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Exploring Methane Capture Potential in Alkaline Coal Mine Drainage: Insight from the Microbial Community Structure and Function Analysis

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Abstract: Alkaline coal mine drainage represents one of the most critical issues in the coal industry, driven by complex hydro-biogeochemical processes. However, the interplay of hydrogeochemical and biogeochemical interactions in alkaline coal mine drainage is still poorly understood. To this end, water samples were systematically collected from alkaline coal mine drainage sites from five coal mining areas in Chongqing coal mining district, located in southwestern China. Hydrogeochemical analyses showed that the main water type of the coal mine drainage sample was HCO_3-SO_4~K-Na, which primarily originated from local meteoric water. The microbial community compositions in the studied alkaline coal drainage were critically associated with sulfate, bicarbonate, DOC, nitrate, and pH, and linked to three putative keystone genera via network analysis (*Thiothrix*, *Methylophilaceae_MM1*, and an unclassified genus from *Comamonadaceae* family). Functional predictions from FAPROTAX suggested a high abundance of metabolic pathways involving the oxidation of sulfide and sulfur compounds, potentially underscoring their importance in controlling sulfate enrichment in alkaline coal mine drainage. Interestingly, members of the *Methylomonadaceae* family (methanotrophs) and the *Methylotenera* genus (methylotrophs) had positive Spearman correlations with both ammonium and sulfate, potentially inferring that the enhanced activities of methanotrophs might help capture methane in the alkaline coal mine drainage. This study further enhances our comprehension of the intricate interplay between hydrogeochemical and biogeochemical interactions in alkaline coal mine drainage, contributing to the carbon budget.

Keywords: alkaline coal mine drainage; carbon budget; methane oxidation; biogeochemical processes; network analysis

1. Introduction

The coal sector is recognized as a key source of power generation and supporting economic growth and infrastructure development. Coal mine drainage has, however, been identified as a critical issue within coal mining environments, adversely affecting the quality of soil, surface water, and subsurface water bodies, thereby impacting overall biodiversity [1,2]. The pH of coal mine drainage can vary from acidic to neutral or even alkaline, contingent upon the characteristics of the coal ore deposit and its associated minerals [1,3–5]. Consequently, it is essential to elucidate the complex interplay between hydrogeochemical and biogeochemical interactions in coal mine drainage.

The pH of waste-rock drainage primarily depends on the relative rates of acid production through sulfide oxidation and acid neutralization via the dissolution of carbonates or silicates [6]. The abiotic and biological oxidation of reduced sulfur-containing minerals, predominantly pyrite that generally exists during coal mining, generates acid mine drainage [4,7–9], while the presence of sufficient carbonate buffering minerals (for example dolomite, calcite, etc.) can quickly neutralize acid mine drainage, resulting in what is termed neutral mine drainage [3,5,7,10]. Neutral or even alkaline mine drainage is thus characterized by increased alkalinity, ionic strength and pH, and frequently contains elevated concentrations of metals reflective of the parent geology [11,12]. Moreover, under near-neutral pH and appropriate redox conditions, various metals and metalloids, such as Fe, Zn, Sb, and As, remain soluble [13,14]. Given the susceptibility of microbial communities to environmental factors, such as salinity, pH, and concentrations of heavy metals, microbial communities in alkaline mine drainage are essentially different compared to those in acid mine drainage [15]. Understanding the distinction of microbial assemblies in alkaline and acid mine drainage may help us to identify the adaption of particular featured groups of taxa to the environmental conditions [16]. Moreover, coal mine drainage microorganisms are involved in diverse metabolic activities that occupy almost all possible microbiome niches, including organic degradation, methanogenesis and methane oxidation, sulfur and iron redox processes, ammonification, nitrogen fixation, nitrification and denitrification [15,17–21]. These characteristics can subsequently be utilized as bioindicators to assess localized chemical and physical conditions [22]. Additionally, underground coal mining activities induce gas emission (especially methane) that is sequestered in the coal seam and adjacent rock formations, accounting for the largest share (approximately 37%) of China's anthropogenic methane emissions [23,24]. However, most studies on microbial communities in coal mine drainage focus on acid mine drainage environments characterized by high metal concentrations. The microbial community structure and their potential functions in neutral coal mine drainage remain poorly understood, and the responses of their diverse metabolic activities to gas emission during the coal mining are not well-documented. Therefore, it is imperative to thoroughly investigate the structure, diversity, and potential functions of microbial communities in neutral coal mine drainage and to ascertain their regulatory effects on the intricate interactions between hydrogeochemical and biogeochemical processes in situ.

The Chongqing mining district, situated in southwestern China, was chosen as the study area. Intensive coal mining activities in this district began in the mid-1950s. The coal seams of the Chongqing coal mining district predominantly originate from the Late Permian and Late Triassic coal-bearing strata. These geological layers are primarily composed of mudstone, argillaceous limestone, fine sandstone, sandy mudstone, calcareous mudstone, and coal seams, which often contain frequent occurrences of pyrite nodules [25], and thus generally host neutral or even alkaline mine drainage with increased alkalinity, ionic strength, and low water quality [26]. In the Chongqing mining district, 95% of the coal mines are high-gas or gas outburst mines [24], with the highest methane emission factor (60.9 m^3/t) in China based on nearly 1840 coal mines [27]. Despite the importance of this mining district, the hydrogeochemical and biogeochemical processes present are not well characterized, and the potential microbial communities have not been studied. These are important knowledge gaps, as indigenous methanotrophic microbial communities may offer the potential to mitigate methane release. Therefore, in the present study, the Chongqing mining district was selected with the following objectives: (i) to characterize the hydrogeochemistry of neutral coal mine drainage; (ii) to determine the structure, diversity, and potential functions of the bacterial community; and (iii) to investigate the hydrogeochemical and biogeochemical interactions and determine the potential keystone taxa and critical methane-related metabolic pathways in neutral coal mine drainage.

2. Material and Methods

2.1. The Study Area

The study area is situated in the Chongqing mining district, where coal seams are predominantly composed of Late Permian coal-bearing strata. These strata were formed as part of the south China interior epicontinental sea basin during the Late Permian period [28,29]. The sedimentary facies within this area are primarily characterized by clastic shoreline sediments, which notably lack a delta sedimentary system. Indications of marine influence are evident in the depositional environment of the Late Permian coal seams in Chongqing. The principal mineral constituents of the Late Permian coals in this mining district include kaolinite, quartz, pyrite, and calcite [25,30,31]. The sulfide contents of the coals mined in the studied area range from 1.9% to 3.5% (Donglin Coal Mine, 1.89%; Yutianbao Coal Mine, 3.46%, Xintianwan Coal Mine, 3.34%; Nantong Coal Mine, 2.74%; Hongyan Coal Mine, 3.20).

In the Chongqing mining district, precipitation and groundwater serve as the primary sources of recharge for mine water. This region experiences a relatively humid climate, with annual precipitation levels varying between 1.2 and 2.0 m. The Permian Maokou limestone underlies the coal seam, while the Changxing limestone forms the roof. Consequently, the hydrogeochemistry of the drainage from coal mining is predominantly influenced by the limestone aquifers of both the Changxing and Maokou formations. [9,32].

2.2. Sample Collection

Five mine drainage samples (DL01, HY01, NT01, XTW01 and YTB01) were collected in December 2020 from mine drains of the seven coal mines in the Chongqing mining district, including Donglin Coal Mine (DL01; producing coal mine), Nantong Coal Mine (NT01; producing coal mine), Hongyan Coal Mine (HY01; producing coal mine), Xintianwan Coal Mine (XTW01; closed coal mine), and Yutianbao Coal Mine (YTB01; closed coal mine) (Figure 1). All chemical samples were filtered through a 0.45-μm membrane and subsequently divided into sub-samples. One sub-sample was acidified with ultrapure 6 M HNO$_3$ to achieve a pH below 2.0, suitable for the analysis of major cations and trace elements. Another sub-sample was adjusted with concentrated H$_2$SO$_4$ to maintain a pH less than 2 for the analysis of dissolved organic carbon (DOC). The sub-sample intended for anion analysis underwent no modifications. All sub-samples were preserved on ice during transport to the laboratory and subsequently stored at 4 °C in a refrigerator until analysis within a seven-day period.
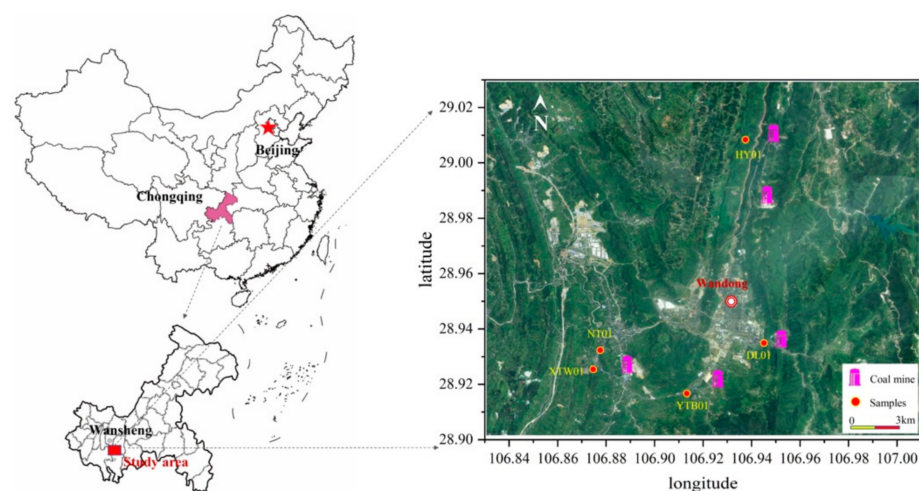
Figure 1. Locations in the study area and water samples sites from the neutral coal mine drainage in five different coal mining areas.

Microbial samples were obtained by filtering 5–10 L of groundwater through 0.22-μm filters (Millipore, Burlington, VT, USA). The biomass-laden filters were handled with autoclaved stainless-steel forceps, enclosed in 50 mL sterile conical centrifuge tubes, and transported to the laboratory (Majorbio Co., Ltd., Shanghai, China) on dry ice. They were then stored at −80 °C until DNA extraction, which was performed within 7 days.

2.3. Laboratory Chemical Analysis

Effluent pH, electrical conductivity (EC), and total dissolved solids (TDS) were measured in situ utilizing a multi-parameter portable meter (HI 9829, HANNA Instruments Ltd., Woonsocket, RI, USA). Alkalinity was determined via titration using a HACH Model 16,900 digital titrator. The ammonium, nitrite, S(-II), and Fe(II) were quantified with a portable spectrophotometer (DR2800, HACH, Loveland, CO, USA) using analytical methods based on 5-aminosalicylic acid, 4-amino-monosodium salt, methylene blue, and 1,10-phenanthroline, respectively. Major cation concentrations were assessed using Inductively Coupled Plasma-Atomic Emission Spectroscopy (ICP-AES, Thermo Fisher Scientific, Waltham, USA), and trace element concentrations were determined using Inductively Coupled Plasma-Mass Spectroscopy (ICP-MS, Thermo Fisher Scientific, Waltham, MA, USA). Unacidified aliquots were subjected to ion chromatography for major anions (DX-120, Dionex, Sunnyvale, CA, USA), achieving an accuracy exceeding 95%. All samples maintained an ionic charge imbalance of less than ±5%. Dissolved organic carbon (DOC) concentrations were measured using a Shimadzu TOC-VCPH analyzer, with a detection limit of 4 μgC/L [33].

2.4. DNA Extraction, High-Throughput Sequencing and Data Analysis

The DNA from the samples was extracted using the FastDNA® SPIN Kit for Soil (MP Biotechnology, Solon, OH, USA) following the manufacturer's instructions. Amplification of the V4–V5 region of the 16S rDNA gene was carried out using the primers 515F (GTGCCAGCMGCCGCGGTAA) and 926R (CCGTCAATTCMTTTRAGTTT) [34]. The PCR reactions were prepared in a total volume of 20 μL, comprising 4 μL of 5× FastPfu buffer, 2 μL of 2.5 mM dNTPs, 0.8 μL of 5 μM primers, 0.4 μL of FastPfu polymerase, and 10 ng of template DNA. The PCR thermal cycling conditions included an initial denaturation at 95 °C for 2 min, followed by 28 cycles of 30 s at 95 °C, 30 s at 61 °C, and 45 s at 72 °C, with a final extension of 10 min at 72 °C and a hold at 10 °C. High-throughput sequencing was performed on the Illumina MiSeq platform, adhering to rigorous quality control procedures. The negative controls were sequenced (there were only <20 reads, which are negligible relative to the 30,000 to 70,000 reads we sequenced). The QIIME software package (version 2021.4.0) was employed to assess sequence quality and to cluster the sequences into operational taxonomic units (ASVs) with a similarity threshold of 99% or higher. For diversity analysis, singleton ASVs were excluded, and sequence reads were normalized to 15,675 reads per sample. To infer the metabolic and ecologically relevant functions of the microbial communities in neutral coal mine drainage, taxon abundance profiles were transformed and mapped onto putative functional abundance profiles utilizing the FAPROTAX v1.2.5 [35]. Specifically, the potential functions at the genus level were assessed manually by comparing the microbes, which were encoded with specific functional genes to corresponding entries within the same genus in the UniProt database, including sulfide-oxidizers (*fccAB* and *sqr*), sulfite-oxidizers (oxidative *dsrAB*) [36], sulfate-reducers (genus name with a *desul* prefix and reductive *dsrAB*) [36,37], sulfur-disproportionators [38], Fe(III)-reducers (*ImcH* and identified) [39,40], Fe(II)-oxidizers [40–42], denitrifiers (*narGHI* and *napAB*), and ammonium-oxidizers (*pmoABC*, *amoABC*, and *hzs*) [43], methane-producer (*mcrABCD*) [44], and methane-oxidizers (*mmoABC* and *pmoABC*) [45].

2.5. Data Visualization

The alpha diversity index at the ASV level was computed and visualized using the Tutools platform (https://www.cloudtutu.com, accessed on 30 June 2024). Hydrogeochem-

ical classifications of groundwater samples were depicted through Piper plots, generated with Grapher 14 version 14.2.371. Heatmaps were created using R version 4.0.5, employing the 'psych' package (version 2.2.5). Redundancy analysis (RDA), utilizing the Hellinger method and the Monte Carlo permutation test ($p = 0.025$), was performed using Canoco 5.0. Pearson correlations were calculated to explore relationships between independent geochemical parameters and the top 40 genera in microbial abundance.

3. Results and Discussions

3.1. Hydrogeochemistry

The potential water origin can be traced using stable oxygen and hydrogen isotopes [46]. The δD (-37.42 ± 1.72 ‰) and $δ^{18}O$ (-6.49 ± 0.16 ‰) of coal mine drainage waters aligned with both the Global Meteoric Water Line (GMWL: $δD = 8.0\ δ^{18}O + 10.0$) [47] and the Chongqing Local Meteoric Water Line (CLMWL: $δD = 8.73\ δ^{18}O + 15.73$) [48] (Figure S1). There were no indications of evaporation or water–rock interactions (i.e., enrichment of ^{18}O), suggesting that the coal mine drainage is derived from local meteoric water [49]. The pH values of the coal mine drainage samples varied from 8.0 to 8.2, with a mean of 8.0, indicative of a neutral to slightly alkaline condition in the coal mine drainage of the study area. Total dissolved solids (TDS) values ranged between 1.1 and 1.5 g/L, averaging 1.3 g/L, as shown in Table 1. These values are comparable to those of underground river water (TDS of 1.0 ± 0.1 g/L) and surface water (TDS of 0.3 ± 0.1 g/L) in the suburban regions of Chongqing [50]. As shown in the Piper triangular diagram (Figure S1 in Supporting Information), a useful method for showing different hydrogeochemical characteristics of water samples [51], the main water type of coal mine drainage samples is HCO_3-SO_4~K-Na. The total alkalinity ranged from 388.1 to 558.9 mg/L with an average of 471.2 mg/L (Table 1), inferring that critical buffering effects of the carbonate minerals in the study area [25,30,31]. The total dissolved carbon ranged from 0.3 to 0.9 mg/L with an average of 0.58 mg/L (Table 1). Concentrations of sulfate ranged from 485.3 to 1355.0 mg/L with an average of 802.7 mg/L, while the sulfide was less than 10 μg/L, which might be due to the effects of biotic and abiotic sulfide oxidation in the effluents from the coal mining area. The obvious enrichment of sulfate highlighted the importance of microbial S-oxidizing processes in the coal mine drainage [4,7–9]. The concentrations of nitrate and ammonium were measured at 7.6 ± 0.4 mg/L and 1.0 ± 0.6 mg/L, respectively, while nitrite concentrations were below the detection limit of 0.01 mg/L (Table 1). The concentrations of total dissolved iron (Fe) and Fe(II) were not detectable, attributable to rapid Fe(II) oxidation and the low solubility of Fe(III) species under conditions of neutral pH [52].

Table 1. Variations in geochemical parameters in neutral coal mine drainage.

	XTW01	NT01	HY01	DL01	YTB01	Average	Stand. Error of Average
TDS (mg/L)	1.5	1.1	1.2	1.1	1.4	1.3	0.1
pH	8.0	8.1	8.2	8.0	8.0	8.1	0.0
Cl^- (mg/L)	30.6	177.5	170.3	45.6	195.9	124.0	35.4
Nitrate (mg/L)	6.9	6.6	8.6	7.5	8.5	7.6	0.4
Sulfate (mg/L)	1355.2	485.3	599.1	800.5	773.2	802.7	149.7
Na^+ (mg/L)	545.4	408.6	467.3	355.9	548.2	465.1	37.7
NH_4^+ (mg/L)	2.7	0.1	0.1	2.1	0.1	1.0	0.6
K^+ (mg/L)	4.9	3.4	3.5	4.6	2.9	3.9	0.4
Mg^{2+} (mg/L)	121.9	57.6	64.7	104.1	75.7	84.8	12.2
Ca^{2+} (mg/L)	40.7	13.5	19.7	34.7	17.8	25.3	5.3
Carbonate (mg/L)	63.8	71.4	98.3	32.1	24.1	57.9	13.5
Bicarbonate (mg/L)	495.1	316.7	416.9	407.9	429.7	413.3	28.6
DOC (mg/L)	0.9	0.6	0.7	0.3	0.4	0.6	0.1

Table 1. *Cont.*

	XTW01	NT01	HY01	DL01	YTB01	Average	Stand. Error of Average
Al (μg/L)	59.2	66. 3	102.1	64.0	107.0	83.1	10.2
As (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ba (μg/L)	73.24	89.42	78.07	75.03	78.1	78.8	2.8
Cd (μg/L)	50.95	55.03	51.61	49.71	54.11	52.3	1.0
Co (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Cr (μg/L)	14.50	14.91	15.48	15.27	15.39	15.1	0.2
Cu (μg/L)	1.44	5.78	0.74	1.57	n.a.	2.4	1.1
Fe (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Mn (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Ni (μg/L)	5.42	2.83	9.46	8.17	5.36	6.2	1.2
Pb (μg/L)	9.71	9.40	8.05	8.60	9.81	9.1	0.3
Sr (mg/L)	7.12	7.92	3.81	7.26	4.61	6.1	0.8
V (μg/L)	33.27	29.58	30.67	32.02	32.03	31.5	0.6
Zn (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sb (μg/L)	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.	n.a.
Sn (μg/L)	7.96	7.57	18.51	4.33	16.89	11.1	2.8
Ti (μg/L)	18.32	18.61	18.57	18.44	18.57	18.5	0.1
δ^2H‰	−39.75	−35.82	−38.60	−37.04	−35.89	−37.4	0.8
δ^{18}O $_{SMOW}$ ‰	−6.70	−6.28	−6.53	−6.53	−6.40	−6.5	0.1

## 3.2. Microbial Ecology and Putative Keystone Taxa

A total of 393,350 sequences of the 16S rRNA gene were acquired from five mine drainage samples following the exclusion of low-quality sequences and chimeras. The quantity of sequences derived from individual samples ranged from 78,012 for sample XTW01 to 79,081 for sample DL01 (Table S1). Bacterial sequence coverage was >99% in the libraries, with a variety of taxa observed at the 99% ASV level, and an average of 514.4 predicted ASVs (ranged from 314 to 684) (based on Chao1) (Table S1), with an average Shannon index of 4.91 and Simpson index of 0.96. The classification of the ASVs yielded 34 phyla, 89 classes, 204 orders, 319 families and 494 genera. Across all samples, Gammaproteobacteria was the dominant class (55.8 ± 15.0%), followed by Bacteroidia (14.7 ± 17.1%), Alphaproteobacteria (6.0 ± 1.3%), Omnitrophia (3.9 ± 4.9%), and an unclassified class of the bacterial domain (2.0 ± 1.7%). Previous studies showed that the Gammaproteobacteria is found in contaminated deep groundwater from coalfields [53] and coal mines in the Red River Basin, Vietnam [54]. At the genus level, the top five genera were affiliated with *Thiothrix* (6.7 ± 3.8%), *Candidatus Omnitrophus* (3.6 ± 3.6%), and three unclassified genera from the *Hydrogenophilaceae* (21.1 ± 22.5%), *Comamonadaceae* family (7.1 ± 4.1%) and *Burkholderiales* order (2.7 ± 1.9%). Of the ASVs, 2.7% were assigned to known species (Figure 2), being consistent with the dominant unknown microorganisms in the subsurface [55].

To identify putative keystone taxa that may contribute to or be influenced by the key biogeochemical parameters at these mine sites, a co-occurrence network was constructed using Spearman correlations ($r > |0.6|$, $\rho < 0.05$) (Figure 3a). The putative keystone taxa were identified based on nodes exhibiting high degree centrality and low betweenness centrality within the network [56,57]. Three putative keystone taxa were determined via the analysis of betweenness centrality and degree for each node (Figure 3b), associated with three bacterial genera (*Thiothrix*, *Methylophilaceae_MM1*, and an unclassified genus from *Comamonadaceae* family). Members of the genus *Methylophilaceae_MM1* are among the most important methylotrophs in aquatic habitats, encoding diverse metabolic modules that are involved in the biogeochemical cycling of carbon [58], nitrogen [59], and sulfur [60]. Members of the genus *Thiothrix* are also very versatile and can incorporate acetate and/or bicarbonate under heterotrophic, mixotrophic and chemolithoautotrophic conditions. It was reported that *Thiothrix* species are filamentous sulfur-oxidizing chemolithotrophs

important in the oxidation of sulfide in anaerobic sediments [61], and could induce the formation of intracellular sulfur globules from thiosulfate [62,63]. However, the potential functions of the *Comamonadaceae* family in the deep subsurface are less clear; members of this family exhibit diverse metabolic capabilities, including denitrification, Fe(III) reduction, hydrogen oxidation, and fermentation [64]. Therefore, the putative keystone taxa played critical roles in the complex interplay of hydrogeochemical and biogeochemical interactions in the coal mine drainage.
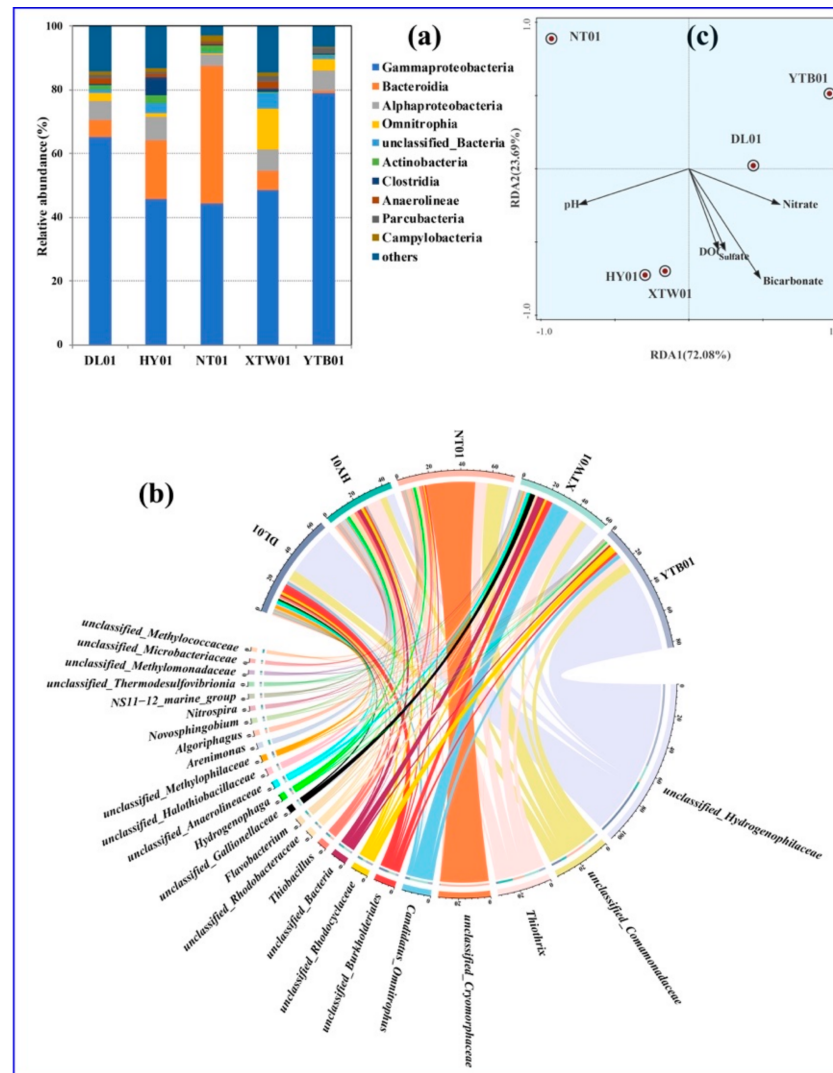
Figure 2. Change in microbial community approximately along the drain flow path at the genus level (others: <1%) (**a**); and Circos diagram visualizing the linkage between coal mine drainage samples and microbial taxa at genus level (top 32 genera) (**b**); Redundancy analysis (RDA) of relationships between microbial community and geochemical parameters (**c**).
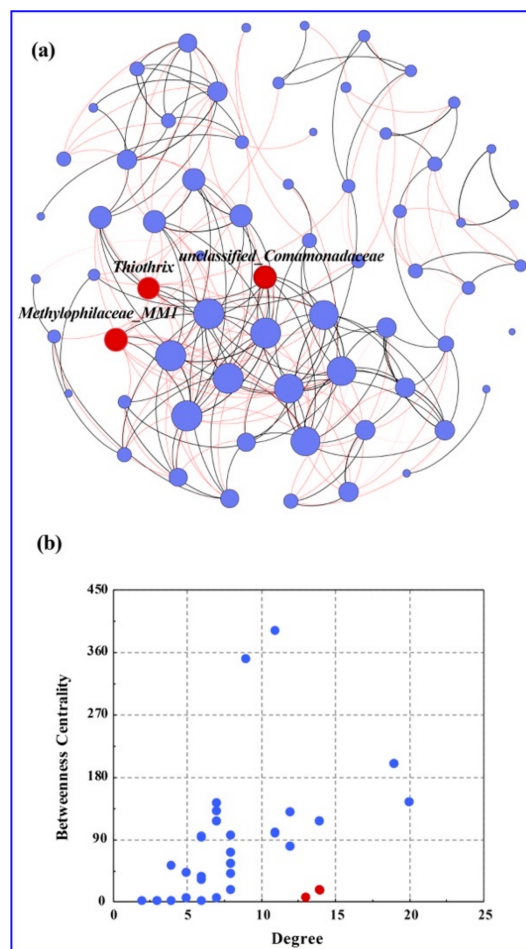
Figure 3. Genus level-based network (Spearman correlations ($r >$ |0.6|, $\rho < 0.05$)) (**a**) and putative keystone taxa identified using betweenness centrality and degree of the nodes (**b**). The 3 key taxa are shown in red in the figure (*Thiothrix*, *Methylophilaceae_MM1*, and an unclassified genus from *Comamonadaceae* family).

3.3. Potential Function of the Microbial Community

To determine the dominant biogeochemical cycles mediated by microorganisms in the alkaline coal mine drainage, the potential metabolic function of the microbial community was predicted using the FAPROTAX (Figure 4). In the alkaline coal mine drainage, the top five metabolisms were anaerobic and aerobic chemoheterotroph, the dark oxidation of sulfide and sulfur compounds, and methylotrophy, followed by methanol oxidation, methanotroph, hydrocarbon oxidation, and fermentation. The high abundance of oxidation of sulfide and sulfur compounds was suggested as the key factors controlling sulfate enrichment in the alkaline coal mine drainage. Moreover, genera that occurred in the alkaline coal mine drainage were also compared to those that encoded the specific functional genes in the Uniprot database (Figure 5). Results supported the hypothesis that sulfite and sulfide oxidation were dominant S-cycling biogeochemical processes, with the total abundance of the genera that encoded these pathways ranging from 3.2% to 12.2% and from 6.1% to 12.1%, respectively. Specifically, 3 genera (*Thiobacillus*, *Sideroxydans*, and *Thiothrix*) were associated with sulfite oxidation and 15 genera were associated with sulfide oxidation, including *Sulfurimonas*, *Sulfuricurvum*, *Sulfurovum*, and *Thiobacillus*, which were reported to be potentially involved in the sulfide oxidation [65–67]. Interestingly, seven genera were potentially associated with methane oxidation, including unclassified_Rhodobacteraceae (UniProt database entry number: A0A651ESV8, A0A2D5J7Y2, A0A7Y7A459, X6L2T0, A0A5P2ZWP6, A0A5P2ZWI7, and A0A5P2ZZW5), *Pseudomonas* [68], *Legionella* (UniProt database entry number: A0A078L5X7 and A0A378LHH6), unclassified_Methylophilaceae,

unclassified_Methylomonadaceae, *Methylomonas*, and *Methylovulum* [69,70], suggesting the significance of potential methane capture in the alkaline coal mine drainage.
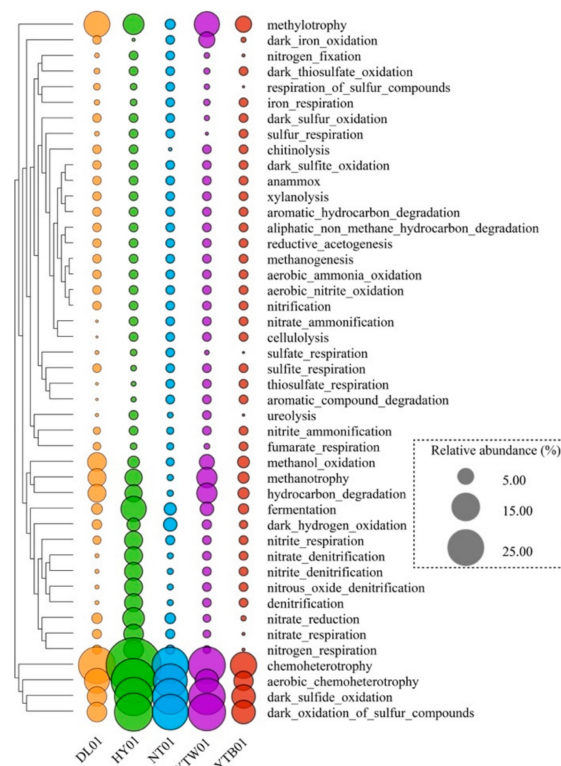

**Figure 4.** Potential function of the microbial community predicted using FAPROTAX (v1.2.5).

3.4. Correlations between Microbial Community and Hydrogeochemistry

Positive Spearman correlations were observed between microbial community compositions at the ASV level and key hydrogeochemical components (Figure 3 and Table S2), indicating a strong interplay between indigenous microbial communities and their associated hydrogeochemical processes. Specifically, the RDA analysis of the key biogeochemical parameters affecting microbial community compositions suggested that microbial community compositions in neutral coal drainage samples were critically shaped by sulfate, bicarbonate, DOC, nitrate, and pH. The correlations between microbial genera (occurred in at least four samples) and the key biogeochemical parameters are shown in a heatmap based on Spearman correlations (Figure 6 and Table S2). These five and nine genera were positively correlated with DOC and bicarbonate, respectively, among which were organisms associated with biogeochemical carbon cycling, such as members of the genus *Thiothrix* and the order Omnitrophales [71,72]. Specifically, members of the genus *Thiothrix* can grow both organoheterotrophically and lithoautotrophically in the presence of reduced sulfur compounds, and they can dominate microbial populations in sulfide-rich waters [71], being consistent with the dominant anaerobic and aerobic chemoheterotroph in the studied alkaline coal mine drainage. *Aeromonas* and *Candidatus Megaira* showed a positive correlation with nitrate (7.6 ± 0.4 mg/L) ($p < 0.05$). It was reported that *Aeromonas* spp. potentially participated in nitrate reduction [73]. The genes associated with nitrate transport were observed in the species of *Candidatus Megaira* (UniProt database: A0A8K1J267), but the exact nitrate pathways are not well understood. Moreover, 11 genera were positively correlated with ammonium and sulfate ($p < 0.05$), such as the *Methylotenera*, *Candidatus Komeilibacteria*, *Sideroxydans*, and unclassified genera of Gallionellaceae family, Thermodesulfovibrionia class, Methylomonadaceae family and Caulobacteraceae family. The potential for sulfide oxidation was noted in the members of *Sideroxydans* [66,74] and the unclassified genera of the Gallionellaceae family [75] and Caulobacteraceae [76], which would be critical in leading to the high sulfate concentrations in the alkaline coal mine drainage (802.7 ± 149.7 mg/L). A previous study

showed that the *Candidatus Komeilibacteria* spp. (UniProt database: A0A1G2BJC5) and unclassified genera of *Thermodesulfovibrionia* may be associated with sulfate reduction [75,77], while sulfide and Fe(II) oxidation were associated with the members of *Sideroxydans* [66,74] and unclassified genera of Gallionellaceae family [75] and Caulobacteraceae [76], which was in line with the low sulfide and Fe(II) concentrations in alkaline coal mine drainage. Interestingly, members of the Methylomonadaceae family (typical aerobic methanotrophs) and the *Methylotenera* genus (typical methylotrophs) had positive Spearman correlations with both ammonium and sulfate. Previous research has indicated that aerobic methanotrophs of the Methylomonadaceae family possess more versatile metabolic capabilities than previously thought. They may also participate in nitrate/nitrite- or mineral oxide-dependent aerobic methane oxidation under conditions of oxygen limitation [76]. Enhanced methane oxidation rates were observed when nitrate or sulfate were added to anoxic water column samples, where the methanotroph *Methylobacter* produced methane-derived carbon compounds that were used by the non-methanotrophic *Methylotenera*, which encoded all genes required for denitrification [78,79]. Therefore, we suggest that nitrate- and sulfate-enhanced microbial activities of methanotrophs may be the potential key methane capturing processes in alkaline coal mine drainage.
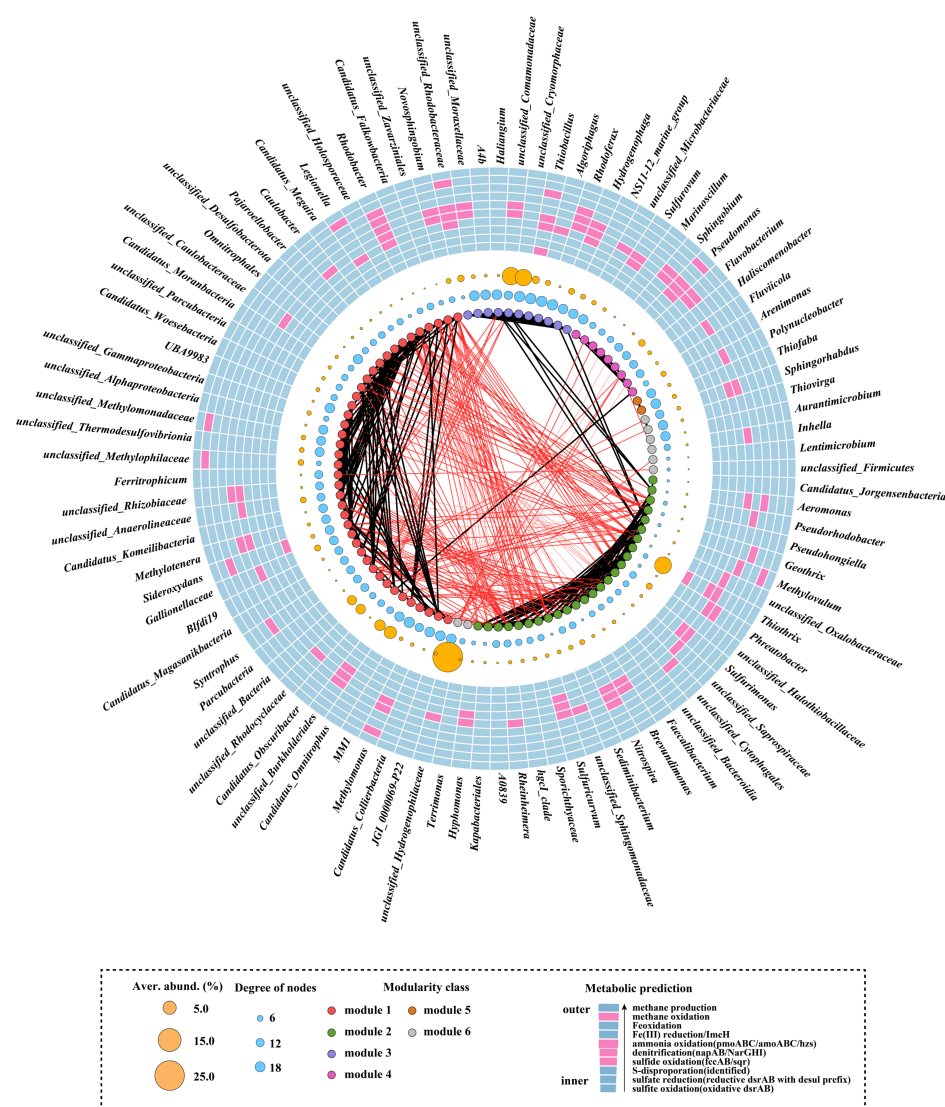
Figure 5. Metabolic potential (outer circle), average relative abundance, degree and modularity class of each genus in the correlation network. The purple and blue bricks in outer circle indicate the presence and absence of the metabolic prediction, respectively.
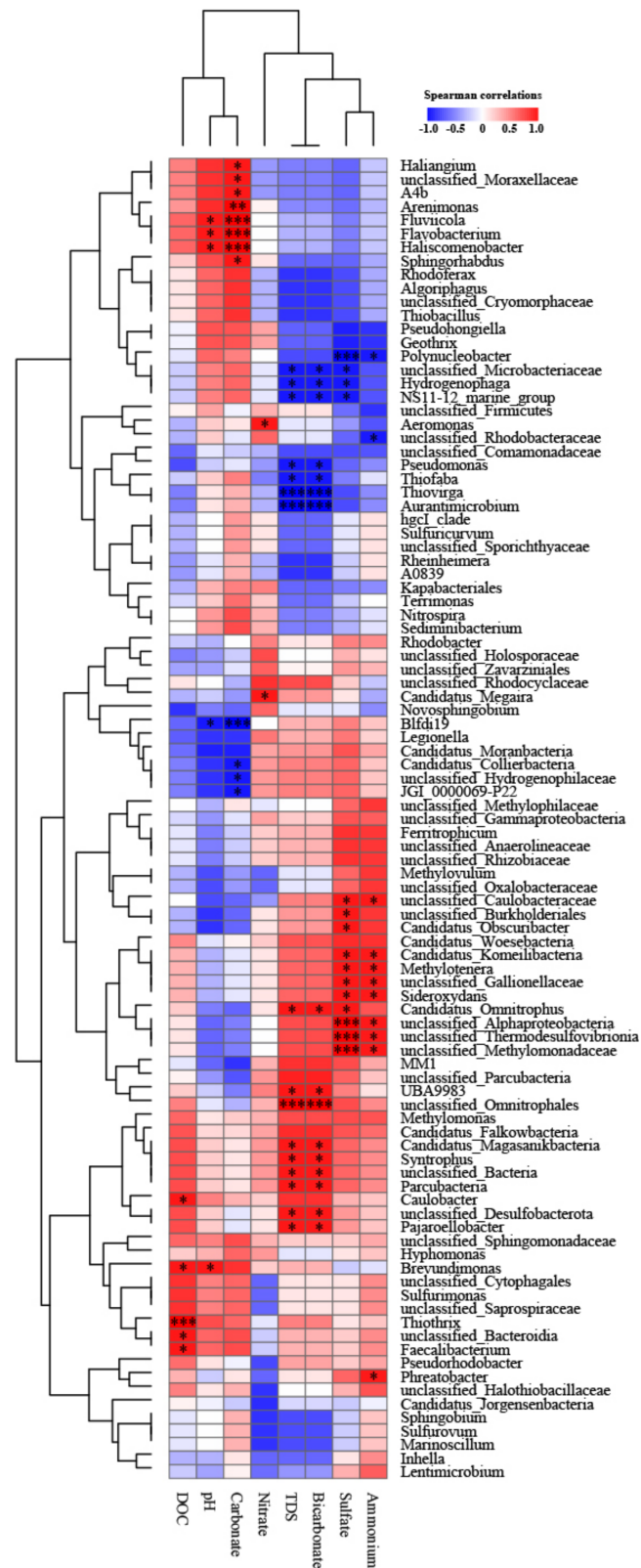
Figure 6. Spearman correlation heatmap with clustered geochemical parameters and dominant genera (occurred in at least three samples). Significant correlations were presented as * ($p < 0.05$), ** ($p < 0.01$), and *** ($p < 0.001$).

4. Conclusions

In this study, to explore the intricate interactions between hydrogeochemical and biogeochemical processes in neutral coal mine drainage, water samples were collected from five different neutral coal mining areas within the Chongqing mining district in southwestern China. The neutral coal mine drainage water was originated from local meteoric water and was of the HCO_3-SO_4~K-Na water type. The microbial community compositions in neutral coal drainage samples were shaped using sulfate, bicarbonate, DOC, nitrate, and pH. Three putative keystone genera were highlighted using correlation network analysis, including organisms affiliated with *Thiothrix*, *Methylophilaceae_MM1*, and an unclassified genus from the *Comamonadaceae* family. Functional predictions showed a high abundance of pathways associated with the oxidation of sulfide and sulfur compounds, potentially suggested as the key factors controlling the sulfate enrichment in the alkaline coal mine drainage waters. Interestingly, members of the *Methylomonadaceae* family (methanotrophs) and the *Methylotenera* genus (methylotrophs) had positive Spearman correlations with both ammonium and sulfate, potentially inferring that the enhanced activities of methanotrophs might capture methane in the alkaline coal mine drainage. Therefore, alkaline coal mine drainage could be a potential contributor for the carbon budgets via their abilities of methane capture, although further studies should highlight the genome resolved information of methane metabolisms and its interactive connections with hydrogeochemical and biogeochemical processes.

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