**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 the constituents of AMD and microbial communities, as well as plants associated with remediation. This complex interaction creates diverse metabolic pathways and mechanisms that are controlled by genes expressed by microbial communities that enhance AMD remediation. Knowledge of these associated genes is essential because it can be used to develop strategies that can promote better/optimal remediation. Although diverse technologies have been employed by researchers to identify numerous microbes and associated genes involved in AMD bioremediation, the advent of next-generation sequencing (NGS) has been shown to be more promising. This review aims to unpack the identified microbes and genes prevailing in AMD conditions that could play a crucial role in remediation. Brief information on the global impact of AMD on crop growth and development was provided. Classes of microbes relevant to AMD and associated genes as well as the role of NGS as a pivotal tool in the advanced discovery of relevant genes. Sulfate-reducing bacteria, iron-reducing bacteria, metal-resistant bacteria, biofilm-forming microbes, and methanogenic archaea are the dominant classes of microbes that play vital roles in AMD remediation. Although diverse genes, such as *dsrAB*, *mtrA,* *mtrB*, *mtrC*, *acn, furA, dpsA*, *copF, actP, copA, mmco, cutO*, *arsT, arsC, aioA/aoxB*, *afeR*, *ISAfe600*, *ISAfe1*, and *IST2* have been identified as genes responsible for the ability of microbial communities to survive and remediate AMD, additional application of NGS is recommended to identify 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 for the mining industry, particularly in areas where metal ores are extracted (Rezaie and Anderson 2020; Nleya et al. 2016). It occurs when sulfide minerals are often found in rocks containing metals, such as iron, copper, zinc, and lead, and encounter air and water (Kumari et al. 2010). AMD, which is highly acidic, disrupts the surrounding ecosystem, 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 solutions for AMD prevention and remediation has been driven by environmental and economic concerns (Rambabu et al. 2020; Kefeni et al. 2017; Kalin et al. 2006).

AMD can be treated using both active and passive remediation technologies. Currently, active and passive remediation methods are considered to be the most practical options for treating AMD (Naidu et al. 2019; Johnson and Hallberg 2005). Although AMD remediation methods are widely used at most mining sites, 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 because of its lower operational and labor costs, as well as its simpler process design and control (Fernandez-Rojo et al. 2017; Jamil and Clarke 2013). The bioremediation of AMD using sulfate-reducing bacteria (SRBs) involves the microbial recovery of metals and sulfates within AMD as metal sulfides (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 metals (Rückert 2016). Desulfovibrio species are particularly capable of this process, leading to the formation of metal sulfides via the precipitation of sulfides with metal ions. This process not only helps to remove metals from water but also reduces acidity (Neria-González and Aguilar-López 2021). The presence of dissimilarities in the sulfite reductase β-subunit genes (*dsrB*) in SRB communities plays a 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, which catalyzes the reduction of sulfite to sulfide, and is hence required by all sulfate reducers. Chen et al. (2015) evaluated microbial diversity in AMD systems and their functional diversity. The authors observed an abundance of members belonging to *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 diversity present in AMD enhanced their survival, and possibly the bioremediation of AMD.

Bioremediation processes involve the application of potential microbial diversity aided by the inherent genes involved in AMD remediation. This necessitates the need to uncover both the culturable and non-culturable microbial diversities present in AMD, which play crucial roles in their remediation through traditional and modern methods 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). Consequently, genomics has rapidly advanced across several fields (Satam et al. 2023; Sijmons et al. 2014). Compared to traditional Sanger sequencing, these new technologies offer incredibly high throughput at 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 our knowledge of bioremediation through high-throughput microbial identification in AMD, thereby enhancing its remediation. This review provides an overview of the global impact of AMD. The microbiological mechanisms adopted by AMD-enriched microbial diversity in AMD remediation of AMD was highlighted. The advancement of next-generation sequencing (NGS) in unveiling important microbial diversities in an AMD-condition, as well as the associated genes that enhance their proliferation in the environment and remediation, was also provided.

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

The environmental impact of AMD depends on the severity of acidic conditions. Elements (such as metals, metalloids, and non-metals) in AMD, as reported by Masindi (2017), are of great importance because of their residual impacts on the environment. In Morocco, Essalhi et al. (2016) reported the deleterious impact of waste from barite mining on the health of people living around the area and the physiognomy of the landscape. Colliery and Metalliferrous mining in South Africa have negatively affected groundwater and surface water quality and sinkholes or dolines, causing severe problems for farmers in these mining areas (Akcil and Koldas 2006; Bell et al. 2002). Campaner et al. (2014) revealed that AMD generated in bituminous coal mining activities in southern Brazil is a potential source of metals such as As in water systems. Silva et al. (2011) stated that the major environmental concern related to coal mining in Brazil is the contamination of the surface and groundwater due to the surface disposal of waste rock. Table 1 provides a list of studies conducted from 2010 to the present, to indicate the impact of AMD on the environment and human health. An overview of the AMD impact is presented 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 AMD remediation have been studied extensively. The use of microbes to change the concentration of heavy metals in soil and improve the ability of plants to deal with elevated metal concentrations has significant economic and ecological benefits (Jin et al. 2018). The microbial community structure and functional capability of 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 the pH, temperature, biomass concentration, and initial metal concentration (Du et al. 2022). Culture-dependent and-independent studies have uncovered the diversity, functions, and metabolic potential 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 in their ability to utilize substrates in the AMD environment for energy production. This process can lead to the ability to remediate the environment. Diverse microbes present in the AMD environment are grouped based on their functional fingerprints and mechanisms of environmental purification, including the following.

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

Sulfate-Reducing Bacteria play a significant role in the control and treatment of AMD through the neutralization of acidic conditions (Ayangbenro et al. 2018). SRB are anaerobic bacteria that use sulfate as a terminal electron acceptor during metabolism (Qian et al. 2019). This process enables bacteria to precipitate metals and possibly increase AMD pH of AMD (Zhang et al. 2021; Neculita et al. 2007). They play a crucial role in the bioremediation of AMD by reducing sulfate to sulfide, which can precipitate heavy metals as insoluble sulfides, thereby mitigating the environmental impact of AMD. The mechanisms adopted by SRB include dissimilatory sulfate reduction, in which bacteria use sulfate as a terminal electron acceptor during 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).

SRB, such as *Desulfosporosinus* and *Sulfobacillus* are often found in AMD environments. *Desulfosporosinus* is known for its acid resistance, metal resistance, and sulfate-reducing capabilities, whereas *Sulfobacillus* is considered a sulfur oxidizer and is used in mineral bioleaching (Zhu et al. 2022). The presence of genes such as *dsrAB* in high-sulfate environments indicates the role these genes play in the survival of this SRB in these habitats and possibly in the sulfate-reducing abilities of the SRB (Zhu et al. 2022). In summary, SRB contribute 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 such as IS, which can affect gene expression and metabolic pathways, enhancing their survival and function under these extreme conditions (Huang et al. 2023).

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

Iron-Reducing Bacteria have been studied for their potential to remove heavy metals from metal-contaminated sites including AMD.IRB can reduce Fe3+ to Fe2+ to form 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 adopts 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 cytochromes and other redox-active proteins that facilitate electron transfer to Fe (III) minerals. The presence of genes such as *mtrA,* *mtrB*, and *mtrC* has been identified in *Shewanella oneidensis*, a model IRB 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 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 is associated with the presence of *aioA*, 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 As oxidation potential in AMD (Tardy et al. 2018).

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

Some bacteria are naturally resistant to high heavy metal concentrations. These bacteria can immobilize metals through processes, such as biosorption, in which metals are adsorbed onto the bacterial cell surface. The metals can then be removed along with bacterial biomass. The ability of MRBs to remove heavy metals from contaminated sites has been studied extensively. Bacteria can adapt to metal toxicity through the synthesis of metallothioneins, production of extracellular polysaccharides and siderophores, and efflux systems (Dong et al. 2023). Various mechanisms have been developed for MRB to cope with high metal concentrations in AMD environments. 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 such as metallothioneins, and changes in cell membrane permeability to reduce metal uptake (Barahona et al. 2020). Additionally, some bacteria form biofilms that can immobilize metals, thereby reducing their bioavailability (Jasu & Ray 2021).

Specific genes involved in metal resistance have been identified. 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 that act as barriers or filters to 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 to remove heavy metals from AMD (Rilstone et al. 2021). Biofilms are aggregates of microbes that form on biotic and abiotic surfaces, and can withstand harsh environmental conditions and toxic contaminants (Mishra et al. 2021; Prabhakaran et al., 2016). Biofilms formed by microbes play a crucial role in the bioremediation of heavy metals by aiding the sequestration of metallic ions by microbes through the release of extracellular polymeric substances (EPS) (Rather et al. 2022). EPS, which contains a large amount of anionic charges, facilitates the sequestration 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 helps in the development of new metabolic pathways that ensure their survival in harsh environments, including AMD (Huang et al. 2023).

***Methanogenic archaea* (MA)**

Under anaerobic conditions, MA plays a role in removing heavy metals by promoting the formation of metal sulfides and carbonates, which are less soluble and more likely to precipitate (Paulo et al. 2015). These microorganisms have been found to be efficient in removing toxic metal ions, such as arsenic, cadmium, chromium, cobalt, copper, iron, lead, and manganese (Ghosh et al. 2021). 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 a bioelectrochemical system, has shown promising results for the efficient removal and recovery of various metals, including heavy metals (Sun et al. 2020). Among the microbes employed in the system is MA, which promotes the oxidation and reduction reactions of heavy metals at the electrodes and provides a flexible platform for their removal (Kumar & Patil 2020). Overall, MA offers 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 typically not capable of growing 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 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 to be useful for metal recovery from waste lithium-ion battery leachates (Gupta et al. 2021). However, limited information is available on the genes responsible for this action.

Members of the genera *Methanohalobium* and *Methanosarcina* have been detected in saline precipitates and have been suggested to belong to the *Methanosarcinaceae* family (Sanz et al. 2021). *Methanosarcina* has been reported in the sediments of an extremely 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 acidic environments, such as AMD, indicates the presence of genes, as well as diverse enzymatic and metabolic pathways that promote survival. Identification of these genes could serve as a tool for AMD remediation.

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

Diagnostic microbiology aims to rapidly and accurately identify microbes in their natural environment. Microbial identification and the associated genes involved are crucial. This helps characterize the microbial communities exploited to mitigate the effects of acid mine water on the environment. General methods routinely employed in microbial identification processes from various samples include the following.

***Microscopic Examination of Organisms***

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

The earliest forms of microbial identification relied heavily on phenotypic techniques, such as observation under light and fluorescence microscopes (Bond et al. 2000a; Ferris et al. 1989; Wichlacz & Unz 1981). These data provide information regarding the microbial morphology and cellular structures. Targeted identification can be achieved by staining specific microbial groups with fluorescent dyes (Emerson et al. 1989; Muyzer et al. 1987). However, there are obvious limitations to microscopic techniques for the examination of microorganisms. Low sensitivity, difficulty in specimen preparation methods, challenges in obtaining microscopic scales on time, and lack of detailed molecular information leading to a paucity of phylogenetic and genetic information are some of the drawbacks of microscopy (Abhishek et al. 2022; Beniac et al. 2014, 2015).

***Isolation and Cultivation of Microbes***

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

Isolation and cultivation of extremophiles present in AMD sites is an important step in environmental microbiology, providing insight into both the identity and adaptive mechanisms of microbes thriving in extreme environments. The process begins with strategic sample collection using the appropriate materials and collection devices, depending on the nature of the analyte. This often includes sterile bottles and containers for storage of collected samples, plastic scoop and 50 mL syringe for sample collection. In addition, parameters such as the pH, water conductivity, redox potential, and dissolved oxygen were measured (Valdez-Nuñez et al. 2022; Aguinaga et al. 2018). Furthermore, the choice of growth medium for microbial samples can mimic the physicochemical parameters obtained in an AMD environment (Connon & Giovannoni 2002). Several growth media modifications and enrichments have been used, such as sulfate-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 a history of active mining activities were considered for the sample collection. Due to a change over time in the microbial community present in the samples, they were best kept at 4°C and were advised to be analyzed within three weeks of collection (Yang et al. 2023). Because appropriate culture media are essential for the growth of microbes for eventual isolation, preliminary studies are often needed to determine the optimal isolation medium (Yang et al. 2023). Pure cultures can be obtained using this method and specific strains of microorganisms can be characterized. This information is useful for targeting possible remediation candidates (Hallberg 2010; Baker & Banfield 2003).

***Molecular Techniques***

Traditional culture-based methods for microbial identification have limitations, such as decreased specificity, higher rate of diagnostic errors, low success 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. Because microorganisms possess unique genetic fingerprints, molecular methods, such as DNA sequencing, polymerase chain reaction (PCR), quantitative PCR for the amplification of specific DNA regions, and DNA hybridization, are widely used to rapidly and precisely characterize microbes (Lukhele et al. 2020; Mohapatra et al. 2011; Bond et al. 2000b). This approach is immensely useful for understanding microbial diversity, because it facilitates the detection and identification of microbes at the subspecies level. Specific regions unique to each microbial genome are being examined as a crucial part of the molecular techniques for understanding microbial diversity. For example, the16s ribosomal RNA (rRNA) gene is widely used to determine the bacterial identity in diverse and complex microbiomes. However, in mycology, the 18s rRNA gene (being an active site for protein synthesis) is extensively used as an important biomarker in phylogenetic studies to identify fungi from different species (Kadnikov et al. 2019; Kock & Schippers 2008).

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

FISH allows direct visualization of target microbes in their native environments (Moter et al. 2000). This technique uses a piece of purified DNA attached to a fluorescent dye called a prob. Prob specificity is important for hybridization of a specific DNA sequence of interest. Pre-fluorescently labelled oligonucleotide probes bind 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 grown 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 obtained using proteomic analysis (Ram et al. 2005). This technique primarily involves the identification and quantification of proteins expressed by AMD microorganisms through their specific protein expression pattern fingerprints and remedial activities in AMD (Méndez-García et al., 2015). For instance, proteomic analyses of acidophilic microbial communities provide invaluable insights into the intricate mechanisms underlying their adaptation to extremely acidic environments (Belnap et al. 2010). Where metagenomic information is available, shot-gun and tandem mass spectrometry-based proteomic analyses have been effective for characterizing 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 proven to be a valuable technique for the study of organisms at the genome level. In the past, the study of microbial communities in diverse environments relied primarily on phenotypic methods, including microscopy and various culture techniques (Ben-Dov et al. 2009; Bradley & Martiny 2007). However, there are significant difficulties in cultivating most microorganisms, thereby limiting the study of microbial ecosystems (Fakruddin 2015).

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

Several next-generation sequencing (NGS) platforms have been developed over the past decade. Pervez et al. (2022) reported that Nanopore PromethION, BGI, Illumina, and Ion Torrent are leading instruments in terms of speed and strength. Although the application of NGS to AMD bioremediation is still in its infancy, its use to date has included an understanding of the microbial roles in AMD remediation. This is possible through microbial gene analysis, which ultimately provides clues regarding metabolic activities (Méndez-García et al. 2015). NGS has been used to determine the functional potential of the microorganisms involved in AMD. Examples include the assessment of bioleaching properties and the potential of microorganisms used for the extraction of metallic compounds, such as iron, to reduce the harmful effects of acid mine drains (Zhou et al. 2018). Furthermore, NGS techniques have been used to determine sulfate-reducing and acid-neutralizing microbial communities present in AMD. Cho et al. (2022) identified bacteria with acid-neutralizing potential, such as *Serratia liquefaciens, Citrobacter youngae, Pseudescherichia vulneris,* and *Serratia grimesii*. Bacteria metabolize nitrogen compounds via the hydrolysis of urea and carboxylation to generate NH4+. In a study by Van Den Berg et al. (2016), which determined the microbial diversity present in domestic wastewater sludge using NGS, analyses revealed sulfate and chemical oxygen demand removal by bacteria, such as *Chlorobium* spp., *Magnetospirillum* spp., and *Ornithobacterium* spp. These are potentially useful organisms for 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 heavy metal toxicity in AMD. This makes them suitable candidate genes for bioremediation. 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 encodes a metal-binding protein. These genes have been reported to have the ability to bioaccumulate copper in microbial cells, resulting in a change in bacterial colony color to blue (Irawati et al. 2016), (ii) sulfur-reducing genes. Sulfates are an important constituent of AMD environments, and sulfur reduction-related bacterial (SRB) genes have been used as bioremediation agents. 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-oxidizing genes. These genes encode enzymes involved in the oxidation of sulfur compounds. Sulfur-oxidizing bacteria (SOB) play a crucial role in acidifying saline and alkaline soils, particularly those rich in calcium carbonate. This acidity is advantageous for plant growth, especially in areas of high salinity (Shuochao et al. 2013). SOB has also been reported to be useful for removing 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). Understanding the genes and organisms implicated in AMD bioremediation can pave the way for more remediation strategies, including genetically engineered microbes with the potential for acid neutralization and elimination of heavy metals in AMD environments through the NGS platform. Figure 2 provides a flow diagram for the identification of microbial communities present in the 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, owing to increased urbanization and industrialization. Global industrialization has resulted in the continuous release of acid mine water, which has drastically devastated the environment. The most promising remediation strategy is the bioremediation approach, which involves the use of microbes and application of their remediation potential. Some microbes with remediation potential are also present in AMD. Identification of these microbes and unpacking their diverse genes are necessary for the development of advanced remediation strategies that are cost-effective and environmentally friendly. The present review revealed the different classes of microbial communities present in AMD as well as the genes associated with their ability to adapt and possibly remediate AMD. 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 different classes of microbial communities in AMD. The advent of NGS has been 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, which can lead to the modification of the genomes of the microbial communities due to mutation, constant evaluation of the genes is required. This could cut across different fields of study, including molecular docking approaches and advances in NGS platforms with in-depth bioinformatics approaches, to provide sustainable remediation technologies for AMD.

**Author’s contribution**

Author Contributions: UVO conceived and drafted the manuscript. UVO, CMK, ELU, and WBA drafted the final version of the manuscript. MT, SAK, KN and PA supervised and edited the final manuscript. All the authors have read and approved the final manuscript.

**Conflicts of Interest**

The authors declare no conflict of interest.

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