

## Review Article

# Application of metagenomics in the study of groundwater microorganisms

Li-qiang Ge<sup>1,2</sup>, Xin Yuan<sup>1,2\*</sup>, Liu Yang<sup>3\*</sup>

<sup>1</sup> National Research Center for Geoanalysis, Beijing 100037, China.

<sup>2</sup> Key Laboratory of Eco-geochemistry, Ministry of Natural Resources, Beijing 100037, China.

<sup>3</sup> Institute of Hydrogeology and Environmental Geology, Chinese Academy of Geological Sciences, Shijiazhuang 050061, China.

**Abstract:** With the rapid development of molecular biology technology, especially the application of metagenomics, many challenges in groundwater microbial research have been overcome. Metagenomics has enabled the exploration of the diversity of unculturable microorganisms in groundwater. This paper reviews macro genomics 16S rRNA and metagenomics sequencing data, highlighting recent applications of metagenomics in investigating groundwater microbial communities. It also examines the relationship between microbial diversity and environmental factors, the identification of functional microbial groups, the role of microorganisms in groundwater pollution remediation, and their contribution to the hydrogeochemical cycle. Finally, it provides insights into future research directions in groundwater microbiology.

**Keywords:** Metagenomics; Groundwater; Functional microbial community; Biogeochemical cycles

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## Introduction

Groundwater is an important strategic resource that supports social and economic development, playing an essential role in both human production and daily life. However, human activities and extensive groundwater exploitation have caused numerous issues, such as declining groundwater tables and groundwater pollution. As a result, increasing attention is being paid to the health and sustainable management of the groundwater systems. Through continued research, the importance of microorganisms in groundwater ecosystems has become evident, and groundwater as an ecosystem has gradually entered the horizon of scientists (Griebler et al. 2014; Yan et al. 2019; Zhang et al. 2010; Humphreys, 2009).

Groundwater ecosystems are significant compo-

nents of the Earth's critical zone, harboring a diverse variety of microorganisms, including bacterial flora, such as heterotrophic bacteria, autotrophic bacteria, aerobic bacteria, anaerobic bacteria, etc. These microbes thrive by harnessing energy from various chemical reactions occurring within the Earth's subsurface, sustaining their growth and reproduction. However, traditional pure culture methods can isolate and culture only about 1.0% of the environmental microorganisms (Alain and Querellou, 2009), severely limiting our understanding of groundwater microbial communities. Consequently, our knowledge of microorganisms in general is limited, and even more so regarding groundwater microorganisms, often referred to as "groundwater microbial dark matter" (Rinke et al. 2013).

With the rapid development of molecular biology technology, especially the application of metagenomics, it has become possible to study unculturable microorganisms in groundwater. Investigating groundwater microorganisms can enrich the groundwater microbial resource pool in China, improve the understanding of the evolution of groundwater environment, and provide insights into the roles of microbial communities in hydrogeochemical processes. This, in turn, deepens

\*Corresponding author: Xin Yuan, Liu Yang, E-mail address: [yuanxin7618@163.com](mailto:yuanxin7618@163.com), [yangliu@mail.cgs.gov.cn](mailto:yangliu@mail.cgs.gov.cn)

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knowledge of groundwater ecosystem services, providing a theoretical foundation for the sustainable use and protection of groundwater resources. This paper reviews the application of metagenomics in the exploration of groundwater microbial resources, the relationship between groundwater microbial diversity and environmental factors, functional groups of groundwater microorganisms, and the role of microorganisms in groundwater pollution remediation. Additionally, it outlines the future research directions in groundwater microbiology.

### 1 Overview of metagenomics

The concept of "metagenome" was first introduced by Handelsman et al. in 1998 (Handelsman et al. 1998), referring to the collective genomes of all microorganisms within an environmental sample. Metagenomics involves treating the genetic information from an entire microbial community as a whole, allowing researchers to directly extract and analyze the genetic information and analyze the DNA of microorganisms from the environment without the need for isolation and cultivation. This method enables a deeper understanding of the microbial community composition and their interactions with the environment through DNA sequencing (Handelsman, 2004; Huson et al. 2011). There are two main types of metagenomics sequencing: Tag sequences sequencing and whole genomes sequencing. The flow of sequencing data is illustrated in Fig. 1.

Tag sequence, also known as amplicon sequence (Shin et al. 2016), refers to species-specific gene sequences that are highly conserved during evolution (Chang et al. 2012). Ribosomal RNA (rRNA) is a typical example of a tag sequences due to its evolutionary conservation. Different types of rRNA include 5S rRNA, 16S rRNA, 18S rRNA and 23S rRNA, etc. Of these, 16S rRNA is widely present in prokaryotic microorganisms, and is often used as a tag sequence in microbial studies.

Data from 16S rRNA sequencing can effectively reveal the composition and phylogenetic relationships of microbial community across different environments.

Whole genome sequencing, also known as metagenomic sequencing, involves sequencing all genetic material within a microbial sample to obtain comprehensive data. This approach allows for analysis of the structure and function of entire microbial communities (Hugenholtz and Tyson, 2008). Unlike tag sequence, metagenomic sequencing retains complete genetic information from the community, providing a more accurate depiction of the microbial system. This comprehensive data supports deeper investigations into interactions within microbial communities and their environment, including the specific functions and metabolic pathways of specific genes within the community.

### 2 Investigation of groundwater microbial community

Groundwater harbors an immense reservoir of biological resources, with groundwater microorganisms representing a valuable source of strains and genetic material. These microorganisms hold both theoretical and practical significance. The application of metagenomics in studying unculturable microorganisms, the investigation of microbial diversity in various ecosystems, including the groundwater environment, has reached a new level. In 2016, the United States launched the "Microbiome Program", recognizing groundwater microorganisms as critical components of habitat microorganism. Following this, the National Natural Foundation of China introduced the "Hydrosphere Microbiome Program" in 2017. Additionally, in 2016, the Chinese Academy of Sciences initiated the "Deep Subsurface Biosphere" strategic research project (Huang et al. 2017; CGS, 2018).

Approximately 40% of Earth's prokaryotes inhabit the unique environments of underground

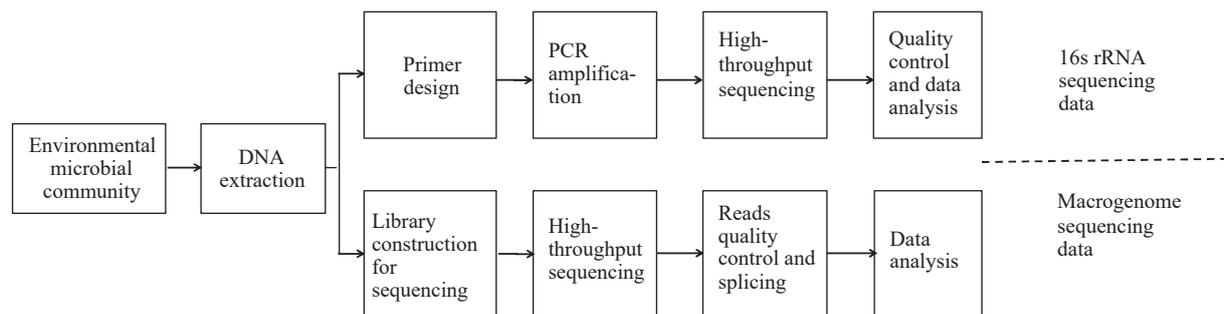


Fig. 1 16s rRNA sequencing data and macrogenome sequencing data

aquifers (He and Chen, 2015), with a significant number of microbial populations still undiscovered. In recent years, the use of 16S rRNA amplification and metagenomic sequencing has greatly expanded our understanding of microbial communities and their functions in groundwater and sediments across various countries. This research has revealed remarkable microbial diversity and uncovered many new species (Li et al. 2021). For example, a variety of Sulfate-Reducing Bacteria (SRB) taxa, such as *Desulfotomaculum*, *Desulfobulbus*, *Desulfotomaculum*, *Desulfosarcina*, and *Desulfobacca* have been identified in groundwater. (An et al. 2016); A fermentative iron-reducing bacterium, *Tepidibacillus decaturensis*, was isolated from groundwater at a depth of 1.7 km (Dong et al. 2016). In addition, microbial genera like *Klebsiella* and *Shewanella* have been found in groundwater with high arsenic concentrations (Liu et al. 2020), along with *Paraclostridium*, *Citrobacter*, *Klebsiella*, and *Bacillus* (Tian et al. 2019; Mohapatra et al. 2017; Biswas et al. 2019; Wang et al. 2021a). These microbial taxa provide valuable insights into the chemical processes within groundwater environments, and their specific functions and genes may serve as crucial resource for addressing ecological challenges in the future.

### 3 Research on groundwater microbial functional groups

Microorganisms are vital components of ecosystems, and the biogeochemical processes they mediate play a key role in the cycling of essential elements such as carbon, nitrogen, phosphorus, sulfur, and others (Falkowski et al. 2008). The complex geochemical conditions found in groundwater environments provide a variety of habitats for microorganisms, which in turn have evolved into complex microbial functional groups (Li et al. 2021). Research has shown that groundwater microorganisms participate in numerous metabolic activities, including organic matter degradation, methanogenesis, methane oxidation, sulfur redox, iron redox, ammonification, nitrogen fixation, nitrification and denitrification (Sonthiphand et al. 2019; Schwab et al. 2016; Jiang et al. 2019; Li et al. 2023).

The development of advanced technologies such as metagenomics enables the reconstruction of complete genomes or nearly complete genomic sketches for organisms without the need for pure laboratory cultures (Alneberg et al. 2014). This technological leap offers new possibilities for

interpreting key biogeochemical processes. For instance, Professor Banfield's research team at the University of California, Berkeley, applied metagenomics to study the biogeochemical processes in shallow groundwater and sediment microorganisms subjected to artificial interference, such as injection of acetate and oxygen. Through this study, they identified 2,540 genomes, representing the metabolic capacities of 1,297 species. Their findings revealed that many organisms are capable of mediating only a single step in the chemical cycle. Instead, metabolic transfer processes are distributed across different microorganisms, and as environmental conditions shift, varying combinations of organisms are selected to adapt and modify the connections between key biogeochemical cycles (Anantharaman et al. 2016).

Microbial activity in groundwater environment is key to catalyzing the geochemical cycling of various elements. Under aerobic or microaerobic conditions, oxidizing bacteria facilitate the conversion of elements from lower to higher oxidation state, while under anaerobic conditions, reducing bacteria reverse this process by converting oxidized elements back to the lower oxidation states, thus completing their geochemical cycle. For instance, microbial processes such as anaerobic oxidation of methane coupled with sulfate reduction (Timmers et al. 2016) and methane oxidation linked with nitrate reduction (Costa et al. 2000) are examples of microbial influence on element cycling in groundwater. Microbial biogeochemistry of arsenic has emerged as a major area of focus in research related to high-arsenic groundwater. In recent years, extensive studies have been conducted to investigate the causes of arsenic contamination in groundwater, its transport and transformation mechanisms, and the metabolic pathways involved in high-arsenic groundwater regions. Countries like Bangladesh, Vietnam, Cambodia, and areas in the Yellow River of China have been at the forefront of these studies, with metagenomics technology playing a key role in uncovering the processes involved in arsenic migration and transformation (Gorra et al. 2012; Guo et al. 2015; Lawati et al. 2012; Liao et al. 2011; Li et al. 2013; Paul et al. 2015a; Paul et al. 2015b; Sarkar et al. 2013; Wang et al. 2021b). In addition, microorganisms are critical in the global iodine cycle through iodine oxidation, reduction, volatilization and deiodization (Jiang et al. 2023).

Incorporating microbial factors into hydrogeochemical modeling significantly enhances the predictive capabilities of these models. There are

four primary types of hydrogeochemical models considering microbial contribution:

(1) Non-equilibrium thermodynamic models: These models, such as the one proposed by Vallino and Algar (2016), have successfully predicted the nitrate reduction pathway across various environments. Based on the "entropy maximization principle", they do not require detailed knowledge of microbial composition or interactions, offering a simplified yet effective approach for predicting biogeochemical processes.

(2) Kinetic models with microbial metabolic pathways: These models combine chemical reactions with groundwater transport, often referred to as reaction-migration models. They thoroughly consider the transport and transformation of solutes at different interfaces, while also incorporating microbial roles in biogeochemical processes. These models also consider spatial scale effects and medium heterogeneity, making them widely accepted and practical in current hydrogeochemical studies (Li et al. 2017).

(3) Kinetic models with metabolic pathways: These models focus on the metabolic pathways of specific functional microbial communities. They require laboratory data on pure bacteria cultures and the fitting of key parameters before being applied at the site scale, but it is often not suitable (Jin, 2012).

(4) Metagenome models: These models were developed recently, classifying groundwater microbial communities by function using metagenomic data. These models consider the gene abundance and expression degree of specific functional microorganisms, allowing for a more accurate representation of microbially mediated geochemical processes (Yan et al. 2016).

#### 4 Research on the relationship between microbial community diversity and environmental factors in groundwater

The metabolism of microorganisms is strongly influenced by environmental factors such as temperature, pH value, dissolved oxygen, ionic strength, partial pressure of carbon dioxide, etc. Different microbial populations exhibit distinct environmental adaptability, including cryophilic, thermophilic, acidophilic, basophilic, neutrophilic, aerobic and anaerobic microorganisms. Microbial diversity and environmental factors interact with each other. The composition of microbial communities differs across various habitats. In turn, these

microbial communities actively shape their environment through metabolic activities. This interplay is particularly evident in groundwater environments, where variations in microbial diversity varies among different groundwater environments, and the population structure of groundwater microorganisms can serve as an indicator of the groundwater environment.

16S rRNA data analysis effectively reflects the characteristics of microbial community composition and phylogenetic relationships, making high-throughput sequencing of 16S rRNA a common method for studying microbial diversity in groundwater environments. The chemical composition of groundwater and the mineral composition of sediments significantly impact the structure of microbial communities in groundwater. For instance, Liu et al. (2022) identified Total Dissolved Solids (TDS) as an important ecological factor influencing the spatial distribution of bacteria, archaea and fungi in shallow groundwater. Wang et al. (2017) analyzed the dynamic changes in microbial community diversity during the interaction of Carbon dioxide with brackish water and sandstone. They found that certain microorganisms could thrive under extreme conditions (high temperature and acidity) after Carbon dioxide was injected into the brackish water layer. Their study revealed that microbial genera tended to become homogeneous during this interaction, with the relative abundance of the phylum Proteobacteria ultimately reaching 99.77%. Zhang et al. (2021) examined the groundwater microbial community structure in Jintan Beach, Qingdao, China, and found that tidal effects significantly altered bacterial community diversity in shallow groundwater. This research demonstrated that microbial abundance and diversity were spatially partitioned, with species abundance influenced by environmental indicators such as alkalinity and high salinity in weakly reducing environments. In contrast, species diversity was related to depth and dissolved oxygen (Zhang et al. 2023). Through 16S rRNA sequencing and hydrochemical analysis, Li et al. (2019a) determined that nitrate ( $\text{NO}_3^-$ ), sulfate ( $\text{SO}_4^{2-}$ ) and depth were the main factors affecting microbial diversity in groundwater. Their findings indicated that microbial community composition varied at different depths, with microbial diversity decreasing as depth increased. Additionally, Huang et al. (2011) studied the temporal and spatial distribution characteristics of groundwater microbial communities and found that human activities had a significant impact on these ecosystems.

## 5 Research on the application of groundwater pollution remediation

As early as the early 20th century, the impact of microorganisms on groundwater quality was acknowledged (Chapelle, 2000). Microorganisms are crucial components of the groundwater environment, utilizing organic matter as electron donors and inorganic matter, such as nitrate, as electron acceptors for their life activities (Feng et al. 2023; Eduardo et al. 2024). In groundwater ecosystems, microorganisms act as decomposers and transformers of nutrients, serving as both storers and producers of matter and energy. They play a significant role in evolution and natural attenuation of geochemical processes related to shallow groundwater pollution (John and Rose, 2005; Griebler and Lueders, 2010). The evolution of microbial community composition not only indicate changes in the groundwater environment (Macler and Merkle, 2000), but also contributes to the purification of groundwater from organic contamination (Bradley, 2000) and nitrate pollution (Groffman et al. 1996) to some extent.

In the study of groundwater microbial pollution remediation, modern molecular biology techniques have been employed to monitor changes in microbial community characteristics and functional gene composition across varying pollution pressures and remediation stages. This approach often involves the addition of degrading bacteria and remediation agents to regulate and restore the environment, employing joint remediation techniques to maximize microbial degradation of groundwater pollutants (Li et al. 2019b). Xue et al. (2017) used 16S rRNA sequencing to identify the differences in microbial diversity and the relative abundance of groundwater microorganisms in various environments, providing a foundation for the ecological evaluation of groundwater environments. Additionally, Li (2018) effectively identified dominant bacterial flora by comparing the composition and abundance of microbial communities under different pollutants and pollution pressures, thereby offering technical and theoretical support for groundwater pollution remediation. Direct injection of additives into groundwater contaminated areas can stimulate and enhance the activity of indigenous microorganisms that degrade pollutants. Consequently, research into the changes in groundwater microbial community and functional gene structure following additive injection has become a hot topic in this field (Zhang et al. 2015a; Zhang et al. 2015b; Kao et al. 2015).

Microorganisms demonstrated exceptional efficacy in remediating organic pollution in groundwater. Organic contaminants serve as metabolic substrates for microbes, providing them with the necessary energy and carbon for growth (Dai, 2006). The composition of microbial communities is closely related to the concentration and types of organic pollutants (Rooneyvarga et al. 1999; Fahy et al. 2005; Wang et al. 2013). Notably, the similarity in microbial community composition diminishes in the sequence of "pollution source - downstream source area - downstream pollution plume - upstream source area - flanking pollution plume". In recent years, researchers have increasingly focused on the remediation of emerging organic pollutants using groundwater microorganisms (Mohr et al. 2020; Zheng et al. 2016). For instance, Wang et al. (2023) investigated the characteristics of petroleum-contaminated groundwater, and established bio-indicators to reflect the status of petroleum contamination, thereby providing a basis for environmental monitoring and management. Chen et al. (2024) successfully extracted and screened native efficient degrading bacteria (BL5) from a chlorinated hydrocarbon contaminated site, developing a CS@ZVI functional material modified with BL5 that enhanced degradation performance. Additionally, Michalsen et al. (2020) successfully used two strains, I-C and KTR9, to rapidly degrade cyclotrimethylene trinitramine in groundwater using in-situ remediation method. Zhang et al. (2018) used biological oxidation involving sulfur (S) or iron (Fe) autotrophs in conjunction with heterotrophic microorganisms to remove vanadium (V) from groundwater. In a different approach, Gibert et al. (2019) implemented Permeation Reaction Barriers (PRBs) to remove nitrate from groundwater in situ, successfully eliminating high concentrations of nitrate ( $\text{NO}_3^-$ ) by providing a carbon source for denitrifying bacteria through active substances in PRB. Liu et al. (2024) identified the synergistic effect of *Dechloromonas*, *Delftia* and *Geobacter* as key contributors to the complete mineralization of phenanthrene. Metagenomics analysis further elucidated the phenanthrene degrading genes, offering molecular insights into the decomposition of refractory organic matter in-situ bioremediation in electric field.

## 6 Conclusion and prospect

Since the dawn of the 21st century, the academic community has progressively transitioned from

single-discipline research to multidisciplinary, cross-disciplinary studies. This shift has significantly broadened the scope of groundwater scientific research, particularly in the study of groundwater microorganisms, which holds substantial theoretical and practical significance. As biotechnology has advanced, research on groundwater microorganisms has developed from focusing on species diversity to exploring functional gene abundance. It has also shifted from examining the relationship between microbial diversity and environmental factors to investigating the roles of microorganisms in hydrogeochemical cycles, resulting in increasingly in-depth and comprehensive studies. However, despite these advancements, numerous issues remain to be addressed in the field of groundwater microbiology. Further research is essential to deepen our understanding of these microorganisms and their interactions with groundwater environment.

(1) Groundwater microorganisms represent an important reservoir of strains and genetic resources; however, they remain underexplored. To address this gap, there is a pressing need for systematic investigation and development of groundwater microbial resources. Building on studies of microbial diversity through metagenomics, efforts should be intensified to cultivate key microorganisms, with the aim of constructing a comprehensive groundwater microbial resource bank.

(2) Microbial communities exhibit complex behaviors influenced by both their micro and macro microhabitats. Key questions remain regarding which environmental factors significantly affect core microbial groups in groundwater and how these microorganisms, in turn, affect their environment. Addressing these questions is crucial for understanding groundwater ecological functions and warrants further in-depth research.

(3) Current research on hydrogeochemical cycle processes has primarily focused on the individual cycling of elements in which microorganisms are involved. However, the urgent scientific challenge lies in understanding the coupling of multi-temporal and multi-element biogeochemical processes. In addition, distinguishing between biological and abiotic processes within these cycles remain a significant challenge. The integrated application of metagenomics, proteomics, and isotope technology provides promising possibilities for addressing these critical scientific issues.

(4) Groundwater microorganisms offer several advantages in the remediation and treatment of emerging pollutants, including low cost, quick

effect, and the absence of secondary pollution. However, challenges remain in cultivating functional communities of groundwater microorganisms, which limits the application of microbial remediation and bioremediation engineering. Therefore, it is essential to enhance collaborative research and development efforts that integrate multiple technologies with existing groundwater remediation approaches.

In conclusion, the emergence of metagenomics has opened new avenues for studying unculturable microorganisms, thereby accelerating research on groundwater microorganisms. This progress fosters interdisciplinary collaboration across various fields, including geology, molecular biology, environmental geology, hydrogeology, biochemistry and bioinformatics. In the future, the integration of additional technologies and methodologies will enhance the study of groundwater microorganisms. By advancing our research in this area, we can enrich China's groundwater microbial resources bank and improve our understanding of the evolution of groundwater environment. This knowledge will ultimately promote the sustainable utilization of groundwater resources and contribute to the protection of groundwater ecosystems.

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