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Long-term and high-concentration heavy-metal contamination strongly influences the microbiome and functional genes in Yellow River sediments

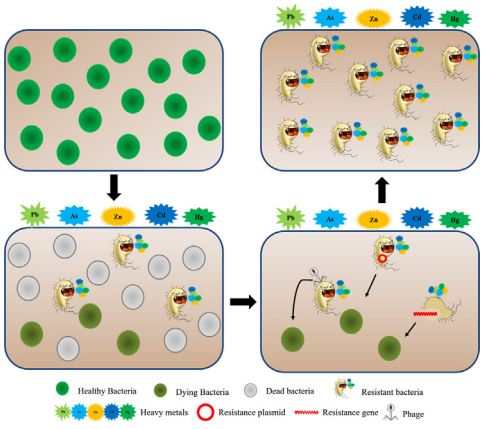
Yong Chen [a,1], Yiming Jiang [a,b,*,1], Haiying Huang [a,b,1], Lichao Mou [c,d], Jinlong Ru [e], Jianhua Zhao [f], Shan Xiao [f]

[a] Ministry of Education Key Laboratory of Cell Activities and Stress Adaptations, School of Life Science, Lanzhou University, Tianshuinanlu #222, Lanzhou, Gansu 730000, PR China
[b] Institute of Virology (VIRO), Helmholtz Zentrum München, German Research Center for Environmental Health, Ingolstädter Landstr. 1, 85764 Neuherberg, Germany
[c] Signal Processing in Earth Observation (SiPEO), Technische Universität München, 80333 Munich, Germany
[d] Remote Sensing Technology Institute (IMF), German Aerospace Center (DLR), 82234 Wessling, Germany
[e] Department of Bioinformatics, Technische Universität München, Wissenschaftzentrum Weihenstephan, Maximus-von-Imhof-Forum 3, D-85354 Freising, Germany
[f] Shanghai Majorbio Bio-pharm Technology Co., Ltd., Building 3, Lane 3399, Kangxin Road, International Medical Zone, Pudong New Area, Shanghai, PR China

HIGHLIGHTS

- Sediments in Dongdagou contained high concentrations of cadmium, arsenic, lead, and mercury.
- The microbiome has acquired resistance to long-term heavy-metal pollution.
- *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* were the core functional phyla.
- The sediment contains genes related to DNA repair and heavy-metal resistance.
- Viral abundance in Dongdagou was higher than in Maqu.

GRAPHICAL ABSTRACT



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ABSTRACT

The world is facing a hard battle against soil pollution such as heavy metals. Metagenome sequencing, 16S rRNA sequencing, and quantitative polymerase chain reaction (qPCR) were used to examine microbial adaptation mechanism to contaminated sediments under natural conditions. Results showed that sediment from a tributary of the Yellow River, which was named Dongdagou River (DDG) supported less bacterial biomass and owned lower richness than sediment from Maqu (MQ), an uncontaminated site in the upper reaches of the Yellow River. Additionally, microbiome structures in these two sites were different. Metagenome sequencing and functional gene annotations revealed that sediment from DDG contains a larger number of genes related to DNA recombination, DNA damage repair, and heavy-metal resistance. KEGG pathway analysis indicated that the sediment of DDG contains a greater number of enzymes associated with heavy-metal resistance and reduction.

* Corresponding author at: MOE Key Laboratory of Cell Activities and Stress Adaptations, School of Life Sciences, Lanzhou University, Lanzhou 730000, PR China.
E-mail address: yiming.jiang@helmholtz-muenchen.de (Y. Jiang).
[1] These authors contributed equally to this work.

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16S rRNA sequencing
Sediment

Additionally, the bacterial phyla *Proteobacteria*, *Bacteroidetes*, and *Firmicutes*, which harbored a larger suite of metal-resistance genes, were found to be the core functional phyla in the contaminated sediments. Furthermore, sediment in DDG owned higher viral abundance, indicating virus-mediated heavy-metal resistance gene transfer might be an adaptation mechanism. In conclusion, microbiome of sediment from DDG has evolved into an integrated system resistant to long-term heavy-metal pollution.

1. Introduction

Due to the long-term toxicological and detrimental effects of most heavy metals on the environment and humans (Kjeldsen et al., 2002; Järup, 2003), heavy-metal pollution has been regarded as an important environmental issue in many countries. Among them, Cadmium (Cd), arsenic (As), lead (Pb), mercury (Hg), and chromium (Cr) are considered to be the most toxic substances (Alloway, 2013), and potentially damage the ecosystems. For instance, Cd can interfere with the metabolism of calcium, which may lead to kidney and skeletal damage (Perfus-Barbeoch et al., 2002; Johri et al., 2010). Therefore, Cd has been classified as a Group 1 human carcinogen by the International Atomic Research Council (Järup, 2003). Arsenic is also widely presented in soil, water, and air. Ingestion of inorganic As causes gastrointestinal symptoms, cardiovascular diseases and neurological disorders (Alam et al., 2003; Järup, 2003). Pb poisoning has various symptoms, including headache, abdominal pain, and neurological disease. In addition, inorganic Pb penetrates the blood-brain barrier of children (Järup, 2003). Acute Hg poisoning can lead to lung damage (Şener et al., 2003). Since these toxic heavy metals are mainly absorbed through the food chain (Duruibe et al., 2007) and drinking water (Wongsasuluk et al., 2014), it is important and necessary to accurately determine heavy-metal concentrations, and to establish effective measures for the elimination of heavy-metal pollutants.

Baiyin City (in Gansu Province, China) is an industrial city rich in mineral resources. Since the 1960s, there has been a considerable rise in development and construction. Prior to 1995, the annual discharge of heavy metals contained acid wastewater from the Baiyin Nonferrous Metal Company and reached approximately 19 million tons. The indigenous soil, water, and sediments have been heavily polluted by various heavy metals for decades. Wastewater from metal mills ran straight into Dongdagou River (DDG), which is a tributary of the Yellow River (Yu et al., 2006). Therefore, the 38-km-long Dongdagou River is the largest source of pollution in the upper reaches of the Yellow River, in which the contents of Cd, Hg, and As exceed the national secondary standard (Yu et al., 2006; Liu et al., 2016; Liu et al., 2016). In particular, long-term sedimentary background values over 1400–2200 times that of Cd pollution. For decades, residents along DDG have suffered from the detrimental effects of heavy-metal contamination. Even more serious is the threat to the environment of the lower Yellow River. However, knowledge about the specific impacts of long-term heavy-metal pollution on natural ecosystems is limited (Si et al., 2014; Liu et al., 2016).

Microorganisms in natural environment play an import role in maintaining soil biological activity and may reduce the levels of pollutants (Torsvik and Øvreås, 2002; Valls and De Lorenzo, 2002; Díaz, 2010). Microbial communities are, additionally, also highly sensitive to environmental changes, therefore, they are considered indicators of local environmental conditions (Blakely et al., 2002; Schloter et al., 2003). However, the characteristics of microbial communities and the specific functions of different microorganisms are not clearly understood (Gillan et al., 2015; Keshri et al., 2015). Although studies have sought to elucidate changes in the diversity of the microbial communities in soil, groundwater, and sediments which were polluted by heavy metals (Kandeler et al., 2000; Kelly et al., 2003; Epelde et al., 2015), changes of microbes' functional genes in long-term heavy-metal contaminated sediments in the Yellow River have rarely been analyzed. Here, we hypothesized that, those indigenous microbial communities that have become adapted to long-term heavy-metal contamination play a key role in the remediation of heavy-metal pollutants.

The main goal of the present study was to examine how the microbial communities and their functional genes respond to the long-term accumulation of heavy metals. To this end, we analyzed sediments from the heavily polluted Dongdagou River, using metagenome sequencing, 16S rRNA sequencing, and qPCR to determine the adaption mechanisms of the microbiome in sediments. The approach adopted in this study is potentially widely applicable to the analysis of microbial communities in sediments contaminated by heavy metals. Moreover, our results may be used to advise on the effective remediation of the tributary river Dongdagou of the Yellow River.

2. Materials and methods

2.1. Sample collection

In early October 2014, sediment samples were collected from three sites of the Yellow River in Gansu Province, China: the Yellow River in the Maqu area (MQ: 33.959° N, 102.082° E), which is the upper reach of the Yellow River; Dongdagou River (DDG: 36.432° N, 104.379° E), which is a heavy-metal contaminated tributary river; and the Yellow River in the Baiying area (BY: 36.429° N, 104.394° E) (Fig. 1), where is located in the lower reach of MQ and DDG. In this study, we employed a nested five-point sampling strategy. In detailed, at each sampling site, surface sediments (0–10 cm) were sampled in 5 points (Chang et al., 2004), and the distance between each point was above 10 m. Within the 5 points, 3 random subsamples out of the standard five-point sampling method were collected in each point. Once sampled, all the samples from each site were manually pooled into one sample mixing to visual homogeneity (Yuan et al., 2002), and were placed in sterile boxes and transported to the laboratory. The air temperature was measured on-site. After mixing, each sediment sample was divided into two parts. One was stored at 4 °C for physical and chemical analyses, whereas the second one was stored at −80 °C until DNA extraction.

2.2. Physical and chemical analyses

Sediment pH was measured after shaking a sediment: water (1:5 wt/vol) suspension for 30 min (Shen et al., 2013). Total organic carbon (TOC), total nitrogen (TN), and total phosphorus (TP) were

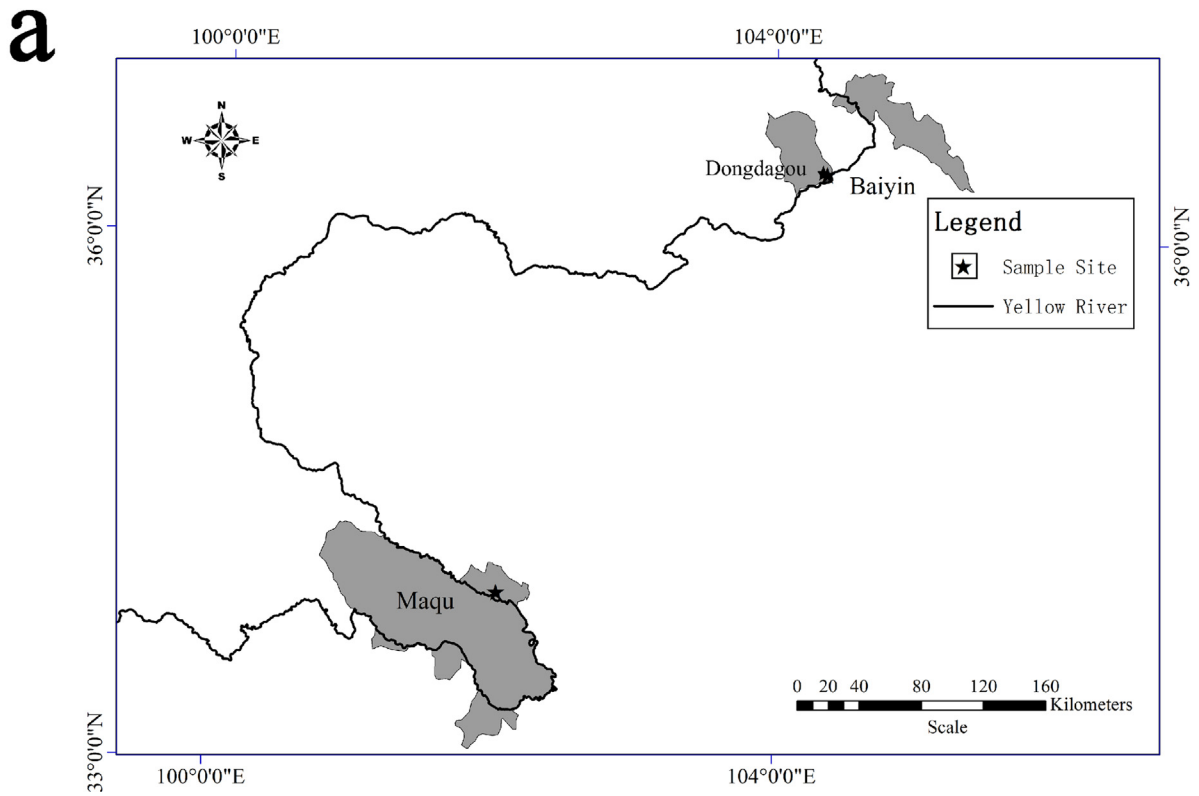

Fig. 1. Map of the sampling site. (a) Sediment samples were collected from the Maqu area (MQ: 33.959° N, 102.082° E, Gansu Province, China), Dongdagou River (DDG: 36.432° N, 104.379° E, Gansu Province, China), and the Yellow River in the Baiyin area (BY: 36.429° N, 104.394° E, Gansu Province, China). (b) Satellite imagery shows location of DDG and BY, which are closed to each 7

measured as described previously (Walkley and Black, 1934). Concentrations of Cr, nickel (Ni), copper (Cu), zinc (Zn), Cd, Pb, As, and Hg were determined by atomic absorption spectrometry (Olmedo et al., 2010; Wu et al., 2016, 2016).

2.3. DNA extraction

DNA was extracted from 0.5 g of wet sediment using A.E.Z.N.A.™ Soil DNA Kit (Omega Biotek, Inc., Norcross, GA) according to the manufacturer's instructions. Ten aliquots of DNA were extracted per sample. The extracted DNA samples were dissolved in 30 μL of Tris-EDTA buffer, and then quantified using a NanoDrop™ 2000 spectrophotometer (Thermo, USA). DNA extractions with $OD_{260/280nm}$ around 1.80, and $OD_{260/230nm}$ above 1.80, were pooled into two aliquots for 16S rRNA sequencing and metagenome sequencing. All DNA samples were stored at −80 °C until sequencing (Zhou et al., 2016; Pei et al., 2018).

2.4. Abundance of total microbes

Total microbial abundance in sediment samples was measured using absolute quantitative polymerase chain reaction (qPCR) as described in a previous study (Wu et al., 2016, 2016; Yu et al., 2016). Bacterial 16S rRNA was amplified using the primer pair P1 (341F) and P2 (534R), the sequences of which are 5′-CCTACGGGAGGCAG CAG-3′ and 5′-ATTACCGCGGCTGCTGG-3′, respectively (Muyzer et al., 1993). After PCR amplification and purification, the purified products were ligated into the pGEM-T Easy Vector (Promega Corporation, Madison, WI, USA). *Escherichia coli* DH5α cells were transformed using the constructs. Subsequently, plasmids were extracted using Plasmid Mini Kit I (OMEGA) according to the manufacturer's instructions. Gene expression levels and absorbance were measured by qPCR using SYBR®Premix Ex Taq™ GC kit (TAKARA, Japan) in a Real-Time PCR Detection System (Bio-Rad). PCR reactions were performed following the manufacturer's instructions under the following conditions: an initial incubation at 95 °C for 30 s, followed by 35 cycles at 95 °C for 60 s, 55 °C for 60 s, and 72 °C for 2 min. Each sample was used in triplicates, and the average values were used for quantification.

2.5. 16S rRNA gene sequencing and analysis

16S rRNA sequencing was performed on an Illumina MiSeq platform by analyzing the V4 region using the barcoded primer pair 515F 5′-GTGCCAGCMGCCGCGGTAA-3′ and 907R 5′-CCGTCAATTCC TTTRAGTTT-3′ (Zhou et al., 2015). Each DNA sample was amplified in 30-mL reaction mixtures using the following PCR program: initial denaturation at 94 °C for 5 min, followed by 30 cycles of denaturation at 94 °C for 45 s, primer annealing at 58 °C for 45 s, and extension at 72 °C for 45 s, and a final extension of 10 min at 72 °C. The PCR products were purified, mixed, and sent to Majorbio, Inc. (Shanghai, China) for sequencing. Raw sequences were demultiplexed and quality-filtered using the QIIME pipeline, according to previously described methods (Caporaso et al., 2010; Mason et al., 2014). Operational taxonomic units (OTUs) were assigned from the reads at 97% identity using an open-reference OTU-picking protocol with UCLUST (Edgar, 2010). Mothur version v.1.30.1 (Schloss et al., 2011) was used to calculate the alpha-diversity indices, including Ace and Chao1, which reflect the richness, and Shannon and Simpson calculators were used to estimate diversity. Other analyses were performed in R using the Vegan package (Wang et al., 2012).

Table 1
Physicochemical and biological profile of the sediment samples.

Samples	Cr	Ni	Cu	Zn	Cd	Pb	As	Hg	TN	TOC	TP	pH	AT	Bacteria numbers
	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	mg/kg	g/kg	g/kg	g/kg		°C	Log copies/L
MQ	59.1 ± 0.44	26.2 ± 0.14	20.8 ± 0.73	66.1 ± 2.41	0.2 ± 0.015	20.1 ± 0.51	12.9 ± 0.34	0 ± 0.002	7.6 ± 0.034*	18.8 ± 0.16	4.2 ± 0.18*	6.3 ± 0.06	1–14	9.16 ± 0.08
DDG	32.6 ± 1.34*	14.7 ± 0.23*	57 ± 0.89*	330.2 ± 8.30*	5.9 ± 0.12*	153.7 ± 2.15*	57.1 ± 0.79*	1.3 ± 0.022*	0.4 ± 0.01	2.3 ± 0.48	2.0 ± 0.04	6.6 ± 0.04	5–21	6.02 ± 0.05*
BY	55.3 ± 1.56	23.9 ± 0.39	19.7 ± 0.98	54.8 ± 2.99	0.3 ± 0.001	21.2 ± 1.40	8.8 ± 0.22	0.1 ± 0.003	0.4 ± 0.01	2.3 ± 0.36	2.1 ± 0.04	6.8 ± 0.02	5–21	8.76 ± 0.06

Data are the means ± standard deviation (SD; n = 3). * indicates a statistically significant difference at $P < 0.05$. TN, total nitrogen; TOC, total organic carbon; TP, total phosphorus; AT, daily min-maximum air temperatures.

2.6. Metagenome sequencing and annotation

Metagenomic shotgun sequencing libraries were prepared and then sequenced by Majorbio, Inc. (Shanghai, China) using the HiSeq 2000 platform. This approach generated 225,247,148 raw reads and 22,749,961,948 total bases for two samples (DDG and BY). Sequences were cleaned and assembled using Seqprep, Sickle, BWA, and SOAPdenovo (Version 1.06), and the length of contigs >500 bp were retained for further bioinformatics analyses. MetaGene software was used for ORF prediction, and CD-HIT was used to build a non-redundant gene catalog. Taxonomic assignment of the predicted genes was carried out using BLASTP alignment against the integrated non-redundant (NR) database of the National Center for Biotechnology Information (NCBI) (Tatusov et al., 2003). BLASTP was also used to search the protein sequences of the predicted genes in the evolutionary genealogy of genes: non-supervised Orthologous Groups (eggNOG) database (Jensen et al., 2008). The NR gene catalog was aligned against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database (Kanehisa et al., 2004) by BLAST (Version 2.2.28+) and assigned KEGG functional annotation by KOBAAS 2.0 according to previously described methods (Qin et al., 2010).

2.7. Statistical analyses

All statistical analyses were conducted using R. Differences in the physicochemical profiles of sediment samples were tested by one-way analysis of variance (ANOVA). $P < 0.05$ was considered to indicate a statistically significant difference.

3. Results

3.1. Physical, chemical, and biological properties of the samples

In early October 2014, sediment samples were taken from three sample sites, which were Maqu (MQ), Dongdagou (DDG) and Baiyin (BY) (Fig. 1). The min-maximum air temperatures on the day of sampling were as follows: MQ (1–14 °C), and (DDG and BY, 5–21 °C). Moreover, there were no significant differences among the three sediment samples in total organic carbon (TOC: 18.88, 2.3, and 2.3 mg/kg, respectively) and pH (6.3, 6.6, and 6.8, respectively), however, the concentrations of Cr, Ni, Cu, Zn, Cd, Pb, As, Hg, total nitrogen (TN), and total phosphorus (TP) of the sediment samples were significantly different ($P < 0.05$; Table 1). The concentrations of TN and TP were higher in MQ than in DDG and BY, whereas the concentrations of most of the heavy metals analyzed (Cu, Zn, Cd, Pb, As, and Hg) were highest in the DDG samples. According to the Environmental Quality standards for Soils of China (GB15618-1995) (Yang et al., 2014), concentrations of heavy metals are classified into five classes (I, II, III, IV, and V, which correspond to clean, relatively clean, normal, polluted, and moderately to heavily polluted, respectively). The concentrations of Cr and Ni in the three sediment samples were classified as class I, whereas concentrations of Hg, Cu, and Pb were classified as class II in the DDG sample, and class I in the MQ and BY samples. The concentrations of Zn, As, and Cd were considerably higher (up to 2- to 5.9-times higher) than the specified safety limits (Fig. S1).

3.2. Overview of 16S rRNA sequencing and metagenome sequencing

In order to investigate the diversity and structure of the microbial communities, we used Illumina high-throughput sequencing technology to sequence the 16S rRNAs. After filtering the low-quality reads and trimming the adapters and barcodes, there were 60,488 effective sequences with an average length of 395.84 bp of microbiota generated from the three sediment samples: 22,359, 26,422, and 23,381 sequences on average for the samples collected from MQ, DDG, and BY, respectively. Coverages of 0.9611, 0.9927, and 0.9856 were achieved, respectively (Table 2). The shape of the rarefaction and Shannon-Wiener curves tended to approach the saturation plateau, indicating that bacterial richness was complete (Data not shown). The sequences were assigned to 1,150 OTUs of bacteria with a threshold value of 0.97.

Metagenomic sequencing was used to detect the functional genes between DDG and MQ. By performing metagenome sequencing, we obtained 225.25 million raw reads with a total of 22.75 billion bps. After trimming, 17.30 million clean reads were remaining, and after these had been assembled, 55,263 contigs comprising 59,341,455 bases were obtained (Supplementary Table 1).

Structure and abundance of bacterial community were analyzed using 16S rRNA sequencing and metagenome sequencing. Cluster tree based on Bray-Curtis distance showed that results from two sequencing methods of each sample cluster into one group, indicating a high similarity of results from 16S rRNA sequencing and metagenome sequencing. In addition, compared to the similarity of MQ, the microbial structure of DDG was more similar to that of BY (Fig. S2).

3.3. Richness and diversity of the bacterial community

Based on the OTUs number, the sediment sample from BY was found to have the richest diversity, with an OTUs number of 525, whereas the samples from DDG and MQ displayed considerably lower richness, with OTUs numbers of 320 and 305, respectively. Chao1 and Ace indices are generally used to calculate OTUs numbers. In this study, the patterns of Ace and Chao1 indices of the three samples were very similar to that of the OTUs numbers. In detailed, the community of BY had the maximum richness, with Ace index of 542 and Chao1 index of 539, whereas, the richness of the samples from DDG (Ace index of 338, Chao1 index of 344) and MQ (Ace index of 363, Chao1 index of 389) was lower than those of the BY sample. Similarly, Simpson and Shannon indices indicated that BY showed the greatest diversity (Simpson index of 0.0125 and Shannon index of 5.29), followed closely by that of MQ (0.0124 and 4.88), whereas the sediment sample of DDG displayed considerably lower diversity (0.0387 and 4.24; Table 2).

3.4. Structure of the bacterial community in different sediment samples

Venn diagram shows unique and shared bacterial OTUs in the three sediment samples. Here, sediment samples from BY, DDG, and MQ contained 297, 139, and 77 unique OTUs, respectively, whereas, the three samples had 140 OTUs in common. Additionally,

Table 2
Statistics of the trimmed sequences and alpha-diversity of the bacterial communities.

Sample ID	Primers	Sequences	Bases (bp)	Average length (bp)	Coverage	Reads	OTU	Ace	Chao	Shannon	Simpson
MQ	515F-907R	10,685	4,235,305	396.38	0.9611	3743	305	363 (341,398)	389 (352,457)	4.88 (4.84,4.92)	0.0124 (0.0117,0.0131)
DDG	515F-907R	26,422	10,438,578	395.07	0.9927	17,245	320	338 (329,356)	344 (330,337)	4.24 (4.22,4.27)	0.0387 (0.0373,0.0402)
BY	515F-907R	23,381	92,6917	396.06	0.9856	11,125	525	542 (534,557)	539 (531,558)	5.29 (5.26,5.31)	0.0125 (0.0118,0.0131)
Total	–	60,488	15,600,800	395.84	–	–	–	–	–	–	–

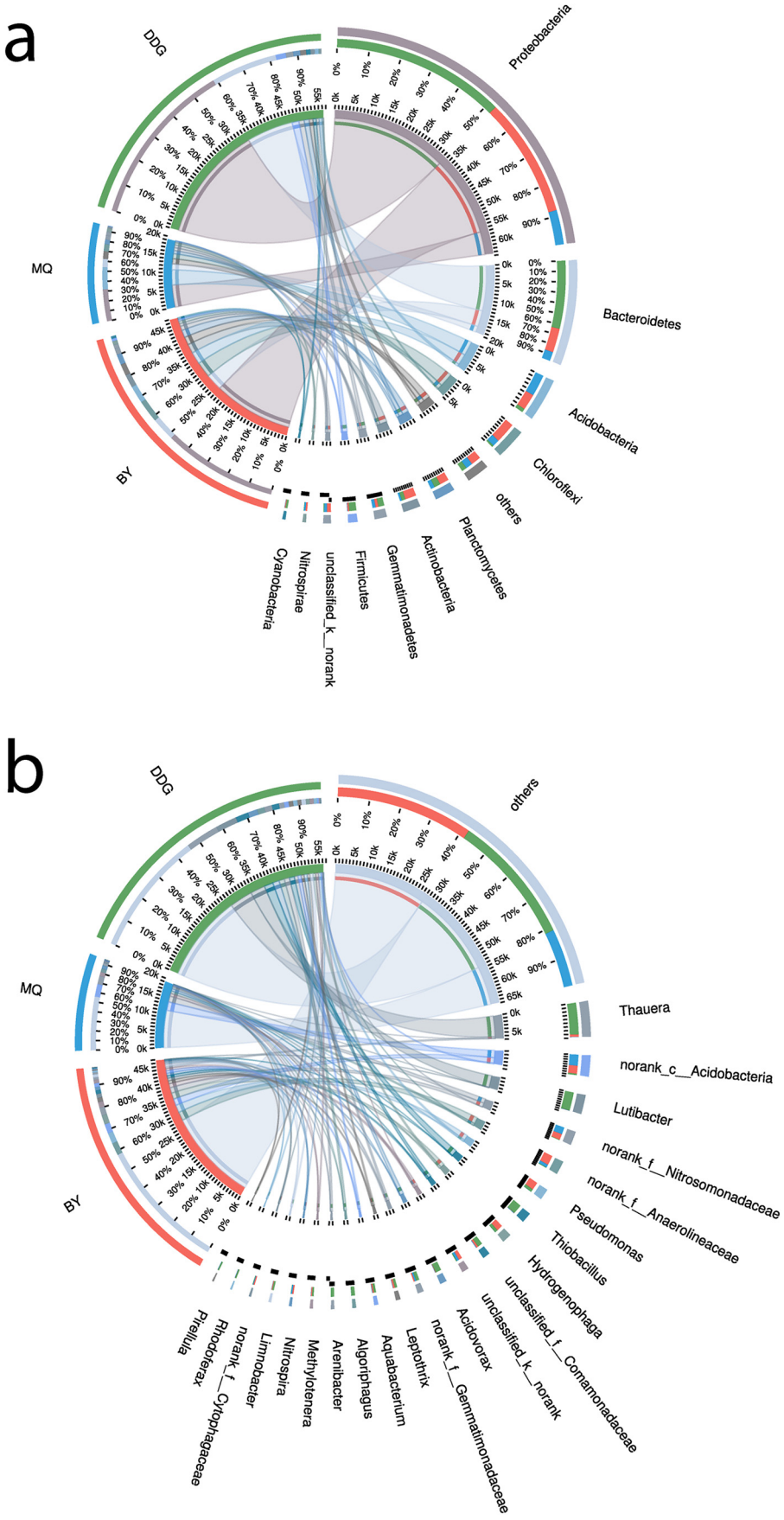

Fig. 2. Composition and abundance of the sediment samples from Dongdagou (DDG), Baiyin (BY) and Maqu (MQ) at phylum (a) and genus (b) levels. Relationships between samples and phyla/genera are showed in Circos figures.

DDG and MQ shared a minimum of 157 OTUs, while the OTUs shared by DDG-BY and BY-MQ were 306 and 366, respectively (Supplementary Fig. S3).

Shared bacterial phylum analysis based on the Venn diagram showed that the three sediment samples had 15 phyla in common, accounted for 99.03%, 98.89%, and 98.56% in total in BY, DDG, and MQ, respectively (Fig. S4). The Circos figure and heatmap show the distribution of bacterial phyla in the three sediment samples (Fig. 2a and Fig. S2). *Proteobacteria* was the most abundant phylum in all samples, accounting for 57.9%, 61.4%, and 36.1% of effective bacterial sequences from BY, DDG, and MQ, respectively. The next most dominant phylum in the BY and DDG samples was *Bacteroidetes*, with a prevalence of 10.7% and 24.06%, respectively, whereas in the sample MQ, the second most dominant phylum was *Acidobacteria*, with a prevalence of 28.61%. Within the phylum *Proteobacteria*, *Betaproteobacteria* was the most abundant class, followed by *Gammaproteobacteria* in sediment samples from MQ and BY, and by *Alphaproteobacteria* in the sample from DDG (Fig. S4f).

There were 122 core bacterial genera, whereas samples BY, DDG, and MQ contained 83, 53, and 10 unique genera, respectively (Fig. S5). Differences in bacterial community structures were seen among the three samples, particularly for the dominant genera. The predominant genera in samples DDG and BY were *Thauera* (13.96%) and *Hydrogenophaga* (9.79%), respectively, both of which belong to *Proteobacteria.* However, in MQ, a genus belonging to norank subgroup-6 of *Acidobacteria* was the predominant genus, with a representation of 19.85%. The next most dominant genera varied among the three samples. Detailly, they were *Luteibacter* (8.73%) in the DDG, *Pseudomonas* (1.50%) in BY, and an unclassified genus belonging to *Nitrosomonadaceae* (13.67%) in MQ (Fig. 2b). Compared with the samples BY and MQ, there were more dominant genera in DDG, including *Luteibacter*, *Thiobacillus*, *Acidovorax*, *Algoriphagus*, *Albidiferax*, *Ramlibacter*, *Methyloversatilis*, and *Pirellula* (Fig. 2b and Fig. S6).

3.5. Correlation between bacterial community, environmental parameters and phyla

In this study, air temperature and twelve physico-chemical parameters, including pH, concentrations of Cr, Ni, Cu, Zn, Cd, Pb, Ag, Hg, TOC and TP, were taken into consideration to evaluate the relative contributions to bacterial community and phyla. Eight parameters with high contributions were listed in CCA (Fig. 3). Among them, heavy metals, including Hg, Cd, Pb, Cu, Zn and As, were positively related with samples from DDG. For correlation between environmental indicators and phyla, results showed that parameters regarding heavy metals contributed positively to *Proteobacteria*, *Cyanobacteria*, *TA06*, *Tenericutes*, *Firmicutes*, *Bacteroidetes*, while contributed negatively to phyla including *Verrucomicrobia*, *Actinobacteria* and *Chloroflexi* etc.

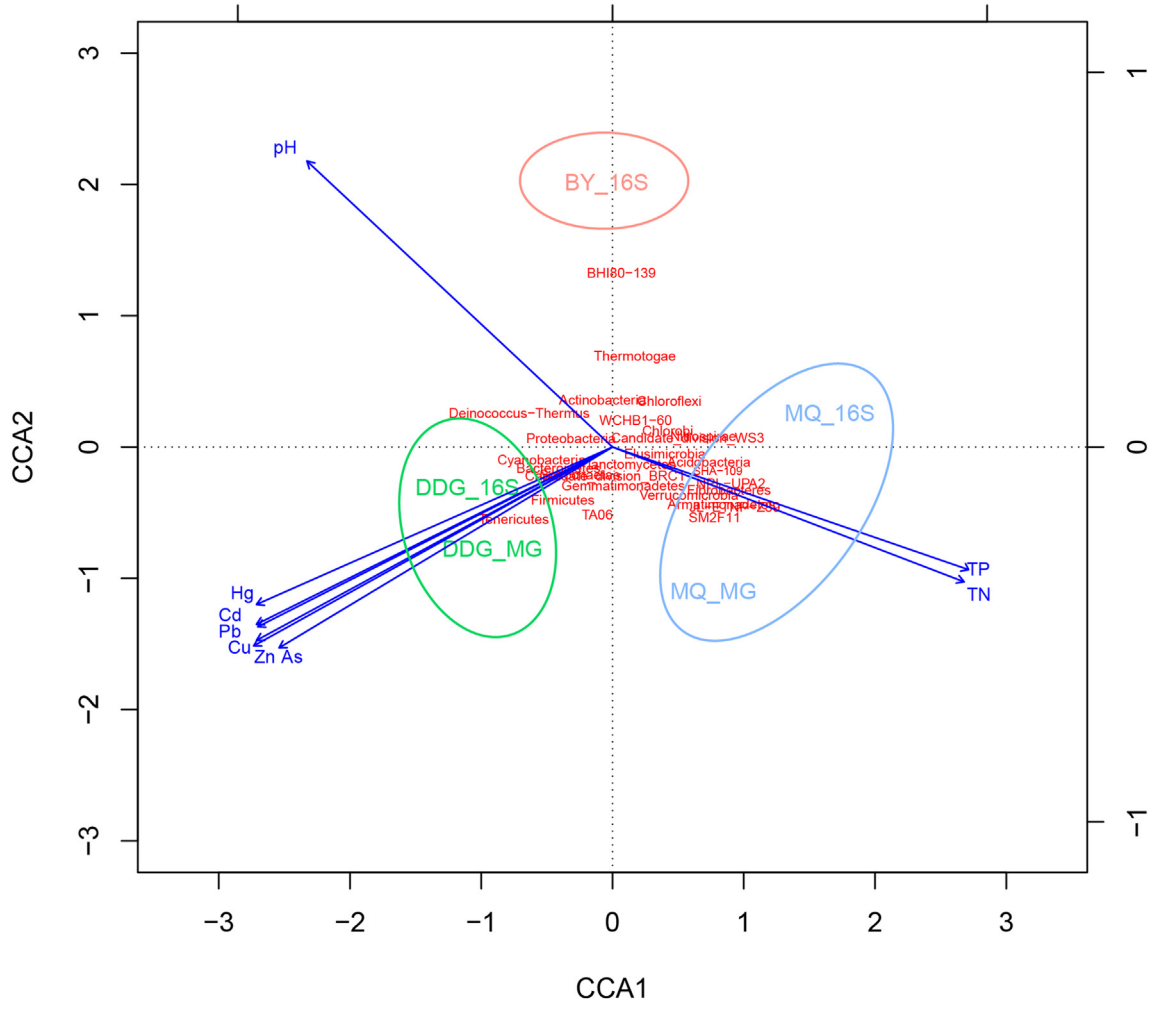

Fig. 3. Canonical correspondence analysis (CCA) of the microbial community, environmental parameters and samples.

3.6. Microbiome structure and function annotation based on metagenome sequencing

Data generated from metagenome sequencing were searched against the NR database to analyze the microbial community structure. Sequences were assigned to archaea, bacteria, eukaryota, viruses and norank. In DDG, 96.27% OTUs were bacteria, while in MQ, relative abundance of bacteria was 20.55%, which was ~50% lower than archaea there (Fig. 4). Intriguingly, abundance of viruses varied greatly between DDG and MQ, with percentages of 2.23% and 0.01%, respectively.

We classified the predicted genes by aligning them to the eggNOG databases and KO (Kyoto Encyclopedia of Genes and Genomes Orthology) database. The results of annotation against the eggNOG database demonstrated that the number of total predicted functional genes was five-times higher in DDG than in MQ. Compared with the predicted functional genes in DDG, the functional genes in MQ were generally associated with functions supporting development and reproduction, such as 'Translation, Ribosomal structure, and Biogenesis' (11.89%), 'Energy production and conversion' (10.5%), and 'Amino acid transport and metabolism' (9.71%). In contrast, in addition to 'Function Unknown' and 'General Function Prediction Only', genes' functions in DDG were more closely associated with 'DNA replication, recombination, and repair' (18.67%) and 'Inorganic Iron Transport and Metabolism' (9.37%) (Fig. 5). Results aligned against KO database illustrated that, in DDG, six types of enzymes participating in heavy-metal resistance or reduction were abundant, they were Cu^{2+}-exporting ATPase, Zn^{2+}-exporting ATPase, Cd^{2+}-exporting ATPase, As-transporting ATPase, Hg^{2+}-reductase, and alkylmercury lyase (Fig. 6a).

The phyla containing genes related to heavy-metal resistance and/or reduction were selected (Fig. 6b). In addition to unclassified phyla, there were 12 phyla harboring heavy-metal resistance and/or reduction genes: *Proteobacteria*, *Bacteroidetes*, *Firmicutes*, *Gemmatimonadetes*, *Euryarchaeota*, *Planctomycetes*, *Parcubacteria*, *Acidobacteria*, *Tenericutes*, *Spirochaetes*, *Thaumarchaeota*, and *Chlorobi*. Among them, *Proteobacteria*, *Bacteroidetes*, and *Firmicutes* owned the most varieties and counts of heavy-metal resistance/reduction genes. All the species (276 in total) harboring heavy-metal resistance/reduction genes were displayed in a phylogenetic tree (Fig. S7), and detailed information of the related functional genes was listed in Supplementary Table 2. Among these heavy-metal resistant/reduced species, 150 of them belonged to *Proteobacteria* (76, 41, 25, 6, and 2 in *Beta-*, *Gamma-*, *Alpha-*, *Delta-* and *Zetaproteobacteria*, respectively), 95 species were *Bacteroidetes*, and 5 species were grouped in *Firmicutes*.

4. Discussion

Although there is considerable evidence that heavy-metal stress influences sediment microbial community structure and function, the relationship between these characteristics in long-term polluted river sediments under natural conditions remains unclear (Hemme et al., 2010; Azarbad et al., 2013; Azarbad et al., 2015). The results of microbial community analyses are often confounded by natural sediment and climatic conditions, including pH and air temperature. Therefore, in this study, we selected sampling sites with similar conditions in order to provide better control for such conditions and constructed a nested five-point sampling strategy to ease the sampling bias (Zhou et al., 2016). Both 16S rRNA sequencing and metagenome sequencing were used to compare the difference between the two sequencing methods.

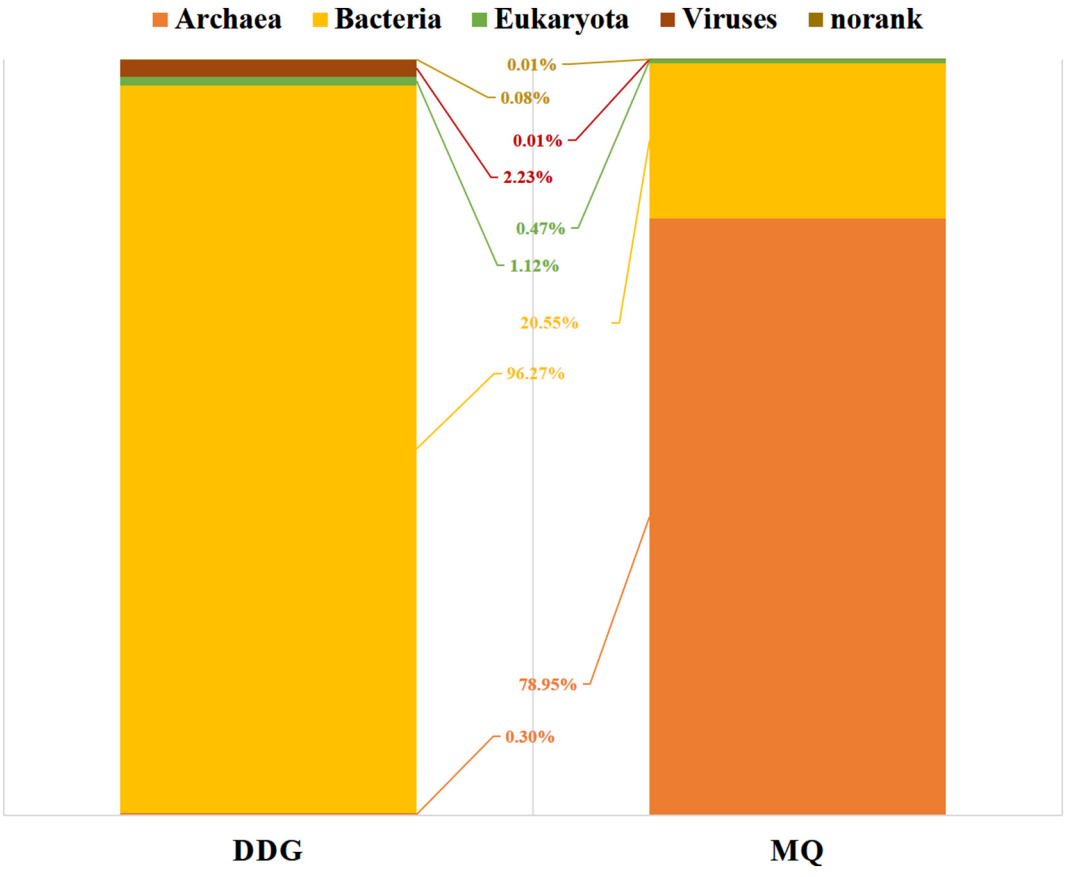

Fig. 4. Bar chart of microbiome structure at domain level. Abundance of bacteria and viruses were higher in Dongdagou (DDG) than in Maqu (MQ).

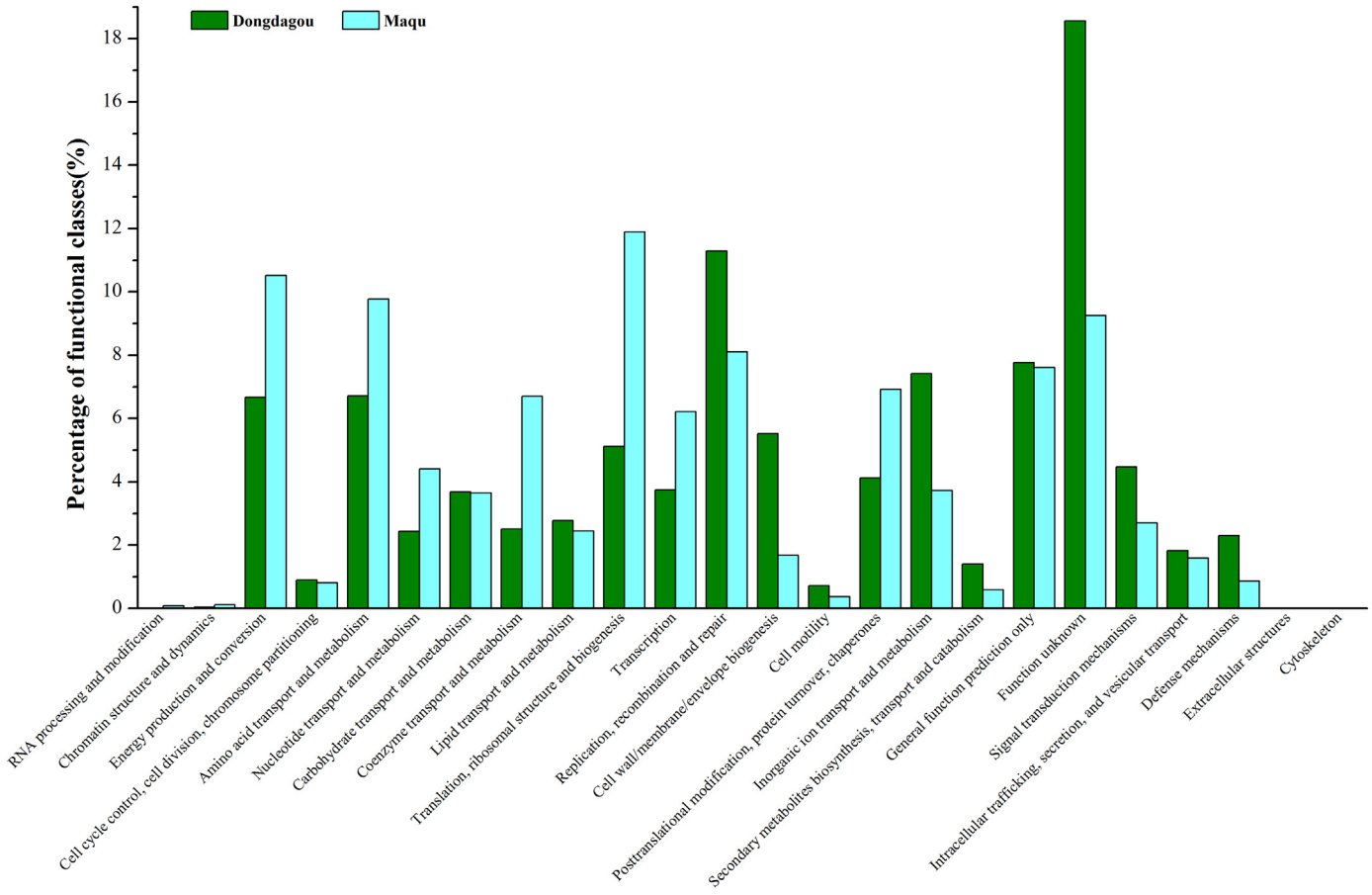

Fig. 5. Clusters of Orthologous Group (COG) annotations of two samples of Dongdagou and Maqu.

Results from the two platforms were aligned (Fig. S2), providing mutual confirmation of their veracity. Thus, we considered the results for bacterial community of 16S rRNA sequencing and metagenome sequencing as replicates and analyzed them using the mean values. Results showed that air temperature and pH revealed no significant differences among the sampling sites (ANOVA, $p > 0.05$, Table 1), whereas CCA analysis showed pH, but not air temperature, was a driver to alter the bacterial community (Fig. 2), which is in a line with the previous study that slightly varied pH shaped the bacterial community largely (Pei et al., 2018). However, almost all the heavy metals owned high correlations with bacterial community in Dongdagou (DDG), indicating heavy-metal contamination is the sharpest force to shape the microbiome in the polluted district.

Our results demonstrated that long-term heavy-metal pollution in DDG has a marked reduction in microbial diversity and biomass. It has been well documented that heavy-metal contamination shifted microbial diversity and biomass, as the bacterial community is sensitive to heavy metals (Giller et al., 1998; Azarbad et al., 2015). Kandeler et al. (Kandeler et al., 1996) studied the effect of Cu, Zn, Pb, and other heavy metals on the sediment microbiome, and found that microbial biomass was severely inhibited. Further, a study by Stefanowicz et al. (Stefanowicz et al., 2008) showed that bacterial diversity was decreased with increasing metal concentration. The most common conclusion is that only high concentrations can significantly decrease sediment bacterial biomass, whereas low concentrations of heavy metals can increase microbial biomass and stimulate microbial growth (Fliessbach et al., 1994; Chander et al., 1995). In the present study, qPCR assays and 16S rRNA gene sequencing showed that sediment samples from BY and MQ had greater bacterial biomass and higher taxonomic richness, whereas the sample from DDG had lower scores for the measured indices (Tables 1 and 2). These findings are consistent with the conclusions, which is concentrations of the analyzed heavy metals, i.e., high concentrations of heavy metals inhibit the biomass and diversity of bacteria.

Some taxons show high ability to adapt to the long-term and high-concentration heavy metals contamination. At the domain level, 96.27% OTUs in DDG were assigned to bacteria (Fig. 4), reflecting their predominance in long-term and high-concentration heavy-metal polluted sediments. However, the relative abundance of eukaryote, especially fungi, in DDG is greater than that in MQ, which is consistent with the findings of previous studies (Rajapaksha et al., 2004; Stefanowicz et al., 2008). At the phylum level, *Proteobacteria* and *Bacteroidetes*, were dominant in all the samples (Fig. 2 and Fig. S2), which are consistent with those of a study by Janssen et al. (Janssen, 2006). Particularly, the sample of DDG exhibited a higher relative bacterial abundance and number of resistance-related genes in *Proteobacteria*, *Bacteroidetes* and *Firmicutes*, therefore, these phyla were considered the core resistance phyla in DDG sediments (Ellis et al., 2003). Previous studies showed that the abundance of phylum *Chloroflexi* in metal-contaminated sites was increased (Chodak et al., 2013; Azarbad et al., 2015), whereas it opposites to our observation (Figs 2 and 3). The abundance of some phyla, for instance, *Verrucomicrobia*, is reported negatively correlated with pollution levels (Berg et al., 2012), which is a relationship that was also observed in our studies (Figs. 2, and S2). *Alpha-* and *Betaproteobacteria* were found to have the highest abundance in the most polluted samples, whereas there was a relatively higher abundance of *Gamma-* and *Deltaproteobacteria* in the sample from MQ, which are consistent with

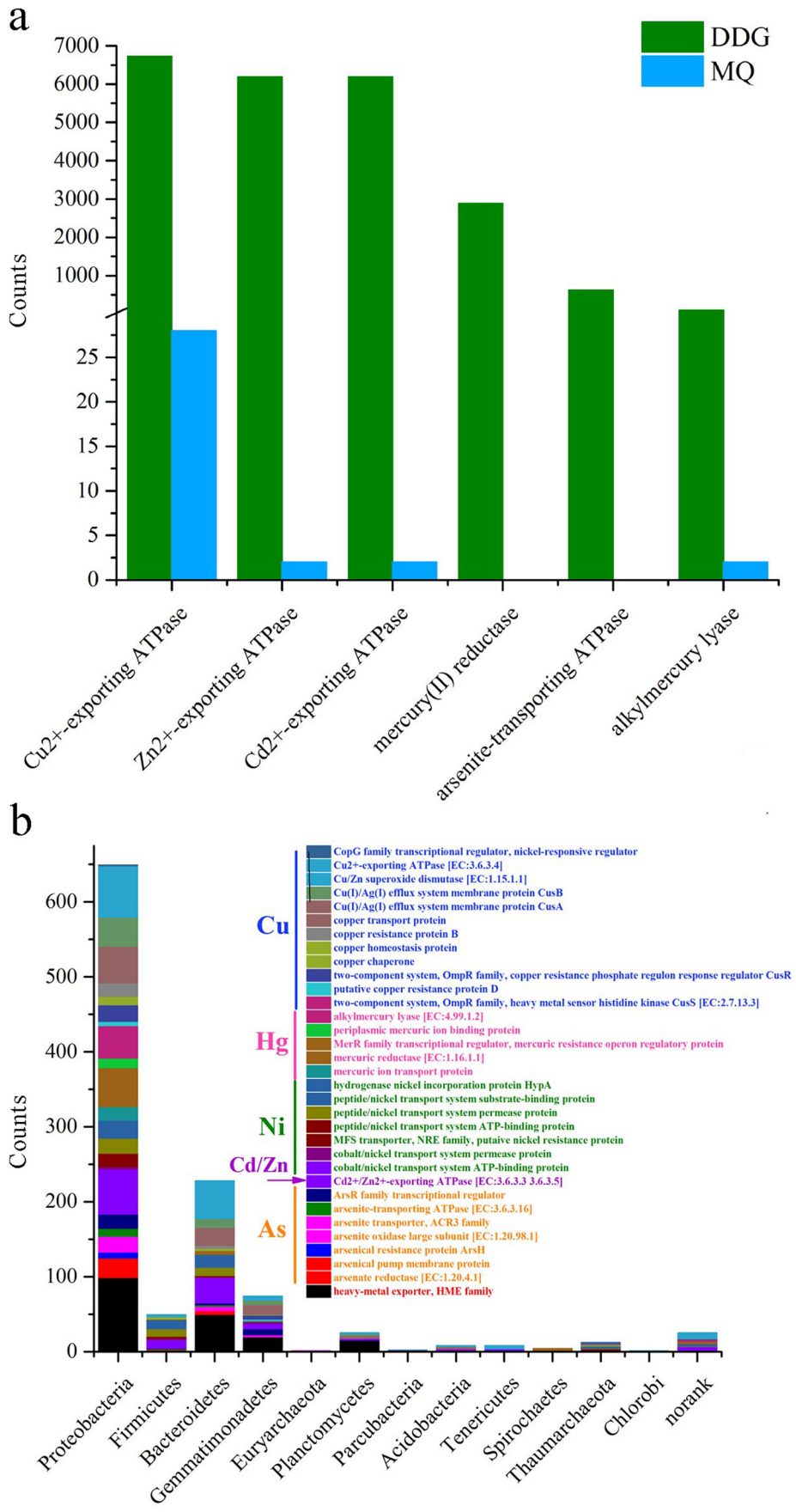

Fig. 6. (a) Enzymes of heavy-metal resistance and reduction in the KEGG pathway were predicted. (b) The counts and varieties of heavy-metal resistance/reduction genes in each phylum from the sediment samples of Dongdagou (DDG).

a previous study by Ellis et al. (Ellis et al., 2003), who showed that *Gammaproteobacteria* have a lower abundance in contaminated samples. In our study, *Pseudomonas*, *Rhodopirellula*, *Thiobacillus*, *Thauera* and *Arenibacter* were the genera owing the highest abundance of heavy-metal resistance genes (Table S2). Studies showed that bacteria strains of *Pseudomonas*, *Thiobacillus* and *Rhodopirellula* isolated from long-term heavy-metal contaminated area harbored ability to resist or/and reduce heavy metals (Blais et al., 1992; Von Canstein et al., 1999; Lage et al., 2012; Huang et al., 2016; Huang et al., 2017).

It is essential that the microbial community adapts to environmental stress in long-term heavy metals polluted area (Hemme et al., 2010). Previous studies have summarized the metabolic mechanisms underlying the resistance to heavy metals, including: (I) export the ion directly and completely, as in the case of Cd^{2+} and Zn^{2+}; (II) export the ion to the periplasm, followed by reduction to a lower toxic and soluble state, as in the case of Cr^{6+}; and (III) convert the ion to a less toxic form, followed by efflux, as in the case of Hg^{2+} (Silver and Phung, 1996). Functional gene annotations showed that, compared with the sediments collected from MQ, the sediment sample of DDG contained more genes related to DNA replication, recombination, and damage repair, and heavy-metal resistance (i.e., inorganic ion transport and metabolism genes) (Fig. 5). Heavy metals cause DNA damage and generate oxidative stress, whereas DNA recombination acts to repair damaged genes, and the related genes are proved located in both the genome and plasmids (Bruins et al., 2000; Xie et al., 2015; Losada et al., 2016) of microorganisms, and thus, these genes could be exchanged via plasmids or gene transfer. Moreover, there were more heavy-metal resistant and reduced enzymes in the DDG than in the sample of MQ (Fig. 6). In addition, genes involved in pathways of Hg, Pb, Cu, As, Zn, Cd and Cr exportation, resistance and reduction, has been observed in our study (Table S2). All the indications evidenced that, with a long-term and high-concentration pollution, microbial community in DDG has been evolved to an adaption strategy obtaining resistant and reduced genes.

Viruses serve as environmental reservoirs for microbial genes to store genetic information of their host population (Ochman et al., 2000). Furthermore, they are able to facilitate the mobile genes movement between microbial hosts by horizontal gene transfer, allowing microbes to quickly adapt to new niches, such as heavy-metal contaminants (Suttle, 2007; Rohwer and Thurber, 2009). It is worth noting that the abundance of viruses in DDG was higher than in MQ (Fig. 4), indicating these genes could be exchanged between species via viruses mediated horizontal gene transfer, such that the community as a whole acquires metal resistance (de Boer et al., 2011).

In conclusion, the microbiome of sediments in the Dongdagou River has evolved into an integrated system that has adapted to and become resistant to long-term heavy-metal pollution. The mechanism underlying this adaptation is shown in Fig. 7. In summary, (I) microbiome in DDG has high efficient of DNA recombination, which can repair DNA damage by toxic heavy metals; (II) microbiome in DDG have ability to resist oxidative stress given by high concentrations of heavy metals; (III) heavy-metal tolerance and resistance are achieved by the exportation of corresponding transporters via pumps and ion channels, or by reduction via redox reactions; (IV) resistant or reduced genes can transfer via plasmids, DNA fragments or virus.

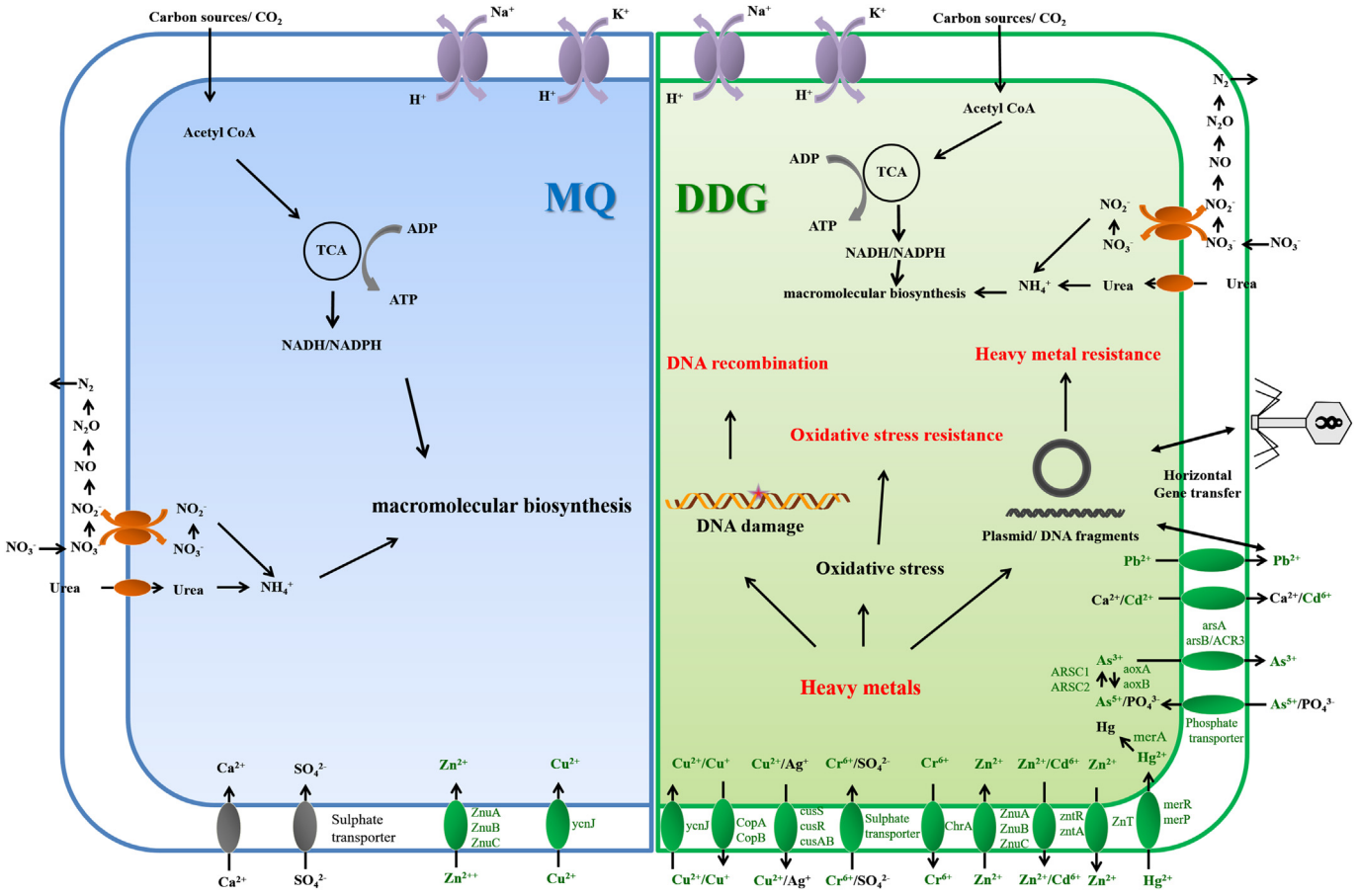

Fig. 7. Schematic map shows how the microbes related to heavy-metal tolerance or resistance. The left cell (light blue) represent the healthy bacteria in Maqu (MQ), play the normal function. The right cell (Light green) show heavy-metal tolerance and resistance in bacteria living in long-term and high concentration heavy-metal polluted sediments in Dongdagou (DDG).

Supplementary data to this article can be found online at https://doi.org/10.1016/j.scitotenv.2018.05.109.

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Data available

The sequencing data has been uploaded to SRA database with accession number: SRP080776.

Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

Conceived and designed the experiments: YJ, YC, and HH; performed the experiments: YJ, HH, SX; analyzed the data: YJ, HH, LM, JR and JZ; drafted the manuscript: YJ, HH.

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