Pollution Intensity of Hazardous Metals, Microbiome and Potential Bacterial-Based Toxic Metal Sequestration of Coal Mine Drainage

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Research Article

Keywords: Acid mine drainage, coal mine, microbial community, bioremediation, ecotoxicology

Posted Date: March 3rd, 2021

DOI: https://doi.org/10.21203/rs.3.rs-279031/v1

Abstract

Drains from coal mine remain a worrisome point source of toxic metal/metalloid pollutions to surface- and ground-waters worldwide, requiring sustainable remediation strategies. Understanding the microbial community subtleties through integrated metagenomic and geochemical data elicit selection of autochthonous bacteria consortium, spurring decommissioning of drains before discharge to hydrosphere. The drains contained characteristic sulphates (313.0 ± 15.9 mg/l), carbonate (253.0 ± 22.4 mg/l), and nitrate (86.6 ± 41.0 mg/l), having extreme tendencies to enrich receiving environments with extremely high pollution load index (3110 ± 942) for toxic metals/metalloid. The drains exerted severe degree of toxic metals/metalloid contamination (3,400,000 ± 240,000) and consequent astronomically high ecological risks in the order: Lead > Cadmium > Arsenic > Nickel > Cobalt > Iron > Chromium. Metagenome of the drains revealed dominance of Proteobacteria (50.8%) and Bacteroidetes (18.9%) among bacterial community, whereas, Ascomycota (60.8%) and Ciliophora (12.6%) dominated the eukaryotic community. A consortium of 7 autochthonous bacterial OTUs exhibited excellent urease activities (≥ 253 µmol urea/min.) with subsequent stemming of acidic pH to > 8.2 and sequestration of toxic metals (approx. 100% efficiency) as precipitates (15.6 ± 0.92 mg/ml). The coal mine drain is a point source for metals/metalloid pollution to surrounding hydrosphere, and its bioremediation is achievable with the bacteria consortium.

Introduction

Coal mining often generate acid mine drainage (AMD) that remains a major global geogenic and anthropogenic sources of heavy metals (HMs) pollution of the surrounding lithosphere and hydrosphere. AMD is a distinctive fluvial wastewater that originates from the natural oxidation of sulphide minerals contained in mining wastes at developmental, operating and derelict mine sites. An insidious feature of AMD is that its sources may remain active for decades or even centuries after mine closure, continuously creating environmentally hazardous sceneries. These sights exist globally in many places that have sometimes depended or still depend on coal for energy generation. Not less than 19,300 km of rivers and 72,000 ha of lakes and reservoirs worldwide are severely affected by mining effluents [1]. A major problem encountered in the supply of potable water to residents of Enugu and other mining areas in Nigeria is the AMD pollution [2].

The environmental stressors contained in AMD include mobile toxic HMs; SO_4^{2-}, and H^+ that are responsible for the acidic pH; and relatively low (< 20 mg l^{-1}) concentrations of dissolved organic matter [3]. AMD causes surface- and ground-water to become acidic (pH as low as 2); triggers eutrophication via sulphate enrichment; and makes metal load become menace that often hampers agriculture and endangers aquatic life. The high metal/metalloid concentrations in AMD from coal mining are of greater concern than the acidity in terms of environmental impairment. The metals/metalloids either wield direct toxicity on the ecosystems [4, 5], or their precipitates cloak the water-beds of receiving hydrosphere [6, 7]. The dissolved toxic metals/metalloids in coal AMD include, but not limited to iron (Fe), aluminium (Al), zinc (Zn), copper (Cu), cadmium (Cd), mercury (Hg), lead (Pb), and arsenic (As). Environmental pollution

with coal AMD makes toxic concentrations of metals/metalloids contained thereof to exacerbate public health hazards that lead to fatality or permanent deformity of humans and even unborn babies in many cases [5, 8]. Cases of metal poisoning of rural dwellers emanate from artisanal mining and consequent AMD pollution of surface waters occur unabated, but rarely reported, in poor countries (personal observation of Nigeria mining villages).

AMD impact on receiving environments has been the focus of extensive restoration efforts with the goals of reducing metal loads, as well as enhancing biotic structure (diversity and abundance) [9]. Remediation of AMD via application of chemical neutralization, ion exchange and construction of wetland has been suggested with some limitations opposing outright removal of AMD effect on water sources [10–12]. Therefore, bioremediation approach through the activities of autochthonous microorganisms has been suggested as the best eco-friendly option to decommissioning AMD stressors [9, 12–13]. In conjunction with oligotrophic heterotrophs (such as *Acidocella* and *Thermoplasma*), the early microbial colonizers indirectly ameliorate HMs toxicity via syntrophic commensal associations with iron- and sulphur-oxidizers [3]. Thus, they utilize organic compounds (cell exudates and lysates) originating from the autotrophic primary producers, thereby essentially “detoxifying” the environment for the later groups of microorganisms [14–15]. Sulphate reducing bacteria (SRB) belonging to the genus *Desulfosporosinus* and *Desulfitobacterium* and genre of Firmicutes and Actinobacteria have been detected in mine lakes reversing the chemical reactions that formed AMD, attenuating toxic metals/metalloids concentrations by sulphide precipitation, redox reactions, and raising the pH of the acidic water [3, 6, 10].

Attempt to ameliorate the harmful environmental effects of AMD necessitated research to evaluate geochemical hazards and characterize microbial structure in AMD stream from coal mine systems. Autochthonous organisms, particularly those microorganisms that produce urease activities [16] are postulated for novel application in the bioremediation of AMD. The search for competent microorganisms with provident insight into pivotal taxa relevant to ecophysiology of stemming AMD stream is apt for knowledge-based bioremediation of AMD from coal. Thus, integrating culture independent metagenomes with geochemical data is sought in this study to decipher underline dynamics in microbial community structure of drains from a coal mine. Furthermore, culture enrichment of the AMD stream will provide information on key players involved in alleviating the metal/metalloid loads contained in the AMD.

Results And Discussion

Geochemistry and ecotoxicology of AMD

AMD systems are important source of metal/metalloid pollution to the receiving hydrosphere with devastating consequences on the biological drivers of affected ecosystems. Environmental menaces of AMD have not been exhaustively reported worldwide with scanty information across Africa and many developing economies. The measured values of the physical properties and contents of selected HMs in drains from a coal mine in Nigeria were as presented in the Supplementary Table A.1 online. Virtually all the measured parameters exceeded the permissible limits of WHO guidelines for potable water. The AMD

water was acidic (pH = 3.1 ± 0.265), and contained characteristic anions that are common to AMD including dissolved sulphides (1.37 ± 0.233 mg/l), sulphates (313.0 ± 15.9 mg/l), carbonate (253.0 ± 22.4 mg/l) and nitrate (86.6 ± 41.0 mg/l) above the allowable limits of WHO. Although the acidic pH of AMD in the present study compares well with those associated with mines in Russia [14], more extreme acidic pH values have been reported in other climes. Negative pH values of -1.56 and -3.6 were observed in AMD from Iberian Pyrite Belt [17] and Richmond Mine at Iron Mountain, USA [18], respectively. The values of physico-chemical parameters associated with the AMD from 'Onyeama' were similar to data reported for other mine wastewaters in Nigeria [19] and elsewhere [4]. It is known that sulphide minerals, in presence of water and oxygen, oxidise to sulphate as observed in the elevated sulphate concentration (313 ± 15.9 mg l^{-1}) in the present study. The sulphate readily form sulphuric acid in presence of protons (H^+) that was responsible for the low pH observed in the AMD and consequently cause leaching of metal/metalloid ions into the drains. The concentrations of dissolved organic matter in AMD tends to be relatively low (<20 mg l^{-1}) [17], but the total organic carbon of the AMD samples from 'Onyeama' coal mine was 25.7 ± 5.96 mg l^{-1} signifying oligotrophic conditions. Moreover, carbonate (253 ± 22.4 mg l^{-1}) in the AMD indicated alkali stemming of acidic pH from the characteristic <2 associated with freshly formed AMD to >3.0 as presently observed.

A comprehensive assessment of HMs is pivotal to evaluating potential of AMD to spur the degree of pollution in receiving environments. The contents of toxic metals and metalloid measured from the AMD water sample were extremely high, ranging from the Cr content (3.87 ± 3.87 mg l^{-1}) to a 326.0 (± 26.8) mg l^{-1} associated with Pb (Supplementary Table A.1 online). Concentrations of Pb in AMD recorded in this study was higher than 12 mg l^{-1} associated with Iron Mountains' AMD [18], and 30 mg l^{-1} in Sao Domingo mine's AMD [20]. Other metals contained in the AMD include Cd (95.0 ± 5.12 mg l^{-1}), Co (27.3 ± 9.25 mg l^{-1}), Ni (28.8 ± 13.4 mg l^{-1}), As (56.7 ± 14.7 mg l^{-1}), and Fe (39.7 ± 22.3 mg l^{-1}). All the metals/metalloid were apparently at toxic concentrations when compared with the WHO permissible limit and the values obtained from the unpolluted surface water located several kilometres away from the mine. Pb and Cd that connoted the highest concentrated HMs in the AMD, for example, have no metabolic importance than rendering havoc to biota [8, 21] in ecosystems the AMD emptied into. These non-metabolic HMs exacerbate ecophysiologies of the receiving milieus with anticipated degrees of public health consequences including mutagenicity, genotoxicity, neurotoxicity etc. [5]. This was reportedly the case with surface waters juxtaposing with AMD from 'Onyeama' coal mine that were reportedly enriched with TOC and toxic concentrations of HMs [22]. Similarly, AMDs have reportedly remained one of the major point sources for anthropogenic HMs pollution of waters globally [2, 11-12].

The awful impact of metals/metalloid poised AMD on the receiving water quality is better modelled via integrating multivariate data into pollution indexes and the functionalities of the ecosystems (Table 1). The added HMs in the AMD from coal mine as determined by contamination factor (CF) was at least 397 (± 223) factors for Fe and up to 2.97 (±0.16) × 10^6 factors for Cd (Table A.2). This implies inordinate tendency of the AMD to contaminate water bodies. The added HMs were in the order: Cd > Co > Pb > As > Ni > Cr > Fe (Table 1). Enrichment of five HMs were exceptionally high (Cd > Co > Pb > As > Ni), while Cr

and Fe were very highly and moderately enriched the AMD water, respectively. The astronomically high contamination and enrichment factors of the AMD signified the enrichment potentials the AMD portends on receiving surface waters. The understudied AMD from 'Onyeama' coal mine has been reported to impact the water qualities of rivers within the location all year round [22]. It is assumed that the extremely high concentrations of toxic metals/metalloids in the AMD dilutes out upon discharges into nearby rivers, contaminating the surface water and raising the bioavailable metals/metalloids beyond safe thresholds. Further reports of toxic metals/metalloids enrichment of surface waters via inflow of AMDs from other mines in Nigeria [23], and other climes [3-4, 24] are worrisome and oblige mitigations.

The HMs-enriched environments inadvertently exert ecotoxicity unto the drivers of the ecosystems. The level of HMs accumulation to organic matter in the AMD, through geo-accumulation (I_{geo}) index of Fe (7.60 ±0.779) to Cd (20.9 ±0.075) (Table A.2), was very severe and in similar order like CF. This possibly implies the organic matter in the AMD harbours the mobile toxic metal/metalloid concentrations and make them available to food web [25]. As such, biomagnification of the toxic metals/metalloids along the trophic level becomes tangible and challenge to biota of any surface water receiving the AMD, and to public health [21, 25]. Ecological risk assessments define and categorise pollution status of ecosystems with the HMs contained in the AMD. Based on potential ecological risk factor (Er), extremely high risk index ($36.3 \pm 1.96 \times 10^6$) was found with Cd and none of the metals/metalloid was postulated with less than 1000 risk index (Table A.2). All the HMs/metalloid contained in the AMD posed very high ecological risks and could be categorised in the order of Cd > Co > Pb > As > Ni > Cr > Fe. The modified potential ecological risk factor (MEr), however, stipulated that five HMs posed very high risk in the order: Cd > Co > Pb > As > Ni, whereas Cr and Fe were determined to be of considerate and low risks, respectively. The HMs exerted high risk to the AMD ecosystem as calculated by ecological risk quotient (RQ) in the order: Pb > Cd > As > Ni > Co > Fe > Cr. The ecological risk index of all the HMs as a whole was very high with 375 000 (± 22 400) index as stipulated by modified potential ecological risk index (Table 1). The prodigiously high ecological risks indexes obtained for the AMD remains a pointer to grave danger the HMs/metalloid portends on surface- and ground-waters the AMD flows/percolates into.

Microbial community structure of AMD from 'Onyeama' coal mine

AMD environment presents an extreme challenge for most forms of life on Earth, making microorganisms co-existing in biofilm as early colonisers since they are able to repulse against the impulses and vagaries of extreme environments. It has been hypothesized that microbial community structures are sensitive descriptors of ecological stressors and very important to understanding ecosystem functions [26]. A total of 26 160 and 40 403 valid sequence reads were obtained for bacteria and eukarya, respectively, after quality check of biofilm-water metagenomic data. The valid sequences were clustered into 2036 and 1002 OTUs of bacteria and eukarya domains of life, respectively, as presented in Table 2. The taxonomic composition and relative abundances of the AMD microbiome, as shown in Fig. 1, revealed that the bacterial community spanned 10 phyla whose sequence reads were at least 1% (Fig. 1a). Whereas, the eukarya domain of life (with sequence reads ≥ 1%) found in the AMD include Fungi, Plantae and Animalia kingdoms (Fig. 1b). Ascomycota, unclassified Fungi phylum (Fungi_p), Basidomycota, and

Mucoromycota represented Fungi kingdom; while Ciliophora, and Arthropoda phyla were Animalia, and Chlorophyta phylum epitomised Plantae kingdom. Association of the domain Eukarya (comprising Alveolates, Chlorophyta and Fungi as observed in this study) with AMD is reportedly of a lesser extent when compared with Bacteria [27]. The Fungi, largely represented by Ascomycota and Basidomycota in this study, are primarily found in sub-surface low-pH biofilms thriving in AMD [28]. While the Alveolates are suggested to have acted as primary/secondary consumers, the amoebae were secondary grazers in the AMD ecosystem [26, 29]. Fungi taxa must have participated in carbon cycling as the main decomposers in the microbial community of the AMD. The taxonomic composition and relative abundance of phyla regarded as 'Others' (sequence reads <1%) were presented in the Supplementary Table A.3 online.

Previous studies revealed that early stage of biofilm development in AMD was dominated by groups of oligotrophic *Leptospirillum* and archaea, which are acidophilic and rely on chemoautotrophic production based on metal oxidation [30]. Acidophilic copiotrophic heterotrophs comprising a surprisingly wide diversity (physiology and phylogeny) with prevailing metabolic traits do succeed the early colonisers [31]. Among the phyla dominating the Bacteria domain of life in the 'Onyeama' AMD were highly diverse classes that are known with AMDs. Low abundance Firmicutes and Actinobacteria lineages have also been previously characterized in AMD metagenomic data sets [6, 10]). Interestingly, families in the Class Bacteroidia including Porphyromonadaceae (12.4%), Prolixibacteraceae (1.6%), and unclassified GU454901 (1.2%) were dominant in the AMD as previously reported [10].

In Eukarya, dominant classes in the kingdom Fungi spread among Ascomycota (Eurotiomycetes, 33.8%; unclassified Ascomycota class, 18.1%; Dothideomycetes, 3.7%; Sordariomycetes, 3.3%; and Saccharomycetes, 1.3%), unclassified Fungi (4.0%), Mucoromycota (Umbelopsidomycetes, 1.2%), and Basidiomycota (Tremellomycetes, 1.2%, and Agaricomycetes, 1.0%). However, dominant Animalia comprised sub-kingdom Alveolata and Metazoa represented by unclassified Ciliophora (12.6%) and unclassified Arthropoda_c (8.4%), respectively. Metabolic CO_2 from protozoan respiration is assumed to further increase the level of dissolved inorganic carbon contributing to carbonate concentration that curtailed acidic pH in the microenvironment [32]. Unclassified Chlorophyta class (5.5%) was the only taxonomic class of Phylum Chlorophyta belonging to kingdom Plantae that formed part of dominant Eukarya in the AMD.

Alpha diversity and phylogeny of microbial OTUs in AMD from coal mine

The number of clustered high quality, non-chimeric sequences as OTUs based on CD-HIT and UCLUST against the sequence reads was depicted as asymptotic rarefaction curves (Fig. A.1). The curves revealed that higher numbers of OTUs were delineated from valid sequence reads of 16S rDNA unlike lesser number of OTUs obtained from valid sequence reads of ITS2 region located between 5.8S and 28S rDNA of eukaryotes. The OTU richness observed in the rarefaction curves established coverage of majority of species and was further validated with the richness and diversity estimations presented in Table 2. Despite the higher number of valid sequence reads obtained from the amplified ITS2 (40 403) than that

of 16S rDNA (26 160), the observed OTUs were more in 16S rDNA (2036) than those of ITS2 (1002). More than 99.8% and about 98.5% of the sequences in the AMD from 'Onyeama' coal mine represented eukarya and bacteria, respectively, based on estimated Good's library coverage. Furthermore, the estimated OTU richness (based on higher values obtained from ACE, Chao1 and JackKnife indexes) showed that bacterial phylotypes were richer than species of eukarya. Alpha diversity indexes (NPShannon, Shannon, and inverse Simpson) phylogenetic diversity index revealed that bacteria in the AMD were more diverse than eukarya OTUs.

The phylogeny, based on evolutionary history, of bacterial OTUs whose relative abundance is ≥1% of the total valid sequence reads was deduced via Neighbor-Joining method as unrooted phylogenetic tree aligned the dominant bacterial OTUs into three clades (Fig. 2). The OTUs have not been reported as dominating bacteria communities in AMD biofilm development other than in AMD undergoing transformation [33]. The relative abundance of the bacterial OTUs with their counts and ratio were presented in Supplementary Table A.4 online. Species of *Paludibacter* are acidophilic and have been associated with reduction of sulphate and Fe^{3+} in an AMD-impacted site [33]. Furthermore, *Rubrivivax gelatinosus* group use NiFe hydrogenase to stem H^{+} to hydrogen, while *Novosphingobium flavum* group degrade coal hydrocarbons and generate hydrogen using formate dehydrogenase enzyme [34]. Moreover, *Thauera selenatis* is known for using selenate or other metals as preferred electron acceptor for respiration, whereas; *Dechloromonas* species are famous for their denitrifying role in extreme ecosystem [35]. Nevertheless, the dominant eukaryotic OTUs (sequence reads ≥ 0.5%) spread across Fungi (7 OTUs), Animalia (3 OTUs) and Plantae (3 OTUs) as presented along with their corresponding counts and ratio (see Supplementary Table A.5 online). The evolutionary relatedness of representative strains of the OTUs were calculated and delineated as unrooted phylogenetic trees (Fig. 3). It is important to note that majority of the dominant OTUs delineated as Eukarya in the AMD were unclassified and their role in carbon fluxes cannot be ascertained for now.

Sequestration of toxic HMs/metalloid from simulated AMD and actual AMD from coal mine

AMD-impacted surface waters have been the motivation for extensive restoration efforts with ultimate goals of stemming acidity, toxic metal loads, and enhancing biotic structure (diversity and abundance) of such polluted hydrosphere. It is essential to collect and decommission AMD of its toxicants since it is impossible to prevent its formation and flow from source. Attempt to ameliorate the stern environmental problem caused by AMD requires selection of competent microorganisms that have capacity to mellow the stressors contained in AMD. Fortification of rich or semi-rich culture broth with toxicants of interest is a common approach to select competent microorganisms [36]. After culture enrichment, 26 373 valid reads from non-chimeric sequences were clustered into 95 OTUs of bacteria. Alpha diversity of the enriched culture revealed low species richness based on estimated values of ACE, Chao1 and JackKnife (Table 2). Estimated diversity further depicted poorly diverse bacteria species in enrichment culture via phylogenetic diversity valued at just 182. This also corroborated the estimates of other diversity indexes (NPShannon, Shannon, and inverse Simpson) presented in Table 2. The consortium of bacteria containing 7 dominant groups of OTUs observed to be involved in toxic metal sequestration of AMD. The

taxonomy and counts, at inoculation and post-incubation comprised two taxonomic classes of bacteria (Table A.6), whose evolutionary relatedness was depicted as unrooted phylogenetic tree (Fig. 4). The classes with their OTUs include Y-Proteobacteria (*Acinetobacter pittii* group, Enterobacteriaceae group, unclassified *FWNZ* species, and *Pseudomonas citronellolis* group), and Bacilli (*Sporosarcina koreensis* group, *Bacillus cereus* group, and *Exiguobacterium aurantiacum* group). The bacteria (particularly *Acinetobacter pittii, Pseudomonas citronellolis,* and *Bacillus cereus*) have been involved in degradation of indole, a heterocyclic aromatic compound found in coal, via attack on either/or both the carbocyclic and N-heterocyclic rings [37]. Studies involving *Acinetobacter* [38], Enterobacteriaceae [39], *Pseudomonas* [40], *Sporosarcina* [7, 41], *Bacillus* [41], and *Exiguobacterium* [38] OTUs for sequestration of toxic metals/metalloids in environmental media have been reported. Bioaccumulations of Cd, Co, and Zn were reported for *Sporosarcina* sp. G3 as sequestration strategy, whereas Cr and Hg were reduced via redox-active enzymatic activities to innocuous forms [42].

Urease-producing bacteria instigate insoluble metal-carbonate micro-precipitation through urease activity [16]. The growth-time courses and urease activities of the bacteria consort in simulated AMD were presented as curves (Fig. 5). It was observed that impact of high concentrations of HMs cocktails was not pronounced beyond early 6 h post-inoculation, which was apparently regarded as lag phase. The bacteria consortium might have activated necessary genes needed to tolerate and sequester the metals/metalloids toxicity during the lag phase without cell multiplications. Afterwards, the bacteria consortium grew steadily with production of urease, based on increasing measurement of urease activity, as incubation continued. At 30 h post-inoculation, 245.3 (±23.7) U ml^{-1} activity of urease was observed in broth without toxic metal cocktail. However, more urease activity (255 ±7.6 U ml^{-1}) by the bacteria consortium was observed in medium amended with low concentrations of metal cocktails unlike less activities of 235 (±7.6) U ml^{-1} and 193.7 (±10.7) U ml^{-1} associated with medium and high metal concentrations, respectively. As the growth remains stationary and pH further increased to >8.2, urease activities were at least 253 U ml^{-1} in all the cultures. Urease activities observed in this report were assumedly the first at acidic pH, which compared favourably with values recorded at alkaline pH in previous studies [7, 16, 41, 43]. Moreover, pH of the culture system kept increasing, alleviating the acidity condition that initially prevailed in the AMD system.

Interestingly, urease activity was observed at acidic pH, although at low quantity unlike the level of activity when the pH inclined towards alkaline. Urease activities could scintillatingly explain principles behind toxic metal/metalloid sequestration in this study among other sequestration processes that may exist in AMD-impacted ecosystems. This must have involved hydrolysis of urea into ammonia and carbamate ($CO(NH_2)_2 + H_2O \rightarrow NH_2COOH + NH_3$), which subsequently released ammonia and carbonic acid ($NH_2COOH + H_2O \rightarrow NH_3 + H_2CO_3$). The products might have equilibrated in water to form bicarbonate, ammonium and hydroxyl ions that serially increased the culture pH. Ultimately, the bicarbonate equilibrium might have shifted to form carbonate ions ($HCO_3^- + H^+ + 2NH_4^+ + 2OH^- \leftrightarrow CO_3^{2-} + NH_4^+ + 2H_2O$) that enhanced the metal-carbonate micro-precipitation ($Me^{2+} + Cell \rightarrow Cell\text{-}Me^{2+} + CO_3^{2-} \rightarrow Cell\text{-}MeCO_3$). The gradual increase in pH could have further indulged the formation of CO_3^{2-} from HCO_3^-,

leading to metal-CO_3 precipitation around cells, and in culture media. Bicarbonates enrichment with inherent ammonia production was thought to have provided additional acid neutralization of the AMD. The growth kinetics after the presumed lag phase in the early 6 h to late exponential phase at 18 h showed that low concentration of HMs cocktails did not have impact on the growth of the bacteria consortium. Consequently, the bacteria consortium exhibited excellent sequestration of multi-component toxic HMs in both the simulated toxic metal-rich AMD and the actual AMD obtained from 'Onyeama' coal mine (Table 3).

The bacteria consort displayed more than 94% efficiency of Cd and Pb sequestration in natural AMD, while 100% efficiency was observed in all the simulated AMD treatments (Table 3). Low performance was found with Ni and As, but not less than 70% sequestration efficiency was observed in all treatments. Efficient sequestrations, up to 100% removal of most toxic metals, have been observed with bacteria consortium (Tamayo-Figueroa et al. 2019) similar to findings in the present study. Mixed-bacterial cultures are known to be able to perform more complex tasks and survive in more unstable environments than a mono-culture. Nevertheless, 89.3-98% removal efficiencies of Ni, Pb, Co, and Cd from solution have been reportedly achievable with urease-producing *Sporosarcina koreensis* [44]. Similarly, *Bacillus* sp. KK1 reportedly mitigated lead-contaminated mines tailings containing mobile Pb (1,050 mg kg^{-1}) to form insoluble precipitates of PbS and $PbSiO_3$ [31]. Growth-dependent sequestration of HMs cocktails by the bacteria consort was adduced to be via precipitation as weight of these precipitates were evaluated to be proportional to concentrations of HMs cocktail present. The bacteria consortium was observed to drive formation of as much as 15.6 (±0.92) mg ml^{-1} precipitates (Table 3) that were assumed to be in form of HMs-carbonates in TGYM supplemented with high concentrations of HMs cocktail within 24 h post-inoculation. In natural AMD bio-stimulated with urea and seeded with bacteria consortium for 24 h, 10.5 (±0.52) mg ml^{-1} HMs precipitates was observed unlike 8.57 (±2.52) mg ml^{-1} precipitates obtained from natural AMD toxic metals sequestration without urea fortification. It appeared that quantity of toxic metal precipitates was proportional to quantities of available toxic metals, which corresponded to the number of heterogeneous nucleation sites on the surface of the bacterial cells. Omoregie et al. [41] reported relatively similar quantum of precipitation as $CaCO_3$ with species of ureolytic Firmicutes isolated from limestone caves. As such, there was no correlation between urease activity and quantum of toxic metal precipitation since there is likelihood that other metabolic activities may be linked to urease activities.

In conclusion, AMD from 'Onyeama' coal mine is a point source of pollution to the surrounding environments because of its richness in anions and toxic metals/metalloids. It has high potential of enriching the receiving hydrosphere with toxic metals/metalloid and exerts severe ecological risks to the biological elements of the ecosystems. Dominance of Proteobacteria, Bacteroidetes, Ascomycota, and Ciliophora characterised the microbial community of the AMD, where unclassified OTUs occurred mostly among the species. Enrichment of the AMDs led to selection of bacteria consortium with excellent potential of stemming the toxicants in the AMD. The bacteria consortium efficiently removed toxic metals/metalloid through precipitation and simultaneously neutralise the acidity of AMD. The bacteria consortium exhibited appreciable urease activity, through which the precipitation was assumed possible

via formation of metal/metalloid-carbonates. The bacteria consortium is suggested as sustainable biotechnological candidate in designing bioremediation strategy to decommissioning AMD before discharge into surrounding environment.

Methods

Study site and sampling

Nigeria, with over 1,200 million tons of coal reserves mostly in Enugu State, had previously depended on coal for energy before it was abandoned upon discovery and boom of petroleum hydrocarbons. 'Onyeama' coal mine is one of the derelict coal fields in Enugu State that has been inactive for more than five decades, and it was the location for the present study (GPS coordinates: Lat., 6^{o} 26' 28.1649" E and Long., 7^{o} 28' 45.156" E). Biofilm samples of AMD were randomly collected from streams emanating from 'Onyeama' coal mine. Biofilms on cobbles were removed using a soft bristle brush into water in a sterile beaker. Biofilms that grow at the solution-air interface in AMD effluent were collected from the surface of AMD solutions in sterile sampling bottles. Pristine freshwater stream in the same locality was collected for reference background geochemical values against calculating pollution and ecological risk indexes of the AMD. The samples (from 10 random locations) were mixed together to represent each sampling replicate, packed in a Ziploc bags, and transported to the laboratory in ice-cooler. Samples for microbiological culturing were stored at 4 ^{o}C, while those for geochemical analysis and metagenomic were stored at -40 ^{o}C. All sampling were performed in three replicates.

Geochemistry of AMD from coal mine and evaluation of hazardous metal pollution

Physico-geochemical parameters of the samples were determined by standard methods earlier reported [25, 45]. While pH, colour and temperature were determined *in situ*, other assays including electrical conductivity, turbidity, chlorides, total dissolved solids (TDS), NO_3^-, SO_4^{2-}, PO_4^{2-}, CO_3^-, total organic carbon (TOC), and dissolved sulphides were determined *ex situ* using standard protocols. Seven HMs including Pb, Cd, Co, Ni, As, Fe and Cr were quantified via Atomic Absorption Spectrophotometry (AAS-Perkin-Elmer Analyst 200; Pelkin-Elmer, Canada) after acidic digestion ($HNO_3/HClO_4$ [4:1, v/v]) of sample (0.1 g) in a microwave oven [21]. AAS normalisation, validation, operational conditions and the limit of detection were as earlier reported [45]. The wavelengths used for Cd, Pb, Co, Ni, Cr, Fe and As measurements were 228.8, 283.3, 240.7, 231.1, 357.9, 248.3 and 193.7 nm analytical lines, respectively.

The pollution indices of the measured HMs were based on contamination factor (CF), enrichment factor (EF), geo-accumulation (I_{geo}), pollution load index (PLI), pollution index (PI), and degree of contamination (C_d) as earlier reported [25], while modified pollution index (MPI), and modified degree of contamination (MC_d) were as stated (see Supplementary Calculations online for details). The ecological risks of the measured HMs in the coal AMD were based and expressed as potential ecological risk factor (Er), and potential ecological risk index (RI) as reported [25], but modified potential ecological risk factor (MEr),

modified potential ecological risk index (MRI), and risk quotient (RQ) were calculated (see Supplementary Calculations online for details). All measurements and calculations were performed in three replicates.

Microbiome analysis of AMD from coal mine

Isolation of total community DNA in AMD

Approximately 0.5 g dry weight of sediments was subjected to total community DNA (tcDNA) extraction, using FastDNA® Spin Kit for Soil (MP Biomedicals, Solon, OH, USA) by following the manufacturer instructions, and mechanical lysis of cells was achieved with the FastPrep® Cell Disruptor FP 120 (Qbiogene, Heidelberg, Germany). Interference of PCR with humic substances in tcDNA was prevented by adding 20 mg of skim milk to sediment sample (0.5 g) in the lysing matrix as earlier reported [25]. The Cell Disruptor was operated at 6.5 speed for 40 s in order to achieve a harsh cell wall disruption. The yield, quality and fragment of the crude and purified tcDNA was checked with Nano-Drop Spectrophotometry along with 0.8 % (w/v) agarose gel electrophoresis and visualized in UV light upon staining with ethidium bromide.

High throughput sequencing and metagenome data analysis

Using tcDNA as template, V3-V4 region of 16S rRNA genes (bacteria) and ITS (Internal Transcribed Spacer) genes between 5.8S and 28S rRNA genes (eukarya) were amplified using primer set 341F and 805R, and ITS3-Mi (forward) and ITS4-Mi (reverse) (ChunLab Inc., Seoul, South Korea), respectively. Libraries were constructed via Illumina MiSeq platform (Illumina, San Diego, CA, USA), qualities checked with Agilent 2100 Bioanalyzer System (Agilent Technologies, Palo Alto, CA, USA) using a DNA 7500 chip, and quantified using Quanti-iT^{TM} PicoGreen^{TM} dsDNA Assay kit (Invitrogen) according to the manufacturer's instructions. Short DNA fragment was removed using CleanPCR^{TM} (CleanNA, Netherlands), and sequencing was performed using Illumina, MiSeq Reagent Kit v2 (500-cycles) at ChunLab Inc.

Quality of sequencing data was checked and low quality (<Q25) reads were filtered with Trimmomatic 0.32 software [46]. The pair-end sequence of the same strand of PCR amplicon were merged based on overlapping sequence information using PANDAseq software [47]. ChunLab's pipeline in-house algorithms were used to remove 16S rRNA PCR primer sequences, and UNITE (https://unite.ut.ee) was used to analyse ITS2 gene. Non-specific amplicons were identified and removed using the HMMER program-based search to exclude Singleton sequences [48]. While sequences denoising were performed with DUDE-Seq software [49], sequences were de-replicated and non-redundant reads were extracted via UCLUST-clustering [48]. UCHIME was used for detection and removal of chimera against BIOiPLUG's chimera-free reference database, while the remaining non-chimeric sequences were clustered into operational taxonomic units (OTUs) as discussed by Lee et al. [49]. Taxonomic assignment was carried out by comparing the sequence reads against the EzBioCloud 16S database (https://www.ezbiocloud.net/), using a combination of the initial BLAST-based searches and additional pairwise 97% similarity comparisons as the cut-off [50].

Culture enrichment and toxic metal sequestration using bacterial consortium

Consortium of bacteria indigenous to coal AMD that tolerate elevated concentrations of HMs mixtures were sought in an attempt to develop bacteria-based bioremediation strategy for alleviating HM-toxicity in AMD. These involved:

Mixed-culture conditions and identification of bacteria consortium tolerant to HMs

The AMD biofilm sample (10 ml) was enriched in a modified sterile Tryptone Glucose Yeast extract (TGY) broth (90 ml) containing (l^{-1}): casein peptone, 5g; glucose, 1 g; yeast extract, 2.5 g (Xebios Diagnostics, Düsseldorf, Germany), dissolved in mineral salts solution instead of distilled water to form Tryptone Glucose Yeast extract (TGYM) broth. The mineral salts solution contained (l^{-1}): K_2HPO_4, 1.775 g; KNO_3, 2g; NaCl, 2g; $MgSO_4$. $7H_2O$, 0.05g; $CaCO_3$, 0.02g; $FeSO_4$. $7H_2O$, 0.01g, and pH adjusted to 3.5 (approx.) in relation to the highest measured pH of AMD from 'Onyeama' coal mine. TGYM broth was amended with 10 mg l^{-1} $CdCl_2$, 20 mg l^{-1} $PbCl_2$, 10 mg l^{-1} $CoCl_2$, 10 mg l^{-1} $NiCl_2$, and 20 mg l^{-1} Na_2HAsO_4. The glucose solution was filtered through 0.2 µm Minisart syringe filters (Sartorius Stedim Biotech, Gottingen, Germany), and added aseptically. Culture enrichment was achieved via four transfers and incubations (30 ^oC; 100 ×g; 48 h), after which 10 ml culture was harvested (10 000 ×g; 10 min) and washed with phosphate buffered saline twice. The washed biomass of bacteria consortium was resuspended (approx. 10^9 cells ml^{-1}) in sterile buffered saline and stored in glycerol (1:1, v/v) mixture at -20 ^oC. Total DNA (tDNA) was extracted from broth culture biomass, bacteria domain amplified based on V3-V4 region of 16S rRNA gene, library constructed and sequenced using Illumina MiSeq system and taxonomic assignments of non-chimera sequences were as explained in Section 2.3.

Preparation of bacteria consortium biomass for sequestration of toxic metals mixture

Prior to further studies, bacteria consortium biomass was resuscitated from the stock by pre-culturing in Erlenmeyer flask containing TGYM broth for 24 h at 30 $^{\circ}C$ and 100 ×g, and the biomass was harvested (7000 ×g; 10 min), washed thrice with phosphate buffer (50 mmol l^{-1} KH_2PO_4, pH 7.2), and suspended in the same buffer. Resuscitated bacteria consortium (approx. 10^6 cfu ml^{-1}) were starved overnight in Tris-HCl (0.1 mol l^{-1}), and re-suspended in sterile Milli-Q water (previously supplemented with mixtures of HMs: 5 mg l^{-1} each of $CdCl_2$, $PbCl_2$, $CoCl_2$, $NiCl_2$, and Na_2HAsO_4, final concentration) as inoculum.

Growth kinetics and urease activity of bacteria consortium

Growth kinetics of the bacteria consortium was performed in TGYM broth (100 ml) supplemented with HMs mixture (5 mg l^{-1} each of $CdCl_2$, $PbCl_2$, $CoCl_2$, $NiCl_2$, and Na_2HAsO_4, final concentration) with and without urea (0.1 M final concentration) fortification, and pH adjusted to 3.5 (approx.). Inoculum (1 ml) as determined in section 2.4.3 above, was added to the set-ups and incubated (100 ×g; 30 ^oC; 48 h). $OD_{(600\ nm)}$ and pH were measured at every 6 h post-inoculation. The urease activity of the mixed culture was

determined as reported previously [7], using phenol-hypochlorite assay where NH_4Cl (50-1000 µM) was used as standard. Readings at 6 h interval were as described [43], and a unit of urease activity (U) was defined as the amount of urease hydrolysing 1 µmol urea min^{-1}.

Determination of bacterial growth-dependent HMs/metalloid sequestration in simulated and natural AMD

For simulated AMD, two sets of 500 ml Erlenmeyer flasks containing 100 ml of TGYM broth amended with HMs/metalloids cocktails (none, low, mid, and high concentrations; see Supplementary Table A.7 online for details) were prepared, divided into two sets where a set was without urea and the other was fortified with urea (2%, w/v). Inoculum of bacteria consortium (1 ml), as prepared above (Section 2.4.2), was added into broth and incubated (100 ×g; 30 ^{o}C; 48 h). Growth of bacteria consortium was monitored every 6 h as turbidity via $OD_{(600\ nm)}$ of 5.0 ml sample using UV-visible spectrophotometer, and the pH was measured simultaneously. The blank was un-inoculated experimental set ups. To determine HMs/metalloid sequestration, biomass of each culture was harvested (10,000 ×g, 10 min), and supernatant was analysed for HMs/metalloid contents using AAS (as explained in Section 2.2). The sequestration efficiency (SE) was determined using: , where MQM is metal concentration in medium before inoculation, SQ is metal concentration in the supernatant after incubation [36]. Evidence of HMs/metalloid sequestration was determined by estimating HMs/metalloid-precipitation using modified methods of estimating $CaCO_3$ precipitates earlier reported [41]. As such, TGYM broth was supplemented with urea 2% (w/v) and salts of HMs/metalloids instead of $CaCl_2$. Experimental set-ups but without bacterial inoculation, and TGYM without HMs/metalloid but inoculated with bacteria consortium were used as negative and positive controls.

For HMs/metalloid sequestration of natural AMD, drains (AMD sample) collected from ‘Onyeama’ coal mine were filtered first through cellulose filter paper (Whatman™ 1001-070 Grade 1; pore size, 11 µm) and then serially through sterile Minisart syringe filters (0.45µm → 0.2µm, sequentially). The sterile AMD, without adjusting its pH but aseptically enriched with sterile casein (5 g l^{-1}) and yeast extract (0.5 g l^{-1}), was divided into four parts of 100 ml AMD in 500 ml Erlenmeyer flasks. Two of the flasks containing sterile AMD were aseptically supplemented with separately autoclaved urea (2%, w/v), while the other two flasks of AMD were without urea treatment. A flask of AMD with urea fortification and another without urea amendment were inoculated with bacteria consortium as stated earlier. Control experiments were un-inoculated AMD with and without urea. Both experimental set-ups and controls were incubated (100 ×g; 30 ^{o}C; 48 h). HMs/metalloid sequestration efficiency of the AMD was determined and HMs/metalloid precipitates were measured as explained earlier. Heterogeneous nucleation sites on bacterial cell surfaces were quantified according to methods of Omoregie and co-workers [41]. All experiments were performed in three replicates.

Statistical analyses and metadata achieving

The mean of three replicates of experimental values or measurements, and standard error of mean (SEM) were performed using the Prism 5 software program (GraphPad Software, San Diego, CA, USA). All charts

(except pie chart that was performed using Microsoft Excel package) and curves were performed using Prism 5 software unless otherwise stated. The estimated coverage of the constructed 16S rRNA gene libraries was calculated as: according to Kemp and Aller [51], where n is the number of Singletons after assembly and N is the total number of sequences in the initial dataset. Richness and diversity statistics of the bacterial community including abundance-based coverage estimator (S_{ACE}), the bias-corrected Chao1 (S_{chao1}) and the Shannon-Weaver diversity index were estimated using pre-calculated program of CLcommunity^{TM} software package (ChunLab Inc.). All statistical tests were considered significant at $p < 0.05$. The sequencing metadata obtained and used in this study have been deposited in the NCBI's sequence read archive (SRA) database under BioProject ID: **PRJNA625753** under the BioSample accession **SAMN14608860** (https://www.ncbi.nlm.nih.gov/biosample/14608860)

Declarations

Conflict of Interest

Each of the Authors has no competing moral or financial interest in relation to the work described.

Funding: This research did not receive any specific grant from funding agencies in the public, commercial, or not-for-profit sectors. The study was self-funded by the authors.

Conflicts of interest/Competing interests: Each of the Authors has no competing moral or financial interest in relation to the work described.

Availability of data and material: Not applicable

Code availability: Not applicable

Authors' contributions: The study conception and design were performed by Ganiyu Oladunjoye Oyetibo. All authors contributed to material preparation, data collection and analysis. The first draft of the manuscript was written by Ganiyu Oladunjoye Oyetibo and all authors commented on previous versions of the manuscript. All authors read and approved the final manuscript.

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Tables

Table 1: Pollution and ecological impact determinants of heavy metals and metalloid contained in the AMD from coal mine.

Indexes	Determinants	Interpretations
Pollution factors		
CF	Very high (> 6)	Cd > Co > Pb > As > Ni > Cr > Fe
EF	Exceptionally high (> 50)	Cd > Co > Pb > As > Ni
	Very high (25 ≤ EF < 50)	Cr
	Moderate (3 ≤ EF < 5)	Fe
I_{geo}	Very severe (≥ 5)	Cd > Co > Pb > As > Ni > Cr > Fe
PLI ($\times 10^2$)	31.1 ± 9.42	Progressively deteriorating (> 100)
PI ($\times 10^5$)	21.3 ± 1.16	Severe (> 3)
MPI ($\times 10^3$)	20.8 ± 1.19	Severe (> 10)
C_d ($\times 10^5$)	34.0 ± 2.40	Severe
MC_d ($\times 10^3$)	33.4 ± 2.44	Severe
Ecological risks		
Er	Very high (Er > 320)	Cd > Co > Pb > As > Ni > Cr > Fe
MEr	Very high (MEr > 320)	Cd > Co > Pb > As > Ni
	Considerate (80 < MEr ≤ 160)	Cr
	Low (MEr < 40)	Fe
RQ	High (RQ > 1)	Pb > Cd > As > Ni > Co > Fe > Cr
RI ($\times 10^6$)	38.1 ± 2.18	Very high (RI > 320)
MRI ($\times 10^4$)	37.5 ± 2.24	Very high (MRI > 320)

Values are mean (± SEM) of triplicate sampling measurements.

The pollution indexes and ecological risk assessment factors include: contamination factor (CF), enrichment factor (EF), geo-accumulation index (I_{geo}), pollution load index (PLI), pollution index (PI), modified pollution index (MPI), degree of contamination (C_d), modified degree of contamination (MC_d), potential ecological risk factor (Er), modified potential ecological risk factor (MEr), potential ecological risk index (RI), modified potential ecological risk index (MRI), risk quotient (RQ)

Table 2: Alpha diversity of microbiome evenness, richness and varieties of species in the sediments

	Bacteria		Eukarya
	Biofilm water (AMD-EB)	Enrichment culture (AMD-EC)	Biofilm water (AMD-EB)
Actual			
Valid reads	26,160	26,373	40,403
OTUs	2,036	95	1,002
Estimated richness			
ACE	2,293.26	133.37	1,033.01
HCI	2,346.43	169.09	1,047.66
LCI	2,244.59	109.57	1,019.11
Chao1	2,174.97	126.23	1,016.11
HCI	2,217.70	173.29	1,029.93
LCI	2,142.28	107.46	1,009.13
JackKnife	2,438	127.45	1,078
HCI	2,438	145.15	1,078
LCI	2,438	109.75	1,078
Estimated diversity			
NPShannon	5.97	1.58	3.54
Shannon	5.86	1.57	3.51
HCI	5.88	1.59	3.54
LCI	5.84	1.55	3.48
Simpson	0.01	0.41	0.13
HCI	0.01	0.42	0.14
LCI	0.0097	0.41	0.13
Phylogenetic diversity	2,779	182	1,186
Good's Lib. Coverage (%)	98.45	99.89	99.81

Clustering of OTUs found was achieved with UCLUST and the open reference method as all taxa were selected for analysis; HCI = High Confidence Interval (95%); LCI = Low Confidence Interval (95%); OTUs = Operational taxonomic units determined at 97%

Table 3: Growth associated sequestration and precipitation of heavy metals/metalloid cocktail and AMD from 'Onyeama' coal mine.

	Sequestration efficiency at 24 h incubation (%)					Precipitate weight (mg ml^{-1})
	Cd	Pb	Co	Ni	As	ΣHMs
Low HMs*	100	100	100	95.9 ± 4.1	91.3 ± 3.3	6.23 ± 0.43
Medium HMs**	100	100	84.6 ± 6.4	89.7 ± 10.3	87.0 ± 2.6	11.9 ± 0.97
High HMs***	100	100	76.6 ± 3.8	89.7 ± 10.3	84.8 ± 2.3	15.6 ± 0.92
Natural AMD + Urea	100	100	70.1 ± 26.8	95.2 ± 4.8	91.2 ± 1.69	10.5 ±0.52
Natural AMD only	96.3 ± 3.18	94.8 ± 2.46	76.9 ± 2.99	90.6 ± 2.76	88.9 ± 0.982	8.57 ± 2.52

Values are mean (± SEM) of triplicate experiments.

*Low HMs broth comprised (per liter) Cd, 27.9 mg; Pb, 118.7 mg; Co, 16.2 mg; Ni, 16.2 mg; and As, 61.5 mg.

**Medium HMs broth contained (per liter) Cd, 55.7 mg; Pb, 237.3 mg; Co, 32.4 mg; Ni, 32.3 mg; and As, 123.1 mg.

***High HMs broth contained (per liter) Cd, 139.3 mg; Pb, 593.3 mg; Co, 81.1 mg; Ni, 80.7 mg; and As, 307.6 mg.

At the start of experiment, Natural AMD from coal mine used in this study contained (per liter) Cd, 95.0 ±5.12 mg; Pb, 326 ±26.8 mg; Co, 27.3 ±9.25 mg; Ni, 28.8 ±13.4 mg; and As, 56.7 ±14.7 mg.

Figures

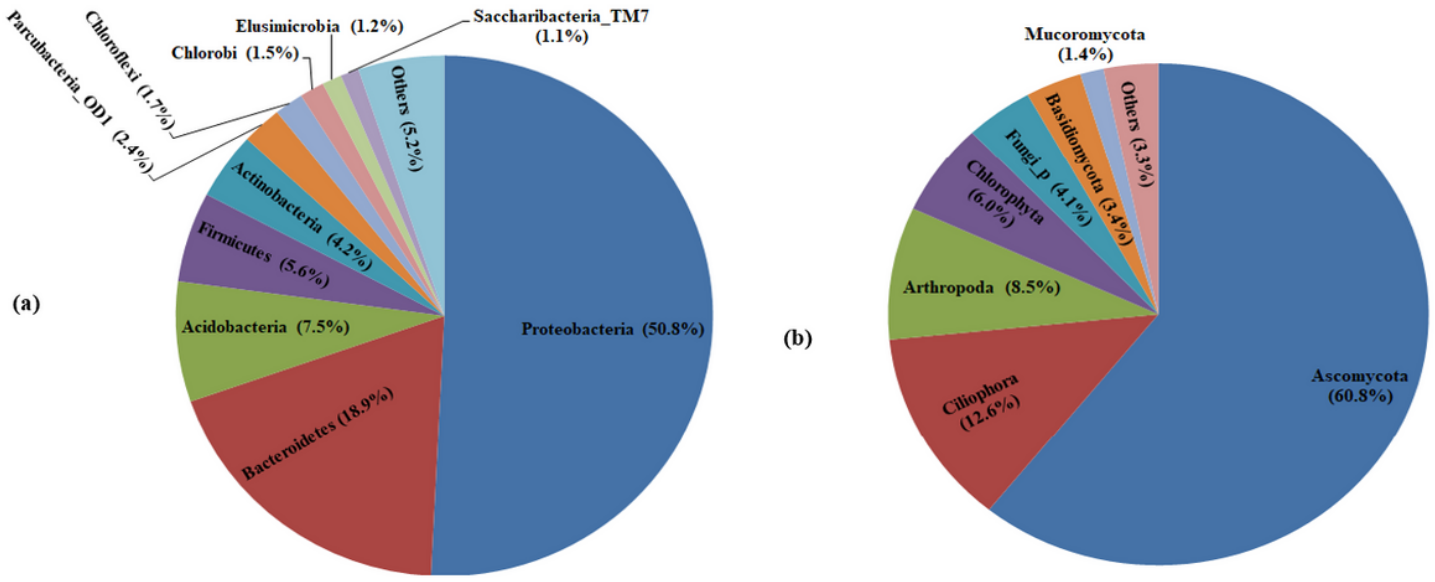

Figure 1

Taxonomic composition of Bacteria (a) and Eukarya (b) domains of life found in the AMD from 'Onyeama' coal mine, showing specific phyla and sub-kingdoms for kingdoms Plantae and Animalia. Phyla and sub-kingdoms that are less than 1% of the total sequence reads were regarded as 'Others'.

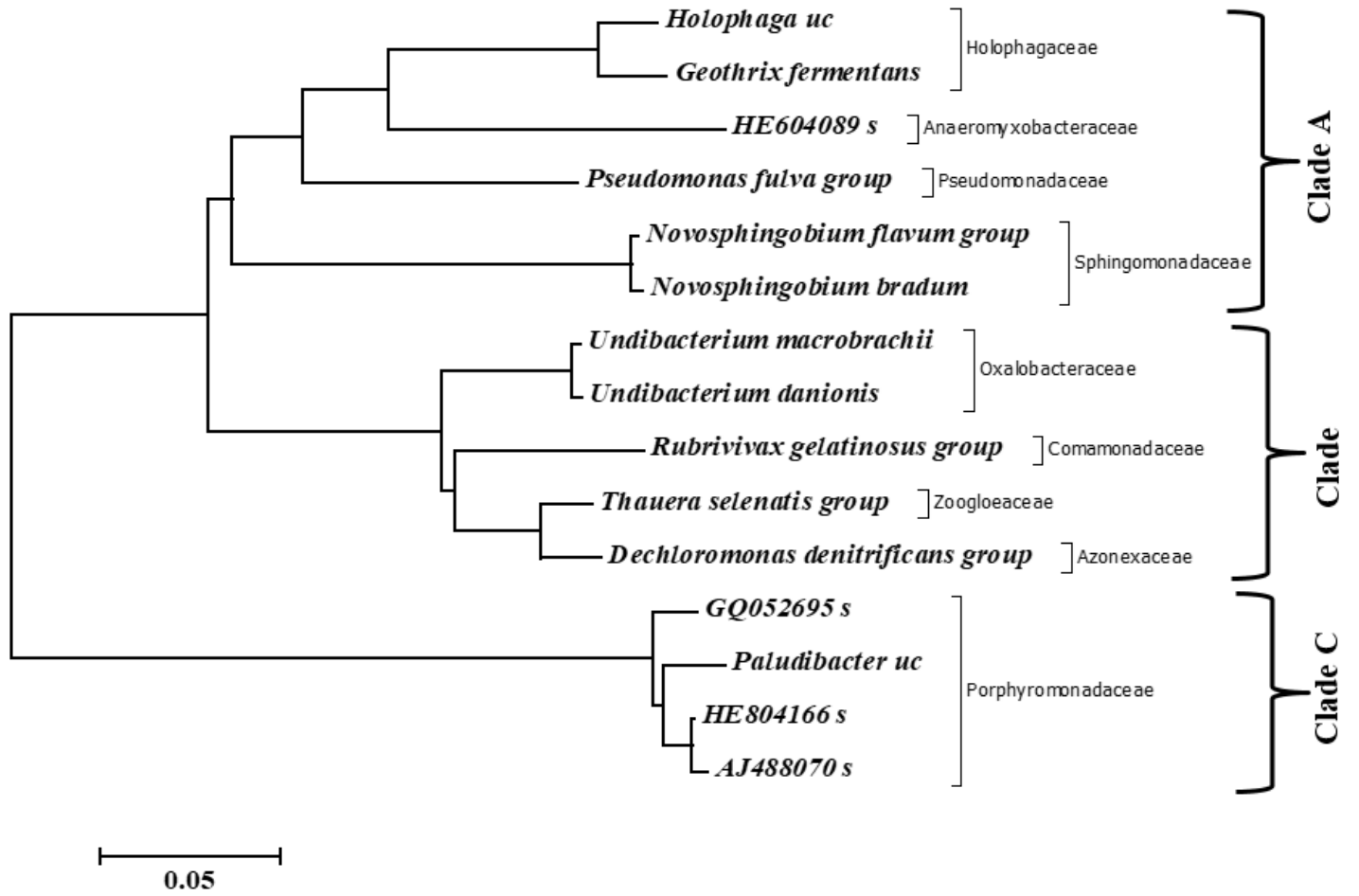

Figure 2

Evolutionary relationships of dominant bacteria taxa in the AMD from ‘Onyeama’ coal mine. The evolutionary history was inferred using the Neighbor-Joining method. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the unrooted phylogenetic tree. The evolutionary history was inferred using the Neighbor-Joining method. Bootstrap test of 1000 replicates were used to cluster associated taxa together. Evolutionary analyses were conducted in MEGA6.

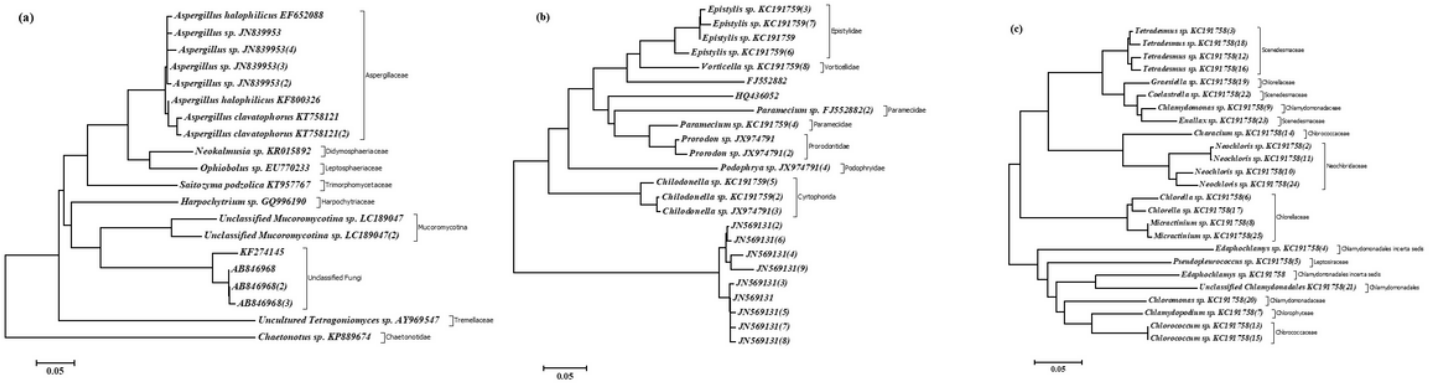

Figure 3

Evolutionary relationships of dominant OTUs of Eukarya showing selected strains of dominant Fungi (a), Animalia (b) and Plantae (c) taxa in AMD biofilm-water from ‘Onyeama’ coal mine. The evolutionary history was inferred using the Neighbor-Joining method. Bootstrap test of 1000 replicates were used to cluster associated taxa together and trees were drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the unrooted phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method Evolutionary analyses were conducted in MEGA6.

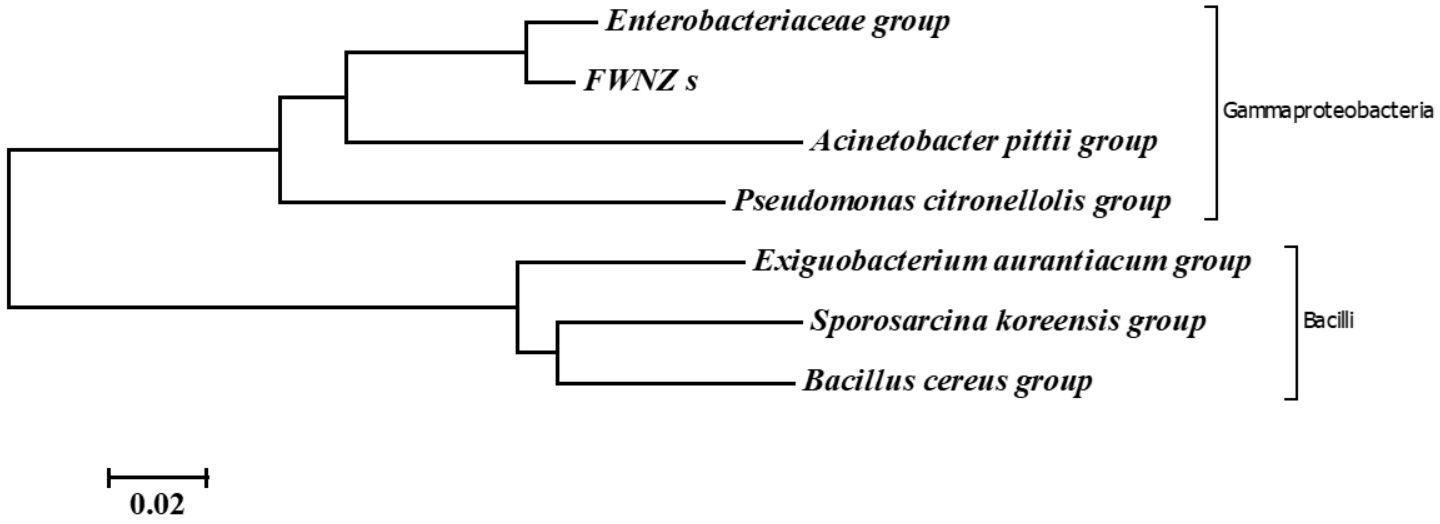

Figure 4

Evolutionary relationships of bacteria taxa that form consortium used in HMs sequestration of AMD from ‘Onyeama’ coal mine. The evolutionary history was inferred using the Neighbor-Joining method upon alignment via MUSCLE. The tree was drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the unrooted phylogenetic tree. The evolutionary distances were computed using the Maximum Composite Likelihood method with pairwise deletion and 1000 bootstrap replicates. Evolutionary analyses were conducted in MEGA6.

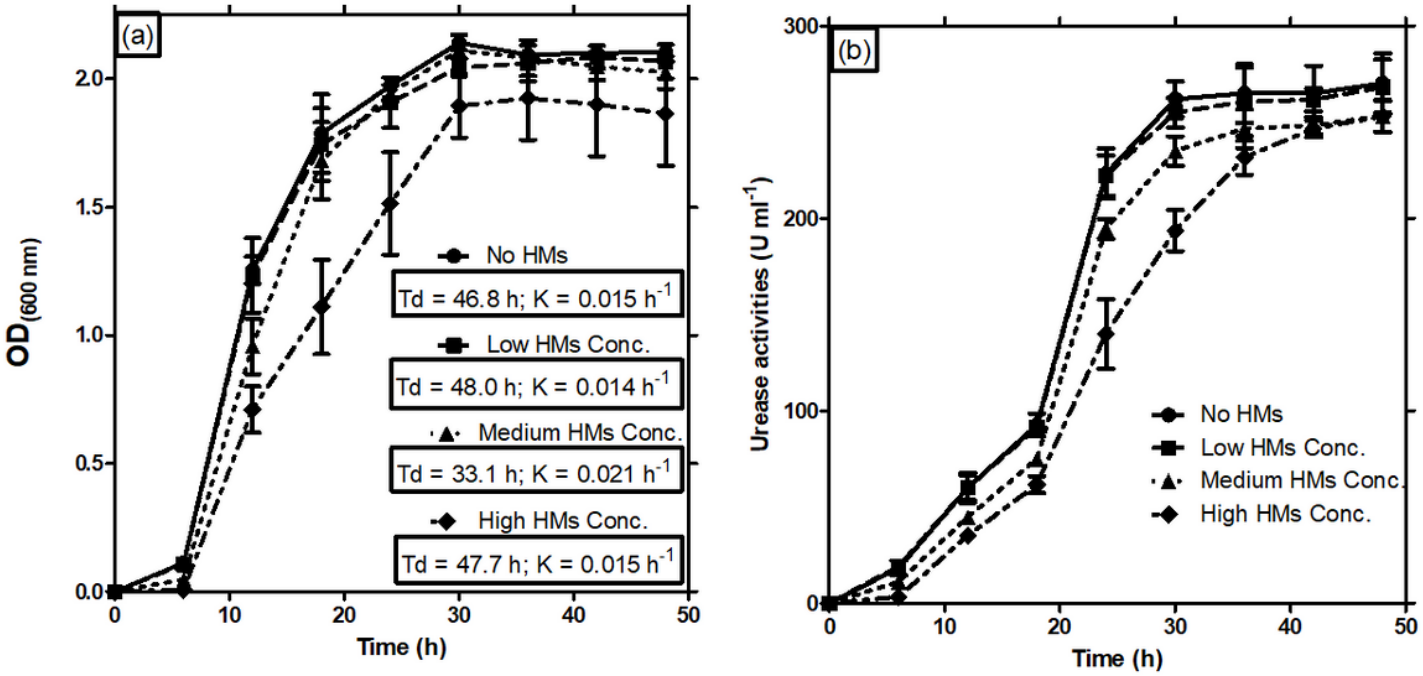

Figure 5

Growth kinetics of bacterial consortium and urease activity in TGYM broth without metal mixture (a), with low concentration (b), medium concentrations (c), and high concentrations (d) of metal mixtures. Growth kinetics is in the inserts, where ‘Td’ represents doubling time and ‘K’ is the growth rate at exponential growth phase. Error bars represent standard error mean (SEM) of triplicate experiments.

Supplementary Files

This is a list of supplementary files associated with this preprint. Click to download.

- Coalminedrainagemicrobiomesupplementarymaterials.pdf