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Analysis of a diverse bacterial community and degradation of organic compounds in a bioprocess for coking wastewater treatment

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ABSTRACT

Coking wastewater is one of the most toxic industrial effluents since it contains high concentration of toxic organic compounds. Biological treatments are widely applied in coking wastewater treatment, pollutants can be degraded completely due to the synergistic effect of the community composition. In this study, the community structure and degradation of organic compounds of a full-scale coking wastewater treatment plant with anaerobic, anoxic, and oxic process ($A_1/A_2/O$) were studied. GC-MS results showed that phenols, indole, quinoline and pyridine, accounting for 61.70, 13.63, 7.71 and, 2.30%, respectively, were the main organic pollutants in the raw coking wastewater. Those pollutants were degraded gradually during the $A_1/A_2/O$ bioprocess, respectively. High throughput pyrosequencing was applied to investigate the bacterial community, the sequences could be affiliated to 21 phylogenetic groups, including *Proteobacteria*, *Chloroflexi*, *Bacteroidetes*, *Planctomycetes*, *Synergistetes*, *Chlorobi*, *Acidobacteria*, *Nitrospira*, *Firmicutes*, and *Actinobacteria*. Diversity and richness indexes, venn analysis and principal component analysis indicated that the diversity and abundance of species in three samples were different. The abundance of the phylum *Proteobacteria* accounted for 84.64% (A_1), 62.73% (A_2), and 83.24% (O) of the total reads, respectively. The corresponding most dominant orders in three samples were *Pseudomonadales* (A_1), *Syntrophobacterales* (A_2), and *Burkholderiales* (O), respectively. While genus *Pseudomonas*, *Desulfoglaeba*, and *Diaphorobacter* was the dominant bacterium in three samples, respectively.

Keywords: Coking wastewater; Aromatic compounds; Biological degradation; Microbial community; Pyrosequencing

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1. Introduction

Pollution caused by coking wastewater is a serious problem, especially in China, where coal is one of the main energy sources for the iron and steel industry [1]. Coking wastewater is considered to be a high organic wastewater, containing a large number of biodegradable and refractory organic pollutants such as polycyclic aromatic hydrocarbons (PAHs), phenols, indoles, quinolones, and pyridines [2,3]. Phenols are the main organic constituents, accounting for about 80% of the total COD [4,5]. Indole and its derivatives form a unique class of toxic and recalcitrant N-heterocyclic compounds that are considered to be environmental pollutants [6]. Quinolinic compounds, including quinoline and isoquinoline, are also considered toxic, carcinogenic, and mutagenic [7]. Pyridine and its derivatives are reported to be toxic, carcinogenic, and teratogenic and are rated as priority pollutants by the USEPA [8]. Hence, coking wastewater is notorious for its toxicity and refractoriness [1], it must be necessary to treat coking wastewater properly to avoid any adverse environmental and ecological impacts.

Biological treatments, such as Anoxic-Oxic (A/O), Anaerobic-Anoxic-Oxic ($A_1/A_2/O$), and Anaerobic-Oxic-Oxic (A/O/O) processes, are widely applied in coking wastewater treatment because of the high treatment efficiency and cost-effective advantages [9–11]. During the biological treatment process, the sludge in the bioreactor comprises a complex microbiological community and the microbial community, which is dominated by bacteria, plays an essential role [12–14]. Microorganisms determine the performance of the biological wastewater treatment process [15]. So, it is necessary to better understand the structure of the microbial community in the coking wastewater treatment process.

$A_1/A_2/O$ process is typically and widely used to biodegrade pollutants in coking wastewater, including organics, ammonia, and other inorganics. Different bacteria play their respective special roles in the $A_1/A_2/O$ bioprocess, and pollutants can be degraded completely due to the synergistic effect of the community. However, little is known about the information of microbial community in $A_1/A_2/O$ process for coking wastewater treatment at present time. Although many a pure culture microorganisms [16–18] from coking wastewater has been isolated and identified by traditional culture-based techniques, the microbial community is a complex system and cannot be adequately studied by traditional methods. Pyrosequencing is a high-throughput analytical method that can generate huge amounts of DNA reads through a massively parallel sequencing-by-synthesis approach, and this approach has so far been used widely to analyze the environmental samples microbial community such as soil and wastewater [19–21].

In this study, degradation of organics in coking wastewater was investigated by detecting concentrations of organics in different stages during the $A_1/A_2/O$ process, and 454 high-throughput pyrosequencing was used to analyze the bacterial population by sequencing the bacterial 16S rRNA gene to understand the composition of bacteria, to identify the richness and diversity of bacteria phylotypes in the three bioreactors, to compare the similarity and difference in the communities. It was expected to elucidate core function microorganisms according to the relationship between microbial community and degradation of organics during the $A_1/A_2/O$ process. This will be beneficial to explore the reaction mechanism, dynamic monitoring, and optimization control of coking wastewater treatment so as to provide a theoretical basis for the stable operation of coking wastewater biological treatment system from the molecular biology level.

2. Materials and methods

2.1. Wastewater treatment plant description and sampling

A full-scale coking Wastewater treatment plant (WWTP) of Angang coking plant in Anshan, Liaoning province of China, with an average treatment capacity of 4,800 m^3/d, which had been steadily employed for the treatment of actual coking wastewater for over 5 years, was investigated. The scheme of coking wastewater was showed in supplementary Fig. 1. Several main qualities of coking wastewater were displayed in supplementary Table 1.

Water samples from the anaerobic tank influent, anaerobic tank effluent, anoxic tank effluent, and aerobic tank effluent, were taken on five separate days on 18–30 April 2014. Two litres of wastewater was taken at 11:00, 14:00, and 17:00, respectively, then mixed in equal proportion as the sample of the day, and analyzed. Sodium azide was added to each sample immediately after collection for preventing microbial degradation.

Sludge samples, which were used to perform 454-pyrosequencing analysis, were gathered from anaerobic tank, anoxic tank, and aerobic tank, respectively. Samples were taken on five separate days on 18–30 April 2014. All sludge samples were briefly settled on site to be concentrated, then fixed in 50% (v/v) ethanol aqueous solution. Samples were freeze dried and kept at −20°C before analysis at the laboratory.

2.2. Analytical methods

2.2.1. Sample preparation, instrumental analysis of wastewater component, and quality control

Duplicate water samples were firstly filtered through 0.45 μm filter paper before analysis. 100 ml of each sample (20 μg/L 1-fluoro-phenol and 2,4,6-tribromophenol were added as the recovery samples) was taken, then extracted according to the method reported by literature [22]. Mass percentage of the organic compounds was calculated according to the peak area by GC-MS method [9,23].

GC-MS analysis was conducted using an Agilent 6890 GC coupled with an Agilent 5973 mass spectrometer detector with a 30 m × 0.25 mm × 0.25 μm filmthickness DB-5 MS column. GC temperature was programmed from an initial temperature of 50˚C up to a maximum temperature of 280˚C, with runs at 10˚C/min and a final holding time of 17 min. The mass spectrometer was operated in selected ion monitoring (SIM) mode with an electron impact ionization of 70 eV, an electron multiplier voltage of 1,288 V, and an ion source at 230˚C.

The average recoveries were 92–108% and the relative standard deviations were less than 8%.

2.2.2. DNA extraction, PCR amplification, and pyrosequencing

Sludge samples for DNA extraction were processed using ezup column genomic DNA extraction kit (Shanghai sangon, China) for soil according to manufacturer's protocols. The concentration and purification of the extracted DNA were determined using agarose gel electrophoresis and the Nanodrop® ND-1000 spectrophotometer (Labtech International, UK). The DNA samples were stored at −20˚C before the next analysis.

For pyrosequencing, the above DNA mixtures of each sample were amplified with a set of primers targeting the V1–V3 region of the 16S rDNA gene. The forward primer (AGAGTTTGATCMTGGCTCAG) [24] and the reverse primer (GTATTACCGCGGCTGCTGGCAC) [25] were used for bacterial sequences. The PCR mixture contained 5 μL of 10 × PCR buffer, 0.5 μL of dNTPs (10 mM), 10 ng of Genomic DNA, 1 μL of Bar-PCR primer F (50 μM), 1 μL of primer R (50 μM), 0.5 μL of Platinum Taq (5 U/μL), H_2O added to 50 μL. PCR conditions were: 94˚C 30 s; 94˚C 20 s, 45˚C 20 s, 65˚C1 min, 5 cycles; 94˚C 20 s, 60˚C 20 s, 72˚C 20 s, 20 cycles; 72˚C 5 min. Barcodes that allowed samples multiplexing during pyrosequencing were incorporated between the 454 adapter and the forward primer. After purification using the UNIQ-10 PCR Purification Kit (Sangon, Shanghai, China) and quantification using a TBS-380 (Turner BioSystems, Inc., USA), DNA samples with different barcodes were mixed in equal concentration and sequenced by a Roche 454 GSFLX sequencer according to standard protocols.

2.2.3. Sequence processing and biodiversity analysis and phylogenetic classification

Following pyrosequencing, the raw reads were treated according to the method [26], the non-chimera reads then formed the database of "effective reads" for each sample. The number of effective microbial sequences was for all the following analysis. The microbial sequences of samples were carried out using the (RDP) classifier. A bootstrap cut-off of 50% suggested by the RDP was applied to assign the sequences to different taxonomy levels [27]. The normalized sequence of sample was aligned by infernal [28] using the bacteria-alignment model in Align module of the RDP. By applying the complete linkage clustering, sequences were assigned to phylotype clusters at cut-off levels of 3% (equivalent to 97% similarity). The operational taxonomic units (OTUs) defined at 97% sequence similarity [27,29] were used to perform and calculate the richness and diversity indices. The Venn diagram with shared unique OTUs was used to depict the similarity and difference in the three communities.

3. Results and discussion

3.1. Performance of the full-scale coking WWTP

Mass percentage of the organic compounds were calculated according to the peak area by GC-MS method [9,23], the results showed that phenols, indole, quinoline, and pyridine, accounting for 61.70, 13.63, 7.71, and 2.30%, respectively, were the main organic pollutants in raw coking wastewater. The degradation of the organics including phenols, indole, quinoline, and pyridine in the coking WWTP were showed in Table 1. After anaerobic digestion, the peak area of phenols (from 10,376,010 to 1,123,969) decreased rapidly, and the peak area of pyridine (from 387,357 to 90,832), indole (from 2,291,359 to 26,016), and quinoline (from 1,296,999 to 0) showed the same trend, this indicated that phenols, pyridine, and indole were degraded, quinoline was degraded completely. Anaerobic reactor plays an important role for removal of the organic compounds in converting refractory or inhibitory compounds into biodegradable organic

Table 1
The degradation of organic compounds obtained from $A_1/A_2/O$ process

Organics	A_1 influent (P A)	A_1 effluent (P A)	A_2 effluent (P A)	O effluent (P A)
Phenols	10,376,010	1,123,969	827,662	—
Indole	2,291,359	26,016	—	
Quinoline	1,296,999	—		
Pyridine	387,357	90,832	6,410	—

Notes: P A (peak area). Symbol "—" represented organic compound could not be detected. A_1, A_2, O represented anaerobic, anoxic and oxic bioreactors of the $A_1/A_2/O$ system, respectively.

substances [30]. Quinoline, indole, 1-methylindole, 2-methylindole, and 3-methylindole were degraded during anaerobic and sulfate-reducing conditions [31,32], phenolics from the coal coking process were degraded in anaerobic digestion [33], it was possible to complete phenol biodegradation into methane and carbon dioxide via benzoate [34]. The anaerobic biodegradation of quinoline was initiated by hydroxylation at position 2 of the pyridine ring [35], while indole was degraded via oxindole and isatin by bacterial consortia under anaerobic condition [32,36].

Through A_1/A_2 bioprocess, the peak area of phenols and pyridine further decreased, indole was degraded completely. After the whole $A_1/A_2/O$ bioprocess, phenols and pyridine disappeared thoroughly. Under aerobic reaction, degradation of phenol was catalyzed by catechol 2,3 dioxygenase or catechol 1,2 dioxygenase, then product was entered into the TCA cycle [37], pyridine ring was cleaved between the C2 and N, and deaminated to glutaric dialdehyde subsequently, followed by successive oxidation to glutarate semialdehyde, glutarate, and acetyl coenzyme A [38,39]. The removal of organics in the anoxic and oxic reactors are due to the requirements of organic materials by microorganisms in the anoxic reactor during denitrification, and the oxidation of the biodegradable organic substances in the oxic reactor [1].

From the result (as shown in Table 1), it was concluded that $A_1/A_2/O$ biological treatment process played a very important role for coking wastewater treatment. Furthermore, microbial community, which is a complex system in the sludge, determines the performance of the biological wastewater treatment process. Therefore, it is essential to gain a detailed insight into the microbial community and explore its relation with the system performance.

3.2. Diversity and abundance of species

The results of each sludge sample, which was repeated three times, were clustered together, which verified that the sequencing results were reproducible and reliable. Pyrosequencing of the anaerobic sludge, anoxic sludge, and oxic sludge samples yielded 8,303, 7,141, and 10,198 effective sequence tags, respectively, this number of sequences was comparable to other studies that also adopted 454-pyrosequencing [13,21]. Total numbers of 638, 790, and 947 OTUs were obtained in the three samples, respectively (Table 2). Samples from the $A_1/A_2/O$ tank displayed considerably less richness, compared with active sludge from sewage treatment plant composed of anoxic and oxic bioreactors [27], who observed there were 3,414 and 3,004 OUTs in the sludge sample at 3% cut-off level, Shannon Index was 7.178 and 7.076, respectively.

Venn analysis was employed to evaluate the similarity of diversity and abundance of species (Fig. 1) in the three sludge samples. The sum of total observed OTUs in three communities was 1975, but only 92 OTUs or 4.66% of the total OTUs were shared by them. 1 and 3 had more common OTUs than any of them. The number of OTUs that were unique to each community was 404, 578, and 685, respectively, and together they accounted for 84.41% of the total number of observed OTUs.

From the result of the venn analysis (Fig. 1), it showed that the abundance of species shared in three samples was less than 5%. This dissimilarity could also be demonstrated by the result from principal component analysis (PCA) as shown in Fig. 2, three samples were quite far in distance. Microbial community determines the performance of the biological wastewater treatment process and the structure of microbial community in wastewater treatment systems can be influenced by many possible factors, including the composition of influent, treatment process, geographical location, season, and so on [15,27]. Deterministic factors, particularly wastewater characteristics, act as the key factor in the community assembly process [29]. The factors impacting the microbial community should deserve more comprehensive and systematic studies in the future work using pyrosequencing.

Table 2
Richness and diversity estimators of the bacterial phylotypes in the three sludge samples

Sample	Seq_num	OTU_num	Shannon_index	Chao1_index	Coverage
1	8,303	638	4.656	1,276.122	0.961
2	7,141	790	5.246	1,532.684	0.920
3	10,198	947	5.642	1,697.126	0.957

Notes: 1: Anaerobic sludge; 2: Anoxic sludge; 3: Oxic sludge.

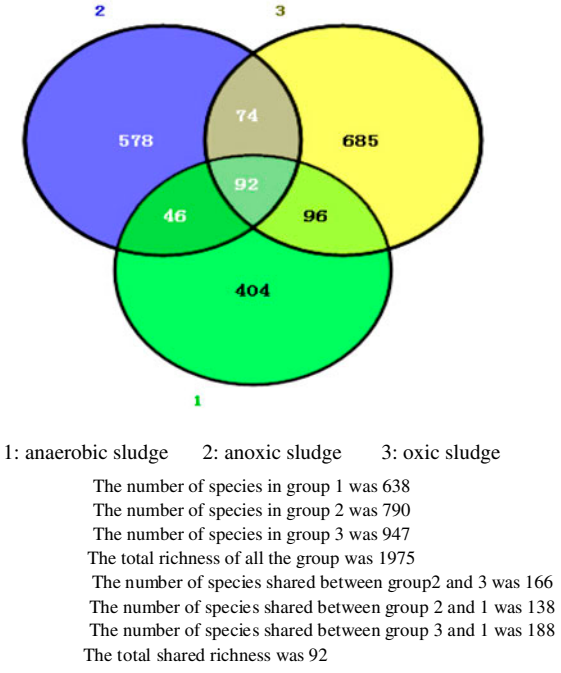

Fig. 1. Venn of the bacterial communities of three sludge samples.

3.3. Taxonomic composition of the bacterial community

To identify the phylogenetic diversities of bacterial community in three sludge samples, qualified reads were assigned to known phyla, orders, and genera. Figs. 3–5 showed the relative bacterial community composition at the phylum, order, and genus level.

The relative abundances of different phyla in three samples were showed in Fig. 3. From the phylum assignment result, there were in total 10 phyla shared by the three samples, which were *Proteobacteria*, *Chloroflexi*, *Chlorobi*, *Bacteroidetes*, *Synergistetes*, *Planctomycetes*, *Acidobacteria*, *Nitrospira*, *Firmicutes*, and *Actinobacteria*. The result was consistent with the previous report of the community composition of the activated sludge from coking wastewater [29], this was also similar with the study reported by Liao [40]. As shown in Fig. 3, *Proteobacteria* was the highest in relative abundance in three communities, the phylum *Proteobacteria* accounted for 84.64% (1), 62.73% (2), and 83.24% (3) of the total reads, respectively. *Firmicutes* was the secondary phylum in the anaerobic sludge and anoxic sludge, corresponding to the percentage of about 10.13 and 9.55%, this was similar to the analytical result of bacterial communities in active sludge, in which *Proteobacteria* (84.53%) was also the most dominant community, followed by *Firmicutes* (13.24%) [40], while this was different from the report, *Firmicutes* accounted for 92.3% of the total sequences in anaerobic sludge [41]. *Bacteroidetes* was 1.02%(1), 5.15%(2), and 4.78%(3) in the three samples, respectively, while *Acidobacteria* was 0.05, 0.04, and 0.46%, respectively, this was different from the report, *Bacteroidetes* and *Actinobacteria* were the most dominant bacteria in the WWTP influent [21]. *Nitrospira*, which was nitrite oxidizing bacterium [42], was the highest in aerobic sludge (0.43%).

Pyrosequencing detected 56 bacterial orders in the three communities and the majority of sequences belonged to 17 orders (Fig. 4). It was found that the most dominant orders in anaerobic sludge, anoxic sludge, and oxic sludge were *Pseudomonadales* (34.17%), *Syntrophobacterales* (8.77%), and *Burkholderiales* (33.19%), respectively. The top six dominant orders in anaerobic sludge were *Pseudomonadales*, *Hydrogenophilales*, *Campylobacterales*, *Clostridiales*, *Burkholderiales*, and *Rhodocyclales*, which was totally different from the dominant population in oxic sludge. It was very interesting to find that *Pseudomonadales*, *Burkholderiales*, *Clostridiales*, *Rhodocyclales*, *Hydrogenophilales*, and *Rhizobiales* were shared by the three sludge samples. *Pseudomonas*, which is described as potential nitrifiers and denitrifiers [18,43], is indole-degrading bacterium [44,45]. *Burkholderiales* and *Rhodocyclales* could be hydrocarbon degrading bacterium, which were also the predominant orders present in the individual metagenomes in the metabolic capacity of hydrocarbon-contaminated groundwater [46], *Burkholderia* is also phenol and pyrene-degrading bacterium [16,17]. The unclassified bacteria at order level increased to 3.59% (1), 38.71% (2), and 29.68% (3) of the total reads.

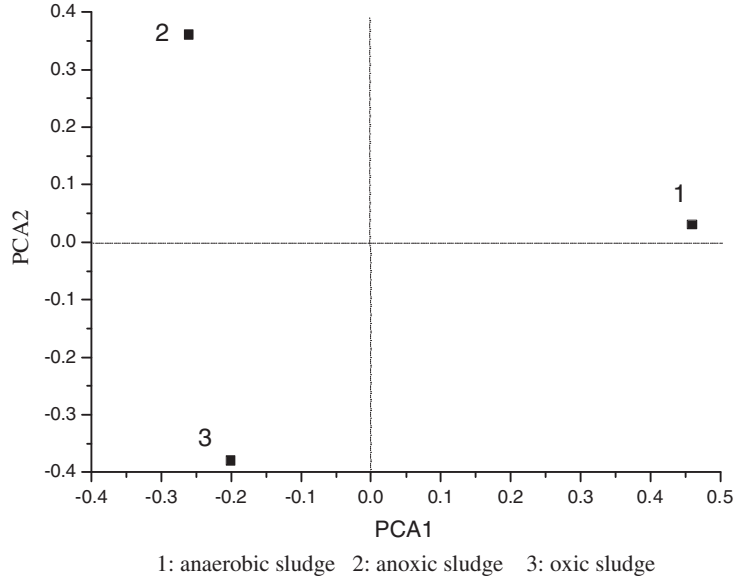

Fig. 2. PCA based on the bacterial communities in the three samples.

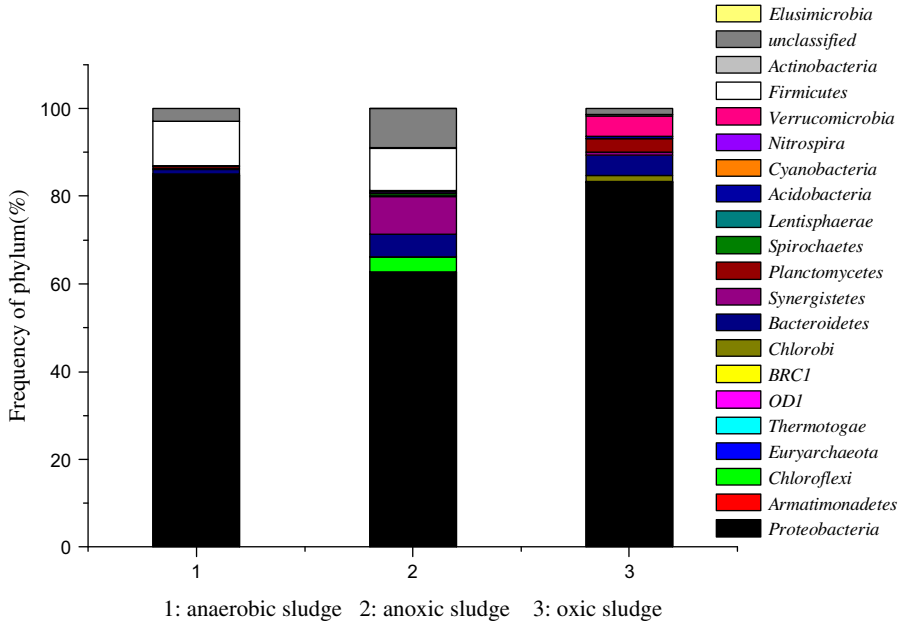

Fig. 3. Proportional distribution of different phyla groups.

According to the comparison of the sequence assignment result at the genus level (Fig. 5), the dominant population in oxic sludge were *Diaphorobacter*, *Thauera*, *Thiobacillus*, and *Roseibacillusa*, this was similar with the study, in which *Thiobacillus* and *Thauera* were the primary genera in the coking wastewater-activated sludge [29], while this was different from the report, in which *Planctomyces*, *Mycobacterium*, *Rhodopirellula*, and *Leptospira* were the dominant genera in the municipal wastewater-activated sludge [13]. Previous studies [47,48] found that *Diaphorobacter* could degrade phenol, pyridine, and pyrene. The predominant *Thauera* was also found in the structure of the quinoline-degrading microbial community [49]. Gunus *Thiobacillus* is responsible for thiocyanate and dimethylsufide biodegradation [29,50]. The most dominant population in anaerobic sludge were *Pseudomonas*, *Petrobacter*, and *Wolinella*, while the most dominant population in the

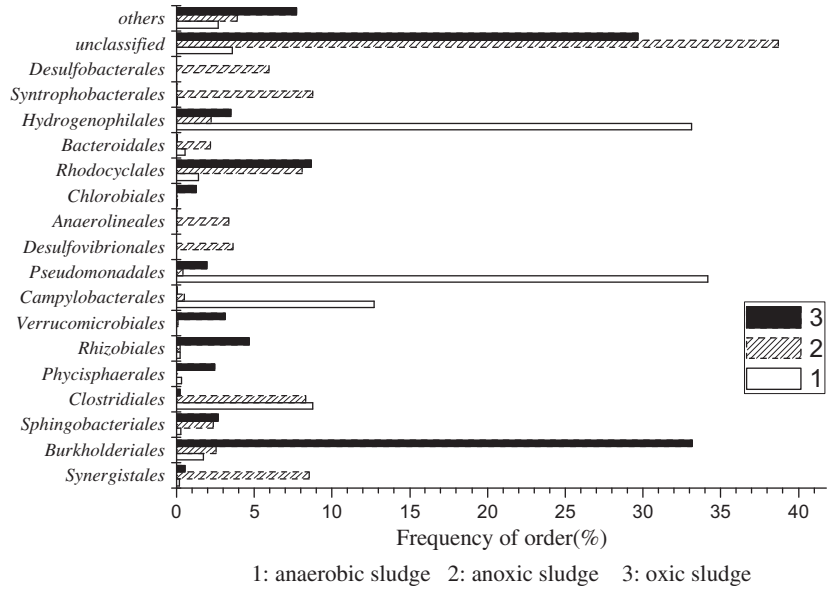

Fig. 4. Bacterial community composition at order level.

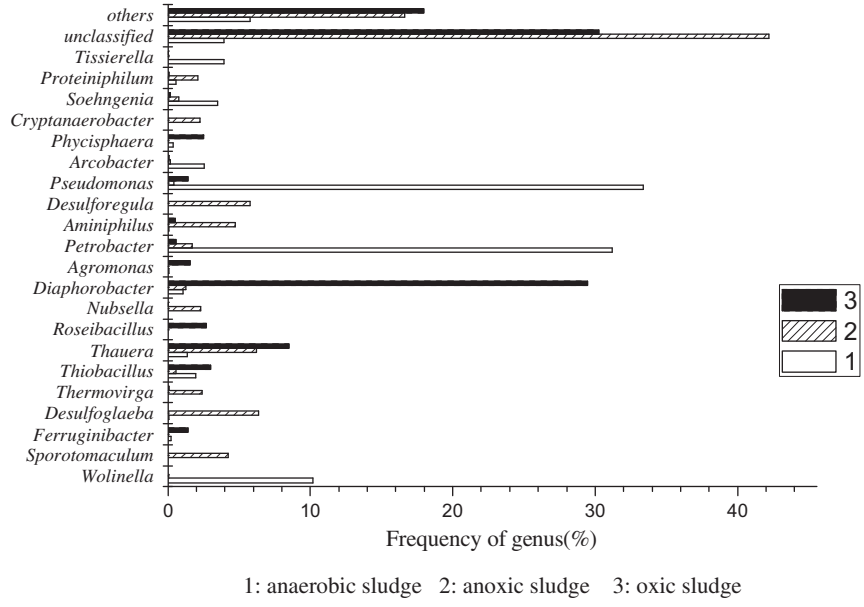

Fig. 5. Genus level distribution of population in three sludge samples.

municipal wastewater digestion sludge were *Kosmotoga*, *Streptococcus*, and *Syntrophus* [13], this difference could be attributed to different wastewater characteristics because wastewater characteristics acted as the key factor in the community assembly process [29]. *Pseudomonas*, which is proved to be capable of degrading phenolic compounds [51], benzene [52], toluene [52], xylene [52], and PAH [53]. There were some genera shared by three sludge samples, including *Sphingobium*, *Brachymonas*, *Ottowia*, *Thermomonas*, *Tistlia*, *Methylocapsa*, *Dongia*, *Sulfurimonas*, *Hyphomicrobium*, *Legionella*, and *Caenimonas* et al. The unclassified bacteria at genus level increased to 3.95% (1), 42.19% (2), and 30.28% (3).

3.4. The role of community composition and correleation with degradation of organics

The main organic pollutants in raw coking wastewater were phenols, indole, quinoline, and pyridine, accounting for 85% of the total organic matters. The degradation of organic compounds (phenols, indole, quinoline, and pyridine) during $A_1/A_2/O$ bioprocess were showed in Table 1. Through $A_1/A_2/O$ bioprocess, those organic pollutants were degraded completely. Different bacteria play their respective special roles in the $A_1/A_2/O$ bioprocess, and pollutants can be degraded completely due to the synergistic effect of the community composition. Microbial community determines the performance of the biological wastewater treatment process, Figs. 3–5 revealed the relative bacterial community composition at the phylum, order, and genus level. Deterministic factors, particularly wastewater characteristics, act as the key factor in the community assembly process. The factors impacting the microbial community should deserve more comprehensive and systematic studies in the future work using pyrosequencing.

4. Conclusions

Phenols, indole, quinoline, and pyridine, accounting for 61.70, 13.63, 7.71, and 2.30% in raw coking wastewater, were the main organic pollutions, $A_1/A_2/O$ process played a very important role in the process of the degradation of aromatic compounds in coking wastewater treatment. Microorganisms determines the performance of the biological wastewater treatment process. 454-Pyrosequencing technology sequence was applied to reval the bacterial community, it showed that *Proteobacteria* was the most dominant phylum in three sludge samples, *Pseudomonadales*, *Syntrophobacterales*, and *Burkholderiales* were the most abundant taxonomic orders in anaerobic tank, anoxic tank, and aerobic tank, respectively. The predominant genera were *Pseudomonas*, *Desulfoglaebas*, and *Diaphorobacter* in three sludge samples, respectively.

Supplementary material

The supplementary material for this paper is available online at http://dx.doi.org/10.1080/19443994.2015.1100556.

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