *Methanosaeta* (Rotaru *et al.* 2014).

*Methanomassiliicoccus* accounted for 2.2% of the Archaea in the P-GS. This genus belongs to a type of methyl methane-producing Archaea with unique metabolic characteristics. Unlike traditional methanogens of the obligate methyl type, these bacteria lack a complete pathway for reducing $CO_2$ to methyl coenzyme. (Lang *et al.* 2015). Therefore, this type of bacterium requires the addition of $H_2$. A genomic analysis has shown that *Methanomassiliicoccus* contains some of the genes that would allow it to utilize $H_2$ in the reduction of methanol, methylamine, dimethylamine, and other substrates.

Therefore, *Methanomassiliicoccus* is not a typical methanogen with regard to using either methyl or hydrogen as nutrients but represents a mix of both types (Zhang *et al.* 2015).

It is hoped that the results of this study provide insights into the biological structure of anaerobic granular sludge to improve the treatment of pulp effluent and to establish a comprehensive database. The results can facilitate a more targeted approach for the treatment of wastewater from different sources using microbial cultures. This will provide benefits to companies treating wastewater.

## CONCLUSIONS

1. In this study, the microbial structures and the physical and chemical properties of two types of granular sludge were compared. They are many differences in terms of heavy metal content, surface functional groups, elemental distribution, microbial variety, and the quantity and relative abundance of the microbes. This is related to the origin of the substrate. However, both types of sludge contained microbial communities capable of degrading various compounds.
2. In the treatment of wastewater by granular sludge, the properties of the sludge are affected by the quality of the influent water. The metal ions in the waste water will bind with anions such as carbonate, sulfate, and phosphate, and some form precipitates attached to the surface of the granular sludge, or adsorb the pores filled with the granular sludge (Alphenaar 1994). These ions or precipitates interact with the extracellular polymer (EPS), the microenvironment of the granular sludge changes, the microbial community of the granular sludge changes, and the physical and chemical properties of the granular sludge eventually change.
3. Anaerobic granular sludge treatment of different wastewater, microbial diversity is quite different, but the main bacteria were Bactericides, Proteobacteria, Firmicutes, Chloroflexi. At the branch level, the major bacteria Anaerolineaceae, Syntrophomonadaceae, Geobacteraceae, Syntrophobacteraceae, *etc*. were quite different.

## ACKNOWLEDGMENTS

The authors are grateful for the National Science Foundation (No.31660182), the Nature Science Foundation of Guangxi (No. 2015GXNSFBA139042; 2017GXNSF-AA198200) and supported by the Dean Project of Guangxi Key Laboratory of Clean Pulp & Papermaking and Pollution Control (No. ZR201607).

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Article submitted: January 8, 2018; Peer review completed: March 17, 2018; Revised version received: March 19, 2018; Accepted: March 20, 2018; Published: March 22, 2018.
DOI: 10.15376/biores.13.2.3511-3524