

ORIGINAL RESEARCH PAPER

## Seasonal variations of microbial community in a full scale oil field produced water treatment plant

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**ABSTRACT:** This study investigated the microbial community in a full scale anaerobic baffled reactor and sequencing batch reactor system for oil-produced water treatment in summer and winter. The community structures of fungi and bacteria were analyzed through polymerase chain reaction–denaturing gradient gel electrophoresis and Illumina high-throughput sequencing, respectively. Chemical oxygen demand effluent concentration achieved lower than 50 mg/L level after the system in both summer and winter, however, chemical oxygen demand removal rates after anaerobic baffled reactor treatment system were significant higher in summer than that in winter, which conformed to the microbial community diversity. *Saccharomycotina*, *Fusarium*, and *Aspergillus* were detected in both anaerobic baffled reactor and sequencing batch reactor during summer and winter. The fungal communities in anaerobic baffled reactor and sequencing batch reactor were shaped by seasons and treatment units, while there was no correlation between abundance of fungi and chemical oxygen demand removal rates. Compared to summer, the total amount of the dominant hydrocarbon degrading bacteria decreased by 10.2% in anaerobic baffled reactor, resulting in only around 23% of chemical oxygen demand was removed in winter. Although microbial community significantly varied in the three parallel sulfide reducing bacteria, the performance of these bioreactors had no significant difference between summer and winter.

**KEYWORDS:** Anaerobic baffled reactor (ABR); Denaturing gradient gel electrophoresis (DGGE); High throughput sequencing; Microbial community; Seasonal variations; Sequencing batch reactor (SBR); Oilfield produced water; Sulfide reducing bacteria (SRB); Wastewater treatment plant (WWTP)

### INTRODUCTION

Oilfield produced water consists of toxic, aromatic, and contaminated hypersaline water, which normally are complex organic compounds, such as phenolics and aromatic hydrocarbons, benzene, toluene, ethylbenzene, xylene, naphthalene, phenanthrene, and dibenzothiophene. The discharges of oilfield-produced water without proper treatment can contaminate soil and water bodies due to the existence of the toxic

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organic compounds. Conventional treatment for oilfield-produced water mainly relies on physical-chemical methods, including gravity separation, dissolved air flotation, hydrogen peroxide treatment, photocatalytic degradation, coagulation and flocculation. Since some halophilic bacteria, yeast and fungi can grow well with crude oil as a source of carbon and energy, some of them were utilized for bioremediation to purify petroleum-produced water because of the low cost. Sequencing batch reactor (SBR) has been widely used in petroleum-produced

water treatment, in which wastewater is initially hydrolyzed under anoxic conditions and then treated under aerobic conditions, by doing this, most of the chemical oxygen demand (COD) can be removed. Ghorbanian *et al.*, (2014) showed that the average petroleum hydrocarbon removal rates in an SBR were 99.9%, 99.6%, and 93.7% at initial concentrations of 950, 1450, and 2500 mg/L. Moreover, the removal rates of COD, total organic carbon (TOC), and oil and grease removed were 86.2%, 90.8%, and 90% respectively in a MBR, with organic loading rate of 1.124 kg of COD/m<sup>3</sup> d and hydraulic residence time (HRT) of 48h. Generally, an anaerobic baffled reactor (ABR) is also used to enhance the COD removal efficiency as a pretreatment. Therefore, an ABR and three SBRs in parallel integrated process (Fig. 1) were employed in the oilfield-produced wastewater treatment plant.

Microbial communities contribute to the biodegradation of hydrocarbons and COD have been investigated in previous molecular studies using sludge obtained from oilfield produced water treatment plants. Molecule microbial analytical methods such as denaturing gradient gel electrophoresis (DGGE) profile and high throughput sequencing, revealed that the bacteria related to organic matter degradation belong to different taxonomic groups, including *Methylobacterium sp.*, *Rhodococcus aetherovorans*, *Achromobacter xylosoxidans*, *Alcaligenes sp.*, *Aquamicrobium defluvium*, *Rhizobium*, and *Stenotrophomonas spp.*. Moreover, temperature plays a major role in biodegradation of hydrocarbon. Changes of the operating temperature in wastewater treatment plants are due to seasonal variations. As removal efficiency of hydrocarbon in a full-scale treatment plant is different in summer and winter, the microbial communities responsible for hydrocarbon degradation during summer and winter are different. While the microbial communities refer to oilfield-produced water is scarce, especially with consideration of seasonal effect on the microbial communities.

The studies aimed at assessment of the effects of temperature and treatment units on microorganisms targeting oilfield-produced water are still unclear. A better understanding of the microbial organisms can lead to a better understanding of the contribution of the microbial organisms in the degradation of hydrocarbons in oilfield-produced water. The aims of the present study were (i) to assess the pollutant removal efficiency of COD in a treatment plant; (ii) to analyze the compositions of

bacterial and fungi in the treatment plant; (iii) to select the dominant species in the process of degrading hydrocarbon in oilfield produced water.

This study has been performed in Beihai City, Guangxi Zhuang Autonomous region during 2012 to 2013.

## MATERIALS AND METHODS

### Study site description

The wastewater treatment plant (WWTP) was established in 2006 to treat 1000 tons of wastewater per day from Weizhou Island Oil Production Plant (China National Offshore Oil Corporation). The influent wastewater composition is illustrated in Table 1. The plant is located in the southeast of Beihai City, Guangxi Zhuang Autonomous Region in China. During the system, an ABR was followed by three sequencing batch reactors. The ABR (21 m long, 16.6 m wide and 6.5 m high) and three SBRs (20.0 m long, 12.0 m wide, and 5.8m high) were connected to enhance the performance of the WWTP. The effective volumes of the ABR and three sulfide reducing bacteria (SRBs) were 1500, 360, 360 and 360 m<sup>3</sup> respectively. All of the SBRs were operated according to the following strategies: filling (1.0 h), reaction (aeration, 8.0 h), settling (2.0 h), extraction (1 h), and idle.

### Sampling and analysis

Water samples were collected from the influent and effluent of the ABR, and three parallel SBRs effluent (1#SBR, 2#SBR, and 3#SBR) between 9:00 and 10:00 during a day in August 2012 and February 2013 respectively. COD was measured according to standard method. The temperature of the samples was also determined. Statistics analysis was employed to investigate the relationship between temperature and COD removal efficiency/microbial diversities using the one-way ANOVA test in the SPSS 17.0 software and difference were considered to be significant when  $p < 0.05$ .

Table 1: Composition of oilfield produced wastewater

Parameter	Range
Cl <sup>-</sup> /mg/L	14000–15000
COD <sub>Cr</sub> /mg/L	384–584
BOD <sub>5</sub> /mg/L	50–127
Total petroleum hydrocarbon /mg/L	11.5–15
S <sup>2-</sup> /mg/L	11.8–20.1
TN/mg/L	9–13
TP/mg/L	7–12
SS/mg/L	140–610
pH	7.8–8.2

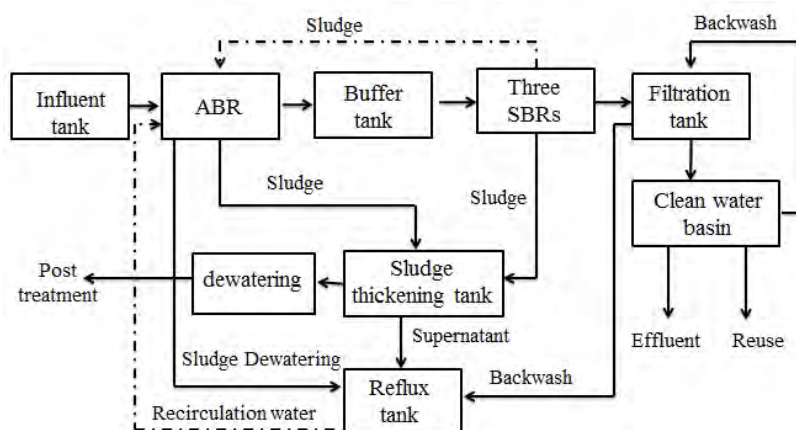


Fig. 1: Simplified system of the Weizhou wastewater treatment plan (pumps were not shown)

Sludge samples were collected in August and February from the ABR (denoted as SA in summer and WA in winter) and three parallel SBRs (denoted as SS1, SS2, and SS3 for samples collected in summer and WS1, WS2, and WS3 for samples collected in winter) for molecular and biological analyses. Six samples at different sites were collected from each reactor, and then fixed and stored at 4 °C before analysis.

#### *Microbial community analysis*

##### *DNA extraction, PCR amplification, DGGE and sequencing*

The total genomic DNA was extracted from sludge samples using Power Soil DNA isolation kit (Mo Bio Laboratories, Inc., Carlsbad, CA, USA). DNA was purified using Universal DNA purification kit (TIANGEN Biotech Co., Ltd., Beijing, China) and quantified using Nano-Drop UV-3000 spectrophotometer (Nanodrop Technologies Inc., Delaware, USA) according to the manufacturer's instructions. Isolated DNA was amplified through PCR using primers specific for fungi (ITS1F CTTGGTCATTTAGAGGAAGTAA and ITS4 TCCTCCGCTTATTGATATGC), targeting the ITS1 region to analyse sludge fungal communities. PCR amplifications of the ITS1 region from genomic DNA were performed in 25  $\mu$ L mixture containing 5  $\mu$ L of 5 $\times$  PrimeSTAR<sup>®</sup> buffer with MgCl<sub>2</sub>, 2  $\mu$ L of deoxyribonucleotide triphosphate mixture, 0.5  $\mu$ L of each primer, 0.25  $\mu$ L of PrimeSTAR<sup>®</sup> HS DNA polymerase, 1  $\mu$ L of DNA, and 15.75  $\mu$ L of molecular-grade water. PCR reactions were performed as follows: 95 °C for 5 min

(one cycle); 95 °C for 30 s, 59 °C for 30 s, and 72 °C for 50 s (30 cycles); and 72 °C for 7 min (one cycle). The amplified products were subjected to electrophoresis on 0.8% agarose gels. All PCR amplification reactions were performed in an S1000™ BioRad model iCycler.

The purified PCR products were analysed on 8% polyacrylamide gels containing gradients of 31%–53% denaturant. DGGE was performed in a DCode Universal Mutation Detection System (Bio-Rad) at a constant voltage of 80 V and temperature of 60 °C for 16 h in 1 $\times$ TAE running buffer. The gels were stained with SYBR Gold nucleic acid gel stain (Molecular Probes, Eugene, OR) and then imaged with the FluoroImager System Model 595 (Molecular Dynamics, Sunnyvale, CA). Gel images were analyzed with Gelcompar II v5.0 (Applied Maths, Sint-Martens-Latem, Belgium) to generate dendrogram profiles.

The individual bands of the DGGE gels were excised and eluted with 30  $\mu$ L of dH<sub>2</sub>O for 48 h at 4 °C before re-amplification using the same set of primers. Sequencing was performed on a 3730 DNA Analyzer using BigDye<sup>®</sup> Terminator v3.1 cycle sequencing kit (Applied Biosystems, United States) according to the manufacturer's instructions. A phylogenetic tree was constructed through neighbor-joining method using the MEGA 4.1 with sequences and bootstrapped for 1000 iterations.

##### *High throughput sequencing analysis*

The purified total community DNA samples prepared in 2.2.2 were submitted to Novogene Bioinformatics Institute (Beijing, China) for high-throughput

sequencing using Miseq Illumina. Primers for sequencing were 515F (5' -GTG CCA GCM GCC GCG GTAA-3') and 806R (5' -GG ACTA CHV GGG TWT CTAAT-3'), with different barcodes for the V4 region of the 16S rRNA gene. Reads with incorrect barcodes, incorrect primer sequences, average quality scores of < 25, homopolymers of  $\geq 6$  and read lengths < 200 bp were excluded from further analysis. High-quality sequences were processed through CD-HIT to generate operational taxonomic units (OTUs) with 97% sequence similarity threshold. Representative sequences from each OTU were aligned using PyNAST. Average data were calculated for each sludge sample before analyzing the unique and shared OTUs/genera.

## RESULTS AND DISCUSSION

### Effect of temperature on COD removal in the full scale ABR-SBR

The average temperature in summer (August, 2012) and winter (February, 2013) were 29 °C and 18 °C. The influent concentration of COD was 535±49 mg/L in summer and 418±34 mg/L in winter (Fig. 2). Statistical analyses of COD removal in ABR system showed significantly ( $P < 0.05$ ) lower efficiency in winter (23±4%) than in summer (30±3%), even though it received lower influent concentration. The corresponding of low temperature and low COD removal in ABR may cause by lower functional microbial activity appearance in winter. However, the removal efficiencies of COD in both season achieved 90±2% in summer and 88±4% in winter, both with effluent concentration lower than 50

mg/L after full-scale ABR-SBR system, which proves the high application performance of ABR-SBR technology for oilfield produced wastewater.

### Variations in fungal community composition

The DGGE fingerprints of the fungal community of sludge sampled from the ABR and SBRs during summer and winter were analyzed and 28 different bands were detected (Fig. 3). Phylogenetic analysis indicated that the majority of these 28 fungal clones belonged to the *Ascomycota*, which consisted of the *Peizomycotina* and the *Saccharomycotina*. The *Peizomycotina* was the dominant taxon and further divided into four distinct groups, including the *Fusarium*, *Bionectria*, *Stachybotrys*, and *Aspergillus* (Fig. 4). The *Mucoromycotina* was also observed in the fungal clones (Fig. 4). Fig. 5 shows proportion of the major fungi in the ABR and SBRs collected in summer and winter, with the *Saccharomycotina*, *Fusarium*, and *Aspergillus* were detected in all the samples and the rest fungi were detected conditionally with consideration of season and treatment units.

It's apparent to see that the *Stachybotrys* was shaped by seasons and treatment units, while the *Saccharomycotina*, *Fusarium*, and *Aspergillus* were ubiquitous in all sludge sampled from ABR and SBRs during summer and winter, although their abundance differed. Previous studies suggested that the filamentous fungi can produce extracellular enzymes and degrade polycyclic aromatic hydrocarbons (PAHs). In the present study, significant difference in the

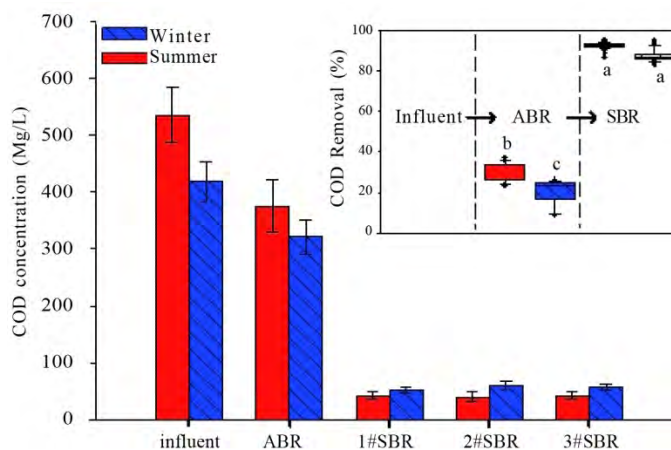


Fig. 2: COD concentration and removal efficiencies of the ABR influent and effluent and three parallel SBRs. Data Bars followed by different letters are significantly different ( $p < 0.05$ )

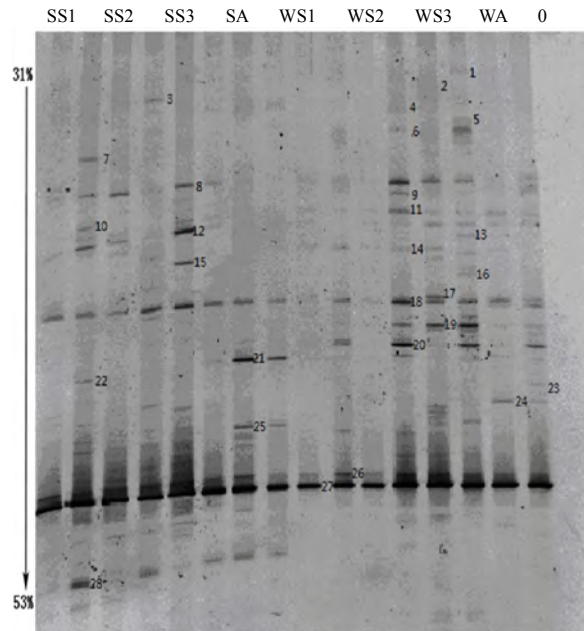


Fig. 3. DGGE analysis of the 16S rRNA gene fragment of fungal population from the ABR (SA for summer and WA for winter) and three parallel SBRs (SS for summer and WS for winter). Sequenced bands are marked on the gel.

removal efficiency of COD were observed between seasons and treatment units, which may due to the difference of microorganisms communities existed in the sludge samples. However, the proportion of the fungi in the ABR and SBRs during summer and winter were not corresponded to the COD removal efficiency, which may imply that fungal community will not influence the COD removal in the present study.

#### Variation in microbial community compositions

The bacterial community diversities of the sludge used in batch tests were analyzed by Illumina sequencing. The number of bacterial species can be estimated using the number of OTUs (Fig. 6). Fig. 6 shows the number of OTUs in summer was higher than in winter, indicating that the bacterial diversity was relatively higher in summer than that in winter in terms of either ABR or SBRs.

#### Microbial community in SRBs

In aerobic sludge, the number of OTUs were 4535 (genus level) and 9203 (species level) in SS and 3647 (genus level) and 7254 (species level) in WS. In agreement with previous reports, the present study

showed that changes in environmental conditions, particularly temperature shock, directly influenced microbial communities. Temperature plays a vital role in biodegradation, and the microbial diversity in the wastewater treatment system reduced as the reactor progressively recovered from low-temperature shock. Extreme temperatures may confer irreversible damages and even death in some bacteria.

Fig. 7 shows that the majority of the bacteria (39.3%) in the treatment plant for oilfield-produced water belonged to the phylum *Proteobacteria*, followed by the phyla *Firmicutes* and *Chloroflexi*, which accounted for 19.6% and 13.5% of the total population, respectively. The phyla *Bacteroidetes*, *Actinobacteria*, and *Planctomycetes* accounted for 5%, 4.7%, and 2% of the total population, respectively. Similarly, previous research showed that bacterial communities at different operating temperatures consisted mostly of the phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, which can effectively degrade organic compounds. Analysis of MBR and conventional sludge produced from PAH-contaminated wastewater showed that *Proteobacteria* could be the potential organism for remediating degraded petroleum.

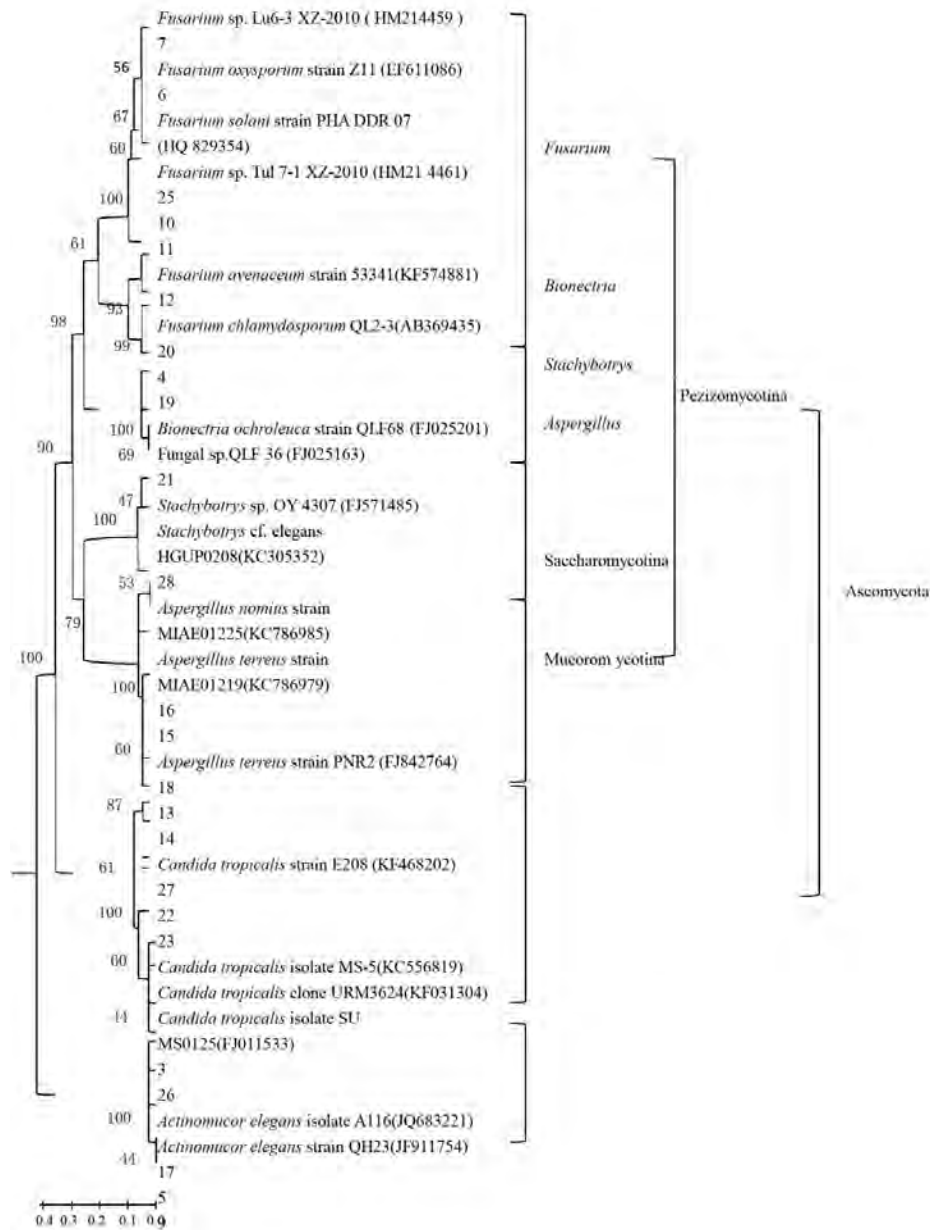


Fig. 4: Phylogenetic tree of the 16S rRNA sequences derived from the DGGE bands

The majority of the OTUs contained only one tag, and tag numbers were counted at different levels to determine the optimal taxonomic level for relevant comparisons. The proportions of assignable tags for the SBR and ABR samples were higher than 70% at the order level, whereas lower than 50% at the family level. Therefore, the order level was selected as the optimal taxonomic level for comparison.

The results demonstrate that bacterial diversity was relatively higher in summer than that in winter (Fig. 7). In SBRs, namely, SS samples, the most abundant order was *DRC31* from the phylum *Chloroflexi* (18.16%), followed by *Lactobacillales* (11.7%) and *Bacillales* (6.9%) from the phylum *Firmicutes*. The relative abundance of *Rhodospirillales* (phylum *Proteobacteria*), and *Gammaproteobacteria*



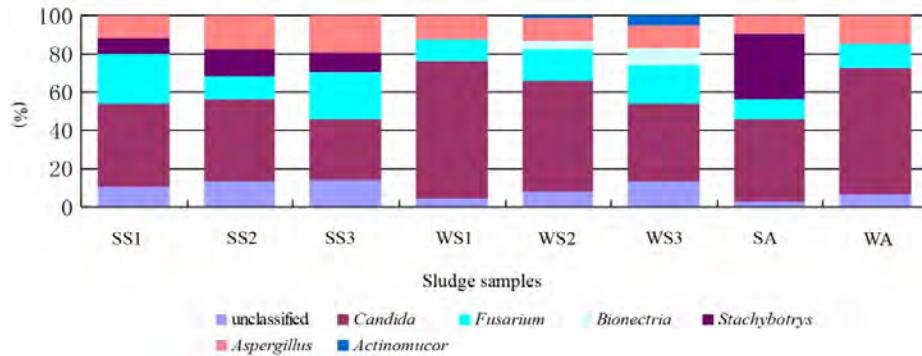


Fig. 5: Relative abundance of the major fungi in each sludge samples from the ABR and three parallel SBRs collected in summer and winter

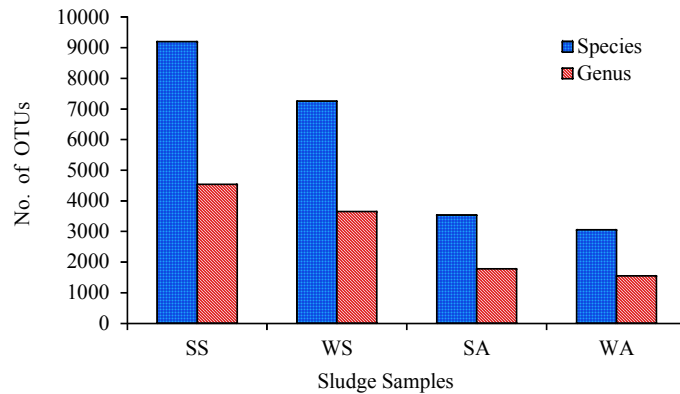


Fig. 6: Analysis of OTUs from sludge sampled from the ABR and SBRs in summer and winter

and *Rhizobiales* (phylum *Proteobacteria*) were comparable, which were 4.6%, 4.3%, and 4.2%, respectively. By contrast, WS samples harbored high relative abundance of *Pseudomonadales* (35.6%, phylum *Proteobacteria*), *Clostridiales* (12.2%, phylum *Firmicutes*), and *Lactobacillales* (8.9%). Furthermore, *Ignavibacteriales* (6.5%), and *Sphingobacteriales* (7.4%) were detected in SS with low abundance and were absent in WS (Fig. 8). The members of *Pseudomonadales*, *Lactobacillales*, and *Bacillales* participate in the biogeochemical transformation of petroleum. Silva *et al.*, (2013) has indicated on the diversity of microbial communities in petroleum samples from Brazilian oil fields showed that eight different phyla (*Actinobacteria*, *Bacteroidetes*, *Deferribacteres*, *Spirochaetes*, *Firmicutes*, *Proteobacteria*, *Thermotogae*, and *Synergistetes*) were the dominant groups involved in hydrocarbon degradation. According to the performance of SBRs, the COD removal

efficiencies were similar (88.5% to 82.2%) when the influent COD concentration were 375.5 and 320.9 mg/L in the summer and winter, respectively (Fig. 2). Although the microbial communities significantly varied between summer and winter, the relative abundance of the dominant hydrocarbon degrading bacteria still remained at a high level (see above mentioned). Therefore, the changes of bacterial communities from summer to winter did not affect the performance of SRBs.

#### Diversity of microbial community in ABR

The OTUs were higher in SA and reached 1787 at the genus level and 3540 at the species level. By contrast, the number of OTUs in WA was 1555 at the genus level and 3057 at the species level in the ABR. In anaerobic sludge, SA samples harbored abundant populations of *Rhizobiales* (13.2%), *Thermotogales* (phylum *Thermotogae*, 10.7%), and *Actinomycetales* (phylum *Actinobacteria*, 10.4%). *Rhodospirillales* and

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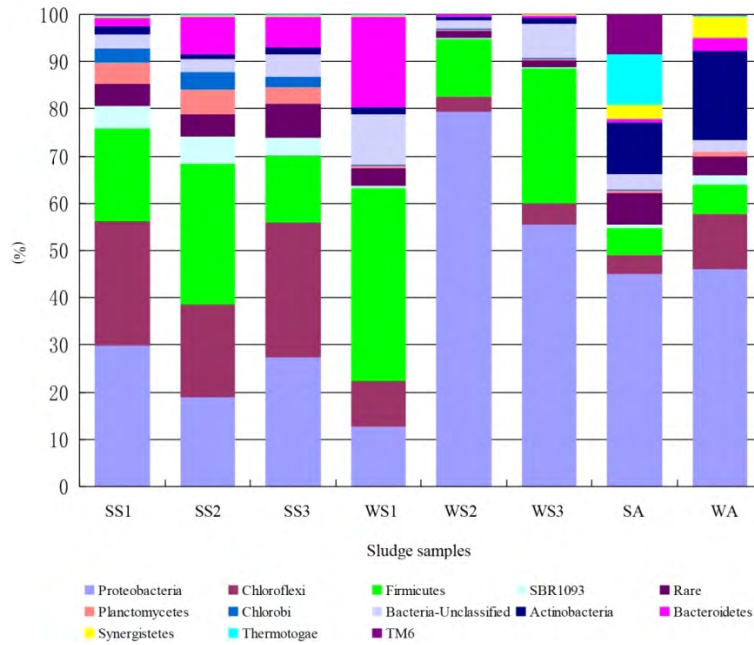


Fig. 7: Microorganism distribution at the phylum level in sludge sampled from three parallel SBRs and ABR in summer and winter

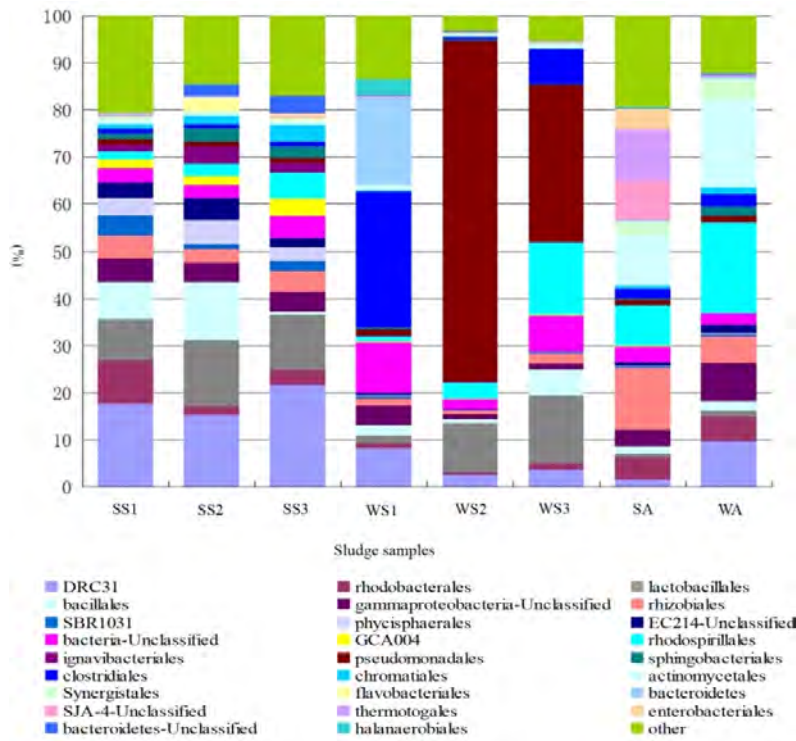


Fig. 8: Microorganism distribution at the order level in sludge sampled from three parallel SBRs and ABR in summer and winter



*Rhodobacterales* accounted for 8.5% and 4.7% of the population, respectively (Fig. 7). *Rhodospirillales* showed the largest proportion (19.1%) of the bacterial population in WA samples, followed by the order *Actinomycetales* (18.4%). *DRC31* and *Gammaproteobacteria* unclassified were also abundant and accounted for 9.7% and 8.1%, respectively (Fig. 7). The mean proportion of *DRC31* in SS was 18.2% and decreased to 5.7% in WS. High temperatures seemed to be more beneficial in *DRC31* survival under aerobic conditions than low temperatures. However, under anaerobic conditions, *DRC31* accounted for higher proportion (9.7%) in the WA samples than that in the SA samples (2.3%). Although information about the role of *DRC31* in petroleum hydrocarbon degradation remains limited, *DRC31* is speculated to positively affect high COD degradation during summer.

*Actinobacteria*, *Thermotogales* and *Rhizobiales* are mainly isolated from petroleum hydrocarbon-contaminated environments, such as marine sediment, beach, and groundwater; and genera within these families contain members with active roles in biogeochemical transformation of petroleum. The relative abundance of *Rhizobiales* and *Thermotogales* were decreased from 13.15% and 10.72% in the summer period to 5.3% and 0.4% in the winter time respectively. However, the relative relative abundance of *Actinomycetales* increased by 8.1%. The results reveal that the total amount of the dominant hydrocarbon degrading bacteria was decreased in the winter period, compared to summer time. Additionally, around 159.1 and 97.4 mg/L of COD, on average, were removed in the ABR in summer and winter respectively (Fig. 2). Therefore, the removed COD was declined resulting from the dominant hydrocarbon degrading bacteria decreasing.

## CONCLUSION

COD removal rates after ABR treatment system were significantly higher in summer than in winter, which conformed to the microbial community diversity. Moreover, COD effluent concentration achieved lower than 50 mg/L level after full-scale ABR-SBR system, which proves the high performance of the technology for oilfield-produced wastewater. The fungal communities in ABR and SBR were shaped by seasons and treatment units, while there was no correlation between abundance of fungi and COD removal rates.

Different seasons affected the microbial community both in ABR and in SRBs. The performance of the ABR was slightly decreased in terms of COD removal. However, the COD removal efficiencies in the three parallel SRBs kept stable between summer and winter.

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## CONFLICT OF INTEREST

The authors declare that there are no conflicts of interest regarding the publication of this manuscript.

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