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High-throughput Sequencing in Soil Microbiology: Challenges and the Path Forward

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ABSTRACT: Plant health, soil fertility, and sustainability all depend on soil microbial populations. However, intensive agriculture, which uses more synthetic inputs, and changing environmental conditions have affected native soil microbial communities. Studying microbial structure, diversity, and activity is essential for understanding plant-microbe interactions. However, microbial complexity, environmental interactions, and technological limitations make comprehensive analysis challenging. Culture-based methods often fail to capture true microbial diversity, necessitating culture-independent approaches like metagenomics, meta-proteomics, meta-transcriptomics, and proteo-genomics, which provide deeper insights into soil microbial ecology and functionality. High-throughput sequencing (HTS) has revolutionized soil microbiology by enabling comprehensive, culture-independent analysis of microbial communities. However, challenges such as DNA extraction biases, sequencing errors, and limited reference databases hinder accuracy. Albeit advancing bioinformatics, improving sequencing technologies, and integrating multi-omics approaches will enhance microbial insights, driving future innovations in soil health and sustainability.

Keywords: Soil microbiology, high-throughput sequencing (HTS), bioinformatics, omics and sustainability.

INTRODUCTION

The most prevalent living things in nature are microbes, which have been on Earth for more than 3.5 billion years. The biosphere is thought to contain an estimated 4-6 × 10^{30} prokaryotic cells (Wei *et al.*, 2018). Despite the immense microbial diversity in soil, much of it remains unexplored, making it crucial to understand microbial biodiversity and ecology (Ahmad *et al.*, 2011). Bacterial populations have been extensively studied using molecular biology techniques, but culturomics**,** which involves isolating bacteria using culture media, identifies only a small fraction of species present in soil (Sarhan *et al.*, 2019). Cultivating and directly observing many soil microbes remains challenging, leaving a significant portion of microbial communities uncharacterized (Jo *et al.*, 2020**).** Riesenfeld *et al.* (2004) highlighted that only 1 per cent of soil microbes are culturable, limiting taxonomic and functional insights into the remaining 99 per cent population**.** Microorganisms play key roles in soil structure, nutrients transformation and organic matter recycling (Ahmad *et al.*, 2011), with root exudates shaping microbial communities and enzymatic activities that drive nutrient cycling (Jacoby *et al.*, 2017). Soil microbes mediate critical biogeochemical processes**,** yet microscopic and biochemical methods provide only partial insights into microbial interactions and functions.

To overcome these limitations, high-throughput sequencing (HTS) has emerged as a revolutionary, culture-independent tool for comprehensively analysing soil microbial communities.

The application of molecular technologies in microbial ecology traces back to the development of molecular phylogeny in the late 1960s (Falkowski *et al.*, 2008). Traditional culture-based and molecular methods, such as Real-Time Polymerase Chain Reaction (RT-PCR), Random Amplified Polymorphic DNA (RAPD), Length of Restriction Fragments and Denaturing Gradient Gel Electrophoresis (DGGE), have provided preliminary insights into microbial diversity (Feinstein *et al.*, 2009). However, these techniques often lack the resolution for comprehensive taxonomic assessment (Rastogi and Sani 2011).

The methodology of such techniques consists of extracting DNA or RNA directly from a soil sample, creating a library which contain the genomes of every microbe that is found in that particular area (Ranjard *et al.*, 2001). For bioinformatic investigations, including taxonomic designations, abundance analysis, and the identification of putative functions for specific genes, this extracted DNA/RNA can be sequenced. Based on sequence differences of conserved genes of DNA *i.e.*,

16S rRNA genes (16S rDNA) for bacteria, 18S rRNA gene (18S rDNA) for eukaryotes and internal transcribed spacer (ITS) for fungi, coding for ribosomal RNA have been identified.

The structural and functional diversity of soil microbial communities can be investigated using metagenomic techniques. High-Throughput Sequencing (HTS) and Next-Generation Sequencing (NGS) have demonstrated a great deal of promise in exposing these communities' hidden diversity. HTS drastically alters the research process and produces a vast amount of data by enabling studies to a particular environment with comparatively low cost and great accuracy (Wei *et al*., 2018).

Soil microbiology has been transformed by high-throughput sequencing (HTS), which makes it possible to identify and analyse microbial communities with great depth and precision. Only a portion of the diversity of microorganisms is captured by conventional culture-dependent methods, which restricts our comprehension of their significance in biogeochemical processes (Ranjard *et al*., 2001). By uncovering formerly unculturable species and their metabolic roles, the advent of HTS, especially next-generation sequencing (NGS) and third-generation sequencing (TGS), has offered hitherto unheard-of insights into soil microbial ecology (Zhou *et al.*, 2023). A thorough examination of microbial relationships, stress responses, and ecosystem resilience is made possible by the large-scale sequencing of 16S rRNA, ITS, metagenomic, and meta-transcriptomic data made possible by HTS platforms like Illumina, PacBio, and Oxford Nanopore (Bhattacharjya *et al.*, 2024).

Our knowledge of soil microbial diversity has greatly increased over the last ten years thanks to developments in HTS, yet there are still large research gaps. Research has indicated that changes in land use, soil management techniques, and environmental stressors have a significant impact on the composition and functioning of microbial communities (Zhou *et al*., 2023; Liu *et al.*, 2022). Through the discovery of novel genes implicated in stress tolerance, metagenomic techniques have uncovered microbial adaptations to harsh environments, such as permafrost, polluted soils, and deserts (Parihar *et al.*, 2022; Rosa *et al*., 2020). Additionally, by connecting gene expression patterns to markers of soil health, meta-transcriptomics has shed light on active microbial roles (Emma *et al.*, 2020; Roychowdhury *et al*., 2023).

Recent research has shown how shotgun metagenomics and long-read sequencing can be used to connect microbial communities to important soil processes such organic matter decomposition, nutrient cycling, and plant-microbe interactions (Xiao *et al.*, 2024; Jiang *et al*., 2023). Notwithstanding these developments, there are still issues, including as biases in sequencing, complicated data processing, and the requirement for better functional annotation databases (Tang *et al*., 2024). Our capacity to utilize soil microbial communities for climate adaption, sustainable agriculture, and environmental restoration will be improved by addressing these constraints with integrative multi-omics methodologies and cutting-edge bioinformatics technologies (Chen *et al*., 2024).

HISTORY OF SOIL MICROBIOLOGY

A. Pre-sequencing era: culture-dependent studies (Before 1970s)

Early microbiologists relied solely on microscopy and culture-based techniques to study soil microbes.

- Only microbes that could be grown in nutrient-rich media were identified.
- Discovery of nitrogen-fixing bacteria (e.g., Rhizobium) and decomposers (e.g., Actinomycetes) advanced Soil Science.

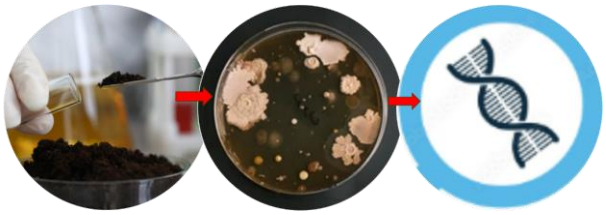

Fig. 1. Advanced procedures are required to study soil microbial diversity (Riesenfeld *et al.*, 2004; Sansupa *et al.*, 2021).

A major challenge in studying soil microbial diversity is that 99 per cent of microbes are unculturable. Despite this, biochemical techniques like the plate count method are still in vogue, involving serial dilution, culturing, and DNA extraction**.** However, traditional culturing techniques not fully encapsulating the variety and usefulness of microbes.

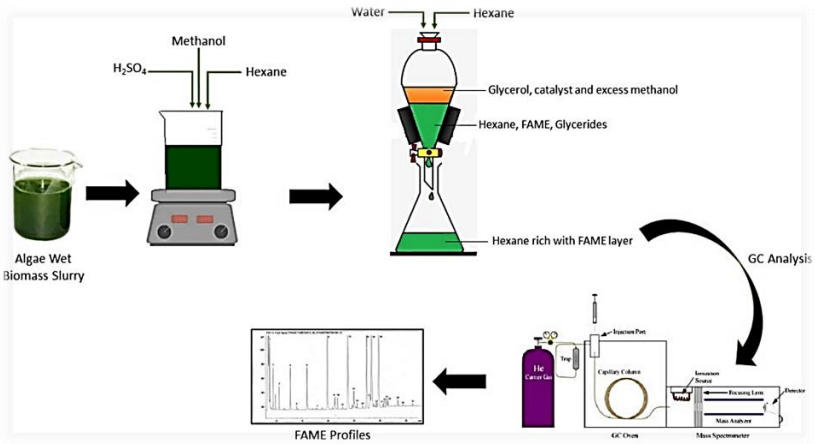

Fig. 2. Fatty acid methyl ester (FAME) analysis (Wallis and Baumgartner 2025).

Advancements in biochemical methods, such as Fatty Acid Methyl Ester (FAME) analysis**,** give information on the microbial community composition based on fatty acid patterns in microbial cell walls. The process involves lipid esterification, fatty acid separation, and identification using GC and Sherlock software**.** However, FAME analysis offers limited diversity insights, pointing out the shortcomings of traditional culturing techniques in uncovering the vast unculturable soil microbial population.

B. The advent of molecular techniques (1970s – 1990s)

The advent of PCR (Polymerase Chain Reaction) and 16S rRNA sequencing allowed identification of non-culturable microbes.

- Scientists discovered previously unknown microbial taxa by the examination of genetic material directly from soil samples.
- Despite advancements, Sanger sequencing was slow, costly, and limited in sequencing depth.

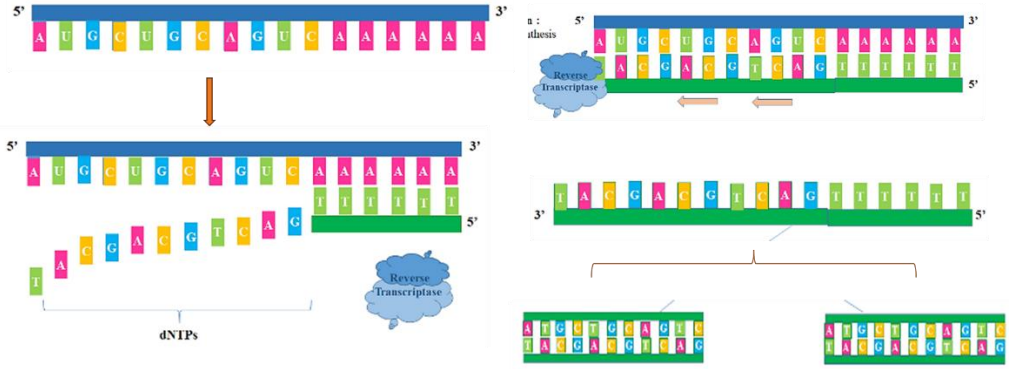

Fig. 3. Advent of molecular techniques (Chen *et al.*, 2023).

The development of molecular techniques like PCR, Denaturing Gradient Gel Electrophoresis (DGGE), and Temperature Gradient Gel Electrophoresis (TGGE) allowed for culture-independent analysis, which significantly advanced soil microbial research. PCR amplifies microbial DNA, while DGGE and TGGE separate bits of DNA depending on sequence variations, helping assess microbial diversity. Benefits include quick detection, high sensitivity, and microbial community profiling. However, limitations involve PCR biases, low-resolution separation, and difficulty in detecting rare taxa. In spite of these obstacles, they offer important information regarding soil health and microbial ecology.

(C) High-throughput sequencing's (HTS) ascent: Revealing the secrets of microbial world (2000s onward)

High-throughput sequencing (HTS), or next-generation sequencing (NGS), revolutionized DNA and RNA analysis through facilitating the quick, large-scale sequencing of millions to billions of fragments in a single run. Unlike labour-intensive Sanger sequencing, HTS provides high-depth, cost-effective insights into genomes, transcriptomes, and microbial communities, transforming microbial, environmental, and clinical research with unmatched precision and effectiveness.

AIM

HTS enables the parallel sequencing of RNA or DNA fragments ranging from millions to billions in a single run, providing unprecedented precision and depth in microbial, environmental, and clinical research (Ranjard *et al.*, 2001).

STEPS OF HTS IN SOIL MICROBIOLOGY

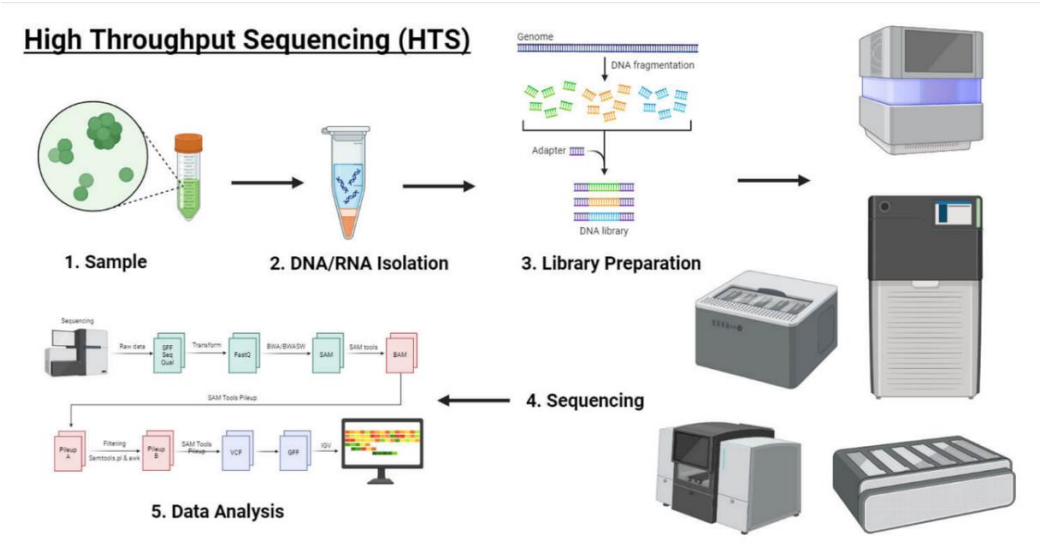

Fig. 4. Steps of HTS in soil microbiology (Muhammad *et al.*, 2024).

Steps of HTS in soil microbiology

1. Collecting soil samples – Collect and store soil samples appropriately.

2. DNA/RNA extraction – Isolate microbial DNA/RNA from the samples.

3. Library preparation and sequencing – Prepare sequencing libraries and perform high-throughput sequencing.

4. Bioinformatics processing – Filter raw reads, classify taxa, and annotate functional genes.

5. Data analysis and interpretation – Analyze and visualize microbial diversity and functions statistically.

A. Commonly used bioinformatics tools

AMPHORA - (Automated Phylogenomic Inference from Uncultured Organisms)- Use of software for phylogenetic analysis of single gene or whole genomes.

ARB - (Itself is the name of the bioinformatics software suite)- Interacting software tools for analysis, maintenance, and sequence databases.

KEGG - (Kyoto Encyclopedia of Genes and Genomes)- Database tools for computational prediction of cellular metabolic processes.

Strain Info.net- (It is the name of an integrated microbial resource)-A bio-portal of information integration services for the microbial community.

UniFrac - (Unique Fraction)- Comparison of microbial communities using phylogenetic information.

B. Need for HTS

From early culture-based studies to HTS, our knowledge of soil microorganisms has drastically expanded, opening up more possibilities for agriculture, climatic resilience and biotechnology.

Why HTS is preferred over conventional methods today?

✔**Captures total microbial diversity**, including unculturable species.

✔**High speed and throughput**, analyzing thousands to millions of sequences at once.

✔**More accurate taxonomic identification** with deeper resolution.

✔ **Functional insights** into microbial roles beyond taxonomy.

APPLICATIONS OF HIGH-THROUGHPUT SEQUENCING IN SOIL MICROBIOLOGY

Microbial Diversity Analysis – Recognizes and categorizes diverse microbial communities.

Assessment of soil health – Monitors microbial indicators of soil quality.

Biogeochemical Cycling Studies – Examines microbial roles in cycles of sulfur, nitrogen, and carbon.

Pathogen Detection – Identifies soil borne plant pathogens.

Impacts of Changes in the Environment – Analyses microbial changes brought on by climate change and land use.

HTS TECHNIQUES FOR UNRAVELING SOIL MICROBIAL DIVERSITY

- **16S rRNA Sequencing** – Identifies bacterial and archaeal diversity.
- **ITS Sequencing** – Analyses fungal community composition.
- **Meta-genomics** – Profiles microbial genomes and potential for use.
- **Meta-transcriptomics** – Studies active microbial gene expression.
- **Metabolomics** – Examines microbial metabolic profiles.
- **Stable Isotope Probing (SIP)** – Links microbial identity with function using labelled substrates.
- **Shotgun Sequencing** – Provides high-resolution, diversity of microbes and their functions.

HTS techniques for soil microbiology

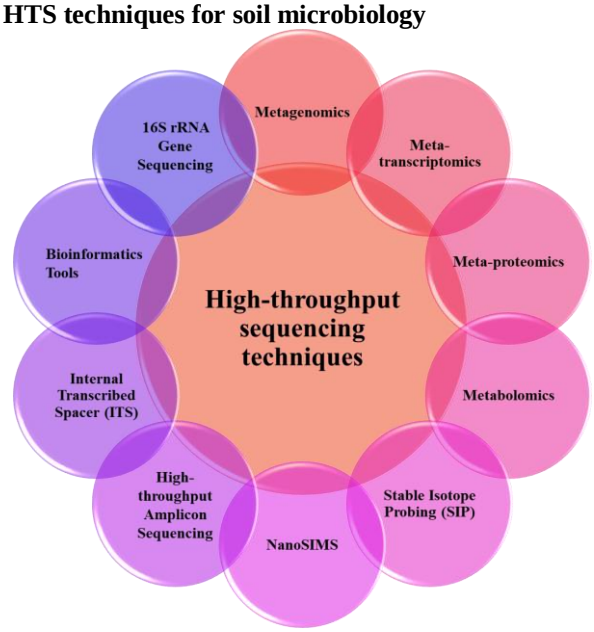

Fig. 5. HTS techniques for soil microbiology (Jiang *et al.*, 2023).

A. 16S rRNA

16S rRNA sequencing is a key high-throughput technique for analysing soil microbial communities. It makes it possible for taxonomic identification, microbial diversity assessment, and ecological function analysis. The process involves DNA extraction, PCR amplification, sequencing, and bioinformatic tools to reveal microbial composition as well as useful possibilities in ecosystems of soil.

B. Emerging multi-omics approaches

Emerging multi-omics approaches are transforming soil microbial research by offering more profound understandings of microbial diversity and functions. Metagenomics deciphers microbial genomes, while meta-transcriptomics reveals active expression of genes. Meta-proteomics identifies functional proteins, and metabolomics examines metabolic interactions. Additionally, stable isotope probing (SIP**)** links microbial identity with function. These integrated methods offer a holistic understanding of microbial ecology, enabling researchers to explore soil health, cycling of nutrients, and ecosystem resilience more effectively.

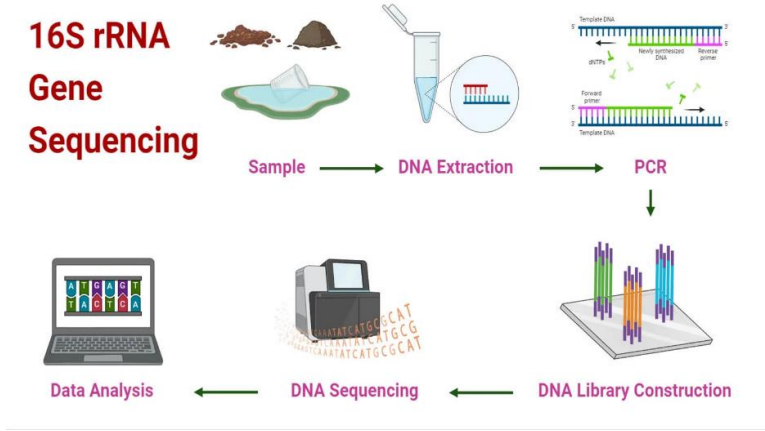

Fig. 6. 16S rRNA (Ranjard *et al.*, 2001).

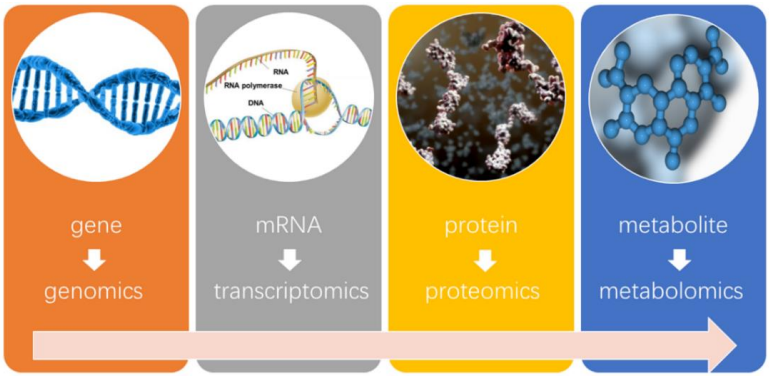

Fig. 7. Omics technology integration (Emma *et al.*, 2020; Roychowdhury *et al.*, 2023).

Numerous investigators have examined the diversity of microorganisms found in soils across various agro-ecological zones. Numerous activities related to carbon, nitrogen, and sulfur cycles in bio-geochemical cycles are linked to protobacteria. For example, Parihar *et al.* (2022) used illumina sequencing to conduct a metagenomic study on microbial diversity in the Thar Desert, Rajasthan, India. The study found that the majority of the bacteria found in the sampled sites were Proteobacteria (19–31%), followed by unclassified bacteria (5–21%), Actinobacteria (3–25%), Planctomycetes (5–13%), Chloroflexi (2–14%), Bacteroidetes (3–12%), Firmicutes (3–7%), Acidobacteria (1–4%), and Patescibacteria (1–4%). Similarly, Rosa *et al.* (2020) assessed soil fungal diversity at Deception Islands, Antarctica. The phyla Ascomycota, the 346 fungal amplicon sequence variants they discovered were dominated by Basidiomycota, Mortierellomycota, and Chytridiomycota. Additionally, they discovered species that were believed to be members of the uncommon phyla Mucoromycota and Rozellomycota, which were difficult to locate in Antarctica using traditional isolation methods.

Tang *et al.* (2017) used a 1H NMR metabolomics approach to examine the metabolic response of earthworms under low soil Pb exposure. They found that earthworms exhibited a toxic response for the first 14 days, and then detoxification mechanisms were triggered for the next 14 days. Group of metabolites such as myo-inositol, 2-hexyl-5-ethyl-3-furansulfonate, scyllo-inositol, succinate, alanine and maltose were found as potential biomarkers of Pb exposure for earthworms. Similarly, 1H NMR metabolomics was used to evaluate the sub-lethal toxicity of earthworms when they were exposed to persistent xenobiotic phenanthrene (Lankadurai *et al*., 2011). Range of metabolites *viz.*, alanine, arginine, lysine, phenylalanine, betaine, and maltose emerged as potential indicators of phenanthrene exposure and also indicated the mode of action of xenobiotic toxicity in the earthworm. Thus, NMR-based earthworm metabolomics appeared to have immense potential in routine eco-toxicity assessments of contaminated soils (Horvath *et al.*, 2023). Similarly, the metabolomics technique can document the metabolic responses of the soil microbial population to soil pollutants in order to ascertain the ecotoxicity response (Jones *et al*., 2014).

Challenges of high-throughput sequencing (HTS) techniques. HTS in soil microbiology faces multiple challenges like DNA extraction biases, sequencing errors, and complex data analysis. Limited reference databases hinder taxonomic and functional annotation.

CONCLUSIONS

High-throughput sequencing (HTS) has revolutionized soil microbiology by enabling high-resolution, culture-independent analysis of microbial diversity and functions. The composition and functional potential of microbial communities are revealed by methods such as 16S rRNA gene sequencing, and metagenomics provide information on the abundance of microorganisms in stress-related biogeochemical and scientific processes. Technological developments in third-generation sequencing, such as enhanced RNA quality and nanopore technology, enable a more thorough

investigation of microbial functions. Agricultural sustainability, environmental monitoring, and ecosystem resilience in the face of shifting global conditions are all supported by HTS and new multi-omics techniques, which improve our understanding of soil microbial ecology despite obstacles such DNA extraction biases and complicated data analysis.

FUTURE SCOPE

Advancing bioinformatics tools, improving sequencing accuracy, and integrating multi-omics approaches can enhance microbial insights. Standardized protocols and AI-driven analytics will drive future breakthroughs in soil microbial ecology and applications.

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Conflict of Interest. None.

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