Understanding the structure and function of microbial community in acid mine drainage system of Malanjkhand Copper Project, India

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Research

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Abstract

Background: Acid mine drainage (AMD) is a worldwide environmental menace with its multifaceted extreme nature, yet, it harbors diverse microorganisms with novel lineages and provides new insights into evolution, adaptation and metabolism. With tight coupling between biological and geochemical processes, AMD microbes play crucial roles in biogeochemical cycles and in constant focus for microbial ecology research through quantitative, genome and metagenome based analysis. This study on microbiome of AMD from Malanjkhand copper mine sheds new light of the general assumptions on microbiome emphasizing the roles of local geochemistry in community assemblages and functions using the samples from an unexplored mine.

Results: Malanjkhand AMD samples showed a regime of acidic pH with elevated levels of iron, sulfate and heavy metals. 16S rRNA gene amplicon and metagenome sequencing revealed that environmental pH controlled the species abundance, richness and assemblages. Extreme acidic niches were predominated by chemolithotrophic iron- and sulphur- oxidizing taxa (*Leptospirillum*, *Acidithiobacillus*, *Ferrithrix*, *Ferrimicrobium*, and *Metallibacterium*) capable of pyrite dissolution. In contrast, the moderately acidic niches were flourished with heterotrophic populations (*Sphingomonas*, *Nitrosomonas*, *Gemmatimonas*, *Meiothermus*, *Novosphingobium*, *Polaromonas*, *Desulfurispora*, *Desulfomonile*) involved in diverse carbon and nitrogen metabolism, sulfate and metal reduction. Relative abundance of taxa, co-occurrence, ANOSIM, and PERMANOVA revealed strength of the selective pressure in habitat-specific microbial guilds. Results indicated that individual species' ability to withstand and flourish under the extreme low pH environment was an important driver for community association, while metabolic interdependencies could be the major factor controlling species interaction within relatively higher pH habitats. Gene abundance in the metagenomes suggested that sulfur/iron oxidizing, carbon fixing, denitrifying, and stress tolerant microbial populations were predominated in both the samples. Metagenome analysis further revealed that only a small set of sulfate/metal reducing populations was abundant in higher pH samples, indicating their potential in natural attenuation of AMD.

Conclusion: Our study provided a deeper understanding about the composition and function of microbial communities within AMD highlighting the roles of local geochemistry in regulating species abundance, distribution, and interrelations. Genomic potential of AMD microbiome in withstanding the extremities and role in biogeochemical cycles, affecting metabolism as well as fate of major hazardous constituents was elucidated.

Background

Acid mine drainage (AMD) is considered to be an extreme environment for life due to its hazardous nature which makes this ecosystem inhospitable for life forms [1]. Microbes mediated oxidation of sulfidic ores in metalliferous mines lead to the generation of toxic, low pH, high sulfate and metal containing acid mine drainage [2–5]. Despite of its extremity, this environment harbors several highly acidophilic, iron- and sulfur- oxidizing microorganisms such as *Ferroplasma*, *Leptospirillum*, *Acidithiobacillus*, *Sulfobacillus*, *Ferrithrix*, etc. which are responsible for acid generation [1, 6–7]. AMD system is unique in its low microbial load and simplicity in geochemical parameters which fascinate the microbial ecologist to understand the ecology, evolution and niche-based functional partitioning of such extreme environment [1]. In recent years, metagenomics based approach has gained a significant importance in the field of microbial ecology to decipher the composition of microbial diversity, their metabolic potential and ecological roles along their succession in such ecosystem [8–15]. With the increasing evidence about the composition of the microbial diversity of this acidic environment, their metabolic attributes have been explored to understand their adaptation mechanism including their roles in diverse biogeochemical cycles [11, 14, 16–17]. In the last decade, several metagenomics based surveys have been conducted in AMD systems including tailings, water, sediments, biofilms, and streamers/mat macroscopic growth to understand the differences of microbial community structure in different niches of AMD [2, 8, 10–15, 18–23] and the role of environmental variables (pH, Depth, EC, ORP, etc.) in governing the niche-specific partitioning of microbes [13, 24–26].

Several omics based studies have capture the metabolic processes of acid mine environment. Tyson et al. [27] performed genomic studies to answer the carbon and nitrogen fixation and energy generation processes within the acidophilic biofilm at Richmond Mine at Iron Mountain, California. Similarly, Bertin et al. [11] unravelled the significant role of seven dominant bacteria in the functioning of an arsenic-rich AMD ecosystem from Carnoules Mines, France using both metagenomic and metaproteogenomic analysis. Microbial stratification and their role in acidic- oxic and suboxic streamer/mat-shaped macroscopic growth have been analysed through metagenomic studies [28]. Fifty-nine physically and geochemically distinct AMD sites in China were explored to predict the role of environmental variations in microbial assemblages [13]. Chen et al. [14] provided a detailed gene transcriptional blueprint of the AMD community, demonstrating their distinct lifestyle and roles in extreme AMD environments of three distinct mine sites of Guangdong Province, China. The natural AMD communities harbor few dominant microbial taxa with several rare microorganisms but the roles of rare taxa in biogeochemical cycling are less known [17]. Goltsman et al. [20] analysed the unexpected high diversity of active members in acidophilic biofilm community which were previously not reported. Recently, few studies were conducted to assess the diversity of three life-forms in AMD environment [21, 29] while change in microbial diversity in AMD with seasonal variation was also investigated [30]. Moreover, microbial community structure of AMD and waste rock was explored to assess the differences in their community composition [31]. Bonilla et al. [32] analysed the response of AMD on river stream sediment through amplicon based analysis while metal resistance gene profile of AMD impacted river sediment was explored by Zhang et al. [33]. All these studies have expanded our knowledge on the microbial community composition and their role in such environment. But still, there are several intriguing questions needed to be answered which help to understand such extreme ecosystem in better way. It is still elusive that low microbial load of AMD is due to less sequencing depth or due to its extreme nature. Till date, interaction of microbial communities in AMD samples is less known. Several studies have explored the metabolic potential and genomic repertoire of AMD or biofilm but less is known in term of functioning of microbiota in AMD sediment [13, 14, 20]. Comparison between the microbial community composition of AMD water and sediment is still less understood.

In the present study, microbial community composition of acid mine drainage of water and sediment of previously unexplored Malanjkhand Copper Project (Asia's largest open-cast copper mine) was explored through 16S rRNA gene based targeted sequencing as well as shotgun metagenomics to decipher the metabolic capacities of microbiota of AMD sediment. Overall, the present study was aimed to understand (i) microbial assemblages or taxonomic composition of AMD water and sediment through amplicon based analysis, (ii) role of environmental variables in microbial community assemblages, (iii) interaction among the indigenous microbiota through co-occurrence based network analysis, and (iv) ecological role and metabolic capabilities of microbial communities in AMD sediment through shotgun metagenomics.

Results

Geochemistry of the samples

Physicochemical properties of 15 samples collected from different sites of AMD or other sites with mine waste contamination showed significant variations (Table 1a and 1b). The pH ranged from highly acidic to moderately acidic (pH 1.92 to 5.9), redox potential (ORP) was positive (268-409 mV) and electrical conductivity varied between 1493-4452 $\mu\text{S}/\text{cm}$. The concentrations of total Fe and soluble SO_4^{2-} were relatively higher in the samples with more acidic pH values (pH <3.5). Several heavy metals were detected, with concentrations varying between 2.1 (Co)–5800 (Cu) mg/kg in sediment and 7.2 (Cr)-559 (Cu) mg/L in liquid samples (Table1a and 1b). The concentrations of NO_3^- were in the range of 100-467 mg/kg sediment or 42-284 mg/L liquid whereas NO_2^- was found to be either at very low concentration or below detection limit. Relatively higher concentrations of NH_4^+ were present in the sediment. Dissolve organic carbon (DOC) concentration varied considerably across the samples, with liquid samples having significantly lower level (order of magnitude) than the sediments (Table 1a and 1b). CHNS analysis of sediments indicated 0.3 to 8.2 % C (w/w), 0.6 to 2.3 % H (w/w), 0.08 to 0.46 % N (w/w) and 0.4 to 3.4 % S (w/w). XRD data indicated presence of Fe (III) oxide phases such as goethite, ferrihydrite, lepidocrocite, hematite, and magnetite as well as Fe sulfate phases (jarosite, natrojarosite, plumbojarosite; schwertmannite; melanterite, rozenite, rhomboclase, etc.) in the sediment samples (Additional File 1 Figure S1). Presence of other crystalline

phases of quartz, cuprite, albite, muscovite, kaolinite, etc. was also noted. XRF analysis confirmed the presence Fe_2O_3 , Al_2O_3 , and SiO_2 and other metal oxides (Additional File 2 Table S1). Principal component analysis (PCA) on the geochemical data showed that samples with lower pH values (pH 1.92-3.5) were well separated from rest of the samples owing to the distinct environmental conditions (Additional File 1 Figure S2).

Quantification of microbial abundance

Quantitative assessment of bacterial and archaeal abundance was done by qPCR of 16S rRNA gene. Due to the abundance of SO_4^{2-} , *dsrB* gene involved in the dissimilatory SO_4^{2-} reduction process was quantified as a measure of microbial metabolic activities (Fig. 1). The gene copy numbers detected for all the three genes were considerably higher in sediments than the liquid samples. In the sediments, 16S rRNA gene copies varied between 3.4×10^6 - 2.3×10^{10} bacterial 16S rRNA gene copies g^{-1} to 1.5×10^4 - 6.1×10^6 archaeal 16S rRNA gene copies g^{-1} (Fig. 1). In the liquid samples the abundance was several orders of magnitude less and in the range of 1.1×10^6 - 5.4×10^7 bacterial 16S rRNA gene copies ml^{-1} with 5×10^2 - 2.8×10^4 archaeal 16S rRNA gene copies ml^{-1} (Fig. 1). With respect to samples' pH, a steady increase in bacterial 16S rRNA gene copy numbers was noted, irrespective of the sample's matrix. Quantitative PCR estimation indicated considerable presence of SRB populations with *dsrB* gene copies varying from 3×10^2 - 3.8×10^7 copies g^{-1} or ml^{-1} . Based on the average 16S rRNA gene copy numbers in bacteria (4.9 per genome) and archaea (1.7 per genome), estimated cell numbers within the AMD microbiome ranged from 10^5 - 10^9 g^{-1} or ml^{-1} sample and up to 1% of the total bacterial cells seemed to harbor *dsrB* gene. The Spearman correlation confirmed a positive correlation between 16S rRNA copy number of bacteria ($R = 0.83$, $p < 0.05$ for sediment; $R = 0.96$, $p < 0.05$ for water) and *dsrB* ($R = 0.90$, $p < 0.05$ for sediment, $R = 1.0$, $p < 0.05$ for water) with pH.

Microbial community structure

Microbial community structures were ascertained through a thorough analysis of 16S rRNA genes retrieved from 15 samples (sediment and liquid). After filtering, a total of 3.77 million (1.17 million from 8 sediments and 2.6 million from 7 liquid samples) usable reads (after filtering of low quality reads and chimeras) was used (Additional File 2 Table S2). A minimum of either 0.1 million reads per sediment sample or 0.2 million reads per liquid sample was obtained. Subsampling of the reads at an even depth was performed considering 0.1 million reads per sample. The total number of operational taxonomic units (OTUs) assigned per sample using UCLUST varied and followed a characteristic trend with pH. High pH samples ($4.0 < \text{pH} < 6.0$) yielded more numbers of OTUs as compared to low pH samples ($1.92 < \text{pH} < 4.0$) (Additional File 2 Table S2). Nonparametric indicator of community diversity, Chao 1 showed a high species richness in high pH samples as compared to that of low pH ones (Additional File 2 Table S2). The relationship was well corroborated by the Spearman correlation test. The number of observed species (OTUs) ($r = 0.7381$, $p < 0.05$ in sediment and $r = 0.8571$, $P < 0.05$ in water) and Chao1 ($r = 0.6190$, $P = 0.1$ in sediment and $r = 0.8571$, $P < 0.05$ in water) were positively correlated with pH (Additional File 2 Table S3a and S3b). In addition, OTU data set covered 94.4% to 98.7% of the species richness as indicated by Good's coverage (Additional File 2 Table S2). Species diversity indices of sediment and water samples as estimated through the Shannon and the Simpson indices, yielded values ranging from 3.58 to 7.99 and 0.75 to 0.98, respectively (Additional File 2 Table S2).

In total, 44 - 47 phyla (both bacterial and archaeal phyla) were detected across the samples with *Proteobacteria* as the topmost abundant taxon (25-82%) (Fig. 2a). *Actinobacteria*, *Chloroflexi*, *Firmicutes*, *Acidobacteria*, *Deinococcus-Thermus*, *Cynaobacteria*, *Euryarchaeota*, *Planctomycetes*, and *Bacteroidetes* represented the other dominant taxa and accounted for 11.70%, 7.0%, 5.88%, 5.45%, 3.51%, 2.89%, 2.70%, 2.62% and 2.46% of all the sequences in sediment samples (Fig. 2a). In the liquid samples, *Actinobacteria*, *Acidobacteria*, *Chlorobi*, *Bacteroidetes*, *Planctomycetes*, *WD272*, and *Chloroflexi* represented the other phyla and constituted up to 28% of all the sequences (Fig. 2a). In order to investigate the possible link between sample's pH and AMD microbiome, the samples were segregated into two groups based on their pH [*viz.*, low pH ($1.92 < \text{pH} < 4.0$) and high pH ($4.0 < \text{pH} < 6.0$)]. Microbial community composition was analysed using the WPGMA based agglomerative hierarchical clustering of both sediment and liquid samples of low and high pH. It was clearly evident that irrespective of sample's matrix (solid or liquid) samples were clustered distinctly based on their pH (Fig. 2b). Irrespective of the sample's

solid (e.g., sediment) or liquid nature *Gammaproteobacteria*, *Acidimicrobiia*, *Acidobacteria*, *Alphaproteobacteria*, and *Nitrospira* represented the major classes in low pH while *Actinobacteria*, *Betaproteobacteria*, *Deltaproteobacteria*, *Planctomycetacia*, *Gemmatimonadetes*, *Ignavibacteria*, *Sphingobacteriia*, *Chloroflexi*, and *Deinococci* dominated the high pH (Fig. 3). It was also noted that although the Malanjhand AMD sites were in general dominated by bacteria, in the samples from M5 (M5S and M5L samples), members of archaea were relatively abundant. *Thermoplasmata* (*Euryarchaeota*) and *Terrestrial group* (*Thaumarchaeota*) were the most dominant archaeal classes present in high abundance in M5S (34.81 %) and M5L (1.46 %).

Difference between microbial community composition across the samples of different pH was investigated using the family level community data. Venn diagrams were generated to understand the uniqueness and commonalities of microbial families present in the samples (Additional File 1 Figure S3). It was evident that out of the total number of families, 509 (61%) were common between sediment and liquid samples whereas 230 (28%) and 94 (11%) families were exclusively present in sediment and liquid samples, respectively (Additional File 1 Figure S3a). To discern the distribution of families based on the two pH regimes and within the sediment and liquid samples, a second Venn was plotted (Additional File 1 Figure S3b) which indicated that 42% of the total families present in the liquid were shared between low and high pH samples. Over 50% of the families were present exclusively in the high pH while for the low pH it was only 7%. A similar trend was observed for sediment samples as well (Additional File 1 Figure S3c). Sediment samples harbored relatively more numbers of families as compared to the liquid ones, and a small fraction of these were unique to low pH, leaving a relatively larger portion unique to high pH samples. This data seemed to highlight an interesting fact that higher pH samples in general harbored more number of taxa (e.g., families) and most of these taxa were unique to the higher pH.

To understand the distribution pattern of microbial groups a heatmap was generated considering the abundance of the major families across the samples (Fig. 4). Based on the relative abundance of individual families, the WPGMA yielded a distinct separation of two clades: one for the low pH and the other for the high pH samples. This clustering further validated that, compared to pH, sediment or liquid nature of the samples had insignificant influence on community assemblages even at lower taxonomic level. Major families like *Xanthomonadaceae*, *Acidobacteriaceae* (Subgroup 1), *Acidimicrobiaceae*, *Acidithiobacillaceae*, uncultured *Acidimicrobiales*, and *Acetobacteraceae* were grouped together and found to be more dominated in low pH. On the other hand, *Sphingomonadaceae*, *Chitinophagaceae*, *Nitrosomonadeceae*, *Hydrogenophilaceae*, *Bradyrhizobiaceae*, *Commamonadaceae*, *Rhodobacteraceae*, *Rhodospirillaceae*, *Rhodocyclaceae*, *Thermaceae*, *Gemmatimonadetes*, *Microbacteriaceae*, and *Sphingobacteriaceae* were present relatively more abundant in high pH samples (Fig. 4).

Analysis of similarity (ANOSIM) confirmed that the microbiomes of low pH AMD were significantly different from their high pH counterparts ($R = 1$, $p < 0.05$; $R = 0.925$, $p < 0.05$) (Additional File 2 Table S4). Similar difference was noted through permutational multivariate analysis of variance (PERMANOVA) (Additional File 2 Table S5), thus validating the influence of pH in shaping AMD microbiomes. Non-metric multidimensional scaling (NMDS) showed the distinctness of the respective communities (Fig. 5). High pH sediment and liquid microbiomes presented two overlapped plots, and remained distinctly separated from the low pH samples. The later (low pH plots), however, showed some degrees of independence between sediment and liquid samples. Similarity percentage (SIMPER) analysis identified the taxa responsible for such differences among the samples based on their pH (Table 2a and 2b). *Acidiphilum*, *Rhodanobacter*, *Methylothermus*, *Acidobacterium*, *Thiomonas*, *Sideroxydans*, *Flavisolibacter*, *Acidithiobacillus*, *Ferrithrix*, *Novosphingobium*, uncultured members of *TRA3-20*, *SR-FBR-L83*, *Acidimicrobiales*, *WD272*, etc. contributed substantially in differentiating the low and high pH liquid samples (Table 2a). Along with the above mentioned taxa *Meiothermus*, *Polaromonas*, *Acidiphilum*, *Thiobacillus*, *Acidiferrobacter*, *Metallibacterium*, and uncultured members of *Xanthomonadales*, *KD4-96*, *Terrestrial group*, *BSLdp215*, *BSV26* etc. contributed for difference in low and high pH sediment samples (Table 2b).

Role of environmental variables in microbial community assemblage

To gain insights into the relationship among community members (as represented by distinct OTUs) and selected environmental factors like pH, Fe, and SO_4^{2-} , the Spearman correlation was calculated. It was noted that pH remained a critical factor in regulating the species abundance as out of the total OTUs identified, 1533 and 1150 OTUs of sediment and liquid samples, respectively were positively correlated with pH at $p < 0.05$. Taxonomic identities of those OTUs which correlated strongly with pH were determined and cumulative number of OTUs affiliated to each taxon (family) was presented (Additional File 2 Table S6). A list of major families was given in Additional File 2 Table S6a and S6b. Among these *Sphingomonadaceae*, *Chitinophagaceae*, *Hydrogenophilaceae*, *KD4-96*, *Comamonadaceae*, *Microbacteriaceae*, *Nitrosomonadaceae*, *Oxalobacteraceae*, *Erythrobacteraceae*, *Planctomycetaceae*, *Rhodocyclaceae* and *Bradyrhizobiaceae* were found to be most dominant. With respect to the OTUs negatively correlated with pH ($p < 0.05$), we could detect 433 and 488 OTUs of sediment and liquid samples, respectively. Taxonomic identities of these OTUs revealed a strong association of *Xanthomonadaceae*, *Acidimicrobiales*, *Acidobacteriaceae*, *Acetobacteraceae*, *Acidimicrobiaceae* and *WD272*, etc. with lower pH (Additional File 2 Table S6c and S6d). With respect to OTUs which positively correlated with Fe, the Spearman correlation showed the importance of *Acidimicrobiaceae*, *Acidimicrobiales*, *Acetobacteraceae*, *Acidobacteriaceae*, *Xanthomonadaceae*, and *Hydrogenophilaceae* members (Additional File 2 Table S7a-d). Interestingly, most of these OTUs (affiliated to the above mentioned taxa) were found to be correlated positively with SO_4^{2-} (Additional File 2 Table S8a-d). Overall, this correlation data identified a set of bacterial taxa (mostly the members of *Acidimicrobiaceae*, *Acidimicrobiales*, *Acetobacteraceae*, *Acidobacteriaceae*, *Xanthomonadaceae*, etc.) positively associated with low pH, high Fe and SO_4^{2-} and could be considered as the core inhabitants of the lower pH AMD environment. The role of other environmental variables like Cu, Fe^{2+} , C, N, DOC, Zn, Ni, NO_3^- , PO_4^{3-} , Al, S, NH_4^+ , EC, etc. in microbial community composition was delineated through canonical correspondence analysis (CCA) (Fig. 6). CCA analysis revealed the close association of DOC, C, N, NO_3^- , PO_4^{3-} , Zn and pH with higher pH sediment samples and these parameters correlated well together with the abundance of *Sphingomonadaceae*, *Chitinophagaceae*, *Thermaceae*, *Hydrogenophilaceae*, and *Comamonadaceae* (Fig. 6a). In the low pH sediment, Fe, Fe^{2+} , S, and SO_4^{2-} and NH_4^+ were positively correlated along with the members of *Acidimicrobiaceae*, *Acidimicrobiales*, *Acetobacteraceae*, *Xanthomonadaceae*, *Acidobacteriaceae* (Subgroup1) (Fig. 6a). Near similar clustering was evident for liquid samples of low and high pH (Fig. 6b).

Analysis of the genes related to dissimilatory sulfate reduction and carbon assimilation

Dissimilatory sulfate reduction and carbon assimilation processes within the communities were investigated by PCR based analysis of two sulfur metabolism related genes encoding for dissimilatory sulfite reductase (*dsrB*) and adenosine-5-phosphosulfate reductase (*aprAB*) and the C-fixation gene ribulose - 1, 5, bisphosphate carboxylase/oxygenase large subunit (*cbbL*) (Additional File 1 Figure S4-S6). All the three genes were amplified from sediment metagenomes, cloned and sequenced. Genes encoding *dsrB* showed similarity with diverse sulfate reducing bacteria belonged to *Desulfobacca*, *Desulfovibrio alkaliphilum*, *Desulforhabdus amnegina*, *Desulfosarcina* and uncultured sulfate reducing organisms (Additional File 1 Figure S4). Adenosine-5-phosphosulfate reductase (*aprAB*) gene sequences showed their affiliation with *Thiobacillus plumbophilus*, *T. denitrificans*, *T. thioparus*, *Sideroxydans lithotrophica* and uncultured adenosine-5-phosphosulfate reducing bacteria (Additional File 1 Figure S5). RuBisCO large subunit (*cbbL*) gene sequences showed similarities with the same gene obtained from different autotrophic bacteria. Among the detected clones, few were affiliated to *T. denitrificans*, *T. thiophilus*, *Thioparus thioxydans*, *Acidithiobacillus caldus*, *Ferrovums* sp., and uncultured *Chlorophyta* while the rest belonged to uncultured bacterial clones containing *cbbL* gene (Additional File 1 Figure S6). All these bacterial taxa harboring the three genes were detected in the 16S rRNA amplicon dataset.

Co-occurrence microbial network analysis

Co-occurrence network analysis demonstrated a high degree of association among the major microbial groups and niche specific partitioning of the taxa. Positive and negative correlations among the taxa were considered to understand the types of their interaction through network analysis for both sediment and liquid samples (Fig. 7 and Additional File 1 Figure S7). Positively correlated network of the sediment samples was generated from 74 nodes with 886 edges, while the same for

liquid samples had 119 nodes with 1338 edges. Each of the positively correlated networks revealed presence of two major sub-networks (Fig. 7). Sub-network 1 of the sediment and sub-network 1 of the liquid samples highlighted similar patterns of inter-connections indicating correlation among the acidophilic taxa including *Ferrithrix*, *Leptospirillum*, *Acidithiobacillus*, *Metallibacterium*, *Acidobacterium*, *Acidobacteriaceae Subgroup 1*, *Acidiphilum*, etc. Each of the sub-networks was connected to second major sub-networks of respective networks through a number of interconnected nodes (Fig. 7a and 7b). Sub-network 2 of both sediment and liquid samples represented similar assemblages with close interdependencies among mostly heterotrophic, moderately acidophilic to neutrophilic bacterial groups. Members of *Rhodanobacter*, *Mucilaginibacter*, *Sphingomonas*, *Novosphingobium*, *Polaromonas*, *Bradyrhizobium*, *Isophaera*, *Rhodobacter*, *Methylothermobacter*, etc. were the major constituents of these sub-networks (Fig. 7a and 7b). It was interesting to note that for both sediment and liquid samples sub-networks representing interactions among the taxa capable of growth at relatively higher pH (sub-network 2) also exhibited significantly higher numbers of connections. In contrast, the taxa present in sub-network 1 showed lesser numbers of edges although many of them were abundantly present in sediments and mostly detected in lower pH samples. The negatively correlated networks highlighted the disclaiming association of strongly acidophilic microbial groups with moderately acidophilic to neutrophilic organisms, thus reflecting the fact that the pH was one of the most critical drivers in microbial community assemblages (Additional File 1 Figure S7a and S7b).

Metagenome assembly and overview of metabolic potential

To gain a comprehensive outlook at the microbial community structure and function of AMD environment, two sediment samples one from each pH regime were selected for whole metagenome sequencing. Shotgun metagenome sequencing resulted into 36,446,038 and 34,961,828 pair-end reads in M8 (pH 3.8) and M16 (pH 5.8), respectively (Additional File 2 Table S9). The metagenome raw reads were assembled into nearly 0.5 million contigs with N50 of 731 and 647 bp in M8 and M16, respectively. Functional annotation through IMG/MER assigned 566,214 and 580,343 protein coding genes including 3,938 (M8) and 4,404 (M16) total RNA genes (Additional File 2 Table S9). Functional annotation through COG and KEGG databases elucidated the metabolic details of the two microbiomes from different pH regimes. Genes involved in energy production and conversion, amino acid metabolism, carbohydrate metabolism, lipid metabolism, inorganic ion transport, signal transduction mechanism and post translational modification were dominated in these two metagenomes (Additional File 2 Table S10 and S11).

Microbial community composition based on protein coding genes, 16S rRNA, *rpoB* and *gyrB* genes

Taxonomic distribution of the microbial taxa was assessed through protein coding genes as well as genes encoding for taxonomic markers (16S rRNA, *rpoB*, and *gyrB*) (Fig. 8). Both the metagenomes exhibited distinct assemblages of microbial taxa. Taxonomic profiling based on the three taxonomic markers and protein coding genes followed a similar pattern with dominance of *Proteobacteria* followed by *Actinobacteria* and *Chloroflexi* (Fig. 8a). *Acidobacteria* and *Actinobacteria* were present in high abundance in M8. In contrast to this, *Firmicutes*, *Bacteroidetes*, *Nitrospirae*, *Deinococcus-Thermus*, *Gemmatimonadetes*, and *Ignavibacteria* were abundant in M16 (Fig. 8a). Archaea represented minor fractions of the two communities (0.25% in M8 and 0.55% in M16). Among the archaeal groups, *Euryarchaeota* was detected in higher abundance followed by *Crenarchaeota* and *Thaumarchaeota* (Fig. 8a). At the class level, *Acidobacteriia*, *Gammaproteobacteria*, *Actinobacteriia*, *Ktedonobacteria*, and *Acidimicrobiia* constituted the major populations of the M8 (Fig. 8b). *Alpha*-, *Beta*- and *Delta*- *Proteobacteria*, *Chitinophagia*, *Planctomycetacia*, *Nitrospira*, *Sphingobacteria*, *Deinococci*, and *Clostridia* showed higher abundance in M16 (Fig. 8b). The whole metagenome based microbiome composition was found to be perfectly in line with the 16S rRNA gene amplicon based community composition.

The sharp contrast in composition of the two communities was more revealing at lower taxonomic level. M8 showed dominance of *Acidobacteriaceae* (*Terracidiphilus*, *Acidobacterium*, and *Granulicella*), *Rhodanobacteraceae* (*Rhodanobacter*), *Acetobacteraceae* (*Acidiphilum*, *Roseomonas*, and *Acidocella*), *Ktedonobacteraceae* (*Ktedonobacter*), *Acidimicrobiaceae* (*Ferrimicrobium*, *Ferrithrix*, and *Acidithrix*), *Burkholderiaceae* (*Thiomonas* and *Burkholderia*), and *Xanthomonadaceae* (*Mizugakiibacter* and *Dyella*) (Fig. 8c and Additional File 1 Figure S8). *Bradyrhizobiaceae* (*Bradyrhizobium*),

Chitinophagaceae (*Hydrotalea* and *Flavisolibacter*), *Oxalobacteraceae* (*Herbaspirillum*), *Hydrogenophilaceae* (*Thiobacillus*), *Mycobacteriaceae* (*Mycobacterium*), *Comamonadaceae* (*Polaromonas*), *Sphingomonadaceae* (*Sphingomonas*), *Rhodanbacteraceae* (*Rhodanobacter*), *Methylophilaceae* (*Methylobacterium*) and *Syntrophaceae* (*Desulfobacca*) were detected in high abundance in M16 (Fig. 8c and Additional File 1 Figure S8).

Metabolic potential and functional annotation of metagenomes

Ecological roles of the two selected microbiomes in AMD environment was thoroughly investigated. Functional potential of these two communities were illustrated based on the protein coding genes (cds) assigned through KO (KEGG Orthology) database (Fig. 9). In particular, genomic potential of the two microbiome to sustain stresses due to pH, low phosphate, high heavy metal content and C, N, S and Fe- metabolisms were analysed in detail.

Genomic potential towards low pH adaptation and sustain phosphate and heavy metal stresses

A number of genes known to be responsible for microbial adaptation to low pH were detected (Fig. 9a). Genes encoding the high affinity K^+ transport system (*KdpABC*), DNA repair *ClpXP* proteins and squalene-hopene cyclase (*sqh*) were most abundant; and their relative abundance was higher in the lower pH sample M8 (Fig. 9a). Additional mechanisms to control cytosolic pH by metabolism of proton buffer molecules were also noted, like the *pst* (phosphate uptake system) and *gad* (glutamate-, lysine-, and arginine- decarboxylase). With respect to the prevailing phosphate limiting state of the AMD environment, both the metageonomes showed presence of a set of genes with definite role in helping the bacterial cells during phosphate starvation (Fig. 9b). Particularly, those, encoding polyphosphates kinase (*ppk*) and high affinity phosphate ABC transporters (*pstSCA*) as well as phosphate regulon response regulator (*phoUB*) known to be upregulated during phosphate limitation were more abundant (Fig. 9b). Genotypic distribution of both pH-, and phosphate starvation-related genes showed potential involvement of *Alpha*-, *Beta*- and *Gamma*- *Proteobacteria*, *Acidobacteriia* and *Actinobacteria* in these two processes (Fig. 10).

Genes/operons providing specific resistance to only Cu (*copAB*, *cusABF*, *cueO*, *pcoC*); Cd, Zn and Co (*czcABC*); Hg (*merABCTE*), As (*arsRABC*, *aoxAB*, *ACR3*), and Cr (*chrA*) were present (Fig. 9c). Cu efflux system encoded by *copA* top the list of metal homeostasis genes followed by *cusA* and *czcA*, both involved in efflux of Cu (Cu^{2+} and other divalent cations like Co, Zn and Cd). Genes involved in cytosolic As^{5+} reduction and efflux of As^{3+} , Hg^{2+} reduction were also found to be relatively abundant (Fig. 9c). Overall abundance of most of the heavy metals homeostasis genes though remained similar among the M8 and M16, the later, showed slightly higher abundance of *copA* and *czcA* (Fig. 9c). With respect to the taxonomic linages of these genes, it was interesting to note that *copA*, *cusA*, and *czcA* had diverse affiliations than other identified metal resistance genes. These heavy metal resistance genes were found to be mostly affiliated to the members of *Alpha*-, *Beta*-, *Gamma*-, *Delta*- *Proteobacteria*, *Actinobacteria*, *Acidobacteriia*, *Nitrospira*, *Clostridiia*, and *Bacilli* in M8 and M16, although the later microbiome showed presence of a few additional taxa (*Planctomycetia*, *Chitinophagia*, *Deinococci*, *Shingobacteriia*, *Flavobacteriia*, etc.) harboring the same genes (Fig. 11).

The other heavy metals resistance genes such as *pcoB*, *copB*, *chrA*, *mcA*, *arsC*, *asrB*, etc. were mostly affiliated to relatively fewer taxa (*Alpha*-, *Beta*-, *Gamma*- *Proteobacteria*, *Actinobacteria* and *Acidobacteriia*) in both the samples (Fig. 11).

Sulfur metabolism

A large number of genes involved in S-metabolism was detected and among these, the sulfide-quinone reductase (*sqr*) that catalyzes the oxidation of sulfide was the most abundant (Fig. 9d), particularly in M8. Phylogenetic affiliation of *sqr* gene showed its differential taxonomic affiliations among the two samples, with predominance of *Alpha*-, *Beta*-, *Gamma*- and *Delta*- *Proteobacteria*, *Actinobacteria*, *Acidimicrobia*, *Bacilli*, *Acidithiobacillia*, *Chloroflexi*, etc. (Fig.12). Genes (*cysNC*, *cysD*, *cysH*, *cysJI*, *cysC*, *cysN*, *sir*, and *sat*) known to coordinate/involved in the sulfate assimilation pathway(s) constituted the major part of sulfur metabolism in the two samples with varied abundance. These genes were mostly harbored by the members of *Alpha*-, *Beta*-, and *Gamma*- *Proteobacteria*, *Actinobacteria*, *Acidobacteriia*, *Acidimicrobiia*, etc. in M8 (Fig. 12). In

addition, genotypes of these genes in M16 were also found to be associated with members of *Chitinophagia*, *Deltaproteobacteria*, *Clostridia*, *Nitrospira*, *Sphingobacteriia*, *Planctomycetia*, *Ignavibacteria*, etc. (Fig.12). Genes involved in oxidation of reduced inorganic sulfur compounds (RISCs) were identified. Within this categories, genes such as *TST* (catalyze oxidation of thiosulfate to sulfite), *doxD* (thiosulfate to tetrathionate), and *ttr* (tetrathionate to thiosulfate) were found to be more prevalent in M8 while *soxB* (thiosulfate to sulfate) and *phsA* (thiosulfate to sulfide) were dominated in M16. (Fig. 9d). Genotypes of these genes were mostly contributed by *Alpha*- and *Beta*- *Proteobacteria*. Furthermore, these genes were also harbored by the members of *Gammaproteobacteria*, *Actinobacteria*, *Ktedonobacteria*, etc. in M8 and *Sphingobacteriia*, *Chloroflexia*, *Ignavibacteria*, *Clostridia* and *Deltaproteobacteria* in M16 (Fig. 12). Genes involved in dissimilatory sulfate reduction process such as *sat* (reduce sulfate to aps), *aprAB* (aps to sulfite), and *dsrAB* (sulfite to sulfide) were detected, but with relatively less abundance and mostly from the M16 (Fig. 9d). All the genes involved in sulfate dissimilation process were mostly affiliated to *Deltaproteobacteria*, *Clostridia* and *Archaeoglobi* (Fig. 12). Interestingly, genes associated with reverse dissimilatory sulfate reduction (*r-aprAB* and *r-dsrAB*) were detected and affiliated to the members of *Proteobacteria* (Fig. 9d and Fig. 12).

Carbon fixation

Out of five identified carbon fixation pathways, key genes associated with the reductive citric acid cycle (Arnon-Buchanan cycle) and 3-hydroxypropionate bicycle were abundant in M8 (Fig. 9e). In contrast, marker genes for reductive acetyl-CoA pathway (Wood-Ljungdahl pathway) and Calvin-Benson-Bassham cycle (CBB cycle) were found to be relatively abundant in M16 (Fig. 9e). Marker genes for reductive citric acid cycle [2-oxoglutarate/2-oxoacid ferredoxin oxidoreductase (*korABCD*), pyruvate ferredoxin oxidoreductase (*porABDG*) and acetyl-CoA synthetase (*ACSS*)] and 3-hydroxypropionate bi-cycle [propionyl-CoA carboxylase and acetyl-CoA carboxylase] were found to be affiliated to *Alpha*-, *Beta*-, *Gamma*-, and *Delta*- *Proteobacteria*, *Actinobacteriia*, *Clostridia*, *Acidobacteriia*, etc. (Fig. 13). Genotypic distribution of RuBisCO (*cbbL*) gene was found to be associated with *Alpha*-, *Beta*- and *Gamma*- *Proteobacteria*., *Anaerolineae*, and *Chitinophagia* while the two most important genes in Wood-Ljungdahl pathway; carbon-monoxide dehydrogenase (*cooS*) and acetyl-CoA synthase (*acsB*) were detected only in M16 and mostly encoded by *Deltaproteobacteria* and *Clostridia* members (Fig. 13).

Nitrogen metabolism

Five modes of nitrogen metabolism (denitrification, nitrification, N_2 -fixation, nitrate assimilation, and nitrate dissimilation) were identified in the two microbiomes with dominance of denitrification and dissimilatory nitrate reduction events (Fig. 9f). Genes encoding for glutamine synthetase (*glnA*), glutamate synthase (*gltBD*) and ammonium transporter (*amt*) were found to be more prevalent in the samples (Fig. 9f). Members of *Alpha*-, *Beta*-, *Gamma*- and *Delta*- *Proteobacteria*, *Actinobacteria*, *Chitinophaga*, *Acidimicrobiia*, *Acidobacteriia*, *Nitrospira*, *Bacilli*, *Clostridia*, and *Sphingobacteria* harbored these genes (Fig. 14). Abundance of two major genes of dissimilatory nitrate reduction: *narG* (catalyze conversion of nitrate to nitrite) and *nirBD* (nitrite to ammonia) were relatively more abundant in M16 as compared to M8 (Fig. 9f). Genotypic distribution of *narG* and *nirBD* was more diverse in M16 and mainly constituted by the members of *Alpha*-, *Beta*-, *Gamma*- and *Delta*- *Proteobacteria*, *Actinobacteria*, *Nitrospira*, *Bacilli*, *Clostridia*, and *Sphingobacteria* etc (Fig. 14). Genes involved in conversion of nitrite to nitric oxide (*nirK*), nitric oxide to nitrous oxide (*norBC*), and nitrous oxide to nitrogen (*nosZ*) in denitrification pathway were abundant in M16 except *norB* (Fig. 9f). Their phylogenetic affiliations showed sharp difference in taxonomic distribution pattern as depicted in Figure 14. Genes contributed in assimilatory nitrate reduction were also noted. The genes, *narB* and *nasA* which converted nitrate into nitrite were detected in M8 and M16 while *nasB* present only in M8 (Fig. 9f). Among the other genes, such as *nirA* which converts nitrite into ammonia was detected only in M16 and found to be affiliated to *Alphaproteobacteria*, *Actinobacteria*, *Acidimicrobiia*, *Nitropsira*, etc. members (Fig. 14); *hao* which converts hydroxylamine into nitrite in nitrification process was present only in M16 and found to be affiliated to *Nitrospira* and *Gammaproteobacteria*. Genes involved in nitrogen fixation pathway were detected in very less abundance in both metagenomes and mostly affiliated to *Alpha*-, *Beta*-, *Gamma*- and *Delta*- *Proteobacteria* (Fig. 9f and Fig. 14).

Iron metabolism

Iron oxidizing bacteria generates energy via the oxidation of Fe^{2+} in acid mine environment and our data showed the presence of several genes responsible for Fe^{2+} oxidation event (Fig. 9g). Cytochrome c oxidase subunits I, II, III, and IV (*coxA*, *coxB*, *coxC* and *coxD*) were most abundant followed by cytochrome c oxidase *cbb3* (*ccoNPOQ*), *pet* operon (*petA*, *petB*, and *petC*) and *cyc1*. Relative abundance of all these key genes involved in iron oxidation was higher in M8 (Fig. 9g). Members of the taxa *Alpha*-, *Beta*- and *Gamma*- *Proteobacteria*, *Acidobacteriia*, *Actinobacteria*, *Acidimicrobiia*, mostly harbored these genes (Fig. 15). Genes responsible for ferrous/ferric ion transport (*feoAB*, *efcJ*, and *fbpABC*), TonB dependent iron transport and iron transport regulator (*furR*) were also identified in both the microbiomes (Fig. 9g and Fig. 15).

Genomes reconstruction from metagenomes

Metagenome assembled genome (MAG) reconstruction was performed with the assembled contigs (at K-mer 63) from both the metagenomes through PATRIC 3.5.25 (<https://www.patricbrc.org/app/MetagenomeBinning>). In total, 30 bins were formed from both the metagenomes (16 bins from M8 and 14 bins from M16) (Additional File 2 Table S12). These 30 bins belonged to the members of *Proteobacteria*, *Firmicutes*, *Acidobacteria* and *Actinobacteria* (Additional File 2 Table S12). Out of 30 bins, only 8 (four from each metagenome) were reconstructed with > 75 % genome completeness based on the presence of single copy marker genes (Additional File 2 Table S12). The genomic inventories of only these 8 bins were highlighted in this study (Additional File 1 Figure S9). Taxonomically these bins were affiliated to seven distinct genera (Additional File 2 Table S12 and Additional File 1 Figure S9).

Bin_1_M8 was taxonomically affiliated to *Acidobacterium capsulatum* with 100% genome completeness (Additional File 2 Table S12). Among the most relevant genes detected in this bin, presence of genes involved in iron acquisition and reduction [iron transport (*FeoB*), siderophore dependent *tonB* and *tonB* dependent receptors; iron reduction decaheme cytochrome (*mtrA*)], squalene hopene cyclase and others genes involved in acid resistance as well as genes conferring metal resistance through efflux transporter (*cusAB*) or resistance protein (*czcABCD* and *copB*) were noticeable (Fig.16). This genome also harbored gene for respiratory nitrate reduction (*narG*) and copper-containing nitrite reductase (Fig. 16). Bin_3_M8 and Bin_2_M16 were affiliated to *Rhodanobacter* sp. with 99.3-98.3% genome completeness (Additional File 2 Table S12). This genome harbored respiratory nitrate reductase, assimilatory nitrate reductase and nitrite reductase genes responsible for assimilatory and dissimilatory nitrate reduction along with denitrification mechanism (Fig.16). Bin_4_M8 belonged to *Thiomonas* sp. with 75.1% genome completeness (Additional File 2 Table S12). The presence of Rubisco gene (*cbbL/cbbS*) in this genome confirmed its autotrophic mode of carbon fixation (Fig. 16). This genome also harbored genes for sulfur oxidation of RISC such as sulfide: quinone oxidoreductase (*sqr*) and sox system (*soxB*) (Fig. 16). Several genes conferring resistance to cobalt, cadmium, zinc (*czcABCD*), copper (*cusA*) and arsenic (*arsC*, *ACR3* and *arsR*) were detected (Fig. 16). Bin_7_M8 was affiliated to *Ferroplasma* sp with 81.6% genome completeness (Additional File 2 Table S12). RubisCO and *prkB* genes for carbon fixation, glutamine and lysine decarboxylase and potassium transporter (*KdpABC*) for conferring pH tolerance and sulfide:quinone oxidoreductase Type III and sulfate adenylyl transferase involved in sulfide oxidation and assimilatory sulfate reduction pathway, respectively were detected (Fig. 16). Assimilatory nitrate reductase gene was detected in this genome indicating the species ability to use of nitrate as the nitrogen source (Fig. 16). Bin_1_M16 was affiliated to *Thiobacillus thioparus* with 97.6% genome completeness (Additional File 2 Table S12). An autotrophic mode of carbon fixation was confirmed by the presence of RubisCO gene (Fig. 16). This genome also harbored genes for sulfur oxidation of RISC such as sulphide: quinone oxidoreductase (*sqr*) and sox system (*soxB*) (Fig. 16). Sulfate adenylylate reductase and sulfite reduction-associated complex *dsrMKJOP* protein were detected in this genome used for sulfate assimilation. Several genes related to heavy metal resistance for cobalt, cadmium, zinc (*czcABCD*), copper (*cusA* and *copB*) and arsenic (*arsC*, *ACR3* and *arsR*) were detected in this genome (Fig. 16). Assimilatory nitrate and respiratory nitrate reductase genes were detected with several nitrate transporter genes (Fig. 16). Nitrite reductase and nitric oxide reductase genes were also identified in this genome. Bin_4_M16 was affiliated to *Pseudolabrys* sp with 84.3% genome completeness (Additional File 2 Table S12). This bin contained the genes related to sulfide oxidation (*sqr*) as well as sulfate assimilation genes (*sat*, *cysJI*) and sulfate/sulfite transporters (Fig. 16). Heavy metals resistance genes such as *czcABCD* for cobalt zinc and cadmium, *cusA* for copper, *aioAB* and *ACR3* for arsenic oxidation and reduction were detected in this bin (Fig. 16). Both

assimilatory and respiratory nitrate reductase genes along with nitrite reductase genes were detected in this genome (Fig. 16). Bin_6_M16 was affiliated to *Meiothermus chliarophilus* with 93.9 % genome completeness (Additional File 2 Table S12). Respiratory nitrate reductase, *nrfA* putative protein and *nirA* were detected in this bin (Fig. 16). Genes involved in sulfate assimilation were also detected in this bin. Universal stress protein and several efflux transporters were detected in this bin (Fig. 16).

Discussion

This study presented the first in depth analysis of microbial ecosystems residing in AMD system of Asia's largest open-cast Cu mine at Malanjkhand, India. An elaborate view of the AMD microbiome across the diverse physicochemical landscape illustrated the role of local environmental variables in shaping microbiome structure and function. Environmental microbiomes were constrained by local physicochemical conditions. The broad range of geochemical data obtained here not only provided the abiotic landscape on which these AMD microbiomes sustain, but illustrated their pattern and highlighted the role in shaping AMD community composition and function. Based on the geochemical data, 15 samples studied in the present work were segregated into two broad categories. Samples collected from the acid generation point (near tailings dam/waste rock heap) exhibited the typical physicochemical characteristics of AMD environments although more extreme pH conditions were previously reported from Iron Richmond mine (pH ranges from 0.3–1.2) [8] and mines from China [13]. Acidic pH values (pH < 3.5), high concentration of dissolve solid (represented as electrical conductivity 2.4–4.4 mS/cm) and elevated SO_4^{2-} , Fe and dissolved heavy metals of our samples indicated the extremities of such sites. Oxidation of Fe^{2+} and/or S^{2-} from sulfidic minerals released soluble Fe, SO_4^{2-} , creates acidity and mobilized the heavy metals [1, 3, 6, 10]. The observed transition in physicochemical characteristics (with respect to pH, SO_4^{2-} , Fe, EC, and HM) of different MCP sites corroborated previous research findings from the other AMD or AMD impacted environments about the environmental gradients generally present there [13, 15, 24, 33, 34]. Samples with relatively higher pH (> 4.0) could be characterized through reduced oxidation coupled with metal precipitation/sedimentation due to altered speciation/microbially catalyzed redox transformations. These sites showed that natural attenuation events were taking place as the water moved away from the AMD source points and could be identified as long-term metal sinks [35, 36].

AMD sites were considered to be an extreme for all life forms with levels of species richness significantly less than those of non-extreme environments [37]. Nevertheless, these sites were inhabited by large communities as confirmed by several quantitative measurements [25, 38–42]. Number of OTUs, the Shannon and the Simpson indices values obtained in this study were comparable or higher to similar reports on other AMD/AMD impacted sites where deep sequencing technology was employed [13, 21, 24, 25, 30, 36]. The alpha diversity indices indicated that extreme pH condition of the sites potentially acting as AMD source or near to the source select for a relatively lower microbial diversity and species richness in comparison to the sites with moderate pH that allowed colonization of a relatively wider variety of taxa. It was noteworthy that although 16S rRNA gene based amplicon sequencing provided much better resolution of species (OTUs) present, the community diversity and species richness remained constrained by local environmental pH and organic carbon content [3, 13]. Although environmental pH has been identified as the single most important factor influencing microbial diversity, presence of relatively higher level of organic carbon as present in AMD samples with increasing pH could be linked to the greater species diversity and richness [13]. Our qPCR based estimation corroborated the alpha diversity data, showing gradual increase in cell number with environmental pH. The total prokaryotic cell numbers remained within the range 10^6 – 10^{10} cells per gram or ml as reported for other AMD environments [40–42, 25]. Within the higher pH samples, the increasing trend in cell abundance could be attributed to the change in pH, organic carbon and reduced extremities which in turn could facilitate the growth of mixotrophic Fe/S reducing populations. In contrast, bacterial abundance in extremely low pH samples highlighted the possibilities of presence of metabolically less diverse but extremophilic chemolithotrophic microorganisms responsible for high sulfide/iron oxidation activities [39].

Microbial community analysis identified Proteobacteria (Alpha-, Beta and Gamma subdivisions), Acidobacteria, and Actinobacteria as the most abundant bacteria along with Firmicutes, Thermoplasmata, Sphingobacteria and Deinococci

covering more than 70% of the individual communities. Near similar patterns of taxonomic composition were previously reported from the global AMD and AMD associated environment, irrespective of geographical location and local environmental variables [6, 7, 12, 13, 25, 34, 43, 44]. Our analysis showed that microbial communities were patterned along the geochemical gradient of the AMD. Family level taxonomic distribution and its statistical analysis showed that communities in the low pH, more extreme AMD areas were distinct from that in the downstream higher pH sites. This was in line with our earlier observation on change in microbial abundance as well as microbial diversity/richness among the zones of low and high pH and highlighted the role of environmental condition such as pH, DOC, Fe, and SO_4^{2-} concentrations acting as the major environmental stressors for microbial adaptation in similar environment [13, 34, 45]. Statistical analyses further validated the role of various environmental parameters mainly those linked with pH influencing the pH-specific niche partitioning of microbial diversity. NMDS, ANOSIM and PERMANOVA analyses suggested the discriminating role of pH in structuring on AMD communities. As evident from SIMPER analysis, highly acidophilic, iron/sulfur oxidizing taxa (Ferrithrix, Acidiphilium, Acidimicrobiales, Xanthomonadaceae, Metallibacterium, Acidithiobacillus, Leptospirillum, and Acidiferrobacter) contributed significantly in distinguishing low pH samples from the high pH samples. In addition to pH, several other environmental variables such as total Fe, Fe^{2+} , SO_4^{2-} , TC, TN, DOC, heavy metals, etc. associated with the prevailing geochemistry influenced the microbial diversity distribution patterns across the samples as clearly indicated by CCA analysis. OTU level correlation analysis confirmed that the OTUs which were positively correlated with pH, Fe, and SO_4^{2-} were highly acidophilic, Fe/S metabolizing and responsible for high Fe/ contents of the low pH samples. Overall, pH, SO_4^{2-} and Fe as well as other geochemical parameters acted as environmental stressors for the change in the observed microbial community composition and functioning in the AMD environment. This observation was consistent with previous reports which clearly indicated that total Fe and SO_4^{2-} were the major environmental factors affecting microbial communities [34]. In AMD/AMD impacted environments, Fe and S compounds were known to influence the microbial communities mainly by controlling the distribution of Fe and S oxidizing microorganisms [13, 34]. Results were self-validating since acidophilic, Fe and S metabolizing bacteria were more frequently detected in low pH samples while sulfate and metal reducing taxa predominated the relatively higher pH samples. A number of recent studies those explored microbial phylogenetics and the relationship between the microbial communities and pH across different acid impacted sites (including different AMD sites across China, Rio Tinto, Black Shale sites, Rice paddy impacted with AMD watershed impacted with AMD) showed the strong influence of pH on microbial survival and activities [12, 13, 46, 47]. Environmental pH may influence the microbial ecology directly or indirectly through controlling of ancillary environmental processes those were closely linked to pH like nutrient availability and solubility of heavy metals [12, 13, 15, 34, 43, 45, 46, 48–50].

The relative abundance of taxa, statistical analysis and co-occurrence networks revealed the strength of selective pressure in habitat specific formation of microbial guilds. Members of the extreme acidophilic taxa (Leptospirillum, Acidithiobacillus, Ferrithrix, Ferrimicrobium, and Metallibacterium) capable of utilizing reduced Fe/S compounds during their chemolithotrophic mode of nutrition flourished in the low pH environment and showed strong interdependency. Abundance of these iron- and sulfur- oxidizing taxa in the low pH communities confirmed their involvement in AMD generation as they were known to enhance pyrite dissolution in highly acidic pH to utilize Fe^{2+} as an electron donor for energy generation [1, 6, 7]. In low pH environment, abilities to occupy similar ecological niche could be considered as more important factor rather than metabolic interdependencies or direct symbioses. Likelihood of the latter possibility seemed to be more appropriate in high pH samples, relatively rich in organic carbon and with reduced extremities. Networks representing low pH samples harbored organisms which had intrinsic abilities towards tolerating the extremities while utilising the available inorganic compounds (Fe/S) through lithotrophic mode of nutrition. Therefore, these organisms which were more capable of growing in low pH environment may not require strong metabolic interdependencies since chemolithotrophy remained the major driver with oxygen as terminal electron acceptor (TEA). However, presence of Acidiphilium, Metallibacterium, and Acidobacterium, which were known acidophilic, chemoorganotrophic members capable of iron reduction within the same sub-network was intriguing [6, 25]. Acidiphilium had more number of connections indicating its requirement for organic carbon from the metabolites or the biomass generated by lithotrophic members [6, 51]. The role of these organisms in iron cycling was also important since they reduce the Fe^{3+} produced by the chemolithotrophic iron oxidizing members. At low

pH, Fe^{3+} ion was more soluble and thermodynamically more attractive electron acceptor for iron reducers thriving in acidic environments [52]. Organic carbon in such environment was provided from phototrophic eukaryotes/prokaryotes activities as well as from autotrophic Fe/S oxidizers [11, 53]. A wide variety of iron bearing minerals (magnetite, jarosite, goethite, amorphous ferric hydroxide etc.) were detected which could be utilized by these iron reducing bacteria under acidic as well as oxic/anoxic condition for their energy metabolism [54, 55]. The low level of organic carbon flux in the AMD environment was maintained by chemoorganotrophic members which helped in flourishing the chemolithoautotrophic populations in such system [6]. In higher pH samples, moderately acidophilic to neutrophilic taxa capable of diverse modes of carbon and energy metabolism namely, chemolithotrophy (*Bradyrhizobium*, *Nitrosomonadaceae*, *Ignavibacteriales*, *Burkholderiales*), chemorganotrophy/mixotrophy (*Sphingomonas*, *Rhodanobacter*, *Rhodoplanes*, *Rhodobacter*, *Novosphingobium*, *Methylobacterium*, *Desulfomonile*, *Prostheobacter*, *Meiothermus*, *Gemmatimonas* and uncultured members of *Peptococaceae*, *Chitinophagaceae*, *Methyobacillaceae*, *Microbacteriaceae*, *Rhodocyclaceae*, *Comamonadaceae*, KD4-96, and *Gaiellales*) and phototrophy (members of *Rhodocyclaceae* and *Chlorobi*) showed strong interdependencies for resources utilization. Most of these members were known to be involved in N_2 -fixation (*Rhizobium*, *Bradyrhizobium*, and *Frankiales*), nitrate reduction (*Gaielleles* and *Rhodanobacter*), denitrification (*Nitropsira*, *Thiobacillus*, *Hyphomicrobium*, *Rhodoplanes*, *Rhodobacter*, etc.), sulfur oxidation (*Rhodanobacter*, *Rhodobacter*, *Mycobacterium*, members of *Rhodocyclaceae*, *Rhodospirillaceae*, *Microbacteriaceae*), ammonia oxidation (members of *Nitrosomonadaceae*) and sulfate/metal reduction (*Anaeromyxobacter*, *Desulfurispora*, *Peptococaceae*, *Pelotomaculum*, *Clostridium sensu stricto* 1, etc.) [56–65]. Co-occurrence networks provide a new outlook to understand the microbial ecology of AMD environment beyond microbial community composition.

Microbial life in oligotrophic and extreme acid mine environment was constrained by a number of physical and chemical factors [1, 6]. Shotgun metagenomics provided a better understanding on the functional characteristics of the inhabiting microbiomes. Our metagenomic inventory corroborated the 16S rRNA gene amplicon based observation highlighting the presence of diverse microorganisms (mostly bacteria of genera. *Acidiphilium*, *Ferrithrix*, *Rhodanobacter*, *Ferrimicrobium*, *Thiomonas* etc. in lower pH sample M8 and *Polaromonas*, *Thiobacillus*, *Sphingomonas*, *Bradyrhizobium*, etc. in higher pH sample M16) which were physiologically and metabolically equipped to sustain under the prevailing conditions.

Abilities of the microorganisms to cope up with low pH, phosphate limiting and heavy metal rich condition were well illustrated from the metagenomic analysis. A number of genes known to be responsible for maintaining the cytosolic pH in acidophilic bacteria such as *sqh*, *kdpABC*, lysine-, arginine-, and glutamine- decarboxylase were detected in both samples, but with relatively higher abundance in low pH M8 sample [66]. Squalene hopene cyclase (*sqhC*) encoded for hopanoid was known to play an important role in stabilizing and regulating cell membrane permeability and maintain pH homeostasis [14, 67, 68]. The other mechanisms through which microbes cope up with pH stress namely (i) generation of inside positive membrane potential by enhancing the amount of K^+ influx through (*kdpABC* system), (ii) amino acid decarboxylation (*gadB*), and (iii) buffering the cytoplasmic H^+ ion via phosphate metabolism (*phoB* and *pstSC*) were all present with relatively higher abundance in lower pH metagenome [14, 17, 66, 69–72]. The detection of phosphate regulon response regulator gene (*phoB*) corroborated well with the phosphate limiting state of the studied AMD samples [14, 73, 74]. In addition, genes for transport of phosphonate (*phnE*) and polyphosphate kinase (*ppk*) further confirmed the abilities of the AMD bacteria in accumulating phosphorous for vital synthesis of macromolecules and support the microorganisms in buffering the cytoplasmic H^+ ions additionally [75, 76].

In addition to the extreme acidity and phosphate limiting state, elevated concentration of toxic heavy metals creates a further stressful condition for the indigenous microorganisms. From the metagenome data it was clearly evident that diverse populations of AMD microbiome were genetically equipped to withstand the heavy metal (Cu, Zn, Cr, Ni, Co, Hg, and As) toxicity. In line with the high Cu concentration present in the MCP samples, most exhaustive genetic repertoire with several genes encoding the Cu resistance strategies was identified. Analysis from the present study indicated that four different modes of microbial Cu resistance systems were operating in the AMD: (i) efflux of Cu using ATPase encoded by *copA*, (ii) periplasmic detoxification systems encoded by *CueO* (multicopper oxidase) that converts Cu^+ to Cu^{2+} (less toxic form) and

cusCBA constituted periplasmic efflux system with cusF as a copper chaperone and providing full resistance against Cu, (iii) plasmid borne Cu resistance system (pco) extruding Cu with pcoBCD and (iv) efflux transporter (copB) [77]. In addition to Cu, genetic determinants conferring resistance to several other heavy metals present in MCP AMD sites viz., czcABCD efflux transporters for Co, Cd, Zn; chrA (Cr), mncA (Ni), arsB and acr3 (As) efflux systems and cytosolic reductases (arsC) (As) and merA (Hg) provided the genomic validation of intrinsic abilities of indigenous microorganisms to inhabit these extreme habitats [78–84].

Acid mine drainage environment was in general organic carbon lean but rich in inorganic compounds of iron and sulfur (reduced inorganic sulfur species) [1, 85]. Paucity of organic carbon, but the abundance of reduced- iron and sulfur compounds allowed strong nexus among the iron- and sulfur- oxidizing chemoautotrophic bacteria, while utilizing their chemoautotrophic mode of carbon acquisition. The metagenomic datasets were specifically mined to understand the mechanism for sulphur- and iron- redox transformations that could potentially drive the lithoautotrophic microbial life. Metagenome based analysis revealed that inhabitant bacteria and archaea were equipped with diverse genetic machineries for sulfur oxidation via reduced inorganic sulfur compounds (RISC) and dissimilatory/assimilatory sulfate reduction processes to generate energy for their growth and metabolism. Genes encoding for sulfide quinone oxidoreductase (sqr) known to oxidize sulfide to elemental sulfur was identified as the primary sulfur oxidation gene in both the samples [86]. This sqr gene was mostly harbored by acidophilic microorganisms (Thioalkalivibrio, Ferroplasma, Acidithiobacillus and Acidiphilium) in low pH sample and moderately acidophilic to neutrophilic members (Thiobacillus, Mycobacterium, Desulfotomaculum, Bradyrhizobium, and Meiothermus) in high pH sample. The varied taxonomic affiliation of sqr gene in low and high pH samples highlighted the recruitment of different taxa for the same process of energy generation. sulfide quinone oxidoreductase gene was previously identified and characterized from most of these taxa and therefore their presence in our samples were congruent [11, 14, 16, 17, 28, 87, 88]. The other important genes of S oxidation, such as sox responsible for oxidation of thiosulfate to sulfate, doxD (thiosulfate to tetrathionate), tst (thiosulfate to sulfite), ttr (tetrathionate to thiosulfate), phsA (thiosulfate to sulfide), etc. indicated the presence of required catabolic system to metabolize the intermediary product of sulfur oxidation (e.g., reduced inorganic sulfur species) by the inhabitant microorganisms [14, 16, 17, 56, 89–93]. With regard to the S reduction, metagenomic inventory confirmed that indigenous microorganisms were equipped with assimilatory sulfate reduction process to use sulfur for their metabolism. Relatively higher abundance of dissimilatory sulfate reduction genes (dsrAB, aprAB, and sat) in higher pH metagenome and their taxonomic affiliation corroborated our observation with co-occurrence network and other analyses that indicated scope for such process in higher pH. Sulfate reduction activity required supply of organic carbon directly or indirectly through fermentation processes carried out by other/same taxa and were known to prefer the environment pH above 5.0 [94, 95]. Subsequently, presence of reverse dissimilatory sulfite reductase and reverse adenylyl sulfate reductase genes in the samples supported the presence of alternative mechanism to oxidize sulfide to sulfite, followed by further oxidation to sulfate [16, 89, 96, 97]. Collectively, the nature and distribution of S metabolizing genes showed a good agreement with our taxonomic data and highlighted the community's potential to utilize the available S resources for their survival and activities.

With respect to iron oxidation, our results indicated the presence of various cytochromes (cytochrome c oxidase subunit I, II, III, and IV (coxA, coxB, coxC and coxD), cytochrome c oxidase cbb3 (ccoNPOQ), pet operon (petA, petB, and petC), and cyc1) in both the metagenomes [98, 99, 100]. Iron oxidation in the AMD environment was considered to be one of the major bioenergetics reactions operated by iron oxidizing bacteria for their energy generation which resulted in generation of ferric iron, a primary oxidant of sulfidic mineral at very low pH [11, 16]. The results suggested that both acidophilic and neutrophilic taxa harbored these genes in both the metagenomes to generate energy for their growth and activities [99]. In addition to solely chemolithotrophic or mixotrophic iron oxidizing populations (Ferroplasma, Acidithiobacillus, Ferrimicrobium, etc.) various nitrate reducing iron oxidizing bacteria (Thiobacillus, Thiomonas, Rhodanobacter, etc.) were detected which could also utilize ferrous iron as electron donor in the AMD system [11]. Together, the sulphur and iron metabolizing (mainly the oxidizing ones) bacteria represented the producers of the communities (first trophic level), providing resources (carbon and energy) for the heterotrophic and mixotrophic members of the communities (second and third trophic levels).

Lack of fixed carbon and nitrogen were the characteristic properties of low pH AMD environment [14, 70, 85]. Inhabiting microorganisms were expected to harbor essential assimilatory pathways for both nitrogen and carbon. Gene for ammonium transporter (amt) was observed in high abundance suggesting the import of ammonium into the cells as nitrogen resource and this corroborated well with an earlier omics based study conducted by Chen et al. [14]. Similarly, the genes encoded for glutamine synthetase (glnA) which would incorporate ammonia to glutamine and then glutamine to glutamate through glutamate synthase (gltBD) were highly abundant in these samples suggesting ammonium as a major source for nitrogen in our AMD system [70]. Abundance of nitrogen fixing genes was found to be considerably low and this well corroborated with other studies where expression of nif genes was detected at very low level in AMD sites [9, 14, 27]. Presence of genes involved in assimilatory nitrate reduction (nasA, nasB, and nirA) confirmed that the microbiome populations were equipped to use nitrate as an alternate source of nitrogen [14]. Furthermore, genes involved in dissimilatory nitrate reduction (narGHIJ, napAB, and nirBD) or denitrification (narGHI, nirK, nirS, norB, norC, and nosZ) were found to play an important role in energy generation by nitrate reducers and/or nitrate reducing iron oxidizers (Thiobacillus, Thiomonas, Acidiphilium, Rhodanobacter, Bradyrhizobium, Nitrospira, etc.) in the two metagenomes [11, 14].

With respect to the autotrophic carbon fixation events, the AMD microbiomes exhibited preferential use of rTCA in low pH sample while CBB and Wood-Ljungdahl pathways in the high pH habitat. This observation suggested that the microorganisms harboring genes for these pathways had a key role as a primary producer of organic carbon in such environment and well corroborated with the type of microorganisms detected through both amplicon and metagenome based approaches [6, 14]. The produced organic compounds were utilized by the heterotrophic microbial populations for their growth, hence reducing the organic substrates from surrounding of autotrophic members [6]. Overall, the synecological interaction among the autotrophs, mixotrophs and heterotrophs in AMD sediment confirmed the exchange of nutrients for proper growth and maintenance of microbial communities [6].

Finally, we have reconstructed 7 different metagenome assembled genomes (MAG) with > 75% genome completeness out of the two microbiome data. These MAGs were affiliated to Ferrovum, Thiobacillus, Thiomonas, Rhodanobacter, Meiothermus, Acidobacterium, and Pseudolabrys which allowed us to explore their ecological functioning in AMD samples. Ferrovum, Thiomonas, Acidobacterium, and Thiobacillus were previously reconstructed from metagenomes of AMD environment and also identified as major populations capable of thriving in AMD sites [11, 17]. It was interesting to note that Ferrovum, a known iron oxidizer and prevalent in many AMD samples worldwide could not be detected through our 16S rRNA gene amplicon based data but its genome got reconstructed from the metagenomes. Deficiency in PCR process (primer bias) or inadequate cell lysis/damage of the genome DNA could potentially hinder their detection in PCR based 16S rRNA amplicon sequencing. Ecological functioning of Ferrovum could be postulated from its bin. Ferrovum bin contained a number of iron oxidizing and carbon fixing genes through which it could oxidize iron and fixed carbon via Calvin Bassaham cycle hence confirming their chemolithoautotrophic mode of life style [101, 102]. Acidobacteria, a known iron reducing bacteria, were reconstructed successfully and found to harbor iron reducing genes and other metal resistance genes which supported their growth in MCP environment. Thiomonas and Thiobacillus, known sulfur oxidizers, harbor enzymatic machineries for thiosulfate (soxB) and elemental sulfur (sqr) oxidation and ability to fix carbon via Calvin Bassaham cycle [56]. Thiomonas sp. were known for their unique capabilities for metal resistance especially arsenic as reported by [103]. The presence of denitrification genes in these two taxa confirmed their abilities to utilized RISCs/Fe²⁺ as electron donors and nitrate as an electron acceptor under anoxic/anaerobic conditions to generate energy [11, 104, 105]. Rhodanobacter sp. was previously reported from various mine environments [106, 107] but its genome was not reconstructed from AMD samples. This was the first attempt to reconstruct genome of Rhodanobacter from such environment. From the bin, it was confirmed that Rhodanobacter could perform denitrification [61] as well as capable of thiosulfate oxidation [108] using nitrate as terminal electron acceptors. Meiothermus was considered as stress resistant bacteria and its presence in AMD samples was well documented [32, 49]. Till date, Meiothermus genome was not reconstructed through MAG from AMD samples. Different universal stress protein and efflux transporters were present in its bin which could provide resistance against stress in AMD environment. Genome reconstruction of Pseudolabrys from AMD samples was not documented yet. Presence of sqr gene involved in sulfide oxidation in its bin indicating its ecological role in AMD environment.

Conclusion

Overall, the present study characterized the microbial community composition of AMD through high throughput sequencing as well as highlighted the relationship between the microbial community members and environmental variables. The results suggested that environmental variables especially pH played an important role in community assemblages. The pH-specific niche partitioning of microbial communities was observed across the samples. The most dominating populations in low pH habitats belonged to Fe/S oxidizing groups, which harbor the essential catabolic genes for sulfur and iron oxidation, and carbon fixation while withstanding the local stress. These populations could be responsible for the AMD generation. Detailed metagenomics inventories revealed the broader spectrum of diverse biogeochemical cycling of AMD environment of MCP. This study also provided an evidence of the members of SRB/IRB which could play a significant role in its remediation. Such deeper knowledge about the microbial diversity of MCP as well as their metabolic potential could be used for the development of AMD attenuation strategy in future.

Methods

Sample collection and geochemical analysis

Malanjkhand copper project (MCP) is located in the central India at Madhya Pradesh. Fifteen samples were collected at different locations along the flow paths of mine drainage system in April 2014 as illustrated in Additional File 3 Figure S1 and Table S1. Samples M6 and M5 were collected from narrow acidic water stream emanating through waste rock piles guarding the tailing dam. M7 was obtained from an AMD sump while M8 was collected from an adjoining field receiving the overflow of AMD sump. Samples M3 and others were collected from downstream flows of AMD water and adjoining impacted field. Especially, M15, M16, and M17 represented the acidic waste released from the mine complex. Both liquid and sediment samples were collected from the same location except M6 where only water was available. M8 and M16 samples were more like slurry and we have considered them as sediment samples. The pH of the samples was measured on-site during the sampling using the Orion 5 star multi parameter meter (Thermo Scientific). For elemental analysis, water samples were filtered through 0.45 μm filter membrane and acidified with 2% HNO_3 to avoid oxidation. Heavy metals from sediment were estimated from acid digestion fraction following US EPA 3050B Methods. Total Fe and Cu were measured by atomic absorbance spectrophotometer (AAS) (AAAnalyst™ 200, Parkin Elmer, USA) while rest were measured through inductively coupled plasma mass spectrometry (ICP-MS) (Varian 810 ICP-MS system California). To measure the pH of the sediment samples, 5 g of the sediment was mixed with 10 ml of distilled water in a 150 ml Erlenmeyer flask. The slurry was mixed with shaking (200 rpm) for 2 h followed by equilibration for 30 min. The pH was measured using a calibrated pH meter (Orion 420A+, Thermo Scientific). Air dried sediment samples were used to estimate total sulfur (S), carbon (C), nitrogen (N), and hydrogen (H) with Vario Micro Cube Elemental CHNS analyzer. Water soluble anions/cations such as SO_4^{2-} , NO_2^- , F^- , Cl^- and NH_4^+ were estimated from homogenized slurry of 1 g of sediment and 10 g of filter sterilized deionized water. Phosphate was extracted from the sediment using 1:10 ratio sediment and 0.5 N sodium bicarbonate while 1 M KCl was used as an extractant for NO_3^- . 0.1 N HCl was used for extraction of Fe^{2+} from the sediment. The slurry was filtered through 0.45 μm filter membrane and the filtrate was used for the analysis. Water samples were directly used for anions and cations analysis. Major anions such as SO_4^{2-} was determined by BaSO_4 -based turbidometric method [109], NO_3^- was estimated by nitration of salicylic acid [110] and PO_4^{3-} (available phosphorous) was determined using ascorbic acid method [111] while NO_2^- , F^- and Cl^- were measured through Ion Chromatography (Metrohm 883 Compact Ion chromatograph, Dionex, USA). Cations like Fe^{2+} was estimated by Ferrozine method [112] and NH_4^+ was measured through Ion Chromatography. Dissolved organic carbon (DOC) was estimated from water soluble fraction of sediment through OI Analytical TOC Analyser. Water samples were directly used for DOC analysis. Both water soluble fraction of sediment and water samples were filtered through 0.45 μm filter membrane prior to measure DOC. For mineralogical analysis, X-Ray fluorescence (XRF) (X'Pert PRO PANalytical X-Ray fluorescence) and X-Ray Diffractometer (XRD) (X'Pert PRO PANalytical, high resolution X-Ray diffractometer) were performed with dried and finely grinded sediment samples. Match 3 was used for identification of

mineralogical phases in the samples. For microbiological investigation, sample containers were stored immediately at 4 °C till it reached to the laboratory and after that samples were stored at -20 °C till further analysis.

DNA extraction, quantification and 16SrRNA gene amplification

Metagenomic DNA was extracted from 8 sediment samples in triplicates using the Power soil DNA isolation Kit (MO BIO Laboratories, CA, USA), following the manufacturer protocol. DNA from 7 Water samples were extracted from 1L each in duplicates using Metagenomic DNA isolation kit (Epicentre, Madison, WI, USA). Quality of the extracted DNA and its concentration were measured using Qubit (Thermo Fisher Scientific). Metagenomic DNA from the extraction replicates were pooled together and used for amplicon sequencing. Metagenomes were subjected to amplification of V4 region of 16S rRNA gene using the V4 specific primer set 515F/806R [113]. Detail of the primer was presented in Additional File 3 Table S2. Each forward primer was tagged with sample specific, 10-12 bp barcode for multiplexing during sequencing run. PCR reaction was performed in 25 µl volume in triplicates using AmpliTaq gold 360 Mastermix, Biosystem, Foster city, CA 94404, 40 picomole of reverse and forward primers, 10-50 ng of DNA. Amplified product was purified and sequenced in Ion Torrent platform (Ion PGM) at Invitrogen Bioservices India Pvt Ltd, Gurgaon, India.

Bioinformatic processing of 16S rRNA sequence data, diversity analysis and phylogeny

Ion torrent data analysis of V4 region of 16SrRNA gene was performed with QIIME 1.9.1 pipeline [114]. Quality filtering was performed by removing barcode, primers, sequences with homopolymers run of >6bp and read length of sequences < 230bp. Operational taxonomic unit (OTU) was assigned at the sequence identity level of 97% using UCLUST algorithm. The taxonomic assignment was attained using RDP classifier trained with SILVA 119 database (www.arb-silva.de/documentation/release-119) with a minimum confidence of 80%. Chimeric sequences were checked and removed using ChimeraSlayer. Alpha diversity indices were performed with rarefied reads sampled to even depth (n=100000). Taxonomic distribution was calculated based on rarefied reads.

Analysis of selected functional genes (*dsrB*, *aprAB* and *cbbL*) and sequence analyses

Three functional genes related to dissimilatory sulfate reduction (*dsrB* and *aprAB*) and carbon fixation (*cbbL*) were amplified from the sediment metagenomes. Details of the primers were provided in Additional File 3 Table S2. Polymerase chain reaction (PCR) reaction was performed in 25 µl volume with 1X Standard Taq buffer (New England BioLabs), 1.5 mM MgCl₂, 200 µM dNTPs, 0.5 µl BSA (10mg/ml stock), 6 picomole each primer, 10-50 ng DNA and 1-unit DNA Taq Polymerase (New England BioLabs). PCR conditions used for amplification of these genes were comprised of first denaturation step at 95 °C for 5 min, followed by 35 cycles at 95 °C for 40 sec, annealing at 55 °C for 40 sec, extension at 72 °C for 40 sec [*dsrB* (350-400 bp) and *cbbL* (~550 bp) gene] but for *arsAB* gene (~ 1100 bp) extension was performed for 1 min 20 sec and final extension at 72 °C for 10 min, performed in Veriti 96 well thermal cycler (Applied Biosystem). PCR amplified products were run on 1% agarose gel for 40 min and were extracted using QIAquick gel extraction kit (Qiagen). Gel purified products were cloned and subsequently transformed into *E. coli* DH10β using InsTA PCR cloning kit (Thermo scientific). Randomly 10-15 colonies were picked and checked for gene of interest using colony PCR with both vector (M13F and M13R) and gene specific primers. The five random transformants of *dsrB* and *apsAB* genes as well as four of *cbbL* gene were selected from all the samples and sent to Xcelris labs limited, Ahmedabad, India for sequencing of insert using vector specific primer (M13R for *dsrB* and *cbbL* while M13F and M13R for *apsAB*). All the sequences were converted into amino acid using Expasy translate tool (<http://web.expasy.org/translate/>) and deduced amino acid was searched against blastp (<http://blast.ncbi.nlm.nih.gov/>). Highly similar sequences (amino acid) were retrieved from NCBI database and were aligned by use of ClustalW program. The alignment was used as the input file for phylogenetic analysis in MEGA 6.0 software [115]. The phylogenetic tree was constructed using the maximum likelihood method with 1000 bootstrap analysis.

Quantification of bacterial, archaeal and *dsrB* population

Quantification of total bacterial and archaeal populations in both sediment and water samples were performed by estimating the copy numbers of bacterial and archaeal specific 16S rRNA genes. Copy numbers of functional gene for dissimilatory sulfate reduction (*dsrB*) was quantified using qPCR based technique to estimate the abundance of sulfate reducing populations. Details of the primers used for qPCR were provided in Additional File 3 Table S2). Quantitative PCR was performed in Quant Studio 5 (Thermo Fisher Scientific) with Power SYBR green PCR mastermix (Invitrogen) containing five picomole each primer and 2 µl of metagenomic DNA with a total volume of 10 µl. Following amplification conditions: 95 °C for 10 minutes, 40 cycles of 95 °C for 15 sec, 55 °C for 30 sec and 72 °C for 30 sec were used. All the reactions were set in triplicates. Melting curve analysis was run after each assay to check PCR specificity. Bacterial 16S rRNA gene copy numbers were determined in each sample by comparing the amplification result to a standard dilution series ranging from 10² to 10⁸ of plasmid DNA containing the 16S rRNA gene of *Achromobacter* sp. MTCC 12117. Archaeal 16S rRNA and *dsrB* genes were cloned from metagenome and different dilution series of plasmid DNA were used as a standard for comparing the amplification result. DNA copy number were used to prepare the standard curve for comparing the amplification result. The efficiency of qPCR was calculated using formula . The standard curve was linear for 16S rRNA gene of bacteria and archaea as well as *dsrB* gene. R² value was greater than 0.993 for all the standard curves while efficiency ranged from 84% to 104% (Additional File 3 Table S3).

Statistical analysis

To understand the relationship among the samples with respect to abundance of major taxonomic groups, weighted pair group mean arithmetic (WPGMA) based hierarchical clustering using Bray-Curtis dissimilarity distance matrix was performed in XLSTAT 2014. Venn diagram was generated to understand the common and distinct taxa across the samples using InteractiVenn (www.interactivenn.net) and Venny 2.0 (<http://bioinfogp.cnb.csic.es/tools/venny/index2.0.2.html>). Analysis of similarities (ANOSIM) and Permutational multivariate analysis of variance (PERMANOVA) were performed by grouping the samples into low and high pH regimes (for both sediment and water) to understand the difference between the samples based on microbial diversity. Similarity percentages (SIMPER) analysis was performed to identify the groups responsible for difference in these two regimes. All the above mentioned analysis was performed in PAST 3.0. Spearman correlation between OTUs and environmental parameters (pH, Fe, SO₄²⁻) were performed in QIIME 1.9.1. Spearman correlation between the environmental variables and/or alpha diversity indices were performed in XLSTAT 2014. Non-metric dimensional scaling (NMDS) was performed to assess the relationship between the samples using family level data (cumulative abundance > 0.001%) in R using vegan package. Canonical correspondence analysis (CCA) was used to correlate the microbial community data with environmental variables and samples using PAST 3.0 with Monte Carlo permutation test (1000 randomized run). Canonical correspondence analysis was performed separately for sediment and water samples. Co-occurrence network analysis was performed at genera level. The genera whose cumulative abundance was less than 0.001% were filtered out in QIIME 1.9.1 for both sediment and water samples. Mothur [116] was used to identify the shared genera (using make.shared command) and the Spearman correlation at p < 0.05 was generated between the shared genera using command (OTU.association). Abundance of the shared genera and its Spearman correlation with p values were used for network analysis in Cytoscape 3.0. [117] Two separate networks were generated for sediment and water samples based on positive and negative correlation values.

Shotgun sequencing, assembly and functional annotation

Two representative samples one from each pH regime of sediment were used for shot-gun metagenome analysis. Metagenomic DNA was extracted from these two samples using the PowerSoil DNA isolation Kit (MO BIO Laboratories, CA, USA), following the manufacturer protocol. Total 8 metagenomic extractions were performed from each sample and pooled together to use for shot-gun metagenome sequencing. Metagenome was sequenced in Illumina NextSeq 500 using 2 x150 bp chemistry. Low-quality bases were trimmed from the sequences using Trimmomatic V0.30 [118] with following parameters (i) adapter trimming, (ii) sliding window trimming of 20 bp (cutting once the average quality within the window falls below a threshold of 20), (iii) bases from the start of a read were removed if the threshold quality was less than 20, (iv)

bases from the end of a read were removed if the threshold quality was less than 20 and (v) Reads < 40 bp were removed. The reads > 40bp were assembled using MetaVelvet assembler [119] at Kmers-63. Based on the number of contigs, its length and distribution assembled reads at Kmer 63 were used for functional annotation. Functional annotation of the assembled reads (contigs) were performed with IMG/MER (<https://img.jgi.doe.gov/cgi-bin/mer/main.cgi>) using IMG Annotation Pipeline v.4.11.4. Sequences which showed matches with COG (Cluster of orthologous group), KEGG (Kyoto encyclopedia of genes and genomes), KO (KEGG orthology) and Pfam (Protein families) databases were retrieved to establish the functional categories along with their taxonomic assignments and to reconstruct the metabolic pathways for diverse biogeochemical cycles. The 16S rRNA genes were picked from the IMG and its taxonomic assignments were performed with SILVA database 119 in QIIME pipeline 1.9.0. Alpha diversity based on 16S rRNA gene was calculated using alpha_diversity.py command in QIIME. The protein coding genes (detected through KO) whose sequence similarity greater than or equal to 50% were considered to establish the functional annotation and pathway reconstruction in both the metagenomes. Relative abundance of a given gene/taxon was calculated as where A was the number of sequences assigned to gene/taxon and B was the total number of sequences assigned to all gene/taxa in the community. Metagenome binning for genome reconstruction from both the metagenomes was performed using PATRIC 3.5.25 (www.patricbrc.org). The genome completeness and taxonomic assignment of the bin as well as functional annotation were also assessed through inbuilt tools of PATRIC 3.5.25.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and material

The amplicon sequences were submitted under BioProject ID PRJNA479957. The SRA accession numbers of each of the sample are M6L (SRR7511721), M5L (SRR7548546), M7L (SRR7545626), M9L (SRR7547199), M3L (SRR7545617), M15L (SRR7545389), M17L (SRR7545602), M5S (SRR7511722), M7S (SRR7511768), M3S (SRR7507877), M9S (SRR7511759), M8S (SRR7511762), M17S (SRR7511779), M16S (SRR7511778) and M15S (SRR7511784). The assembled contigs from each metagenome were submitted to IMG database (M16: GOLD biosample ID: Gb0144254; GOLD Project ID: Gp0175552 and GOLD analysis project ID: Ga0151623) and (M8: GOLD biosample ID: Gb143025; GOLD Project ID: Gp0155277 and GOLD analysis project ID: Ga0136806).

Competing interest

Authors declare no competing interests

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Author's contributions

PS conceived and designed the experiments as well as arranged funds. AG performed all the experiments. PS and AG were responsible for manuscript preparation. MP, PS, and AG arranged sampling from MCP. AG and AD performed the bioinformatics analysis for deciphering microbial diversity. AG and JS performed the qPCR and amplicon preparation.

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Tables

Table 1a Details of the physicochemical parameters of AMD sediment

Samples	pH	C	H	N	S	Al	Cr	Co	Ni	Zn	Fe	Cu	THM	SO ₄ ²⁻	NO ₃ ⁻	F ⁻	Cl ⁻	PO ₄ ³⁻	NO ₂ ⁻	Fe ²⁺	NH ₄ ⁺	DOC
M7 (S)	2.52	62.30	123.4	10	54.2	15.7	29.2	88.6	2.97	5.8	8.95	4.5	467	20.2	13.7	BDL	4.7	329	65.7	11.5		
M9 (S)	3.10	60.60	080.8	2.2	18.4	3.2	6.4	32.9	28.3	0.9	29.26	2.7	142	8.2	9.9	3.6	BDL	414	313.4	7.05		
M5 (S)	3.40	41.70	33	2	1.9	9.3	1.9	5.7	117.36	65	5.0	11.78	4.2	231	24.7	16.9	0.75	5	186	20.7	13	
M8 (S)	3.50	51.40	270.5	13.1	17.2	10.6	22.2	157.22	21	2.8	5.21	4.8	100	28.8	14.5	1.66	BDL	870.6	185.2	28.1		
M3 (S)	4.30	31.30	120.2	5.7	12.6	5.9	11.3	116.33	03	4.0	7.14	0.2	189	BDL	7.8	0.43	BDL	344	44.8	37.7		
M17 (S)	5.58	21.80	460.4	12.5	13.1	9.6	20.4	117.8	1.0	5.6	6.76	1.2	272	7.6	10.8	3.62	BDL	295.6	56.7	40.1		
M16 (S)	5.82	12.10	460.7	25.1	15.6	15	31.5	206.91	76	5.6	7.62	1.5	105	11.3	9.9	3.86	BDL	359	197.6	105.7		

M15 (S) 5.92 90.70 140.4 2.5 22.6 2.1 8.2 46.8 1.7 10.15 2.28 0.8 411 7.9 9.1 3.62 2.3 704 26.7 23.8

Note: S in the suffix of the sample name stands for sediment. C, H, N, and S are represented in % (w/w). Fe, Cu, Al, SO₄²⁻, and THM are represented

in g/kg while rest are presented in mg/kg. BDL: Below detection limit. pH is represented in SI unit. THM denotes total heavy metals (sum of Cr, Co, Ni, Zn, Fe, and Cu) and represented in g/kg. DOC: Dissolved organic carbon. Average of the triplicates is presented in the table.

Table 1b Details of the physicochemical parameters of the AMD liquid

Samples	pH	EC	ORP	Temp	Al	Cr	Co	Ni	Zn	Fe	Cu	THM	SO ₄ ²⁻	NO ₃ ⁻	PO ₄ ³⁻	NO ₂ ⁻	Fe ²⁺	NH ₄ ⁺	DOC
M6 (L)	1.93	702	332	26.7	1151	7.3	118	33.9	293	1193	524.1	2.17	3.8	87.2	BDL	BDL	2.3	8	1.4
M7 (L)	2.54	452	480	29	2230	7.5	214	54.3	377	922.9	553.8	2.19	5.4	54.5	BDL	BDL	4.1	4.4	0.7
M9 (L)	3.12	418	410	33.3	3870	13.4	102	43.2	305	958.9	555.1	1.97	2.3	80.3	BDL	BDL	1.3	2.2	1.6
M5 (L)	3.43	892	375	25.9	1532	7.2	135	41.1	284	1081	559	2.1	3.9	52.8	BDL	BDL	2.8	1.7	1.8
M3 (L)	4.31	493	337	29.6	300	7.5	23.2	12.7	196	24.4	497	0.76	0.3	61.4	BDL	BDL	0.2	1.2	3.7
M17 (L)	5.52	680	268	20.7	577	8.1	42.4	24.2	261	37.1	554.9	0.92	1.6	283.8	BDL	BDL	BDL	7.4	5.6
M15 (L)	5.93	620	294	23	153	9.1	13.1	14.1	157	39.3	549.7	0.78	1.3	41.8	BDL	BDL	BDL	16.6	5.7

Note: L in the suffix of the sample name stands for liquid samples. EC is represented in $\mu\text{S}/\text{cm}$, ORP is represented in mV. Temperature (Temp) represented in degree Celsius. SO₄²⁻ and THM are represented in g/L while rest are presented in mg/L. BDL: Below detection limit. pH is represented in SI unit. DOC: Dissolved organic carbon. THM denotes total heavy metals (sum of Cr, Co, Ni, Zn, Fe, and Cu) and represented in g/L. Average of the triplicates is presented in the table.

Table 2a Similarity percentage analysis (SIMPER) results displaying top taxa responsible for dissimilarity between low and high pH liquid samples

Taxon	Avg. # Diss. %	#Contrib %	Cumul.* %	High pH (L)	Low pH (L)
<i>Acidiphilium</i>	12.81	13.63	13.63	0.03	17.25
<i>Rhodanobacter</i>	7.21	8.67	21.3	4.22	12.4
<i>Betaproteobacteria_TRA3-20</i>	6.81	47.24	28.55	12.6	3.46
<i>Ignavibacteriales_SR-FBR-L8</i>	4.62	24.91	33.46	9.26	0.03
<i>Acidimicrobiales</i>	4.31	24.58	38.05	0.04	7.38
<i>Methylothermobacter</i>	3.82	14.06	42.11	7.64	0.00
<i>Acidobacterium</i>	3.58	9.81	45.93	0.07	3.71
<i>Thiomonas</i>	2.64	6.28	48.74	0.77	5.37
<i>Sideroxydans</i>	2.5	2.65	51.4	0.03	8.3
<i>Sandaracinobacter</i>	2.27	5.42	53.82	4.55	0.00
<i>WD272</i>	2.23	12.37	56.2	0.01	2.7
<i>Flavisolibacter</i>	2.20	6.34	58.54	4.41	0.00
<i>Acidithiobacillus</i>	2.10	4.23	60.78	0.00	13.42
<i>Ferrithrix</i>	1.85	6.97	62.75	0.00	13.37
<i>Acetobacteraceae</i>	1.78	9.90	64.66	0.64	9.4
<i>Nitrosomonadaceae</i>	1.59	1.69	66.35	3.19	0.01
<i>Novosphingobium</i>	1.46	5.57	67.9	2.94	0.00
<i>Comamonadaceae</i>	1.36	8.45	69.36	2.74	0.00
<i>BSV26</i>	1.16	9.24	70.6	2.34	0.01
<i>Xanthomonadaceae</i>	1.11	5.18	71.79	0.01	8.25
<i>Porphyrobacter</i>	0.87	8.93	72.72	1.76	0.00
<i>KD4-96</i>	0.87	4.92	73.65	1.76	0.00
<i>Acidicapsa</i>	0.79	4.84	74.5	0.00	2.67
<i>Gemmatimonas</i>	0.73	4.78	75.28	1.47	0.00
<i>Leptospirillum</i>	0.65	8.70	75.98	0.00	2.32

Note: #Contribution of OTU to overall dissimilarity between groups.

*Average abundance of OTU in each group.

Cumul.: cumulative and Contrib. contribution,

Avg. : average

Table 2b Similarity percentage analysis (SIMPER) results displaying top taxa responsible for dissimilarity between low and high pH sediment samples

Taxon	Avg. Dissim.	#Contrib. %	Cumul. %	*High pH (S)	*Low pH (S)
<i>Xanthomonadaceae</i>	8.186	9.071	9.071	0.0165	16.4
<i>Acidimicrobiales</i>	4.372	4.845	13.92	0.281	9.02
<i>Rhodanobacter</i>	4.304	4.77	18.69	10.1	1.86
<i>Meiothermus</i>	3.481	3.857	22.54	6.98	0.0215
<i>Polaromonas</i>	2.917	3.232	25.77	5.84	0.0112
<i>Acidiphilium</i>	2.808	3.112	28.89	0.0065	5.62
<i>KD4-96</i>	2.337	2.589	31.48	5.3	0.634
<i>Thiobacillus</i>	1.929	2.137	33.61	4.13	0.577
<i>Ferrithrix</i>	1.758	1.948	35.56	0.00525	3.52
<i>Xanthomonadaceae</i>	1.736	1.924	37.48	0.0163	3.49
<i>Terrestrial group</i>	1.707	1.891	39.38	0.0307	3.44
<i>Acidiferrobacter</i>	1.655	1.834	41.21	0.978	2.92
<i>Cyanobacteria</i>	1.605	1.779	42.99	3.3	0.29
<i>WD272</i>	1.586	1.757	44.75	0.00675	3.18
<i>BSLdp215</i>	1.516	1.68	46.43	0.00575	3.04
<i>Flavisolibacter</i>	1.423	1.577	48	2.85	0.004
<i>JG30-KF-CM66</i>	1.258	1.394	49.4	2.54	0.0205
<i>Metallibacterium</i>	1.239	1.373	50.77	0.0045	2.48
<i>BSV26</i>	1.228	1.361	52.13	2.46	0.046
<i>Acidithiobacillus</i>	1.211	1.341	53.47	0.0005	2.42
<i>Acidobacteriaceae (Subgroup 1)</i>	1.2	1.329	54.8	0.474	2.87
<i>JG30-KF-AS9</i>	1.173	1.3	56.1	0.401	2.43
<i>Xanthomonadales</i>	1.061	1.176	57.28	0.299	2.35
<i>Acetobacteraceae</i>	1.059	1.174	58.45	0.134	2.25
<i>Betaproteobacteria_TRA3-20</i>	1.028	1.139	59.59	0.239	2.13

Note: #Contribution of OTU to overall dissimilarity between groups.

*Average abundance of OTU in each group. Cumul.: cumulative and Contrib. contribution,
Avg. : average

Figures

Figure 1

Quantitative PCR of 16S rRNA and *dsrB* genes for estimation of bacterial, archaeal and sulfate reducing populations in sediment and liquid samples.

Figure 2

Relative distribution of major phyla and hierarchical clustering of samples based on their abundance. Relative abundance (%) of major phylum (cumulative abundance greater than 0.1%) across the sediment and liquid samples (a). Samples are

arranged from low to high pH values. Weighted paired group arithmetic average (WPGMA) based agglomerative hierarchical clustering of the low and high pH samples based the average abundance of the major phyla in each pH regime (b).

Figure 3

Relative abundance (%) of major bacterial and archaeal classes in low and high pH samples. Average values of these taxa were used to represent the relative abundance for each of the pH regime. The bars showed the standard errors of relative abundance of the taxa in each pH regime samples.

Figure 4

Heat-map based distribution of major families (a) WPGMA based clustering of samples (b) and families (c) using Bary-curtis dissimilarity matrix. Families with cumulative abundance > 0.2% were considered. Dotted line marked boxes indicate distinct associations of families within the samples from higher pH (purple line) and lower pH (red line) samples.

Figure 5

Non-metric multidimensional scaling (NMDS) plot based on abundance of major families ($> 0.0001\%$) of liquid and sediment samples using Bray-Curtis matrix. Stress = 0.01. Samples name in green and black color denoted low pH sediment and liquid samples, respectively. Samples name in blue and brown color represented high pH sediment and liquid samples respectively.

Figure 6

Canonical correspondence analysis (CCA) triplot based on abundance of major taxa, sampling sites, and environmental data of sediment (a) and liquid (b) samples. Eigen values for each axis generated by CCA indicate how much of the variation seen in species data can be explained by that canonical axis. Taxa are represented at family level (blue circles). Environmental variables used in the analysis are shown by vectors (green lines). Sampling sites are indicated by black circles with their names.

Figure 8

Taxonomic composition derived from shot-gun metagenomes. Major phylum (a), class (b), and genera (c) were considered for the analysis.

Figure 9

Abundance of genes detected from metagenomes involved in various biogeochemical cycles. Genes involved in pH stress (a), phosphate metabolism (b), heavy metal stress (c), sulfur metabolism (d), carbon fixation (e), nitrogen metabolism (f), and iron metabolism (g).

Figure 10

Taxonomic composition of pH stress and phosphate metabolism genes.

Figure 11

Taxonomic composition of major heavy metal resistance genes.

Figure 12

Taxonomic composition of genes involved in sulfur metabolism. Genes involved in dissimilatory sulfate reduction and reverse-dissimilatory sulfate reduction were represented together.

Figure 13

Taxonomic composition of genes involved in carbon fixation.

Figure 14

Taxonomic composition of genes involved in nitrogen metabolism.

Figure 15

Taxonomic composition of genes involved in iron metabolism.

Figure 16

Heat-map based distribution of major genes in metagenome assembled genomes (MAGs).

Supplementary Files

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