RESEARCH ARTICLE

Diverse and distinct bacterial community involved in a full-scale A/O1/H/O2 combination of bioreactors with simultaneous decarbonation and denitrogenation of coking wastewater

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Abstract

Taking into account difficulties in exhaustive simultaneous decarbonation and denitrogenation in biological treatment of coking wastewater (CWW), a novel full-scale CWW biological treatment sequentially combining anaerobic, aerobic, hydrolytic, and aerobic reactors (A/O1/H/O2) was designed performing excellent removal of carbon-containing pollutants in the bioreactors A and O1, while the nitrogen-containing compounds in the bioreactors H and O2. To provide an effective tool for the CWW treatment monitoring and control, the succession of microbial community in this unique toxic CWW habitat should be established and characterized in detail. The results of 16S rRNA genes revealed *Acidobacteria* dominating in the unique CWW habitat. The dominant groups in bioreactors A and O1 include *Proteobacteria*, *Firmicutes*, and *Acidobacteria*, while *Proteobacteria*, *Acidobacteria*, *Nitrospirae*, and *Planctomycetes* dominate in reactors H and O2. The genera of *Rhodoplanes*, *Bacillus*, and *Leucobacter* are rich in genes responsible for the xenobiotics biodegradation and metabolism pathway. The Mantel test and PCA results showed the microbial communities of A/O1/H/O2 sequence correlating strongly with SRT, and COD load and removal. The co-occurrence network analysis indicated decarbonation and denitrogenation driven by two network modules having the keystone taxa belonging to the *Comamonadaceae* and *Hyphomicrobiaceae* families. The results significantly expanded the knowledge on the diversity, structure, and function of the CWW active sludge differentiating the relationships between bacterial communities and environmental variables in CWW treatment.

Keywords Coking wastewater · Microbiology · 16S rDNA · Functional prediction · Diversity

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Introduction

China producing more than 60% coke in the world faces severe problems with coking wastewater (CWW) generated annually in huge amounts of 3.12×10^8 m^3 (Wei et al. 2019). The wastewater is characterized by complex composition of pollutants making it one of the most toxic industrial effluents causing dramatic problems to the ecosystems and human health (Zhao et al. 2018). The toxicity of CWW inhibits bacterial activity thus reducing the removal efficiency of carbon- and nitrogen-containing pollutants in traditional CWW biological treatment processes.

Coking wastewater treatment presents a multi-billion-dollar industry in the world widely applying active sludge (AS) in various CWW treatment processes removing more than 95% of the pollutants (Zhu et al. 2019). Biological processes comprise the key step in removing carbon- and nitrogen-containing pollutants at high removal efficiency and low operational cost. The current target of engineering design

in CWW treatment is decarbonation and denitrogenation achieved with minimum consumption of dissolved oxygen (DO) in aerobic treatment and with nitrification–denitrification sequence, respectively, for minimizing operational costs. Simultaneous decarbonation and denitrogenation, however, is achieved by rear combinations of anaerobic (A), aerobic (O), and hydrolytic (H) biological processes. For example, A/O, A/O/O, and A/A/O processes show good performance in nitrogen removal, whereas O/H/O and O/A/O provide excellent decarbonation results (Fukushima et al. 2013; Li et al. 2019a; Wei et al. 2021). As a rule, satisfactory denitrogenation is difficult to realize for the lack of available carbon source, making the structure and function of CWW microbial community unstable (Zheng et al. 2019). This task becomes particularly hard in respect to the nitrogen hidden in toxic substances—thiocyanates (SCN^-) and cyanides (CN^-)—present in CWW in high concentrations: this nitrogen has to be transferred to ammonium (NH_4^+-N) before nitrification and denitrification processes. In order to simultaneously remove carbon- and nitrogen-containing pollutants with high efficiency, the A/O1/H/O2 sequence of biological processes was designed showing good simultaneous decarbonation and denitrogenation at stable operation for the last 7 years at the discharge quality meeting the requirements of the national standard (GB16171-2012). The idea of the $A/O_1/H/O_2$ design was a combination of decarbonation achieved in the A/O1 bioreactors with subsequent nitrogen removal with high efficiency in the H/O_2 sequence. Compared to other CWW treatment processes, the performance of A/O1/H/O2 demonstrated significant advantages in total organic carbon (TOC) and total nitrogen (TN) removal (Qin et al. 2022). The microbial community of the A/O1/H/O2 sequence, however, is not clearly known in diversity and composition of biota, the knowledge of which may provide theoretical justification of the A/O1/H/O2 combination performance.

The functionality and efficiency of CWW treatment are largely determined by the composition of microbial communities (Joshi et al. 2017). Thus, a thorough knowledge of the ecology of the CWW active sludge communities is required to improve the feasibility and stability of a biological treatment system. The advent of high-throughput sequencing technology provides new opportunities addressing the knowledge gaps by comprehensive characterization of the microbial communities in their relationships under the pressure of environmental variables. The authors failed to find surveys on microbial diversity in communities with their variations for the A/O1/H/O2 full-scale CWW treatment systems in relation to their performance in decarbonation and denitrogenation.

The structure and function of the full-scale A/O1/H/O2 microbial communities were studied using the 16S rRNA genes approach. The physicochemical characteristics of

CWW measured by standard methods together with operational parameters were registered during the long-term stable operation matched with the analysis of microbial communities. Illumina MiSeq high-throughput sequencing was used to reveal the distinct species and the diversity of microbial community in the A, O1, H, and O2 bioreactors. An exploratory bioinformatics tool, phylogenetic investigation of communities by reconstruction of unobserved states (PICRUSt), was used to predict the CWW active sludge community function based on 16S rRNA genes information. The objectives pursued by this study include (1) establishing the variations in the abundance, diversity, and function of microbial communities in the A/O1/H/O2 bioreactors; (2) identification of the dominant bacterial groups and presumption of the biodegradation potential in the A/O1/H/O2 process combination in simultaneous decarbonation and denitrogenation; (3) elucidating the relationships between the bacterial communities and the impact of environmental conditions on the bioreactors. The results of the study are applicable to monitoring of the CWW active sludge's biodegradation potential and its treatment efficiency.

Materials and methods

Full-scale A/O1/H/O2 sequence of CWW treatment: collection of samples

An industrial-scale CWW treatment in A/O1/H/O2 sequence exploiting the three-phase fluidized bed reactors providing simultaneous decarbonation and denitrogenation at the Baowu Group Guangdong Shaoguan Iron and Steel Co., Shaoguan city, Guangdong Province, China, was studied. The raw CWW was treated in three stages including the primary stage, the biological stage, and the advanced stage (Fig. 1). The biological treatment consists of four sequential fluidized bed bioreactors: anaerobic (A), aerobic-1 (O1), hydrolytic (H), and aerobic-2 (O2). The biological reactors sequence operates at daily capacity of approximately 2800 m^3 of CWW, providing stable performance at a satisfactory result with the total hydraulic retention time (HRT) of 76 h.

The active sludge samples were collected in June, 2019, on daily basis from separate biological reactors three times a day with 8-h intervals. Samples taken during the day were mixed together to form an average sample of the day. Mixed liquor was also sampled in triplicate from separate bioreactors. The samples were kept in ice boxes during the sampling and transportation to the laboratory. Aliquots of 2–3 mL of each sample were centrifuged at 12,000 g for 10 min at 4 °C. The cell pellets were rinsed twice with 120 mM sodium phosphate buffer at pH 8.0 and stored at − 20 °C prior to the DNA extraction.
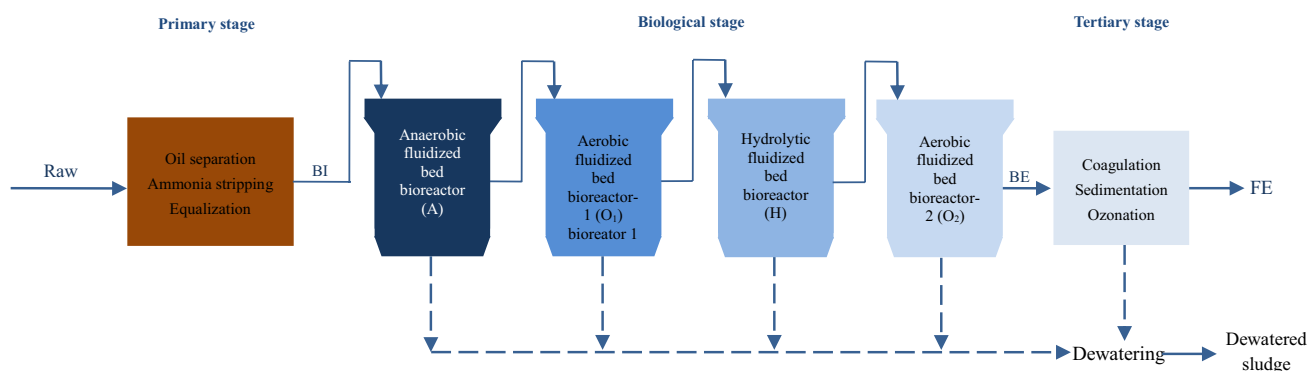
Fig. 1 Flow outline of the full-scale A/O$_1$/H/O$_2$ coking wastewater treatment combination: BI, biological influent; BE, biological effluent; FE, final effluent

Physicochemical properties and operational parameters

The collected CWW samples were centrifuged at 3500 g for 3 min and the supernatants were analyzed using standard methods for the chemical oxidation demand (COD) and biochemical oxygen demand (BOD). The contents of phenolic compounds, sulfides (S^{2-}), SCN$^-$, and total phosphorus (TP) were analyzed by the colorimetric methods with a spectrophotometer (Genesys TM-5, Spectronic Inc. USA). Total nitrogen (TN), ammonium (NH$_4^+$-N), nitrate (NO$_3^-$), and nitrite (NO$_2^-$) were measured using their respective standard methods (Chinese SEPA 2002). After distillation, the CN$^-$ concentration was determined by the pyridine-pyrazolone method. The contents of PAHs were analyzed using the 7890A-5975C GC/MS (Agilent, USA). Total organic carbon (TOC) was measured using a TOC analyzer Shimadzu TOC-VCPH (Japan). The DO concentration was measured using the CellOX 3310i DO meter (WTW, Germany), and the pH was monitored with a pH meter (PHS-3D, China).

The design of bioreactor is known to directly influence the pollutants removal efficiency and the distribution of microbial population species. In order to deal with a high COD load rate (1.88 kg COD m^{-3} day^{-1}), an internal-loop multiphase airlift–fluidized bed reactor was developed by the authors and applied in the full-scale reactors under consideration (Wei et al. 2000). The temperatures, pH, DO, hydraulic retention times (HRTs), and sludge retention times (SRTs), at which the reactors are operating, are given in Table 1. Long-SRTs are beneficial for the development of specific functional microorganism species, such as ammonium-oxidizing bacteria (AOB) and nitrite-oxidizing bacteria (NOB). The concentrations of active sludge varied across the range of 2620 to 4,490 mg L^{-1} over the course of operation. Sludge retention times are controlled by the removal rate of excess sludge, resulting in different mixed liquor suspended solids (MLSS) concentrations in the biological treatment processes.

DNA extraction and MiSeq sequencing of 16S rRNA genes

Microbial genomic DNA extraction was conducted using the PowerSoil™ DNA isolation kit (Mobio, USA) according to the manufacturer's protocol. Concentration and purity of extracted DNA was determined by the ratios of 260/280 and 260/230 nm absorption measured by the ND-2000 spectrophotometer (Nanodrop Inc. Wilmington, DE, USA).

Table 1 Operational parameters of the full-scale coking wastewater A/O1/H/O2 treatment system

Bioreactor unit	T,°C	pH	DO, mg L^{-1}	HRT, h	SRT, d	MLSS, mg L^{-1}	Volume, m^3	COD load rate, kg COD m^{-3}d^{-1}	COD removal rate, kg COD m^{-3}d^{-1}
A	38 (36–39)	7.9 (7.8–8.1)	0.26 (0.1–0.3)	28±8	180±19	3420±160	2800	1.88	0.34
O$_1$	35 (34–36)	7.6 (7.4–7.9)	1.36 (1.0–2.0)	12±3	10±5	4210±280	2400	1.90	1.26
H	32 (32–33)	7.5 (7.3–7.6)	0.46 (0.3–0.8)	20±4	100±14	3320±150	2600	0.64	0.21
O$_2$	30 (30–32)	7.1 (6.9–7.2)	3.08 (2.5–3.5)	16±5	45±5	2730±110	2500	0.45	0.31

T, temperature; *DO*, dissolved oxygen; *HRT*, hydraulic retention time; *SRT*, sludge retention time; *MLSS*, mixed liquor suspended solids

The V4 hyper variable region of 16S rRNA gene was PCR-amplified (triplicate reactions for each sample) using primers F515 (5′-GTGCCAGCMGCCGCGGTAA-3′) and R806 (5′-GGACTACVSGGGTATCTAAT-3′) (Fierer et al. 2008). A single composite DNA sample for MiSeq sequencing was prepared by combining approximately equimolar amount of purified PCR products from each sludge sample. The composition of the PCR products of V4 region of 16S rRNA genes was determined by Illumina MiSeq platform (Illumina Inc., San Diego, CA, USA). The sequences have been deposited in the NCBI Sequence Read Archive under the accession number PRJNA615645.

MiSeq sequence analysis

After MiSeq sequencing, the raw data were processed and analyzed following the pipelines of Mothur (v1.35.1) and QIIME (v. 1.9.1). Raw data were denoised using the Mothur implementation of PyroNoise algorithm. Operational taxonomic units (OTUs) defined at the 97% sequence similarity level were picked using average neighbor method after the Needleman alignment and a single-linkage pre-cluster procedure. Taxonomic assignment was obtained using RDP Classifier at the 80% confidence threshold by default. Based on the OTU information, alpha-diversity indices, including the Shannon, Simpson, Chao 1, ACE, and number of OTUs index, were calculated using R (v 3.6.2) (http://www.r-project.org/). Good's coverage estimators were calculated using the Mothur software (v 1.35.1) and rarefaction curve was analyzed by R (v 3.6.2). The PcoA was used to show the beta-diversity, which explores differences in the microbial communities between the bioreactors A, O_1, H, and O_2 by R software (v 3.6.2).

To investigate the functional profiles of the microbial community dataset, a bioinformatics tool PICRUSt predicting gene family abundances was used based on 16S rRNA gene surveys, given a database of phylogenetically referenced genomes (Langille et al., 2013). For the analysis, OTUs were closed-reference picked against the May 18, 2012 Greengenes database using QIIME (v. 1.9.1) according to the online protocol. The resulting dataset was rarefied at 10,570 sequences per sample. These data were used to assess the difference between gene functions of the sequential A/O1/H/O2 active sludge.

Network analysis of microbial communities

The co-occurrence networks are used to statistically identify keystone taxa (Berry and Widder 2014). Microbial association networks were constructed to investigate the possible biotic interactions using the Molecular Ecological Network Analyses Pipeline (MENAP) (http://ieg4.rccc.ou.edu/mena) (Deng et al. 2012). The OTUs with the relative abundance exceeding 0.3% in the samples were used for network construction avoiding poorly represented OTUs and reducing network complexity. Network was visualized using Gephi (http://gephi.github.io/), and the topological property of betweenness centrality in the networks was also determined by Gephi. Local property measures degree and betweenness centrality of microbe can efficiently identify the key species in a microbial association network. In this study, positive edges correlation implied a mutualistic interaction, whereas negative edges correlation indicated competition.

Statistical analysis

The relationships between microbial community and coking wastewater characteristics/operational parameters were examined by Mantel tests analysis and principal component analysis (PCA) using R 3.6.2 (http:// www.r-project.org/) with the vegan packages. The redundancy analysis (RDA) was carried out using R (v 3.6.2) to discern possible associations among the major genera and variable wastewater characteristics. The ANOVA was performed to assess the significance of relative abundance of phyla, genera, and genes in bioreactors, having a, b, c, and d indicating significant differences at the $p < 0.05$ level.

Results and discussion

Performance of the A/O1/H/O2 sequence

Functional operation performance of the full-scale CWW treatment A/O1/H/O2 sequence was studied in variation of fifteen CWW indicators including COD, BOD_5, concentrations of phenolic compounds, CN^-, SCN^-, ammonia, NO_2^-, NO_3^-, TN, total phosphorus (TP), total suspended solids (TSS), TOC, sulfides, polyaromatic hydrocarbons (PAHs), and oil. Table 2 shows the CWW quality indices inside the bioreactors comprising the A/O1/H/O2 sequence illustrating its functional stability. The COD removal in the reactors sequence comprised approximately 97%. The ratio of BOD_5/COD (B/C ratio) used as a key parameter in wastewater biodegradability evaluation is also a measure of available energy and toxicity to microorganisms in active sludge. The ratio is hypothesized being responsible for the composition of the active sludge microbial community (Zhang et al. 2019a). The B/C ratios in A/O1/H/O2 sequence comprised 0.42, 0.51, 0.10, 0.27, 0.08, and 0.10 for the influent to the biological treatment, the bioreactors A to O2, and the effluent from the biological treatment, respectively, showing the majority of biodegradable organics removed in the A and O1 bioreactors (Table 2). Noteworthy, the B/C ratio increased in the bioreactor H due to the hydrolytic acidification transferring the refractory substances to the biodegradable ones. The removal of phenolic compounds reached 99.99% in the final

Table 2 Characteristics of coking wastewater treated at the full-scale A/O1/H/O2 biological reactors sequence

Wastewater parameter	Primary treatment effluent	A	O_1	H	O_2	Effluent
COD, mg O_2 L^{-1}	1738 ± 94	1550 ± 96	443 ± 58	418 ± 48	194 ± 8	42 ± 5
BOD_5, mg O_2 L^{-1}	728 ± 88	794 ± 69	46 ± 12	114 ± 12	15 ± 6	4 ± 2
Phenolic compounds, mg L^{-1}	598..0 ± 68.0	419.0 ± 21.0	2.1 ± 0.8	0.8 ± 0.2	0.2 ± 0.1	0.1 ± 0.1
CN^-, mg L^{-1}	25.6 ± 3.1	15.9 ± 2.5	1.3 ± 0.3	0.7 ± 0.1	0.3 ± 0.1	0.1 ± 0.1
SCN^-, mg L^{-1}	260..0 ± 31.0	215.0 ± 33.0	17.5 ± 2.1	5.2 ± 0.6	0.3 ± 0.1	0.1 ± 0.1
TN, mg L^{-1}	256 ± 31	201 ± 18	164 ± 9	41 ± 7	30 ± 6	28 ± 4
NH_4^+-N, mg L^{-1}	153 ± 14	165 ± 16	83 ± 4	12 ± 2	2.6 ± 1.2	2.6 ± 1.2
NO_2^-, mg L^{-1}	8.0 ± 4.0	2.6 ± 0.5	26.3 ± 3.4	5.0 ± 2.5	1.2 ± 0.3	0.1 ± 0.1
NO_3^-, mg L^{-1}	5.0 ± 3.0	7.0 ± 3.0	4.5 ± 3.8	3.2 ± 0.5	24.0 ± 3.8	26.1 ± 2.5
Sulfides, mg L^{-1}	260.0 ± 31.0	165.0 ± 5.0	2.0 ± 0.5	3.2 ± 0.6	0.1 ± 0.1	0.1 ± 0.1
TOC, mg C L^{-1}	1012 ± 89	924 ± 72	136 ± 32	118 ± 20	67 ± 4	31 ± 4
TSS, mg L^{-1}	195 ± 18	179 ± 16	98 ± 11	178 ± 15	36 ± 4	25 ± 3
TP, mg L^{-1}	7.5 ± 0.5	4.5 ± 0.4	3.4 ± 0.3	4.3 ± 0.4	1.9 ± 0.2	0.2 ± 0.1
Oil, mg L^{-1}	178.0 ± 20.0	127.0 ± 17.0	5.9 ± 0.8	4.3 ± 0.5	2.1 ± 0.3	0.3 ± 0.1
PAHs, µg L^{-1}	287 ± 31	214 ± 21	163 ± 16	58 ± 11	37 ± 6	8 ± 4

effluent with the major eliminations of 29.9% and 69.7% in the bioreactors A and O1, respectively. The average removals of SCN^- and CN^- were also excellent reaching 99.96% and 99.53%, respectively. Most of the CWW quality indices were improved for more than 70% in the bioreactors A and O1. Interestingly, in the bioreactor A, the concentrations of NO_3^- and NH_4^+-N both increased from 5 to 7 mg L^{-1} and from 153 to 165 mg L^{-1}, respectively, implying that nitrogen-containing heterocyclic compounds, being partly decomposed, may release nitrogen in the NH_4^+-N form. At the same time, the concentration of NO_2^- in the bioreactor O1 increased for an order of magnitude from 2.6 to 26.3 mg L^{-1}, while NH_4^+-N decreased twofold from 165 to 83 mg L^{-1} demonstrating the performance of ammoxidation at this stage. In the bioreactor H, the concentrations of TN, NH_4^+-N, and NO_2^- dramatically dropped showing a good nitrogen removal (Table 2). Due to the hydrolytic acidification in the H-unit, the subsequent bioreactor O2 shows the removal of small-molecular pollutants: the content of NO_3^- increased substantially implying the complete nitrification taking place in the unit. In a word, most of the carbon-containing pollutants are removed in the bioreactors A and O1, while the denitrogenation takes place in H and O2. The results showed that the physicochemical characteristics describing the pollutants removal reflect and determine biological properties of active sludge in the reactors, determining the CWW treatment performance.

Analysis of phylogenetic diversity

High-throughput MiSeq sequencing was used to analyze the bacterial and archaeal 16S rRNA genes across the 12 active sludge samples from four bioreactors of the A/O1/H/O2 system. A total of 209,522 effective sequences and 1887 OTUs were retrieved from the samples. Based on the subset of 10,570 randomly selected sequences, the average of 688, 368, 654, and 655 OTUs were identified in the sludge samples taken from A, O1, H, and O2 bioreactors, respectively (Table 3). The OTU numbers detected in the sludge of the A/O1/H/O2 reactors are smaller than those observed at municipal WWTPs (Wu et al. 2019) as was shown earlier in the treatment of CWW and other industrial wastewaters (Zheng et al. 2019; Zhu et al. 2019). The rarefaction curves reached the plateau and the Good's coverage ranged from 97.06 to 99.02% in all samples (Table 3), indicating that the sampling depth was sufficient to characterize the majority of microbial community.

The diversity indices, Shannon or Simpson index, showed that the biodiversity increased in the bioreactors in the sequence of O1, H, and O2. The first in the operation sequence bioreactor A, however, exhibited the highest bacterial diversity among all the treatment stages (Table 3). The Chao1 and ACE indices were used to estimate the number of OTUs present in communities; both indices are positively related to the community richness. Considering the indices of Chao 1, ACE, and the number of OTUs, one can see that the lowest and the highest community richness numbers were shown by O1 and A reactors, respectively. In general, the CWW active sludge samples demonstrate moderate biodiversity, which is more complicated than those of extreme habitats, e.g., hydrothermal and acid mine drainage (Wang et al. 2013; Zhang et al. 2019b), but simpler than soil (Nottingham et al. 2018; Sun et al. 2019). Heatmap of distance matrix and principle co-ordinates analysis (PcoA) were

Table 3 Number of quality sequences, OTUs at 0.03 cutoff, richness estimates, and diversity indices of the microbial communities in the full-scale A/O1/H/O2 coking wastewater treatment system

Sample ID	Number of quality reads	Number of OTUs	Good's coverage (%)	Chao 1	ACE	Shannon (H')	Simpson (1-D)
A-S1	12,037	708	97.51	1058.39	1131.37	5.74	0.92
A-S2	10,874	676	97.06	1140.00	1204.77	5.57	0.90
A-S3	12,109	679	97.42	1132.42	1159.44	5.47	0.89
O1-S1	12,547	346	98.67	598.02	595.14	2.97	0.64
O1-S2	13,825	355	98.71	611.95	672.22	2.94	0.63
O1-S3	19,016	402	99.02	661.57	674.89	2.60	0.56
H-S1	25,057	696	98.75	1071.12	1190.28	3.81	0.75
H-S2	27,374	698	98.86	1124.59	1168.9	3.76	0.73
H-S3	16,101	567	98.29	1032.12	1021.59	4.11	0.81
O2-S1	24,891	724	98.70	1201.09	1211.6	4.46	0.84
O2-S2	16,991	607	98.28	1245.48	1071.28	4.41	0.82
O2-S3	18,700	634	98.49	1014.03	1063.76	4.38	0.82
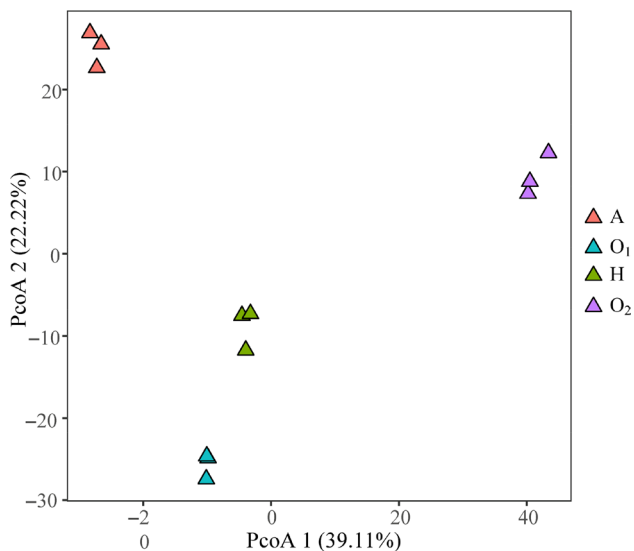
Fig. 2 Beta-diversity PcoA based on the OTUs abundance in active sludge samples

calculated to compare beta-diversity among the CWW bioreactor samples. The results of PcoA showed the beta-diversities of microbial communities in the bioreactors being at far distances from each other (Fig. 2), which implied the bioreactors harboring unique microbial populations. The microbial diversity in the CWW treatment reactors showed distinct community compositions highly coherent with the wastewater characteristics and environmental variables (Joshi et al. 2018).

Taxonomic composition of microbial communities

The Ribosomal Database Project (RDP) Classifier was used to assign the effective sequences to different OTUs and phylogenetic taxa with 3% of nucleotide cutoff. Almost all the classified sequences (99.73–100%) in the A/O1/H/O2 treatment were assigned to bacteria, while only $\leq 0.27\%$ sequences were identified as archaea in anaerobic A and hydrolytic H bioreactors (Table S1). The DO is kept at low levels in bioreactors A (0.1–0.3 mg L^{-1}) and H (0.3–0.8 mg L^{-1}) on the course of their long-term operation making the detection of minor anaerobic archaea in these facultative anaerobic bioreactors reasonable (Zhu et al. 2016). The relative abundance of the microbial communities was summarized at the phylum and genera levels for each sample in Tables S1, S2 and S3. A total of 40 bacterial phyla, including 6 subphyla of *Proteobacteria*, and 148 classified genera were identified in the A/O1/H/O2 sequence. The identified genera retrieved from the bioreactors appeared being dramatically different from each other with a long tail of low-abundance genera (Fig. 3c and S1).

In the anaerobic bioreactor A, the predominant phylum *Proteobacteria* was quantified including subphyla *β-Proteobacteria* and α-*Proteobacteria* in amount of 38.32% and 8.68%, respectively. *Firmicutes*, the subdominant phylum with its relative abundance of 19.34% is consistent with the results of previous study identifying *Firmicutes* as the dominant group in the first stage of anaerobic treatment of CWW (Zhu et al. 2016). *Acidobacteria* and *Bacteroidetes* follow the dominant phyla with their 7.28% and 7.11% relative abundance, respectively (Fig. 3a). Most identified OTUs of *β-Proteobacteria* and *Acidobacteria* showed positive associations with COD removal from wastewaters by means of RDA and network analysis (Jia et al. 2019). Yang et al. (2015) reported the major role of anaerobic *Bacteroidetes* in fermentation systems breaking down macromolecules of proteins, starch, and cellulose. At genus level, *Bacillus* (6.01%) was the predominant genus being able to degrade diesel oil possessing inherent potential of biosurfactant production concomitant with hydrocarbon degradation (Parthipan et al. 2017). Besides, *Bacillus* exhibits also high ammonification
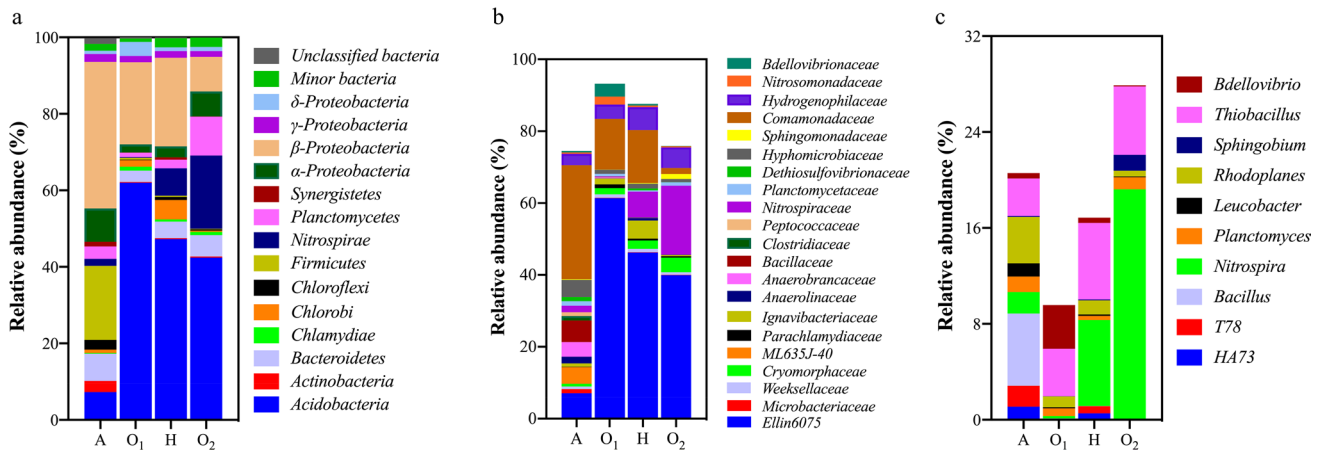
Fig. 3 Relative abundance (%) of major phyla, genera, and families in the bioreactors: the relative abundance > 1% in at least one sample defined the phyla, genera or families as major; taxa accounted for < 1% were defined as minor phyla; the average of three parallels represent the value of the corresponding sample); refer to Fig. 1 for abbreviations; **a** phylum (Proteobacterial subphyla); **b** family; **c** genus

activity in the N-cycle process (Huang et al. 2019). *Rhodoplanes* (3.87%), *Thiobacillus* (3.15%), *Nitrospira* (1.79%), and *T78* (1.75%) comprised the subdominant groups. *Thiobacillus* is able to degrade toxic pollutants such as CN⁻ and SCN⁻ in the first anaerobic reactor of CWW treatment (Joshi et al. 2017; Zhou et al. 2017). Additionally, the genus of *T78*, prosperous in the first anaerobic bioreactor at its relative abundance of 1.75%, is capable of anaerobic degradation of benzene compounds and oil hydrocarbons (Xu et al. 2018).

In the aerobic bioreactor O1, *Acidobacteria*-related sequences were the predominant phylum while over 60% of the sequences were assigned to the *Ellin6075* family (Fig. 3b). *Acidobacteria*, particularly *Gp4* and *Gp6*, was frequently detected in active sludge being the core genera shared by dozens of wastewater treatment systems analyzed by pyrosequencing (Ma et al. 2015). Surprisingly, *Acidobacteria* was found thriving in the O1 stage with its relative abundance of 62.02%. Previous study showed that the fluctuations of *Acidobactera*, the slowly growing species strongly associated with the biochar mineral complex and sludge, are related to the removal activity of NH_4^+-N (Jia et al. 2019). *Proteobacteria* was the subdominant group with 28.94% abundance. Especially, the relative abundance of *β-Proteobacteria*-related sequences also comprise more than one-fifth in O_1 indicating the major function of the aerobic bioreactor as decarbonation. At the genus level, *Thiobacillus* was the predominant genus with relative abundance of 3.96%. *Thiobacillus* is known for its sulfur metabolism and denitrifying ability, making it related to the degradation of S/N-containing pollutants, including SCN⁻, CN⁻, N-heterocyclic carbenes, and PAHs (Joshi et al. 2018). At the same time, interesting finding was made in respect of *Bdellovibrio* genus burst out from 0.45 to 3.64% thus ranked as the second dominant group. *Bdellovibrio*, affiliated with

Deltaproteobacteria, is a major group of predatory bacteria preying on Gram-negative ones (Niu et al. 2016). Therefore, prosperous *Bdellovibrio* inhibits the growth of Gram-negative bacteria, such as, e.g., *Bacillus*. The predatory relations thus reduce the OTU number and the diversity indices in the O_1 active sludge.

In the hydrolytic bioreactor H, 47.28% of the classifiable sequences were also assigned to the predominant phylum *Acidobactera*. *β-Proteobacteria*, *Nitrospirae*, *Chlorobi*, *Bacteroidetes*, *α-Proteobacteria*, and *Planctomycetes* comprised the subdominant groups with the relative abundance values of 23.13%, 7.18%, 5.06%, 4.34%, 2.87%, and 2.22%, respectively. The occurrence of complex community with a large fraction of the *Chlorobi*, *Bacteroidetes*, *Chloroflexi*, and *Proteobacteria* phyla was reported in anammox bioreactors (Lawson et al. 2017). Besides, *Bacteroidetes* are involved in nitrification, including autotrophic metabolism with subsequent nitrite oxidation (Wu et al. 2018). *Chlorobi*-affiliated bacteria are highly active protein degraders, catabolizing extracellular peptides while recycling nitrate to nitrite (Lawson et al. 2017), thus making their rise in the bioreactor H reasonalbe. *β-Proteobacteria* play a key role in denitrification under low toxicity conditions (Zhou et al. 2019), and, being combined with the prosperous *Nitrospirae*, explains why the total nitrogen was removed by denitrification for more than 50% in the bioreactor H. At the genus level, *Nitrospira* dominates with the relative abundance of 7.18%, playing a crucial role in the nitrogen cycle enhancing the ammonia oxidation, thus contributing to the NH_4^+-N removal (Zhang et al. 2020). The subdominant genera groups include *Thiobacillus* and *Rhodoplanes* with their relative abundances of 6.38% and 1.17%, respectively. The previous study reported *Thiobacillus* and *Rhodoplanes* being

the key microorganisms in the FeS-driven denitrification process removing both carbon- and nitrogen-containing compounds (Ma et al. 2019; Zhu et al. 2019). The *Rhodoplanes* genus is also diverse in metabolic capabilities playing an important role in denitrification (Sun et al. 2017).

Finally, in the aerobic bioreactor O2, *Acidobacteria*, *Nitrospirae*, *Planctomyces*, *β-Proteobacteria*, *α-Proteobacteria*, *γ-Proteobacteria*, and *δ-Proteobacteria* comprised the dominant phyla each containing 42.40%, 19.17%, 10.17%, 9.01%, 6.54%, 1.52%, and 1.06% of detections, respectively (Table S1). Noticeably, *Nitrospirae* and *Planctomycetes* boomed in O2 to the highest among the reactors, showing 19.17% and 10.17%, respectively. *Nitrospirae* species may catalyze the second step of

nitrification, while the major function of phylum *Planctomycetes* consists of the removal of nitrogen-containing pollutants (Sorokin et al. 2012). Species of the phylum *Proteobacteria*, including *α-, β-*, and *γ-Proteobacteria*, are capable of autotrophic denitrification (Miao and Liu 2018). Interestingly, the relative abundance of *β-Proteobacteria* decreased steadily from 38.32% in the reactor A to 9.02% in the reactor O2 along the reactors' sequence A/O1/H/O2 (Fig. 3a). The decreasing trend in *β-Proteobacteria* abundance is explained by its function to remove organic compounds and nutrient elements from CWW diminishing along the treatment from stage to stage in the reactors combination with decreasing decarbonation and denitrogenation rates. The genera detected in the bioreactor O2 were

Fig. 4 Microbial metabolic pathways in active sludge samples of A/O$_1$/H/O$_2$ coking wastewater treatment process. a. second-level pathways in the KEGG; b. sub-pathways in xenobiotics degradation pathways
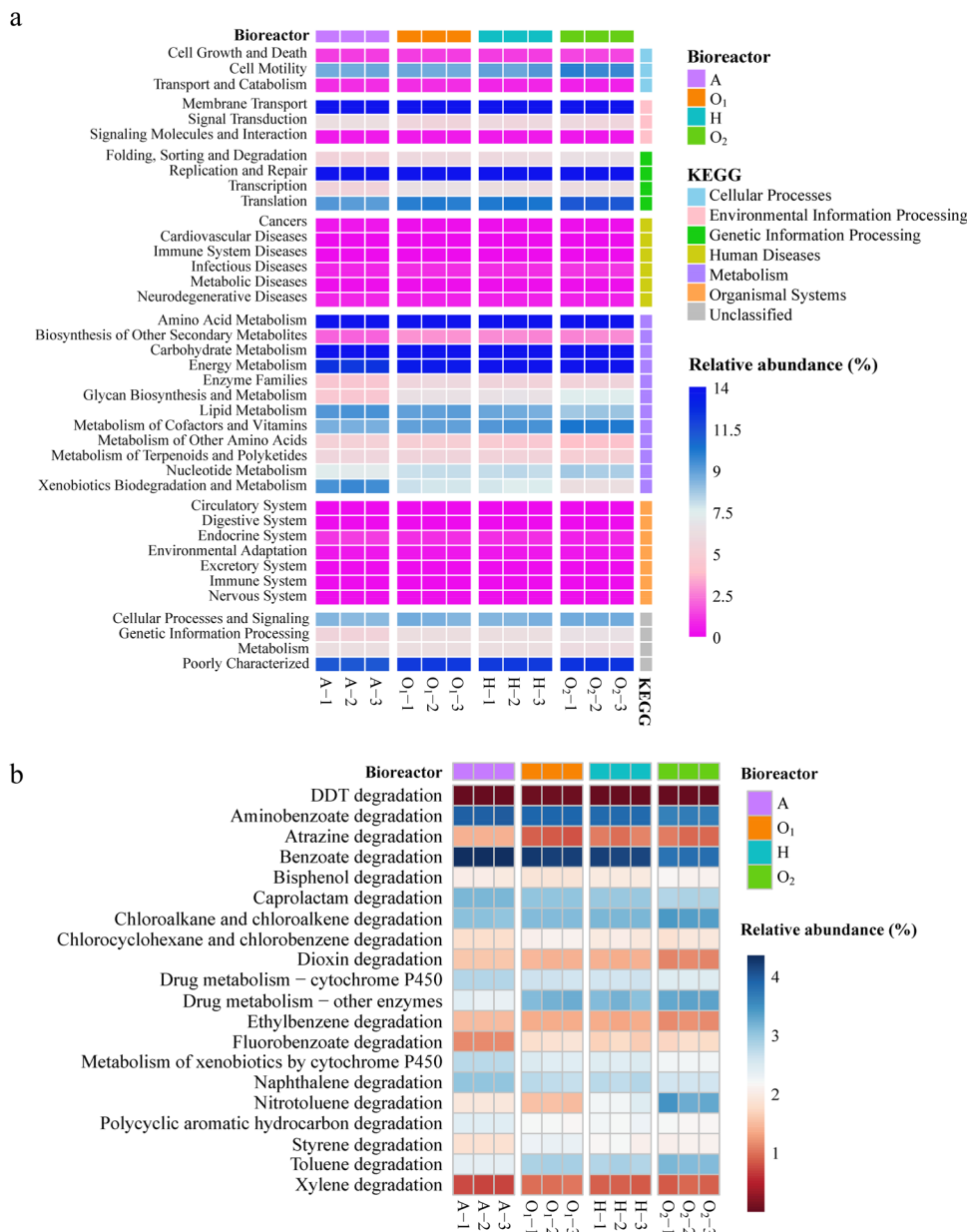
dominated by *Nitrospira* (19.16%), *Thiobacillus* (5.71%), *Sphingobium* (1.32%), and *Planctomyces* (1.01%). The most important finding in the bioreactor O2 consists of the prosperous NOB genus *Nitrospira* accounting for nearly a fifth part of the whole community and finalizing nitrification by oxidation of nitrite to nitrate (Song et al. 2020). Since more than 70% of sequences detected in the CWW active sludge were not classified to specific genera, one can consider the knowledge about the microbial resource in CWW active sludge as a limited one. Summarizing, the microbial composition analysis showed the bioreactors A and O1 responsible for the removal of carbon-containing pollutants, while the nitrogen-containing pollutants were removed mostly in the bioreactors H and O2.

Function profiles involved in removal of carbon- and nitrogen-containing pollutants

Function prediction was proposed to explore the functional pathways related to the removal of carbon- and nitrogen-containing pollutants in the A/O1/H/O2 biological CWW treatment system. The accuracy of metagenome predictions was quantified by means of the Nearest Sequenced Taxon Index (NSTI) having the lower NSTI values indicating a closer mean relationship (Langille et al. 2013). The weighted NSTI scores ranged from 0.14 to 0.25 with the average of 0.19 ± 0.06 providing a suitable data set for examination of PICRUSt predictions. The predicted results in this study provide a specific insight into the bacterial functions in the removal of carbon- and nitrogen-containing pollutants.

In the predicted metagenomes, 41 out of 43 level 2 KEGG Orthology groups (KOs) were represented. The

dominating metabolism pathways providing survival of microorganisms include amino acid ($10.29 \pm 0.41\%$), carbohydrate ($10.07 \pm 0.22\%$), and energy ($6.08 \pm 0.75\%$) metabolisms (Fig. 4a). These three dominating pathways, essential for the growth of microorganisms, imply the pollutant degradation mechanisms in CWW treatment (Qiu et al. 2019). The energy metabolism pathway and the pathway of xenobiotics biodegradation and metabolism accounted for the high proportion of $5.35 - 6.74\%$ and $2.48 - 4.21\%$ in the A/O1/H/O2 process, respectively, contributing to the removal of carbon- and nitrogen-containing pollutants, such as NH_4^+, CN^-, SCN^- and phenols.

Degradation pathways for carbon-containing pollutants are crucial for any wastewater treatment (Sun et al. 2014). In order to unveil those in CWW active sludge, the biodegradation and metabolism pathways of xenobiotics were considered (Fig. 4b). Generally, the xenobiotics biodegradation and metabolism pathway consists of 20 specific metabolic sub-pathways. In this case study, the relative abundance of the degradation pathways of aminobenzoate, benzoate, and caprolactam decrease along the sequential A/O1/H/O2 process (Fig. 4b), implying that these pathways are important in the removal of major carbon-containing pollutants, aromatic compounds, CN^-, and phenols (Deng et al. 2020). These functional pathways are attributed to the previously detected major genera of *Bacillus*, *Rhodoplanes*, *Planctomyces*, and *Leucobacter*. So combined with the microbial community composition results, the major function of the bioreactors A and O1 consists of the removal of carbon-containing pollutants. At the same time, the pathways of drug metabolism—other enzymes, caprolactam, and nitrotoluene degradation intensify in the bioreactors H and O2, showing the pathways being important in the removal of nitrogen-containing

Fig. 5 Relative abundance (%) of nitrogen recycling genes involved in nitrification and denitrification in the A/O1/H/O2 biological treatment system: within each group, bars with different lowercase letters are significantly different from each other ($p < 0.05$, ANOVA)
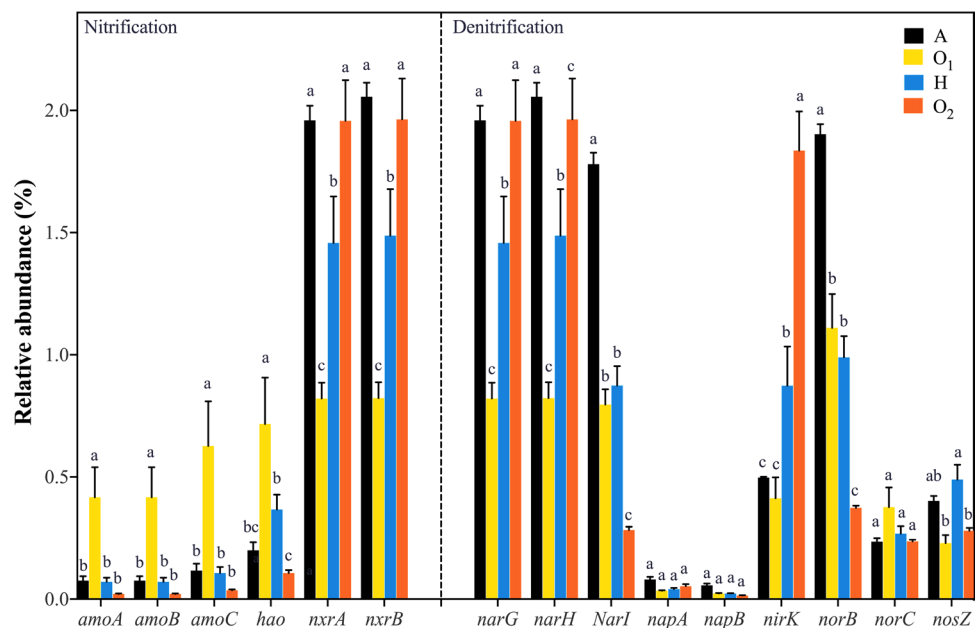
pollutants (Fig. 4b). The predominant genus *Nitrospira* belonging to the *Nitrospirae* phylum is known for nitrification in the removal of nitrogenous pollutants and provides the gene *porB* for the nitrotoluene degradation pathway.

Nitrification and denitrification comprise the major TN removal processes in general, and also in the A/O1/H/O2 biological CWW treatment process combination. The predicted genes in the nitrogen metabolism sub-pathway are also related to the nitrogen recycling in nitrification and denitrification. Six nitrification-related genes, *amoA*, *amoB*, *amoC*, *hao*, *nxrA*, and *nxrB*, and 11 denitrification-related genes, *narG*, *narH*, *narI*, *napA*, *napB*, *nasA*, *nasB*, *nirK*,

norB, *norC*, and *nosZ*, are involved in nitrogen removal in the considered reactors sequence. The nitrification-related genes of the *amoCAB* and *hao* show the highest abundance in O1 (Fig. 5). The ammonium monooxygenase and hydroxylamine oxidoreductase, which convert ammonia to nitrite by ammonium oxidation, are encoded by the *amoCAB* operon (*amoC*, *amoA*, and *amoB*) and *hao* gene, respectively (Mendez-Garcia et al. 2015; Sun et al. 2014). It is well known that NOB oxidize nitrite to nitrate by the NXR enzyme, which is membrane-associated and contains an alpha-subunit (*nxrA*) with the catalytic site, and a beta-subunit (*nxrB*) that channels electrons derived from nitrite
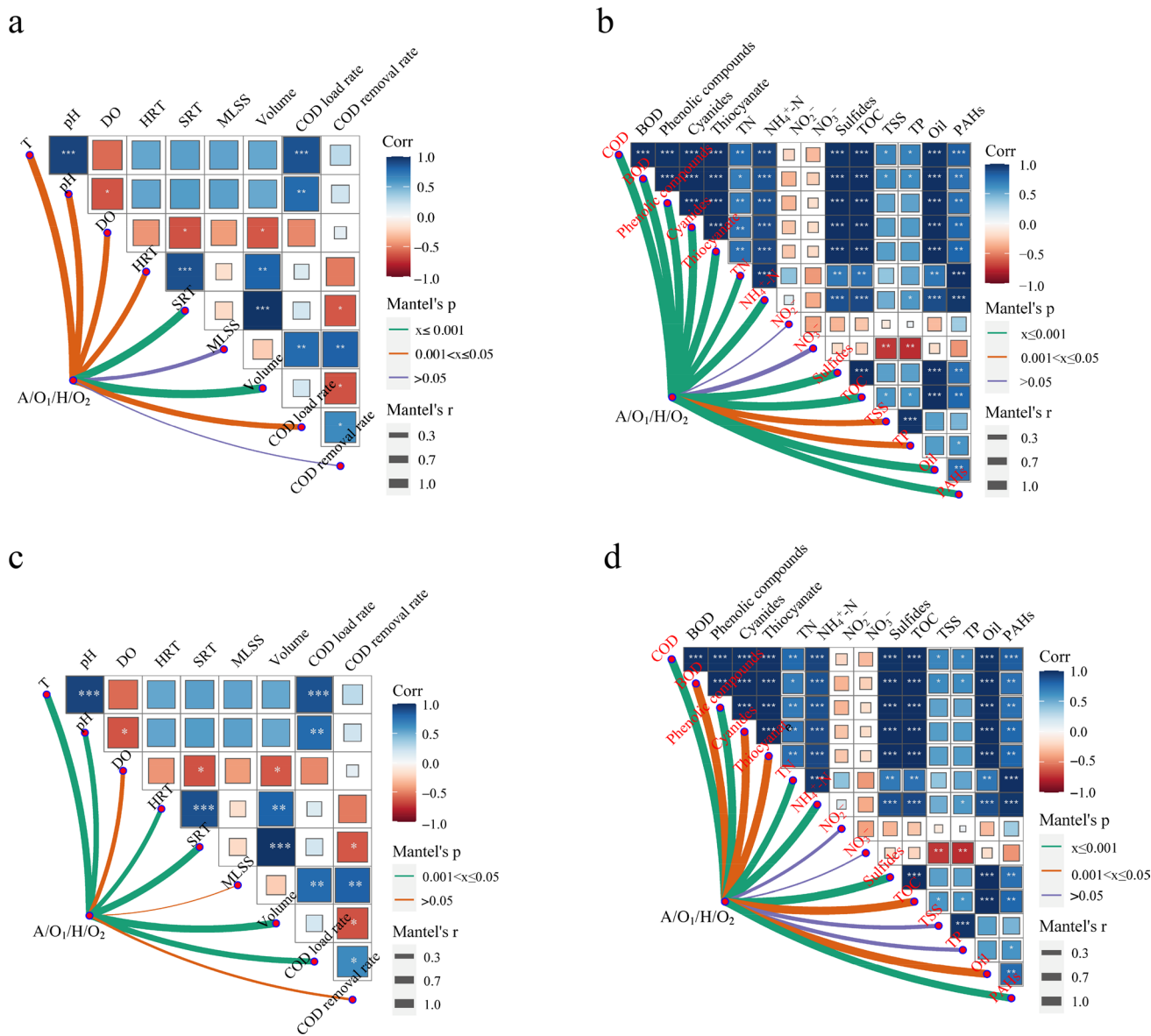

Fig. 6 Mantel test between operational parameters and microbial community (**a**), wastewater characteristics and microbial community (**b**), operational parameters and functional pathways (**c**), and waste-water characteristics and functional pathways (**d**) in the A/O₁/H/O₂ biological treatment process

to downstream components of the respiratory chain (Sorokin et al. 2012). The nitrifying genes *nxrA* and *nxrB* responsible for the oxidation of NO_2^- to NO_3^- demonstrate higher relative abundance in the bioreactors A and O2, as compared to O1 and H (Tang et al. 2020a). This is in a good accordance with the high abundance of *Nitrospira* and *Planctomyces* in the A- and O2-active sludge. Both denitrification and dissimilatory nitrate reduction start with the reduction of NO_3^- to NO_2^- either by means of the periplasmic nitrate reductase *NapAB* or the cytoplasmic nitrate reductase *NarGHI* (Mendez-Garcia et al. 2015; Jia et al. 2019). Subsequently, the resulting NO_2^- is reduced to nitrogen gas by nitrite reductase (*NirK*), NO reductase (*norB* and *norC*), and nitrous oxide reductase (*nosZ*) (Tang et al. 2020a). The denitrification *nirK* gene was prevalent in the bioreactors H and O2, and the relative abundance of *nosZ* gene was the highest in H showing the most nitrogen-containing pollutants removed in the bioreactor H by denitrification.

Relationships between microbial community and the environmental factors

The PCA- and RDA-analyses and the Mantel test were conducted to illustrate the influence of environmental factors on the microbial community composition and functions. Based on the results of Mantel test (Fig. 6), the indices of COD, BOD, TOC, phenolic compounds, CN^-, SCN^-, sulfides, NH_4^+-N, TN, and oil were strongly related with microbial community composition ($p < 0.005$, $r > 0.7$) as the main driving factors in the microbial community succession. The SRT operational parameter also appears to be the major driving factor ($p < 0.001$, $r > 0.7$). The PCA analysis clarified the main impact factors (Fig. S2), showing SRT, phenolic compounds, CN^-, sulfides, and oil influencing the microbial community composition in the bioreactor A. The microbial community in the bioreactor O1 was shaped by the factors of MLSS, COD removal rate, NO_2^-, and TN. In the bioreactor H, the microbial community was shaped by SRT, HRT, and NO_3^-. In addition, NO_3^-, COD, and DO regulated the microbial community composition in bioreactor O2 stronger than in the others. Generally, the microbial communities of the bioreactors A and O1 were mostly affected by the factors relating to carbon-containing pollutants, while in the bioreactors H and O2 the concentration of NO_3^- was the principal factor.

The RDA analysis was conducted to quantify the impacts of the environmental factors and showed the wastewater characteristics determining the microbial community composition at phyla and genera level: the weighted average values for the first two axes comprised 93.88% and 94.22% of the total variance (Fig. 7a and b), respectively. The phylum *Nitrospirae* is in positive correlation with NO_3^-, although negatively related to NO_2^-, SCN^-, TN, and $NH_4^+ - N$

illustrating the ability of *Nitrospirae* species to oxidize NO_2^- to NO_3^- in the second step of nitrification, thus contributing to the metabolism of nitrogen-containing pollutants (Fig. 7a). At the genera level, *Nitrospira* and *Sphingobium* also show similar correlation (Fig. 7b) explained by enhanced ammonia oxidation helping the NH_3-N removal (Tang et al. 2020b). Besides, the RDA results also showed the phylum *Acidobacteria* and the genus *Thiobacillus* being negatively related with TOC, BOD_5, and concentrations of phenolic compounds, sulfides, CN^-, SCN^-, and oil.
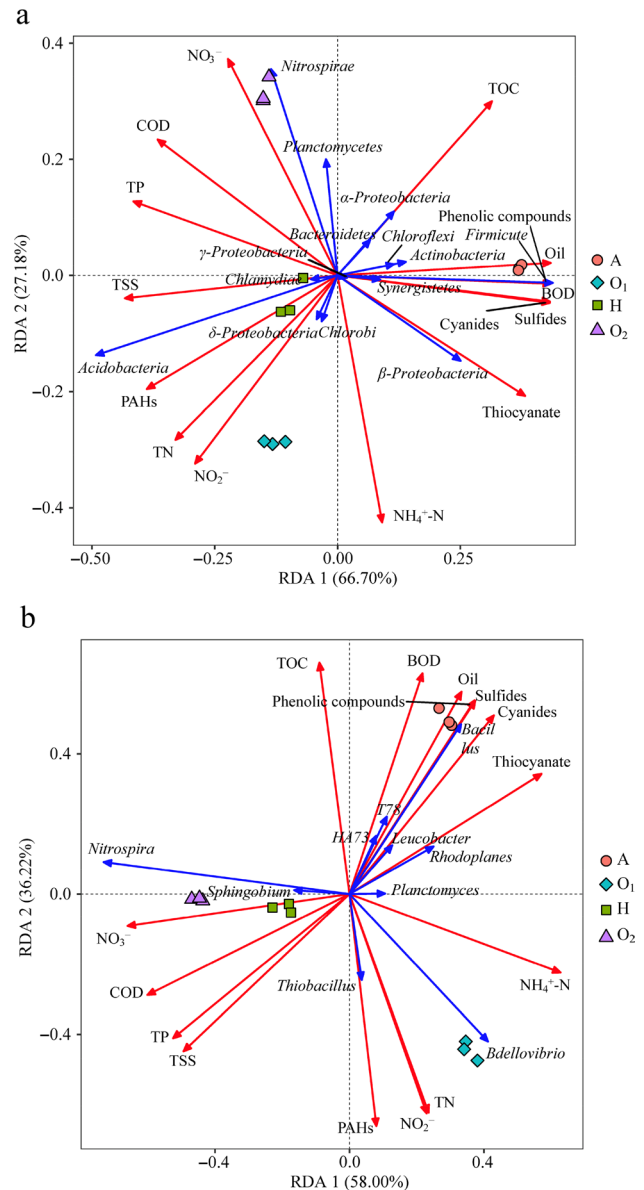

Fig. 7 Redundancy analysis (RDA) of microbial species under the impact of environmental factors in the bioreactors: arrows indicate the direction and magnitude of environmental parameters associated with phylum and genus; the relationships between bacteria at phylum (**a**) and genus (**b**) level and wastewater characteristics
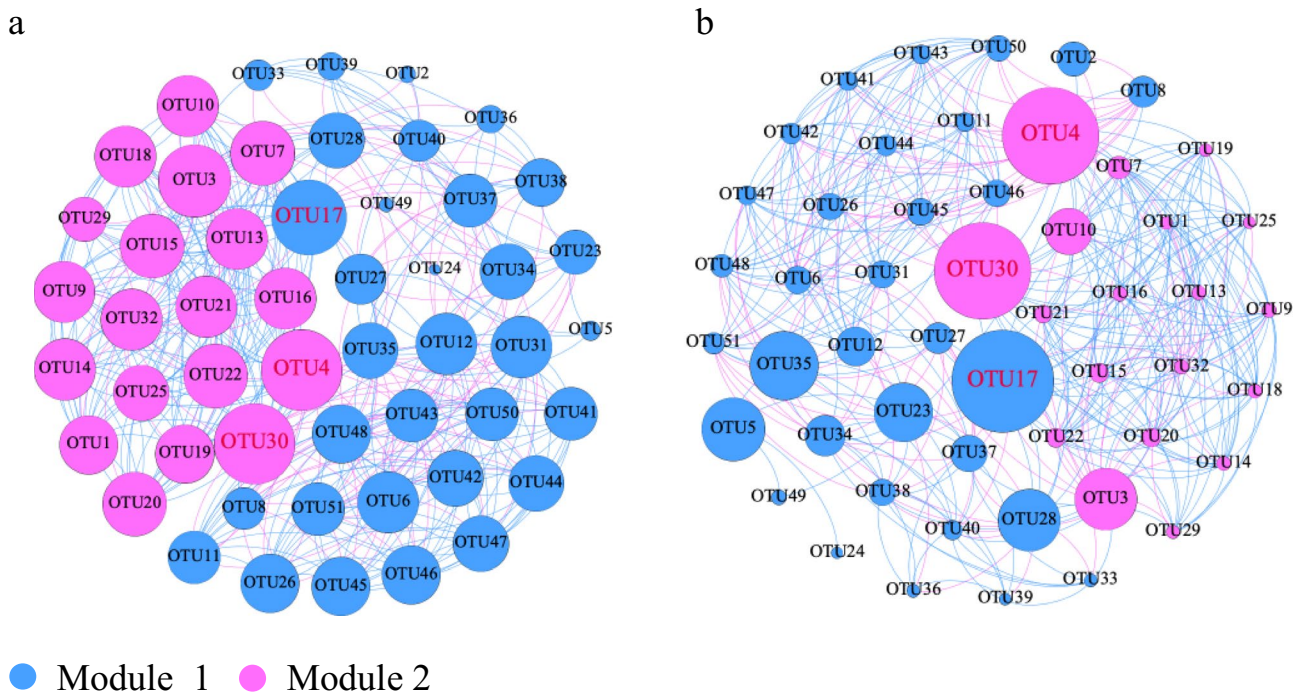
Additionally, the *β-Proteobacteria* and *Firmicutes* together with genera of *Thiobacillus*, *Bdellovibrio*, *HA73*, *T78*, *Leucobacter*, and *Rhodoplanes* showed negative relationships with COD, indicating an important role played in the removal of carbon-containing pollutants. All of the RDA results coincide with the structure and function of the microbial communities in the A/O1/H/O2 combination providing good simultaneous decarbonation and denitrogenation.

Network patterns reveal microbial community characteristics

Interaction of microorganisms is another deterministic factor for diversity and function of microbial community, understanding of which is achieved by identifying the keystone taxa (Zhang et al. 2019a). The association network of the microbial community consists of 51 nodes and 411 edges being separated into two modules, module 1 of positive correlations (62.75%, blue), and module 2 of negative correlations (37.25%, pink) (Fig. 8). The positive and negative edges accounted for 69.83% and 30.17% in the network

analysis, respectively, suggest the microbial community being dominated by symbiotic relationships in the active sludge. Each module in the network considered a subset of species from similar ecological niches and may perform similar functions (Zhang et al. 2019a). The functions of species in the module 1 were mainly related to nitrification (*Nitrosomonadaceae* and *Nitrospira*), denitrification (*Thiobacillus*, *Ignavibacteriacea*, and *Hyphomicrobiaceae*), and anammox (8 OTUs belonging to *Planctomycetes*). Most of the OTUs in module 2 were assigned to the phylum *Proteobacteria* and *Firmicutes* responsible for the removal of carbon-containing pollutants. The modules in the network visualize different niches indicating important ecological processes (Rottjers and Faust 2018).

In this study, the threshold of nodes with degree > 23, betweenness centrality values > 100, and relative abundances > 0.3% of the microbial communities were outlined as keystone taxa (Li et al. 2019b). The keystone taxa of OTU4, OTU30, and OTU17 comprised the network hub in the co-occurrence network of the A/O1/H/O2 system. The OTU17 in the module 1 belongs to the *Hyphomicrobiaceae*

● Module 1 ● Module 2

	Empirical networks								Random networks		
	Similarity threshold (St)	R square of power-law	Avg. connectivity	Avg. geodesic distance	Geodesic efficiency	Connectedness	Maximal eigenvector centrality	Avg. clustering coefficient	Modularity	Avg. geodesic distance ±SD	Avg. clustering coefficient ±SD	Modularity ± SD
A/O₁/H/O₂	0.7	0.210	16.118	1.983	0.613	1	0.226	0.730	0.377	0.650 ± 0.002	0.370 ± 0.014	0.123 ± 0.019

Fig. 8 Network visualizing the OTU-OTU interactions in the A/O₁/H/O₂ reactors combination: positive correlations—blue, negative correlations—pink; nodes are colored for types of modularity classes; size of node is proportional to the degree (**a**) or betweenness centrality (**b**); labels of keystone OTUs are colored with red with amplified fonts

family contributing to the denitrification process. The OTU4 and OTU30 in the module 2 belong to the *Comamonadaceae* family known for its contribution to the organic pollutants removal. The high-degree keystone OTUs control the microorganisms in the module 1 directly by negative reaction, while doing this by positive reaction in the module 2. The keystone taxa control the microbial community by a range of strategies being able to promote or suppress the effector groups by secreting metabolites, antibiotics, or toxins to selectively regulate community structure and functioning (Banerjee et al. 2018). Besides, the higher betweenness centrality of key OTUs indicates their higher control potential exerting over the interactions of other species in the network (Li et al. 2019b).

Conclusions

The microbial communities in the full-scale A/O1/H/O2 bioreactors combination, showing efficient simultaneous decarbonation and denitrogenation in CWW treatment, were comprehensively studied in their diversity of distinct microbial species. More than 40% of sequences in bioreactors were assigned to the phylum of *Acidobacteria* with its majority belonging to the taxa family *Ellin6075* without specifically identified function. *Nitrospira*, *Thiobacillus*, and *Planctomyces* were found being enriched in genes encoding the nitrogen removal pathway. The microbial association network composed of two modules provides decarbonation and denitrogenation of CWW driven by the keystone taxa of the *Comamonadaceae* and *Hyphomicrobiaceae* families. The study demonstrated the diverse and distinct bacterial communities are determined by the composition of CWW governing the biochemical reactions. The microbial composition in bioreactors may be controlled to achieve more modes for simultaneous decarbonation and denitrogenation of CWW by regulation of operation parameters including DO, pH, SRT, organic load rate, and reflux ratio.

Declarations

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