**UNVEILING MICROBIAL DIVERSITIES AND ASSOCIATED GENES INVOLVED IN BIOREMEDIATION OF ACID MINE DRAINAGE USING NEXT GENERATION SEQUENCING**

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**Running Title**: Optimizing Bioremediation of Acid Mine Drainage: Unlocking the Potential of Microbial Diversity through Next-Generation Sequencing

**Abstract**

Bioremediation of acid mine drainage (AMD) involves a complex interaction between constituents of AMD and microbial communities as well as plants associated with the remediation. This complex interaction creates diverse metabolic pathways and mechanisms controlled by genes expressed by the microbial communities that enhance the remediation of AMD. The knowledge of these associated genes is essential as it can infer the development of strategies that can promote better/optimal remediation. Although diverse technologies have been employed by researchers to unveil the numerous microbes and associated genes involved in the bioremediation of AMD, advent of next generation sequencing (NGS) has shown to be more promising. This present review aimed at unpacking the identified microbes and genes prevailing in an AMD condition that could play crucial role in the remediation. A brief information on the impact of the AMD globally was provided on crop growth and development. Classes of microbes relevant in AMD and the associated genes were discussed as well as the role of NGS as a pivotal tool to the advanced discovery of the relevant genes. Sulfate-Reducing Bacteria, Iron-Reducing Bacteria, Metal-Resistant Bacteria, Biofilms forming microbes, and Methanogenic archaea are the dominant classes of microbes that play vital role in AMD remediation. Although diverse genes such as *dsrAB*, *mtrA,* *mtrB*, *mtrC*, *acn, furA, dpsA*, *copF, actP, copA, mmco, cutO*, *arsT, arsC, aioA/aoxB*, *afeI*, *afeR*, *ISAfe600*, *ISAfe1*, and *IST2* have been unveiled as genes responsible for the ability of the microbial communities to survive and remediate AMD, more application of NGS is recommended to unveil other unidentified genes that could pivot sustainable remediation of AMD.

**Keywords:** Acid mine water; next generation sequence; genes; microbial communities; remediation; heavy metals

**Introduction**

Acid mine drainage (AMD) is a significant environmental problem associated with the mining industry, particularly in areas where metal ores are extracted (Rezaie and Anderson 2020; Nleya et al. 2016). It occurs when sulfide minerals, often found in rocks containing metals like iron, copper, zinc, and lead, encounter air and water (Kumari et al. 2010). AMD, known to be highly acidic, disrupts the ecosystem around it including ground and surface water, hinders the growth of plants, and hampers microbial communities especially when left untreated (Adeniy et al. 2022; Roy 2021; Castillo 2020; Okereafor et al. 2020; Lopez et al. 2018). The development of cost-effective solution for prevention and remediation of AMD has been driven by concerns due to its environmental and economic impacts (Rambabu et al. 2020; Kefeni et al. 2017; Kalin et al. 2006).

AMD can be treated with a variety of active and passive remediation technologies. Currently, active and passive remediation methods are considered the most practical option for treating AMD (Naidu et al. 2019; Johnson and Hallberg 2005). Although AMD remediation methods are widely used at most mining sites today, their suitability and performance can vary depending on site-specific factors such as geographical conditions and weather (Naidu et al. 2019; RoyChowdhury et al. 2015). Among the various approaches for AMD remediation, conventional pH control using cost-effective neutralization reagents is the most widely used and cost-effective method (Zvinowanda and Caliphs 2023; Maree et al. 2013). The active treatment approach employs various chemical compounds as stated by different authors (Kim et al. 2022; Masindi et al. 2022; Rambabu et al. 2020; Lopez et al. 2018; Masindi et al. 2017; Masindi et al. 2015), while passive treatments rely on natural and biological processes such as constructed wetlands, anaerobic sulfate-reducing bioreactors, anoxic limestone drains, vertical flow wetlands, limestone leach beds, open limestone channels, and different organic materials (Carrillo-González et al. 2022; Gumede and Musonge 2022; Ji et al. 2022; Vasquez et al. 2022; Villegas-Plazas et al. 2021; Martins et al. 2011).

Microbial remediation strategies, known as bioremediation, involve the use of microorganisms to mitigate the environmental impact of AMD (Anekwe and Isa 2023). However, it has been found to be a more cost-effective option than chemical-based treatments due to their lower operational and labour costs, as well as their simpler process design and control (Fernandez-Rojo et al. 2017; Jamil and Clarke 2013). Bioremediation of AMD using sulfate-reducing bacteria (SRBs) involves the microbial recovery of metals and sulfates within AMD as metalsulfides (Luptakova and Kusnierova 2005). Sulfate-reducing bacteria (SRB) differ from other bacteria by utilizing sulfate as their final electron acceptor and transforming it into sulfide, which can bind with metals (Rückert 2016). Desulfovibrio species are particularly capable at this process, leading to the formation of metal sulfides through the precipitation of sulfide with metal ions. This process not only helps in removing metals from water but also reduces acidity (Neria-González and Aguilar-López 2021). The presence of dissimilarity sulfite reductase β-subunit genes (*dsrB*) in the SRB communities play crucial role in enhancing their function in AMD remediation (Geets et al. 2006). According to Geets et al. (2006), the *dsr-*gene encodes the dissimilatory sulfite reductase enzyme in sulfate reduction that catalyzes the reduction of sulfite to sulfide and hence is required by all sulfate reducers. Chen et al. (2015) evaluated the microbial diversity in AMD system and the functional diversity. The authors observed the abundance of members belonging to the *Acidithiobacillus*, *Leptospirillum*, and *Acidiphilium* taxa with high transcriptional activities controlled by diverse genes (genes for housekeeping function, low pH adaptation, carbon assimilation, nitrogen assimilation, phosphate assimilation, energy generation, and environmental stress). The presence of these genes in the microbial diversities present in AMD enhanced their survival and possibly the bioremediation of AMD.

The bioremediation processes involve the application of the potentials in microbial diversities aided by their inherent genes involved in the remediation of AMD. This necessitates the need to uncover both culturable and non-culturable microbial diversities present in AMD that play crucial roles in their remediation through traditional and modern method of microbial identification. The introduction of next-generation sequencing (NGS) has revolutionized genomics research, providing unprecedented capabilities for the high-throughput and cost-effective analysis of DNA and RNA molecules (Satam et al. 2023). As a result of this technology, genomics has made rapid advancements across several fields (Satam et al. 2023; Sijmons et al. 2014). Compared to traditional Sanger sequencing, these new technologies offer an incredibly high throughput for a much lower cost per base, eliminating the need for laborious cloning (Koboldt et al. 2013; Zhang et al. 2011; Metzker 2010). Through NGS, DNA fragments can be sequenced rapidly and simultaneously, providing insights into genome structure, genetic variations, gene expression profiles, and epigenetic modifications (Satam et al. 2023). NGS has extended the knowledge of bioremediation through high-throughput microbial identification present in AMD that enhance its remediation. This present review provided an overview of the impact of AMD globally. Microbiological mechanisms adopted by AMD enriched microbial diversities in the remediation of AMD was highlighted. The advancement of the next generation sequencing (NGS) in unveiling important microbial diversities in an AMD-condition as well as the associated genes that enhanced their proliferation in the environment and remediation was also provided.

**Overview of the Environmental Impact of Acid Mine Drainage**

The environmental impact AMD is enormous depending on the severity of the acidic condition. The elements (such as metals, metalloids, and non-metals) in AMD as reported by Masindi (2017) is of high importance because of the residual impacts to the environment. In Morocco, Essalhi et al. (2016) reported the deleterious impact of the waste from the Barite mining to the health of people living around the area and the physiognomy of the landscape. Colliery and Metalliferrous mining in South Africa have negatively affected the groundwater and surface water quality and sinkhole or dolines, which causes severe problem to farmers around these mining areas (Akcil and Koldas 2006; Bell et al. 2002). Studies of Campaner et al. (2014) revealed that AMD generated in the bituminous coal mining activities in southern Brazil is a potential source of metals such as As to water systems. Silva et al. (2011) stated that the major environmental concerns related to coal mining in Brazil is the contamination of surface and ground waters because of surface disposal of waste rock. Table 1 provides a list of some studies done from 2010 to present to indicate the impact of AMD on the environment and human health. An overview of the impacts of AMD is elucidated in Figure 1.

Table 1: List of studies from 2010 on the impact of AMD to the environment and human.

|  |  |
| --- | --- |
| Countries | References |
| South Africa | McCarthy (2011); Ochieng et al. (2017) |
| India | Equeenuddin et al. (2010) |
| China | Chen, et al. (2021) |
| USA | Skousen et al. (2019) |
| Canada | Ramasamy and Power (2019) |
| Slovakia | Singovszka (2020) |
| Asia | Wei et al. (2013) |
| Burkina Faso | Kagambega (2014) |
| Europe | Balci and Demirel, (2018); Antivachis et al. (2016) |
| New Zealand | Trumm and Ball (2014) |

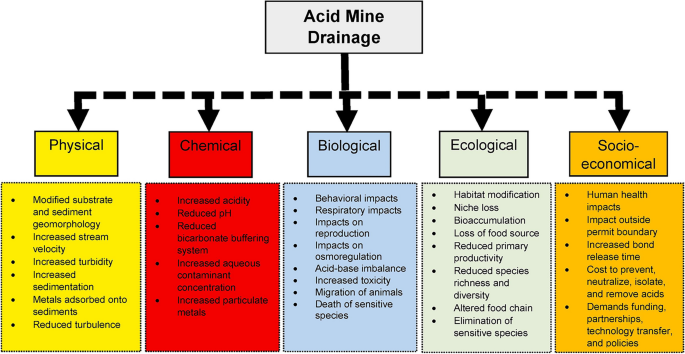


Figure 1: An overview of the physical, chemical, biological, ecological, and socio-economic impacts of AMD

**Microbial Communities Associated with the Remediation of AMD and the Mechanisms of Remediation.**

Microbial communities associated with the remediation of AMD have been extensively studied. The use of microbes to change the concentration of heavy metals in soil and improve the ability of plants to deal with elevated metals concentrations has significant economic and ecological benefits (Jin et al. 2018). The microbial community structure and functional capability in passive remediation systems have been evaluated, revealing shifts in microbial community structure from acidophilic bacteria to a more diverse set of taxa (Ly et al. 2019). Bio-based treatment methods using algae, biochar, and bacteria have been identified as effective treatments for AMD, and their performance is affected by parameters such as pH, temperature, biomass concentration, and initial metal concentration (Du et al. 2022). Culture-dependent and culture-independent studies have uncovered the diversity, functions, and metabolic potentials of AMD microorganisms, leading to a better understanding of microbial diversity and interactions in AMD ecosystems (Villegas-Plazas et al. 2019; Chen et al., 2016). The survival, growth, and regrowth of diverse microbial communities present in AMD could be a factor of their ability to utilize the substrate in the AMD environment for energy production. This process could lead to their ability to remediate the environment. Diverse microbes present in the AMD environment are grouped based on their functional fingerprints and mechanisms of environmental purification and they include the following:

***Sulfate-Reducing Bacteria* (SRB)**

Sulfate-Reducing Bacteria plays significant role in the control and treatment of AMD through the neutralization of the acidic condition (Ayangbenro et al. 2018). SRB are anaerobic bacteria that use sulfate as a terminal electron acceptor in their metabolism (Qian et al. 2019). This process enables the bacteria to precipitate metals and possibly increase the pH of AMD (Zhang et al. 2021; Neculita et al. 2007). They play crucial roles in the bioremediation of AMD by reducing sulfate to sulfide, which can precipitate heavy metals as insoluble sulfides, thus mitigating the environmental impact of AMD. The mechanisms adopted by SRB include dissimilatory sulfate reduction, where the bacteria use sulfate as a terminal electron acceptor in their metabolism. This process is mediated by a complex enzymatic system, including enzymes such as adenosine-5'-phosphosulfate (APS) reductase and dissimilatory sulfite reductase, encoded by the *aps* and *dsr* genes, respectively (Gupta et al. 2022; Zhu et al. 2022 Baker and Banfield 2003).

In AMD environments, SRB such as *Desulfosporosinus* and *Sulfobacillus* are often found. *Desulfosporosinus* is known for its acid resistance, metal resistance, and sulfate-reducing capabilities, while *Sulfobacillus* is considered a sulfur oxidizer and is applied in mineral bioleaching (Zhu et al. 2022). The presence of genes like *dsrAB* in high-sulfate environments indicates the role these genes play in the survival of this SRB in these habitats and possibly, the level of sulfate-reducing abilities of the SRB (Zhu et al. 2022). In summary, SRB contributes to the natural attenuation of AMD through sulfate reduction, which is facilitated by specific genes and enzymes. The adaptation and evolution of these bacteria in AMD environments are influenced by genetic elements like IS, which can affect gene expression and metabolic pathways, enhancing their survival and function in these extreme conditions (Huang et al. 2023).

***Iron-Reducing Bacteria* (IRB)**

Iron-Reducing Bacteria have been studied for their potential in the removal of heavy metals from metal-contaminated sites including AMD.IRB can reduce Fe3+ to Fe2+ forming insoluble metal sulfides, leading to the precipitation and removal of heavy metals from the environment (Jamaluddin et al. 2022; Shylla et al., 2021; Neria-González et al. 2021). The mechanisms involved in metal removal by IRB include sulfide production through elemental sulfur reduction, alkali precipitation, biosorption, immobilization, and enzymatic reduction (Zhang et al., 2022; Sun et al., 2020).IRB adopt various mechanisms to survive and remediate environments affected by AMD through the aid of diverse genes (Gupta & Sar, 2020). The genes involved in iron reduction by IRB include those encoding for cytochromes and other redox-active proteins that facilitate electron transfer to Fe (III) minerals. The presence of genes like *mtrA,* *mtrB*, and *mtrC* has been identified in *Shewanella oneidensis*, a model IRB, which are involved in the reduction of metal oxides including iron minerals (Cooper et al. 2016).

The microbial oxidation of iron and arsenic followed by their co-precipitation leads to the natural attenuation of these elements in As-rich AMD. The bacterial communities responsible for this mitigation include iron-oxidizing bacteria related to *Gallionella* spp. and As-oxidizing bacteria related to *Thiomonas* spp. (Tardy et al. 2018). The latter are associated with the presence of the *aioA* gene, which is involved in arsenic oxidation. Temperature and nutrient supply can influence the rate of iron and arsenic oxidation and precipitation, as well as the bacterial diversity and the As oxidation potential in AMD (Tardy et al. 2018).

***Metal-Resistant Bacteria* (MRB)**

Some bacteria are naturally resistant to high concentrations of heavy metals. These bacteria can immobilize metals through processes such as biosorption, where metals are adsorbed onto the bacterial cell surface. The metals can then be removed along with the bacterial biomass. MRB have been extensively studied for their ability to remove heavy metals from contaminated sites. Bacteria can adapt to metal toxicity through the synthesis of metallothioneins, production of extracellular polysaccharides and siderophores, and efflux systems (Dong et al. 2023). MRB in AMD environments have developed various mechanisms to cope with high metal concentrations. These mechanisms include efflux pumps that actively transport metals out of the cell, enzymatic transformation of metals into less toxic forms, intracellular sequestration where metals are bound to proteins or peptides like metallothioneins, and changes in cell membrane permeability to reduce metal uptake (Barahona et al. 2020). Additionally, some bacteria form biofilms that can immobilize metals, reducing their bioavailability (Jasu & Ray 2021).

Specific genes have been identified that are involved in metal resistance. For instance, genes encoding transposase proteins, which are part of insertion sequences (IS), can contribute to genetic variation and bacterial adaptation to environmental stress, including metal resistance. These IS elements can affect the expression of surrounding genes, potentially enhancing bacterial resistance to metals (Navas et al. 2021). Certain microbial communities have been found to possess metal-resistance genes related to iron (*acn, furA, dpsA*), copper (*copF, actP, copA, mmco, cutO*), and arsenic (*arsT, arsC, aioA/aoxB*) metabolism, which are influenced by the gradient of soil contamination (Navas et al. 2021). In the case of copper resistance, genes such as copA, which encodes a copper-transporting P-type ATPase, and genes involved in the copper efflux system, are critical for the survival of bacteria in environments with high copper concentrations, such as those found in AMD (Barahona et al. 2020). These genes and mechanisms are essential for the survival and function of metal-resistant bacteria in harsh environments characterized by acid mine drainage.

***Microbial biofilms* (MB)**

Microorganisms can form biofilms on surfaces, which acts as a barrier or filter for heavy metals. Biofilms provide a matrix for the precipitation of metal minerals and can trap and immobilize metals in the environment (Koechler et al. 2015). Biofilm formation is an emerging and efficient microbiological mechanism used for the removal of heavy metals from AMD (Rilstone et al. 2021). Biofilms are aggregates of microbes that form on biotic and abiotic surfaces, and they can withstand harsh environmental conditions and resist toxic contaminants (Mishra et al. 2021; Prabhakaran et al., 2016). These biofilms formed by the microbes play a crucial role in the bioremediation of heavy metals by aiding the sequestering of metallic ions by the microbes through the release of extracellular polymeric substances (EPS) (Rather et al. 2022). The EPS, which contains a large amount of anionic charge, facilitates the sequestering of metallic ions, and enhances the efficiency of metal removal from the environment (Jasu et al. 2021).

In *Acidithiobacillus ferrooxidans*, a model organism for studying *acidophiles* in AMD, the quorum sensing (QS) system involving the genes *afeI* and *afeR* plays a role in biofilm formation and ore colonization (Mamani et al. 2016). The QS system regulates the expression of genes involved in exopolysaccharide production, which is essential for biofilm development on mineral surfaces (Mamani et al. 2016). Furthermore, the presence of multiple copies of IS elements such as *ISAfe600*, *ISAfe1*, and *IST2* in the genomes of *A. ferrooxidans* strains help in the development of new metabolic pathways that ensures their survival in harsh environment including AMD (Huang et al. 2023).

***Methanogenic archaea* (MA)**

In anaerobic conditions, MA play a role in removing heavy metals by promoting the formation of metal sulfides and metal carbonates, which are less soluble and more likely to precipitate (Paulo et al. 2015). These microorganisms have been found to be efficient in the removal of toxic metal ions such as arsenic, cadmium, chromium, cobalt, copper, iron, lead, and manganese (Ghosh et al. 2021). The microbial biosorbents produced by methanogenic archaea have metal-binding functional sites that enable them to effectively bind and remove heavy metals from aqueous solutions (Ghosh et al. 2021). The introduction of a system that combines microbiology and electrochemistry known as bioelectrochemical systems has shown promising results for the efficient removal and recovery of various metals, including heavy metals (Sun et al. 2020). Among the employed microbes in the system are the MA, which promotes the oxidation and reduction reactions of heavy metals at the electrodes, providing a flexible platform for their removal (Kumar & Patil 2020). Overall, MA offer potential microbiological mechanisms for the removal of heavy metals from AMD sites (Kumar et al. 2021).

Methanogenic archaea have been identified in AMD environments, although they are not typically capable of growth at pH levels below 3, and no methanogens or enzymes pivotal for *methanogenesis* have been isolated or detected in such systems. In AMD systems, archaea from the filterable fraction are suggested to have a heterotrophic lifestyle, and their contribution to the gross carbon turnover in community metabolism is proposed to be low (Méndez-García et al. 2015). The archaeal community in AMD habitats includes members of the *Methanobacterium* genus, which has been reported useful in metal recovery from waste lithium-ion battery leachates (Gupta et al. 2021). However, limited information is provided on the genes responsible for this action in them.

Members belonging to the genera *Methanohalobium* and *Methanosarcina* have been detected in saline precipitates and are suggested to belong to the *Methanosarcinaceae* family (Sanz et al. 2021). *Methanosarcina* has been reported in the sediments of an extreme acidic river, with pH values ranging from 4.2 to 4.8, and was the only active methanogen in an anaerobic reactor fed with methanol and operating at pH 4.2 (Sanz et al. 2021; Conrad 2020; Sanz et al. 2011). Their survival in such acidic environment like the AMD indicate the presence of genes as well as diverse enzymatic and metabolic pathway to promote their survival. Identification of those genes could serve as a tool that could be adopted in the remediation of AMD.

**Next Generation Sequencing as a Tool to Reveal Diverse Microbial Communities in AMD Remediation and the Associated Genes**

Diagnostic microbiology aims to identify microbes rapidly and accurately within their natural environment. In the remediation of AMD, microbial identification and associated genes involved is crucial. This helps in the characterisation of the microbial communities exploited in mitigating the effect of acid mine waters on the environment. General methods routinely employed in microbial identification process from various samples include:

***Microscopic Examination of Organisms***

The examination of living organisms is both a fundamental and an indispensable technique in life sciences because it reveals the identity, origin, classification, biodiversity, and evolution of life forms on earth. Microscopic examination uncovers intricate details of organisms that are unobservable to the unaided eye. In food microbiology for instance, microscopic examination helps in the identification of food spoilage contaminants (Shan et al. 2019; Oh & Park 2016; Bracke et al. 2014). The microscopic identification and subsequent isolation of pathogenic microorganisms is also a common procedure in medical microbiology (Wang et al. 2022; Golding et al. 2016), while microscopy in microbial ecology assists with characterizing biodiversity (El Mujtar et al. 2022).

Earliest forms of microbial identification relied much on phenotypic techniques such as observation under light and fluorescence microscopes (Bond et al. 2000a; Ferris et al. 1989; Wichlacz & Unz 1981). These can give information regarding the microbial morphology and cellular structures. More targeted identification can be achieved by staining specific groups of microbes with fluorescence dyes (Emerson et al. 1989; Muyzer et al. 1987). There are however obvious limitations to microscopy techniques in the examination of microorganisms. Low sensitivity, difficulty in specimen preparation methods, challenges with obtaining microscopic scales on time, and lack of detailed molecular information leading to paucity of phylogenetic and genetic information are a few of the drawbacks reported in microscopy (Abhishek et al. 2022; Beniac et al. 2014, 2015).

***Isolation and Cultivation of Microbes***

AMD sites exhibit unique characteristics such as extreme acidity of pH level between 2 and 4 (RoyChowdhury et al. 2015), presence of high metal concentration which includes zinc, copper, arsenic, manganese, magnesium, cadmium, and lead (Fuchida et al. 2020), and scarcity of organic matters resulting in low availability of nutrients for agricultural purposes (Agegnehu et al. 2021; Dong et al. 2018). Since AMD poses a significant threat to soil and environmental health, it is important to investigate the unique characteristics that have made the evolution of the microbial communities present in such harsh conditions possible.

The isolation and cultivation of extremophiles present in AMD sites is an important step in environmental microbiology, providing insight into the understanding of both the identity and adaptive mechanisms of microbes thriving in extreme environments. The process begins with strategic sample collection with the right materials and collection devices depending on the nature of analyte. This often includes sterile bottles and containers for storage of collected samples, plastic scoop and 50 mL syringe for sample collection. Also, parameters such as pH measurements, water conductivity, determination of redox potential and dissolved oxygen are carried out (Valdez-Nuñez et al. 2022; Aguinaga et al. 2018). Further to collection, the choice of growth media for the microbial samples is such that can mimic the physico-chemical parameters obtainable in the AMD environment (Connon & Giovannoni 2002). There are several growth media modifications and enrichments that have been used such as sulphate-reducing bacteria growth media (Nguyen et al. 2018; Ayangbenro et al. 2018), and iron-oxidizing acidophilic bacteria solid and liquid media (Ňancucheo et al. 2016; Johnson 1995), which also include modified 9k growth media for cultivating iron-oxidizing bacteria (Mustafa Engin Kocadagistan et al. 2023).

Abandoned mines and sites with history of active mining activities are best considered for sample collection. Due to a change over time in the microbial community present in the samples, they are best kept at 4°C and advisedly analysed within three weeks of collection (Yang et al. 2023). Since appropriate culture media is essential to the growth of microbes for eventual isolation, preliminary studies are often needed to determine the optimal isolation media (Yang et al. 2023). Pure cultures can be obtained from this method and specific strains of microorganism can be characterised. This information is useful in targeting possible remediation candidates (Hallberg 2010; Baker & Banfield 2003).

***Molecular Techniques***

Traditional culture-based methods for microbial identification have limitations which include, decreased specificity and higher rate of diagnostic errors, low successful culture rate of multipathogen samples, and failure to detect microbial presence at low densities among others (Abayasekara et al. 2017; Lee et al. 2013). The emergence of molecular techniques has revolutionized the identification of microbes involved in AMD. Since microorganisms possess unique genetic fingerprints, molecular methods such as DNA sequencing, polymerase chain reaction (PCR) and quantitative PCR for the amplification of specific DNA regions, and DNA hybridization are widely harnessed for the rapid and precise characterisation of microbes (Lukhele et al. 2020; Mohapatra et al. 2011; Bond et al. 2000b). This approach is immensely useful in understanding microbial diversity since it facilitates the detection and identification of microbes down to the subspecies level. As a crucial part of molecular technique in understanding microbial diversity, specific regions unique to each microbial genome is being examined. For example, the16s ribosomal RNA (rRNA) gene is widely used in determining bacterial identity in diverse and complex microbiomes. In mycology however, the 18s rRNA gene (being an active site for protein synthesis) is extensively used as an important biomarker in phylogenetic studies for the identification of fungi from different species (Kadnikov et al. 2019; Kock & Schippers 2008).

***Fluorescence In Situ Hybridization (FISH)***

The use of FISH allows for direct visualization of target microbes in their native environment (Moter et al. 2000). This technique makes use of a piece of purified DNA attached with fluorescent dye combinedly called prob. Prob specificity is important for hybridization to specific DNA sequence of interest. Pre-fluorescently labelled oligonucleotide probs binds to specific rRNA of target species resulting in fluorescence of the molecules (Nicomrat et al. 2006). It is widely accepted as the most convincing technique for locating specific DNA sequences in organismal genomes (Shakoori 2017). Isolates collected from AMD environments and growing in solid media can be identified using appropriate oligonucleotides (Mahmoud et al. 2005).

***Proteomic Analysis***

Valuable insights into the intricate mechanisms underlying the adaptation of AMD microbes can be provided using proteomic analysis (Ram et al. 2005). This technique primarily involves the identification and quantification of expressed proteins of AMD microorganisms through their specific protein expression pattern fingerprint and remedial activities in AMD (Méndez-García et al., 2015). For instance, the proteomic analyses of acidophilic microbial communities provide invaluable insights into the intricate mechanisms underlying their adaptation to extreme acidic environments (Belnap et al. 2010). Where metagenomic information is available, shot-gun and tandem mass spectrometry-based proteomics analysis has been effective for the characterization of microbial communities (Li & Wen 2021; Bharagava et al. 2019; Belnap et al. 2010; Ram et al. 2005).

***Next Generation Sequencing (NGS)***

In the past two decades, new generation sequencing (NGS) has proved a valuable technique in the study of organisms at the genome level. The study of microbial communities in diverse environments in time past relied primarily on phenotypic methods which included microscopy and different culture techniques (Ben-Dov et al. 2009; Bradley & Martiny 2007). There are however significant difficulties in cultivating most of the microorganisms and therefore posing limitations in studying microbial ecosystems (Fakruddin 2015).

NGS is a robust platform that ensures a high throughput sequencing of both DNA and RNA due to its scalability and speed at a comparatively low cost (Kulski 2016; Behjati & Tarpey 2013). In nearly two decades that NGS was introduced, it has emerged as a beneficial tool in investigating microbial populations in different environmental conditions, including the remediation of AMD (Sajjad et al. 2023; Aguinaga et al. 2018; Méndez-García et al. 2015). NGS is particularly useful for the identification of functional genes involved in the bioremediation process of AMD (Zhang et al. 2019; Yelton et al. 2013).

There are several NGS platforms that have been developed in the last decade. Pervez et al. (2022) reported that the Nanopore PromethION, BGI, Illumina, and Ion Torrent are the leading instruments with respect to speed and strength. Although the application of NGS to AMD bioremediation is still at its infancy, its use till date has included the understanding of microbial roles in AMD remediation. This is possible through microbial gene analysis which ultimately provides clues about their metabolic activities (Méndez-García et al. 2015). NGS has been used to determine the functional potentials of microorganisms involved in AMD. Examples of such is the assessment of the bioleaching properties and potentials of microorganisms used for the extraction of metallic compounds such as iron to reduce harmful effects of acid mine drains (Zhou et al. 2018). Furthermore, NGS techniques have been used to determine the sulphate reducing and acid neutralizing microbial community present in AMD. Cho et al. (2022) identified bacteria with acid neutralizing potentials such as *Serratia liquefaciens, Citrobacter youngae, Pseudescherichia vulneris,* and *Serratia grimesii*. The bacteria were found to metabolize nitrogen compounds via hydrolysis of urea and carboxylation to generate NH4+. In a study by Van Den Berg et al. (2016) that determined the microbial diversity present in domestic wastewater sludge using NGS, analyses revealed sulphate and chemical oxygen demand removal bacteria such as *Chlorobium* spp, *Magnetospirillum* spp. and *Ornithobacterium* spp. These are potentially useful organisms for the bioremediation of AMD. Specific microbial genes already identified as important bioremediation agents through the NGS platform include: (i) metal resistance genes. Microbes possessing these genes can resist the toxicity of heavy metals in AMD environments. These makes them candidates’ genes for bioremediation purposes. Examples include Metallothionein (MT) genes used as tools for metal bioremediation development (Li et al. 2020), and *copA* gene found in *Pseudomonas syringae* and *Acinetobacter* sp. IrC1, which encodes metal-binding proteins. The genes have been reported to have the ability to bioaccumulate copper in microbial cells, resulting in the change of bacterial colonies colour to blue (Irawati et al. 2016), (ii) Sulfur reducing genes. With sulphates been an important constituent of AMD environments, sulfur reduction-related bacteria (SRB) genes have been used as bioremediating agents in the past. Examples of SRB include *Desulfosporosinus* spp (Alazard et al. 2010; Lee et al. 2009) and *Desulfovibiro* sp. strain TomC (Karnachuk et al. 2015; Sahinkaya et al. 2015). (iii) Sulfur-oxidising genes. These genes code for enzymes involved in sulfur compounds oxidation. Sulfur oxidising bacteria (SOB) plays a crucial role in acidifying saline and alkaline soils, particularly those rich in calcium carbonate. This acidity is advantageous for plant growth, especially in area of high salinity (Shuochao et al. 2013). SOB have also been reportedly useful in removal of insoluble toxic heavy metals (Maini et al. 2000). (iv) Iron Oxidation Genes. Autotrophic iron-oxidizing bacteria such as *Thiobacillus ferrooxidans*, *Ferrovum myxofaciens* and *Acidithiobacillus ferrooxidans* possess genes that code for key proteins such as rusticyanin for iron oxidation, an important component of AMD (Demir et al. 2021; Hedrich & Johnson 2012; Bengrine et al. 1998). The understanding of genes and organisms implicated in AMD bioremediation can pave the way for more remediation strategies, which may include genetically engineering microbes with potentials for acid neutralization and elimination of heavy metals in AMD environment through the NGS platform. Figure 2 provides a flow diagram for the identification of microbial communities present in samples including AMD.

Sample Collection

DNA Extraction

Library Preparation

Sequencing

Bioinformatics Analysis

Functional Gene Analysis

Taxonomic Assignment

Data Interpretation

Figure 2: Flowchart diagram representing the identification of microbial diversity in AMD bioremediation.

**Conclusions**

The environmental impact of AMD is still ongoing due to increase urbanization and industrialization. The increase in the level of industrialization globally results in the continuous release of acid mine water that drastically devastate our environment. The main promising remediation strategies is the bioremediation approach which involve the use of microbes and the application of their remediation potentials. Some of these microbes with remediation potentials are present in the AMD. Identification of these microbes and unpacking their diverse genes is necessary for the development of advance remediation strategies that is cost effective and environmentally friendly. The present review unveiled the different classes of microbial communities present in the AMD as well as the genes associated with their ability to adapt and possibly remediate the AMD condition. Diverse genes such as *dsrAB*, *mtrA,* *mtrB*, *mtrC*, *acn, furA, dpsA*, *copF, actP, copA, mmco, cutO*, *arsT, arsC, aioA/aoxB*, *afeI*, *afeR*, *ISAfe600*, *ISAfe1*, and *IST2* have been implicated in the different classes of microbial communities in AMD. The advent of NGS has shown to be a promising tool as it promotes the identification of both cultivable and non-cultivable microbes in AMD. Although some of these genes have been identified and due to changing environmental conditions especially in the AMD-environment that can lead to the modification of the genomes of the microbial communities due to mutation, constantly evaluation of the genes is required. This could cut across different field of study which include molecular docking approach and advance of NGS platform with in-depth bioinformatic approach to provide sustainable remediation technologies for AMD.

**Author’s contribution**

Author’s contributions UVO conceived and drafted the first manuscript, UVO, CMK, ELU and WBA redesigned and drafted the final version of the manuscript. MT, SAK, KN and PA supervised and edited the final version of the manuscript. All authors read and approved the final manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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