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Molecular characterization of microbial communities in bioaerosols of a coal mine by 454 pyrosequencing and real-time PCR

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ABSTRACT

Microbial diversity and abundance in bioaerosols of a coal mine were analyzed based on 454 pyrosequencing and real-time polymerase chain reaction (PCR). A total of 37,191 high quality sequences were obtained and could be classified into 531, 1730 and 448 operational taxonomic units respectively for archaea, bacteria and fungi at 97% sequence similarity. The Shannon diversity index for archaea, bacteria and fungi was respectively 4.71, 6.29 and 3.86, indicating a high diversity in coal mine bioaerosols. Crenarchaeota, Proteobacteria and Ascomycota were the dominant phyla for archaea, bacteria and fungi, respectively. The concentrations of total archaea, bacteria and fungi were 1.44×10^8 , 1.02×10^8 and 9.60×10^4 cells/m³, respectively. Methanotrophs observed in bioaerosols suggested possible methane oxidation in the coal mine. The identified potential pathogens to coal miners, such as *Acinetobacter schindleri*, *Aeromonas cavernicola*, *Alternaria alternata*, *Aspergillus penicillioides*, *Cladosporium cladosporioides*, and *Penicillium brevicompactum* were also observed. This was the first investigation of microbial diversity and abundance in coal mine bioaerosols. The investigation of microbial communities would be favorable in promoting the progress of methane control based on microbial technique and concern on coal miners' health.

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Introduction

Bioaerosols in workplaces have been growing focus in recent years (Duquenne et al., 2013; Goyer et al., 2001). The microorganisms in bioaerosols of numerous industrial workplaces have been characterized, e.g., metalworking factories, breeding plants, and wastewater treatment plants (Gorny et al., 2004; Karra and Katsivela, 2007; Nehme et al., 2008; Oppliger et al., 2008). Surprisingly, the microorganisms of coal mine bioaerosols have never been studied although coal mining is considered as one of the most dangerous industries worldwide.

For microbial analysis of coal mine, microbial diversity and functional microorganisms such as methanogens and methanotrophs are often concerned (Han et al., 2009; Jiang et al., 2010; Johnson and Hallberg, 2003; Krüger et al., 2008), which are associated with coal bioconversion and methane metabolism. Methanogens are responsible for biogenic methane formation. In contrast, methanotrophs are associated with methane oxidation, which may be used to decrease the methane concentration in coal mines. For example, the injection of a methanotrophic community into coal seam could potentially remove a large fraction of the methane (Mao et al., 2011). However, these research results of microorganisms obtained are based on the analysis

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of the coal and water samples of coal mine. To get more comprehensive understanding of microorganisms in coal mine environment, the microorganisms in bioaerosols of coal mine should be deeply analyzed, especially for methanogens and methanotrophs.

In addition, occupational diseases of coal miners have become a major research focus on environmental health. Several studies have revealed that long-term exposure to a coal mine environment is responsible for many occupational diseases (Dai et al., 2012). The respiratory diseases were commonly occupational diseases of coal miners (Ross and Murray, 2004). It has been reported that the abiotic hazards, e.g., coal dust are responsible for them. The inhalation of coal dust can cause several lung disorders (Graber et al., 2014; Petsonk et al., 2013), e.g., pneumoconiosis, silicosis, mixed dust pneumoconiosis, emphysema and chronic bronchitis. However, the biological hazards were also important factors for the infections (Donoghue, 2004). The potential pathogens and microbial metabolic products, e.g., mycotoxins and endotoxins, may be hazardous to health (Cho et al., 2011). Such health effects of microorganisms in bioaerosols have been investigated in occupational fields such as medicine, agriculture, and industry (Basinas et al., 2012; Lee et al., 2006). However, to our knowledge, the potential pathogens in coal mine bioaerosols have never been concerned.

Historically, microbial diversity and abundance in bioaerosols have been investigated by culture-dependent methods. However, it was difficult to detect the most microbes, especially rare potential pathogens and other functional microbes. Microbial diversity is therefore largely underestimated by culture-dependent methods. However, the culture-independent methods as 454 pyrosequencing and real-time polymerase chain reaction (PCR) analysis facilitate evaluation of uncultured microbes in a complex environment. 454 pyrosequencing enables sensitive detection of complex microbial communities (Gobet et al., 2012). It has been widely used for characterizing microbial communities in a variety of samples (Buee et al., 2009; Zhang et al., 2011). Real-time PCR is utilized for microbial quantification in environmental samples including microbial analysis of bioaerosol samples (Oppliger et al., 2008). Therefore, microbial communities and their relative abundance in environmental samples could be achieved by combining pyrosequencing with real-time PCR.

In this study, 454 pyrosequencing and real-time PCR were used to deeply investigate the microbial communities of coal mine bioaerosols for the first time. Based on the comprehensive understanding of the microbial communities, methanogens, methanotrophs and potential pathogens in coal mine bioaerosols would be especially concerned.

1. Materials and methods

1.1. Study area and sampling

Bioaerosol samples were collected from a coal mine in Hubei Province, China, which is a representative gassy coal mine with an annual coal production of about 90,000 tons, near the coal mine in the previous studies in Hubei (Wei et al., 2013). The coal has been classified as anthracite according to Chinese classification (Appendix A Table S1). The blasting method used for coal mining results in a large gas emission together with a high explosion risk. The gas emission is

18.07 m³/ton. An uninterrupted ventilation system is adopted for gas drainage. In the coal mining area, the methane content in the ventilation air varied from 0.12% to 1.84% (V/V) based on the annual data. Bioaerosol samples were collected from the coal mining area with an explosion-proof bioaerosol sampler (Quick Take 30, SKC, USA). The sampling was done after the drilling of coal. To avoid the sample contamination, the mining activities stopped and the bioaerosol sampler was placed at 1.5 m above the ground and at least 2 m away from coal miners. A sterilized filter housing containing a 47 mm diameter and 0.22 µm pore size filter (Whatman Japan Ltd., Tokyo, Japan) was connected to the sampler used to collect microbes in the bioaerosols. Air flow rate through the filters was controlled in 20 L/min for 1.5 hr. The sampling site in the coal mining area was narrow with the average basal area of 2.7 m² (1.8 m × 1.5 m). To avoid sampling bias, at least three sample locations were covered, so the air volume of 5.4 m³ was representative. Samples were collected in triplicates in each sampling site (Fig. 1, sampling sites A, B, C), and a total air volume of 16.2 m³ was collected in the coal mine. The membranes with the bioaerosol content were folded with sterile forceps and wrapped in parafilm (Griffin et al., 2001), and then put in a 50 mL sterilized centrifuge tube and stored in an icebox until transported to the laboratory.

The concentrations of methane and carbon dioxide were detected with portable gas detectors GJC4/100 (China Coal Industrial Equipment Corp., Ltd., China) and GRG5H (China Coal Technology & Engineering Group Corp., Ltd., China), respectively. The *in situ* environmental conditions in the sampling coal mine were: temperature 33 ± 5 °C, air moisture 72.5% ± 12.5%, ventilation air methane 0.85% ± 0.1% (V/V) and carbon dioxide 0.19% ± 0.06% (V/V).

1.2. DNA extraction and PCR

Genomic DNA of each site sample was extracted with a Fast DNA SPIN kit for soil (Bio101 Systems, Carlsbad, USA) according to the manufacturer's instructions. The final DNA sample was a DNA mixture of three site samples with a volume of 50 µL. The DNA concentration was measured using a NanoDrop ND-1000 UV-Vis Spectrophotometer (NanoDrop Technologies, Wilmington, Delaware, USA) with a concentration of 5.8 ± 1.3 ng/µL and then stored at –20 °C.

Nested PCR was performed for archaeal, bacterial and fungal communities because the DNA yield was low and direct PCR products were not perceptible on agarose gels (Stark et al., 1998). Primer details and references are listed in Table 1. For archaea, primer sets 8F/958R and 344F/915R were chosen for the first and the second PCR amplification. 27F/1492R and 27F/533R were carried out for the PCR of bacteria. NS1/FR1 and 3NDF/V4_euk_R2 served as the first and second PCR amplification for fungi. The PCR reaction was initiated at 95 °C for 2 min, followed by 25 cycles of 95 °C for 30 sec, 55 °C for 30 sec, and 72 °C for 60 sec, extended at 72 °C for 5 min. PCR was performed in triplicate to reduce the amplification variability. The final PCR product was a mixture of the triplicate. PCR products were purified with a PCR purification kit (MP Bio-medicals, CA, USA) and then prepared for pyrosequencing on a GS-FLX pyrosequencer (454 Life Sciences, Branford, CT, USA).

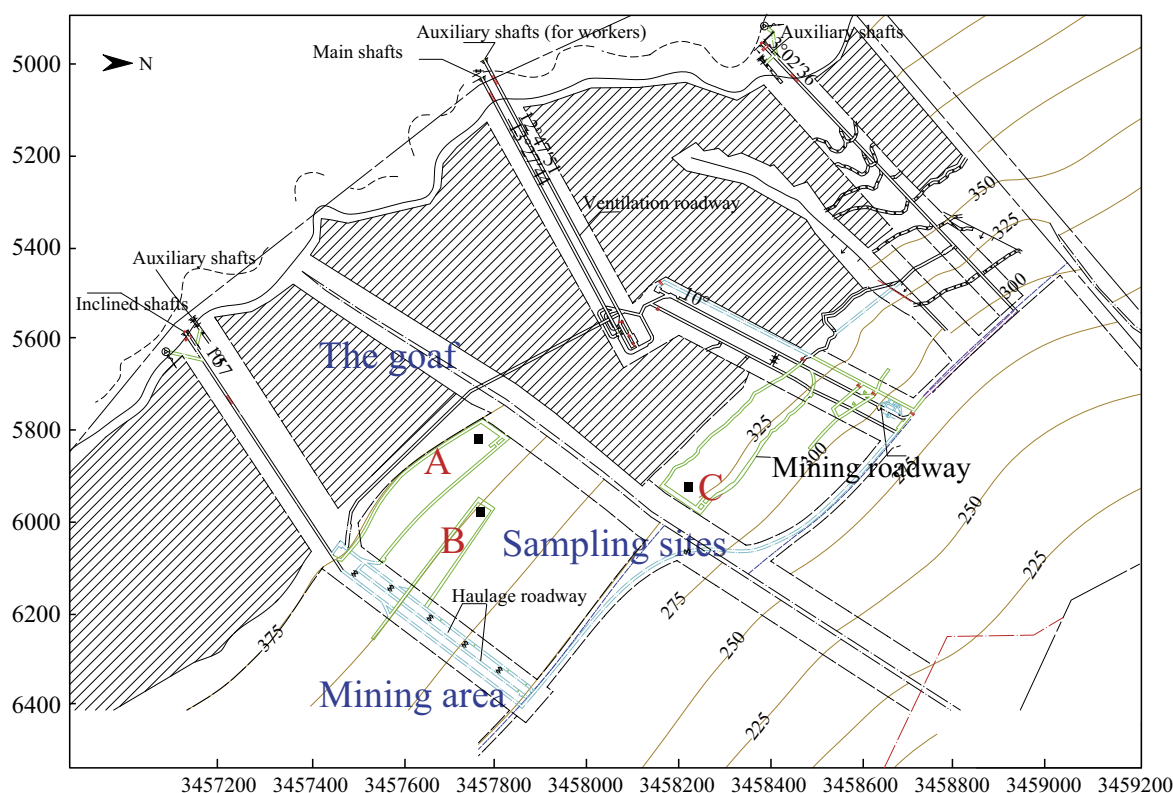


Fig. 1 – Coal mining area and sampling sites (A, B, and C) in the underground coal mine. In the coal mine, the uninterrupted ventilation system is adopted for gas drainage. Main shafts and auxiliary shafts (ventilating shafts) of the underground coal mine are on the slope of a hill. The distance from the entrance to the sampling site is about 1000 m. Average wind speed at main shaft, auxiliary shaft (ventilating shaft) and coal mining area (sampling site) is 3–4 m/sec, 4–5 m/sec and 0.5–1.3 m/sec, respectively.

Table 1 – Primers used in this study.

	Target gene	Primer (5'-3')	Reference
Pyrosequencing Archaea	16S rRNA	8F: TCCGGTTGATCCTGCC	Harrison et al. (2009)
		958R: YCCGGCGTTGAMTCCAATT	
		344F: <u>ACTGACTGACGGGGYGCAGCAGGCGCGA</u>	
Bacteria	16S rRNA	915R: GTGCTCCCCCGCCAATTCCT	Gantner et al. (2011)
		27F: AGAGTTTGATCCTGGCTCAG	
		1492R: GGYTACCTTGTTACGACTT	
Fungi	18S rRNA	533R: <u>CTCAGCTACATTACCGCGGCTGCTGGCAC</u>	Lane (1991)
		NS1: GTAGTCATATGCTTGTCTC	
		FR1: GCATCACAGACCTGTTATTGCCTC	
Real-time PCR Archaea	16S rRNA	3NDF: <u>ACTCTATATAGGCAAGTCTGGTGCCAG</u>	Hoshino and Morimoto (2008)
		V4_euk_R2: ACGGTATCT(A/G)ATC(A/G)TCTTCG	
Real-time PCR Bacteria	16S rRNA	519F: CAGCCGCCGCGGTAA	Bråte et al. (2010)
		915R: GTGCTCCCCCGCCAATTCCT	
		338F: ACTCCTACGGAGGCAGC	
Fungi	18S rRNA	518R: ATTACCGCGGCTGCTGG	De Bok et al. (2006)
		NS1: GTAGTCATATGCTTGTCTC	
		Fung: ATTCGCCGTTACCGGTG	

The barcode sequences for the pyrosequencing of archaea, bacteria and fungi were underlined.

1.3. Pyrosequencing data analysis

Raw sequences were processed by the microbial ecology community software program Mothur (<http://www.mothur.org/wiki>) (Schloss et al., 2009). Barcodes, primers, and reads with lengths less than 200 bp, and ambiguous bases more than or equal to 1 with a quality score below 25 were removed by Mothur. Homopolymers were also processed with Mothur by the screen command. Further steps for sequence analysis included grouping identical sequences and aligning representative sequences against the SILVA database (<http://www.arbsilva.de>) (Pruesse et al., 2007).

Chimeric sequences were detected using UCHIME and deleted (Edgar et al., 2011). The minimum sequence similarity was 99% to species, 95% to genus, 90% to Class/Order/Family, and 80% to Domain/Phylum (Giongo et al., 2010). A distance matrix and clustering were generated with the furthest neighbor algorithm, and operational taxonomic units (OTUs) defined at 97% and 95% sequence similarity were obtained. The total number of species was estimated with a Chao1 estimator (<http://www.mothur.org/wiki/Chao>). Coverage (<http://www.mothur.org/wiki/Coverage>), rarefaction curves (<http://www.mothur.org/wiki/Rarefaction>) and Shannon index (<http://www.mothur.org/wiki/Shannon>) were also calculated with Mothur at the 97% and 95% similarities, respectively. The 16S/18S rRNA gene sequences derived from PCR and 454 pyrosequencing were submitted to the NCBI Sequence Read Archive under accession number SRA 065107.

1.4. Real-time PCR for archaea, bacteria and fungi in coal mine bioaerosols

Real-time PCR was carried out with the ABI 7300 Sequence Detection System (Applied Biosystems, Foster City, CA, USA). A 25- μ L volume of the system with 12.5 μ L of 2 $\times$ SYBR green mix (Fermentas, USA), 1 μ L of each primer (10 nmol/L), 1 μ L of sample DNA, 0.3 μ L BSA (10 mg/mL, New England Biolabs) and 9.2 μ L of ddH₂O was prepared for amplification. Primer sets 519F/915R, 338F/518R and NS1/Fung were respectively chosen for archaea, bacteria and fungi (Table 1). The real-time PCR was initiated at 95 °C for 2 min, followed by 40 cycles of denaturation for 15 sec at 95 °C, annealing for 30 sec at 58 °C, and elongation for 30 sec at 72 °C. Fluorescence signals were collected at 72 °C during the elongation step. Each DNA template was performed in triplicate. Negative controls with ultra-pure water were added to evaluate PCR reagent contamination.

To estimate archaeal, bacterial and fungal 16S/18S rRNA gene abundances, 10-fold serial dilutions of plasmids were used to generate the standard curves, which contain the full-length copy of the *Methanosarcina* sp. (KC215420) and *Desulfosporosinus* sp. (KC215425) 16S rRNA gene, and the *Geomyces* sp. (KC215423) 18S rRNA gene. Plasmids were extracted using a BioMed Mini Plasmid Kit (BioDve-tech, China) and quantified with the NanoDrop ND-1000 spectrophotometer. Standard curves were acquired and analyzed using the 7300 Real-Time PCR System Sequence Detection Software ver. 1.3 by cycle threshold (Ct) values with the original copy number of the plasmids ($\log_{10}C_0$). The detection limit was defined as the lowest concentration of the 16S/18S

rRNA genes within the linear range of a standard curve for the real-time PCR assay. The slope of the standard curve was appraised to determine the PCR amplification efficiency (E), which was estimated by the formula $E = 10^{-1/\text{slope}} - 1$ (Klein et al., 1999). Copy numbers and total cells were calculated according to the description of Smith et al. (2006), Klappenbach et al. (2001) and Van Doorn et al. (2007). The average rRNA gene copy numbers for archaea, bacteria and fungi were 1.7, 4.2 and 200, respectively.

2. Results

2.1. Microbial diversity and richness determined by pyrosequencing

Microbial communities in bioaerosols were analyzed based on 454 pyrosequencing of 16S or 18S rRNA genes. The 37,191 high quality reads were obtained after optimization by Mothur. The reads of archaea, bacteria and fungi were respectively 6456, 17,986 and 12,749. The average lengths of their sequences were respectively 496, 477 and 433 bp. Diversity indices Chao1, Shannon and Coverage are listed in Table 2. Chao1 suggested that the estimated total species in the bioaerosols were 1.3–1.9 times greater than the observed OTUs. The Shannon diversity indices for archaea, bacteria and fungi were, respectively, 4.71, 6.29 and 3.86 at 97% sequence similarity, and 3.17, 6.00 and 3.18 at 95% similarity. The coverage for all samples was more than 96%. The rarefaction curves for archaea, bacteria and fungi had an increasing trend at 97% sequence similarity, whereas rarefaction curves of archaea and fungi approached saturation at 95% similarity (Appendix A Fig. S1).

Archaea was mainly comprised of Crenarchaeota and Euryarchaeota, among which Crenarchaeota was the dominant class accounted for 99.92% of archaeal reads. Euryarchaeota accounted for 0.03%. At the genus level, the vast majority of sequences were classified as uncultured Crenarchaeota. Sequences belonging to Euryarchaeota were classified as *Methanosarcina* spp. at 0.03%.

For bacteria, a total of 22 phyla, including candidate divisions, were identified, but the full extent of genetic diversity remained unachieved (Fig. 2a). Proteobacteria were the predominant bacteria with 49.7% of total sequences, followed by Bacteroidetes (10.96%), Firmicutes (9.53%), Planctomycetes (7.57%), Acidobacteria (5.73%), Cyanobacteria (3.83%), Actinobacteria (3.71%), Verrucomicrobia (3.25%), Chloroflexi (1.22%) and Armatimonadetes (1.02%). Those phyla with abundance less than 1% were classified as "others".

The top 20 most abundant bacteria and closest genera are shown in Fig. 2b. The most abundant genus belonging to Proteobacteria was *Beijerinckia* (6.62%), followed by *Paracoccus* (6.28%), *Limnohabitans* (2.13%), *Hyphomicrobium* (1.12%), *Methylocystis* (1.07%), *Alcaligenes* (0.9%), *Alysiosphaera* (0.85%), *Nitrosomonas* (0.76%), *Arenimonas* (0.73%) and *Desulfocapsa* (0.63%). Such other genera as *Peptostreptococcus* (1.01%), *Bacillus* (3.48%) and *Sporacetigenium* (1.19%) were from the phylum Firmicutes. *Saprospira* (1.39%), *Flavobacterium* (1.16%), *Flaviumibacter* (0.87%) and *Lacibacter* (0.69%) were from the

Table 2 – Summary of OTUs, diversity and richness of microbial communities in coal mine bioaerosols.

	Reads ^a	97% sequence similarity				95% sequence similarity			
		OTUs ^b	Chao1	Coverage	Shannon	OTUs	Chao1	Coverage	Shannon
Archaea	6456	531	673	97.44%	4.71	209	253	99.23%	3.17
Bacteria	17986	1730	2751	96.35%	6.29	1271	1802	97.82%	6.00
Fungi	12749	448	683	98.53%	3.86	176	224	99.55%	3.18

^a The reads means the high quality reads passed quality controls from 454 pyrosequencing.

^b OTUs: operational taxonomic units.

phylum Bacteroidetes. *Planctomyces* (2.99%), SM1A02 (0.86%) and *Gemmata* (0.96%) were present from the phylum of Planctomycetes.

Methanotrophs involved in the methane oxidative metabolism were part of the bacterial community. Only aerobic methanotrophs were found in the bacterial community in the present study. Aerobic methanotrophs (including both type I and type II methanotrophs) identified by pyrosequencing are listed in Table 3. At the genera level, methanotrophs accounted for 1.97% of total bacterial sequences in the bioaerosol samples. Type I methanotrophs were about 0.90% of total bacteria, including *Methylobacter*, *Methylocaldum*, *Methylomicrobium*, *Methylomonas*, *Methylosarcina* and *Methylosoma*. Type II methanotrophs containing the genus of *Methylocystis*, accounted for 1.07% of total bacteria.

To survey possible pathogens for the miners in the coal mine, the genera containing pathogenic bacteria in the bioaerosol samples were identified (Table 4), which were 1.87% of total bacterial sequences. They were composed of the Gram-positive Actinobacteria (0.02%) and Firmicutes (0.73%) and the Gram-negative γ -Proteobacteria (1.12%). The Actinobacteria bacteria included the potential pathogens of *Corynebacterium* and *Mycobacterium*. However, most of the identified species in the coal mine bioaerosols were attributed to unclassified *Staphylococcus* sp., which are from Firmicutes. *Clostridium tunisiense* and the uncultured *Clostridium* sp. are potential pathogens from the Firmicutes as well. The Gram-negative γ -Proteobacteria contained a series of potential pathogens, e.g., *Acinetobacter*, *Aeromonas*, *Coxiella*, *Enterobacter*,

Escherichia, *Legionella*, *Pseudomonas*, *Serratia* and *Shigella* (Berman, 2012). Among them, *Legionella* and *Coxiella* are common airborne pathogens. *Enterobacterium*, *Scherichia*, *Serratia*, and *Shigella* are typical intestinal infectious pathogens (Guentzel, 1991).

Fungal communities are listed in Fig. 3a. They were dominated by Ascomycota (78.11%), including the orders Capnoidiales, Eurotiales, Hypocreales, Onygenales, Pleosporales and Xylariales. A secondary proportion of pyrosequencing sequences (19.81%) belonged to unclassified fungal phyla. Basidiomycota (1.18%) were another fungal component, including the orders Agaricales and Polyporales.

The genera belonging to the dominant Ascomycota were affiliated to *Alternaria*, *Aspergillus*, *Boeremia*, *Cladosporium*, *Paraconiothyrium*, *Penicillium*, *Pleosporaceae*, *Pseudonectria* and *Trichocomaceae* (Fig. 3b). Potential pathogenic fungi in the bioaerosol samples were identified as *Alternaria alternata*, *Aspergillus penicillioides*, *Cladosporium cladosporioides*, *Penicillium brevicompactum*, as well as the majority of uncultured genera (Table 5).

2.2. Microbial concentration determined by real-time PCR

Standard curves based on serial dilutions of plasmids were obtained (Appendix A Fig. S2). The slopes of the curves for archaea, bacteria and fungi were respectively -3.48 , -3.39 and -3.41 . The PCR amplification efficiency ($E = 10^{-1/\text{slope}} - 1$) between 0.938 and 0.972 was within the theoretically anticipated range of 0.9–1.1, which represented valid amplification reactions. The amplification plots showed that plasmids at

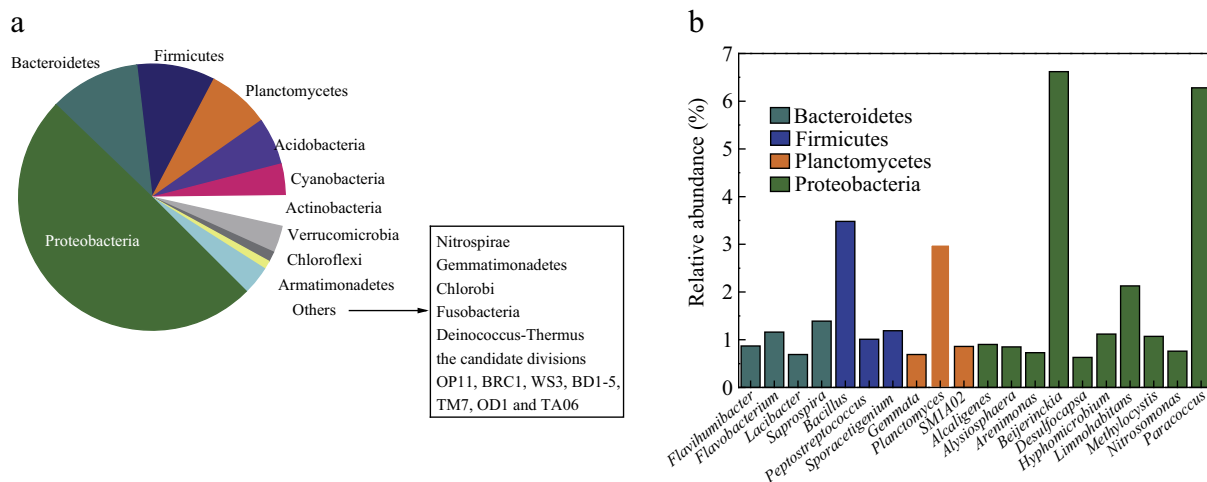


Fig. 2 – Bacterial communities at the phylum level (a) and the top 20 most abundant bacteria and the closest genus (b) identified by pyrosequencing. Those phyla with abundance less than 1% were classified as “others”.

Table 3 – Methanotrophs identified in coal mine bioaerosols.

Methanotrophs	Phylum	Genus	Species	Proportion
Type I	γ -Proteobacteria	<i>Methylobacter</i>	Uncultured <i>Methylobacter</i> sp.	0.43%
		<i>Methylocaldum</i>	Uncultured <i>Methylocaldum</i> sp.	0.31%
		<i>Methylomicrobium</i>	<i>Methylomicrobium album</i> BG8	0.01%
			Uncultured <i>Methylomicrobium</i> sp.	0.10%
		<i>Methylomonas</i>	Uncultured <i>Methylomonas</i> sp.	0.01%
		<i>Methylosarcina</i>	Uncultured <i>Methylosarcina</i> sp.	0.01%
		<i>Methylosoma</i>	Uncultured <i>Methylosoma</i> sp.	0.03%
		<i>Methylocystis</i>	Uncultured <i>Methylocystis</i> sp.	1.07%
Type II	α -Proteobacteria			
Total				1.97%

100, 10 and 100 copies/ μ L were respectively within the linear range of standard curves for archaea, bacteria and fungi. However, the concentrations of plasmids at 10, 1 and 10 copies/ μ L fell outside of the standard curves. Therefore, the detection limit for archaea, bacteria and fungi was 100, 10 and 100 copies/ μ L, respectively. Negative controls were below the detection limits. Based on the standard curves, total archaea, bacteria and fungi in the bioaerosol samples were $1.44 \times 10^8 \pm 0.74 \times 10^8$, $1.02 \times 10^8 \pm 0.58 \times 10^8$ and $9.60 \times 10^4 \pm 1.27 \times 10^4$ cells/ m^3 , respectively (Fig. 4).

3. Discussion

The purpose of the study was to investigate the microbial communities in coal mine bioaerosols. However, we have to emphasize that the results were based on analyses of the samples collected from only one coal mine and one time sampling. The sampling in different time and coal mines had been considered before, but it was not allowed by the management department of coal mines due to the security in the underground coal mine, especially the gassy mine. The 454 pyrosequencing and real-time PCR revealed that microbial communities with high diversity and abundance were present in bioaerosols at the coal mine. The microorganisms, such as methanogens and methanotrophs associated with methane metabolism, and possible potential pathogens closely related to the health risk for coal miners, were especially concerned

in the present study. The research results of coal mine bioaerosols would be helpful in promoting comprehensive understanding on microorganisms in coal mines. To the best of our knowledge, it is the first study on microbial communities in coal mine bioaerosols using such culture-independent methods.

3.1. Microbial diversity and richness in bioaerosols by pyrosequencing

Although air is not a suitable environment for microbial growth, large number of microbes was identified in the bioaerosols of the coal mine by 454 pyrosequencing. It should be pointed out that nested PCR was performed to obtain DNA samples for analysis of microbial community. The nested PCR may introduce a bias and consequently overestimate the diversity in the aerosols (Suzuki and Giovannoni, 1996). However, it was performed in triplicate and low cycles to reduce amplification variability. Furthermore, the nested PCR has been demonstrated to be feasible in the analysis of microbial community (Guo et al., 2012; Klein et al., 2008; Stark et al., 1998).

The diversity index Chao1 was higher than the identified OTUs (Table 2). Rarefaction curves at 97% sequence similarity had an increasing trend and did not reach a plateau, indicating that the full diversity was not captured (Appendix A Fig. S1). Further sequencing might produce more unique OTUs, but high coverage indicated that the additional unique OTUs might be a result of pyrosequencing errors and thus

Table 4 – Abundance of potential pathogenic bacteria identified in coal mine bioaerosols.

Gram	Phylum	Genus	Species	Proportion
G ⁺	Actinobacteria	<i>Corynebacterium</i>	Uncultured <i>Corynebacterium</i> sp.	0.01%
		<i>Mycobacterium</i>	Uncultured <i>Mycobacterium</i> sp.	0.01%
	Firmicutes	<i>Clostridium</i>	<i>Clostridium tunisiense</i>	0.12%
			Uncultured <i>Clostridium</i> sp.	0.09%
G [−]	γ -Proteobacteria	<i>Staphylococcus</i>	Uncultured <i>Staphylococcus</i> sp.	0.52%
		<i>Acinetobacter</i>	<i>Acinetobacter schindleri</i>	0.06%
			Uncultured <i>Acinetobacter</i> sp.	0.07%
		<i>Aeromonas</i>	<i>Aeromonas cavernicola</i>	0.29%
			Uncultured <i>Aeromonas</i> sp.	0.10%
		<i>Coxiella</i>	Uncultured <i>Coxiella</i> sp.	0.02%
		<i>Enterobacter</i>	Uncultured <i>Enterobacter</i> sp.	0.34%
		<i>Escherichia</i>	Uncultured <i>Escherichia</i> sp.	0.01%
		<i>Legionella</i>	Uncultured <i>Legionella</i> sp.	0.01%
		<i>Pseudomonas</i>	Uncultured <i>Pseudomonas</i> sp.	0.17%
		<i>Serratia</i>	Uncultured <i>Serratia</i> sp.	0.04%
		<i>Shigella</i>	Uncultured <i>Shigella</i> sp.	0.01%
Total				1.87

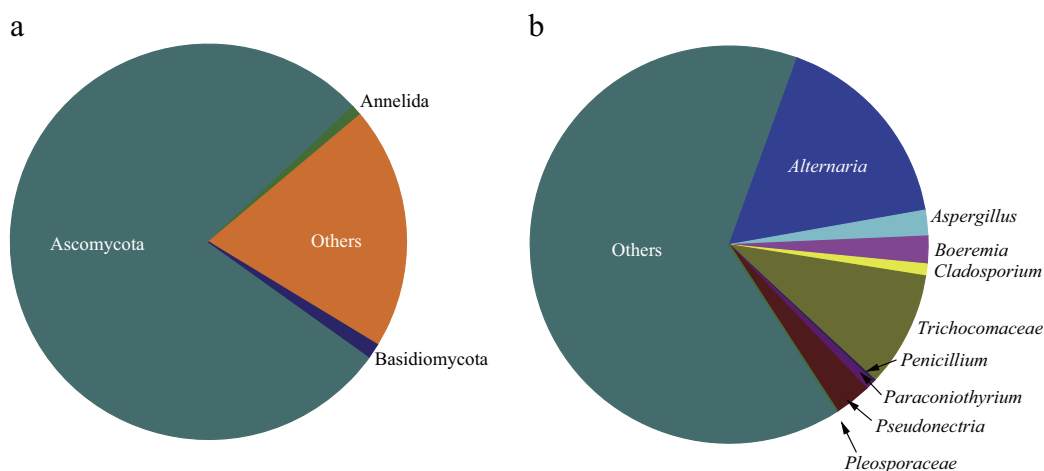


Fig. 3 – Fungal communities at the phylum level (a) and the closet genera of Ascomycota (b) identified by pyrosequencing.

might cause overestimation of microbial diversity in samples. Archaea were dominated by Crenarchaeota in the coal mine bioaerosols. A previous study has suggested that Crenarchaea is ubiquitous to most natural environments and played an important ecological role in complex ecological environments (Dawson et al., 2006). However, the function of Crenarchaeota in coal mine bioaerosols was unknown due to its insufficient information. For the Euryarchaeota, *Methanosarcina*, reported here for the first time in coal mine bioaerosols, is an extremely strict anaerobe utilizing a variety of substrates to produce methane, e.g., acetate, methylamine, hydrogen and carbon dioxide. In previous studies, *Methanosarcina* has been found in coal and produced water in coal mines (Beckmann et al., 2011; Wei et al., 2013), which might be introduced into the aerosols by blasting mining.

Proteobacteria was the dominant bacterial phylum (Fig. 2a). Its genera were affiliated with *Beijerinckia*, *Paracoccus*, *Limnhabitans*, *Hyphomicrobium* and *Methylocystis*. The predominant bacteria identified in the coal mine bioaerosols may be significantly affected by environmental factors in the coal mine. The aforementioned bacteria were reported to be involved in the metabolism of polycyclic aromatic hydrocarbons, e.g., the

predominant genus of *Beijerinckia* and *Paracoccus* are related to degradation of polycyclic aromatic hydrocarbons (Khan et al., 1996; Teng et al., 2010). *Limnhabitans* and *Hyphomicrobium* have been found in coal mine environments (Hahn et al., 2010; Han et al., 2009). *Methylocystis* is methylotrophic bacteria (Dam et al., 2012). Coal fly ash and airborne particulate matter from coal mining processes contain high concentrations of organic and inorganic compounds (Hansen and Fisher, 1980; Korfmacher et al., 1980), which might be the available substrates for microorganisms in coal mine bioaerosols. *Bacillus* and *Planctomyces* were two representative genera from the phyla of Firmicutes and Planctomycetes, which were respectively the common microorganisms potentially present in the air of workplace (Bru Adan et al., 2009; Gilbert and Duchaine, 2009). The *Bacillus* species are obligate aerobes or facultative anaerobes, which can survive under stressful environmental conditions, e.g., desiccation and extreme temperatures. The *Planctomyces* from the phylum of Planctomycetes is an aerobic bacterium, which is typically found in water habitats. In the present study, the high

Table 5 – Abundance of potential pathogenic fungi identified in coal mine bioaerosols.			
Phylum	Genus	Species	Proportion
Ascomycota	Alternaria	<i>Alternaria alternata</i>	16.66%
		<i>Aspergillus penicillioides</i>	2.09%
	Aspergillus	Uncultured <i>Aspergillus</i> sp.	0.01%
		<i>Cladosporium</i>	0.96%
	Cladosporium	<i>cladosporioides</i>	0.01%
		Uncultured <i>Cladosporium</i> sp.	0.01%
	Penicillium	<i>Penicillium brevicompactum</i>	0.05%
		Uncultured <i>Penicillium</i> sp.	0.14%
	Trichocoma	Uncultured <i>Trichocoma</i> sp.	9.40%
	Total		29.32%

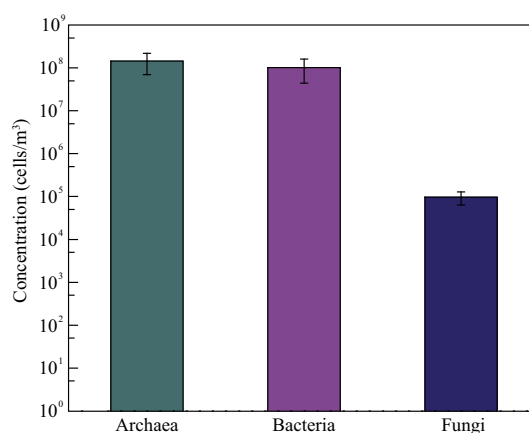


Fig. 4 – Concentrations of archaea, bacteria and fungi in coal mine bioaerosols determined by real-time PCR. Each DNA template was performed in triplicate. Error bars are labeled in the figure representing the standard deviation.

moisture (60%–85%) may be an important factor responsible for the presence of *Planctomyces*.

The majority of fungal sequences were attributable to Ascomycota (Fig. 3a). Various studies have indicated that Ascomycota has been the most common fungi in bioaerosol samples in residential and industrial environments (Balasubramanian et al., 2012; Teixeira et al., 2013). The main genera of fungi identified in this study were *Alternaria*, *Aspergillus*, *Cladosporium*, and *Penicillium*. They have also been found in the bioaerosol samples from the agricultural industries, lumber industries and wastewater treatment plants and are known to be allergenic (Gilbert and Duchaine, 2009).

3.2. Microbes involved in methane metabolism

Methanogens and methanotrophs are the main functional microbes associated with methane metabolism. In the coal mine, both of them were detected in the bioaerosol samples.

As noted above, the *Methanosarcina* spp. belonging to the methanogenic archaea was rare (0.03%) in archaeal sequences. Due to the strict anaerobic characteristics of *Methanosarcina*, they may inhabit in the coal (Beckmann et al., 2011; Tang et al., 2012). The identified *Methanosarcina* may be dead in the aerobic environment and may be derived from coal in the coal mine and brought in to air during coal exploitation.

The aerobic methanotrophs oxidize methane to carbon dioxide in strictly aerobic environments. Here, methane was the carbon and energy resource for the growth of methanotrophs. The aerobic methanotrophs are typically divided into two types, Type I and Type II. These two types were both found in the bioaerosol samples, but Type I methanotrophs were slightly less than Type II (Table 3). Previous studies had demonstrated that oxygen and methane were two significant factors for the distribution of methanotrophs (Amaral and Knowles, 1995). High O₂ and low CH₄ concentrations were more suitable for Type I methanotrophs. By contrast, Type II methanotrophs grew better in low O₂ and high CH₄ concentration environments. Interestingly, our research showed that these two different microbes coexisted in the coal mine.

For the Type I methanotrophs from the γ -Proteobacteria, the ribulose monophosphate (RuMP) pathway for formaldehyde fixation was the metabolic pathways. Methane was fixed by these microbes in the form of formaldehyde and then oxidized to formyl and ultimately to CO₂. Type II methanotrophs belong to α -Proteobacteria, and the *Methylocystis* fixed formaldehyde by the serine pathway (Lidstrom, 2006).

Methanotrophs in gas habitat have never been previously reported. However, the utilization of methanotrophs to remove methane from coal mine has proved to be viable (Mao et al., 2011). A previous study demonstrated that methanotrophs grown in gas phase bioreactors were capable of removing 90% of the methane from methane/air mixtures (Apel et al., 1991). In the coal mine, methanotrophs might be potentially associated with methane oxidation, and could partially alleviate the risk of gas explosion in coal mines.

3.3. Potential pathogens in the bioaerosol sample

As aforementioned, long-term exposure to a coal mine environment is responsible for many occupational diseases

(Dai et al., 2012; Donoghue, 2004). The abiotic hazards such as coal dust, and pathogens are both important pathogenic factors for coal miners. A previous study had demonstrated that microorganisms adhered to sand dust were associated with allergic lung inflammation (Ichinose et al., 2008). However, the relationship between coal dust and microorganisms was unclear due to shortage of relevant research literatures.

In the present study, the genera containing potential pathogens were identified by 454 pyrosequencing. For bacteria, the identified Gram-positive and Gram-negative bacteria included a wide variety of potential pathogens (Table 4). Exotoxins and endotoxins could be produced by these bacteria. Long term exposure to exotoxins or endotoxins might cause respiratory disorders such as chronic airway obstruction, byssinosis and bronchitis, and eye and skin irritations (Basinas et al., 2012; Liesivuori et al., 1994). For fungi, the potential pathogens (Table 5) were known to be responsible for many invasive infections, as both immunocompromised and healthy individuals may be infected by them. Previous researchers have reported that the inhalable hypha and spores from such fungi species were associated with infections as skin allergy and asthma (De Pauw et al., 2008). Such by-products as mycotoxins were implicated in lung, gastrointestinal and carcinogenic diseases (Fung and Clark, 2004). Lung diseases are typically diagnosed in the coal mining industry. The accumulation of coal dust in the lungs was an important pathological incentive. However, the pathogens were also associated with many respiratory and pulmonary symptoms, e.g., dry cough, cough with phlegm, hoarseness and atopy (Simpson et al., 2006).

At the species level, the identified bacterium *Acinetobacter schindleri* in the present study has been reported as an opportunistic pathogen in hospitalized patients with underlying predispositions (Dortet et al., 2006). *Aeromonas cavernicola* could cause opportunistic systemic disease in immunocompromised patients. Infections caused by the *A. cavernicola*, including acute gastroenteritis, sepsis and other diseases in the respiratory tract, biliary tract and urinary tract, have been an issue in recent years (Janda and Abbott, 2010). *A. alternata* was the most abundant potential pathogenic fungi observed in bioaerosols (16.66%). It is recognized as a major cause of fungal allergies, e.g., upper respiratory tract infections and asthma (Neukirch et al., 1999). The identified *A. penicillioides* in the present study was viewed to be associated with mites and dust lice in damp environments (Kort et al., 1990). The *C. cladosporioides* is typically viewed as a fungal plant pathogen that affects wheat. However, it may cause phaeohyphomycosis in clinical entity as well (Vieira et al., 2001). The spores of *P. brevicompactum* were reported to be capable of causing inflammatory and cytotoxic responses (Rand et al., 2005).

However, it should be pointed out that the SILVA or NCBI database has a preference to human pathogens only with cultured species, but most of the sequence information obtained was based on uncultured microorganisms of the coal mine bioaerosols in this study. Therefore, the large number of sequences was classified as uncultured at the genus level (1.87% and 29.32% for bacteria and archaea, respectively), which included both pathogenic and non-pathogenic microbial strains.

3.4. Microbial concentrations determined by real-time PCR

Microbial concentrations in workplaces are a significant factor in industrial health assessment (Macher et al., 1999). Microbes which are considered harmless may become pathogenic at higher concentrations in specific environments (Eduard, 1996).

In the present study, real-time PCR revealed high archaeal and bacterial concentrations in coal mine bioaerosols (10^8 cells/m³, Fig. 4). The archaeal and bacterial concentrations were similar with studies of swine confinement building (Nehme et al., 2009; Nehme et al., 2008), which were up to 10^8 . The fungi concentration was 9.60×10^4 cells/m³ in bioaerosols. The high microbial concentrations in coal mine bioaerosols could have a health effect for coal miners exposed year round to it. For example, long term exposure to high bacterial concentration may cause respiratory symptoms (Douwes et al., 2003).

It should be emphasized that the microbial concentrations of coal mine bioaerosols were only determined by real-time PCR due to the difficulty of cultivation in lab. Moreover, this was the first study on microbial abundance of coal mine bioaerosols, and no relevant literatures were available for comparison of studies. Furthermore, real-time PCR-based occupational exposure limits have not been established. Nevertheless, the exposure to such high airborne microbial levels might be unsafe to coal miners although it is necessary to obtain further experimental data to support this point.

4. Conclusions

In summary, 454 pyrosequencing combined with real-time PCR provided deep and comprehensive survey of microbial communities in bioaerosols of the coal mine. It was clear that: (1) There were abundant and diverse microorganisms in coal mine bioaerosols based on these molecular techniques; (2) Microorganisms associated with methane metabolism including the methanotrophs of Type I and Type II coexisted in coal mine bioaerosols, which might be a possible factor in the reduction of methane emissions to the atmosphere; (3) Bacterial and fungal genera containing potential pathogens and several identified pathogens such as *A. schindleri*, *A. cavernicola*, *A. alternata*, *A. penicillioidea*, *C. cladosporioides*, and *P. brevicompactum* were observed in coal mine bioaerosols; (4) Microbial concentrations in the bioaerosols were significantly high.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found in online version at <http://dx.doi.org/10.1016/j.jes.2014.07.035>.

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