2024

A Literature Review of Factors Contributing to Archaeal Diversity

Anna R. Carmen
University of Northern Iowa

A LITERATURE REVIEW OF FACTORS CONTRIBUTING TO ARCHAEAL DIVERSITY

A Thesis Submitted

in Partial Fulfillment

of the Requirements for the Designation

University Honors

Anna R. Carmen
University of Northern Iowa
May 2024

This Study by: Anna Carmen

Entitled: A Literature Review of Factors Contributing to Archaeal Diversity

has been approved as meeting the thesis or project requirement for the Designation

University Honors

Approved by:

Dr. Marek Sliwinski, Honors Thesis Advisor, Department of Biology

Dr. Jessica Moon Asa, Director, University Honors Program

Abstract

Archaea, being classified as its own separate domain only within the past fifty years, has earned a reputation of being notoriously difficult to culture in a laboratory setting. Because of this, their functions in ecosystems and potential for use in bioremediation is largely unknown and untapped. In order to further develop methodologies to successfully cultivate these microbes, an analysis of past research is needed to help understand where to lead research. After analyzing past studies on archaea and recalcitrant microbes as a whole, it is found that other factors are often overlooked and a label of recalcitrance is added. This leads to a cycle with uncultured archaea not having primers that amplify them, which leads to measures of archaeal diversity as a whole being inaccurate. Further research developments based on past successes are necessary to determine the previously untapped potential of archaea.

Table of Contents

Introduction

The discovery and classification of archaea broadened the scope of microbiology by introducing a third domain of life. Biology had previously categorized organisms into two different branches of life- prokaryotes and eukaryotes. Prokaryotes are single celled organisms that can be both pathogenic and beneficial to other organisms, where eukaryotes contain larger organisms and cells with membrane-bound organelles, and now a third domain of life, archaea, that did not fit into the previous two categories. Archaea are unicellular organisms lacking membrane-bound organelles like prokaryotes but are distinctly different in their chemical composition and behaviors. Archaea is a unique domain because, unlike the microbes in the other two domains, there is little known financial gain to growing them, and they are not known to directly harm or benefit human health.

More recently, a specific group of archaea have become of interest to scientists thanks to their ability to oxidize nitrogen in their environments, which is of interest to both naturalists and microbiologists alike. The ability to take a substance that is so detrimental to other organisms and convert it into a less toxic substance is an intriguing venture into using archaea as a potential for bioremediation of polluted ecosystems. These nitrogen-oxidizing archaea, known as *Thaumarcheota*, have been found in all environments, spanning from common agricultural soils, to extreme conditions, deep in the ocean, to even the dust circulating the globe thanks to jet streams. To fully capitalize on the potentials this phylum can reach, a deeper understanding of archaeal research and history is necessary to further progress the field.

First, an understanding of the difficulties of growing this notoriously recalcitrant type of cell needs to be reached in order to push towards a more effective method for laboratory growth.

Understanding how diversity is measured in archaea allows for increased knowledge about specific types of archaea and further comprehension of archaeal roles. This literature review allows for deeper comprehension of common struggles in culturing archaea in laboratory settings to further progress an understanding into the unique and necessary role archaea play in an ecosystem.

Research Questions

Why are some microbes recalcitrant to growth?

How are primers chosen for Polymerase Chain Reaction (PCR)?

How is archaeal diversity measured?

Methodology

Initial research was conducted to formulate the premise of this literature review and establish a background. Literature was chosen deliberately to avoid bias, avoid redundancy, and continue to refine and answer research questions. Some papers were found to be using their research in efforts to advertise a product a laboratory was developing, so those were purposely not chosen. Literature comparing various products without incentive was used. Some papers had a section added after the conclusion asking researchers to express bias. Only papers proclaiming no bias were utilized.

Searches were conducted via scholarly databases using keywords from research questions. Articles were chosen based on their relevance to this literature review, as well as the recency of each publication as microbiology is a quickly changing field. Some papers that are

older were included because of the revolutionary discoveries about archaea and served as a crucial case study to provide background information that shaped this literature review.

Papers were chosen according to how often they were cited in other publications as a step to ensure credibility. In some cases, looking into papers that cited the initial article allowed for more recent papers with novel discoveries to come to light.

Information from articles that are deemed relevant, unbiased, and credible was laid out in a crude outline of a literature review to determine areas in which information was lacking to continue to supplement this literature review so that it may serve as background information for researchers continuing to study archaea and the role they play in various ecosystems.

Literature review: Why are some microbes recalcitrant to growth?

Previous perceptions

Archaea were believed to grow exclusively in extreme environments, with conditions impossible to replicate in a lab due to severe chemical, temperature, or barometric requirements. In 1992 this theory was debunked when testing of coastal waters yielded evidence of archaeal DNA. Of the vast majority of DNA that was recovered from various coastal water sites in California, up to 2.3% of the DNA found was classified as archaeal, proving that archaea have a far more significant presence in mesophilic ecosystems than previously thought (DeLong, 1992). Diversifying the sources of inoculum for archaeal research opened new doors to microbiology research as the microbes that were previously considered elusive were now more accessible.

Utilizing Genetic Identity

Microbes with specific requirements for growth may seem to struggle to grow in a laboratory setting, when in fact, they hold the key to their own growth. As an emphasis in the field of omics, or utilizing genetic identity to further research, has boomed, more information about requirements for laboratory growth of microbes has come to light. Archaea, though likely previously cultured in labs, could not be proven because there were no known gene sequences and databases to compare genomes to. Advances have been made in using sequencing to produce a better media for microbes and encourage laboratory growth. For instance, sequencing of *T. whipplei* showed that the bacteria lacks genes for amino acid production, leading to a media being developed to supplement that deficit within the microbe (Prakash et. al, 2013). Previously unknown metabolic pathways can be uncovered to find the best "food" for microbes recalcitrant to growth to thrive. Nutrients can also be the downfall of a culture- weed-like microorganisms can reproduce in impure cultures and serve as competitors for resources against the targeted recalcitrant microbes. Methods to eliminate competitors for resources are being researched. One method of growing Thaumarcheota in a laboratory setting added a wide array of antibiotics to media in attempts to reduce bacterial competition for resources proved effective (Liu, 2019). This technique has been used alongside media that is used specifically for growth to obtain a culture of previously recalcitrant microbes.

Some microbes that are seemingly resisting growth in a lab are not necessarily lacking the ability to grow, but more the ability to identify said microbes is lacking. Sequencing more microbes leads to the potential of more microbial groups being grown in laboratories to be successfully identified and added to databases to serve as a reference strain. As of 2012, it was estimated that about 30 phyla of 100 established through a phylogenetic analysis were able to

serve as a cultured representative of microorganisms found within nature (Prakash et. al, 2012). Other microbes have been found in laboratory settings, but not in cultures pure enough to serve as a reference point for future research. Using universal primers that were effective for some microbial cultures does not take into account genetic differences that allow for species diversity. A primer that searches only for one specific sequencing reduces the ability to amplify DNA or RNA that may differ due to different species and growth conditions.

Though utilizing sequencing has yielded strong results of pure cultures, there is no way to account for interspecies relationships, dormant states of bacteria, or other behavioral factors affecting growth using exclusively omics to uncover methods of cultivating the previously uncultured microbes (Prakash et. al, 2012). Omics refers to the area of biology that focuses on specific molecules, whether that be DNA itself or other chemical portions of the cells, like the lipids that make up a cell membrane. Omics provides ample insight into the realm of microbiology, but must be considered in tandem with other research efforts to find ways of culturing the uncultured. An estimate of diversity of microbes ranges from $3*10^4$ to $3*10^{12}$, showing that only a small fraction of microbes have been successfully cultured and identified using some form of metagenomics (Yarza et. al, 2014).

Databases compile the various methods of combining omics and culture techniques to create isolated and pure cultures, allowing researchers to compare and contrast data about DNA sequences.

Time

Many previously isolated microbes are fast-growing, which makes them appear more accessible to researchers. Though the speed of replication from bacteria varies depending on other environmental factors, more is known and identified about those that replicate more

quickly. For instance, E. Coli doubles about every twenty to thirty minutes during room temperature conditions, making it an organism with less complex needs to maintain studies for. Less is known about microbial species that take longer to grow, causing difficulties in running sequencing on these organisms (McCully et. al, 2023). Special nutritional requirements for microbes may aid in reducing competition from microbial weeds, such as double emulsions of nutrients. This is taxing for researchers and not as cost effective, making it more complex for researchers to grow these organisms. Archaea, being notoriously slow to grow, are a prime example of microbes that are often discounted as being recalcitrant to growth. The closest archaeal relative to eukaryotes, *Candidatus* Nanohaloarchaeum antarcticus, took over 12 years of enrichment efforts and research to yield results (Lewis et. al, 2020). Further research into fine-tuning conditions ideal for growth and a modified perception of anticipated time changes the narrative that microbes are recalcitrant to growth altogether, and in fact may just have more specialized needs.

Pure Cultures

Cultures with archaeal DNA are enough to prove a presence of archaea in various ecosystems, but not as beneficial for further unpacking the role they play. Microbes that are seemingly recalcitrant to growth may just be in mixtures and difficult to isolate as a pure culture. Pure cultures allow statistical certainty of results from experiments, as the experiment is now able to be duplicated (Lewis et. al, 2020). There are believed to only be about 11,000 pure cultures of all bacteria and archaea, which leaves endless microbes with unknown impacts because results cannot be successfully duplicated (Yarza et. al, 2014). Various methods, spanning from utilizing omics to specific tools designated for pure cultures, are being studied in efforts to increase the number of pure cultures available for research.

Figure 1

Table 1 Approaches for cultivation of uncultured diversity

From: Microbial cultivation and the role of microbial resource centers in the omics era

Cultivation strategies	Methods	References
Simulated natural environment	Combined use of polycarbonate membrane, soil extract, and viable staining (soil substrate membrane system)	Ferarri et al. 2008; Ferrari and Gillings 2009
Oligotrophic condition and extinction to the dilution of the sample	Filtered marine water with extra source of nitrogen and phosphorous used as cultivation medium and extinction to the dilution of the samples	Connon and Giovannoni 2002; Rappe et al. 2002
Single cell separation and oligotrophic environment	High-throughput cultivation using microgel-droplet encapsulation and continuous flow of low-nutrient medium	Zengler et al. 2002
Extension of growth substrate, electron acceptors, inoculum size, and incubation time	Used wide sets of electron donors and acceptors. Expanded the range of incubation temperatures, increased inoculum size, and extended the incubation time of plates	Joseph et al. 2003; Davis et al. 2005; Köpke et al. 2005; Song et al. 2009
Simulated natural environment	Provided simulated natural environment using diffusion chamber	Kaeberlein et al. 2002
Single-cell separation	Used optical tweezers for single-cell separation and cultivation	Huber et al. 1995; Frohlich and Konig 2000
Filtration–acclimatization method (FAM)	Removed the fast growers, then gradually acclimated the cells to complex medium conditions	Hahn et al. 2004
High-throughput cultivation and screening	Used micro-Petri dish (a million-well growth chip) for the culture and high-throughput screening of microorganisms	Ingham et al. 2007
Inclusion of additional nutritional requirements and signaling molecules in culture medium	Co-culture, addition of culture supernatant, growth-promoting factors	Hughes and Sperandio 2008; Kim et al. 2008; Nichols et al. 2008; Ohno et al. 1999; Tripp et al. 2008

The table above (Figure 1) shows effective methods of growing previously recalcitrant microbes in a laboratory setting, with an emphasis on isolating cultures to be pure (Prakash et. al, 2012). Optical tweezers are a method of physically separating cells from each other, allowing a single cell to be placed into a culture. A small laser is used to separate the cells from each other and reduces the likelihood of contamination from a researcher or environmental conditions. Other methods tested were simulating a natural environment and modifying the media of recalcitrant microbes to imitate their inoculum source, whether that be in the composition of the media or utilizing a diffusion chamber to get closer insight into microbial behaviors.

Though many worked for culturing these microbes, many were dependent on other species or an emulation of a natural environment. When trying to replicate interspecies relationships within a laboratory, oftentimes the sample ends up mixed, so the DNA of a one specific culture cannot be accurately updated to databases.

Literature review: How are primers chosen for archaeal PCR?

What are primers?

Primers are necessary for polymerase chain reactions, which makes it possible to take cells of a sample and create millions of copies of DNA so they can be further studied. In PCR, cells are heated up to break the bonds between DNA. Primers are added to target a desired portion of the DNA to replicate. The primers adhere to the targeted section and encourage DNA polymerase to occur. The primer extends onto the DNA, creating new strands. This cycle repeats 20-40 times, each time doubling the amount of DNA in a sample.

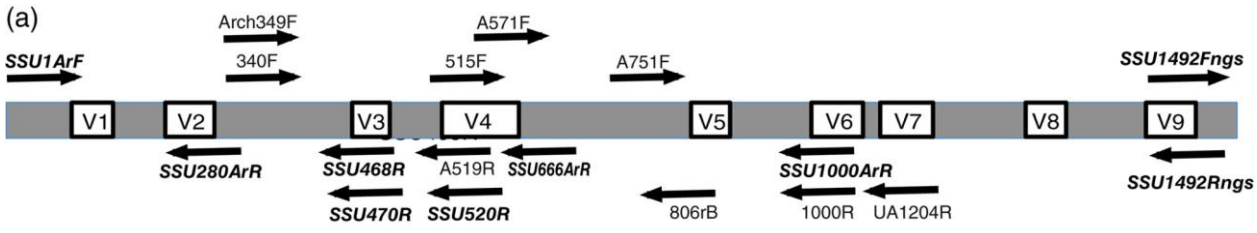
In PCR there are normally at least two primers chosen, one working forward and one working in reverse. The forward primer begins working on one strand of the DNA, the 3' end, and the reverse primer works on the opposite 5' end. The opportunity to utilize two primers can add more variance to which traits are highlighted and can further play into why having diverse primers is so important to archaeal research.

16S rRNA

16S rRNA is a gene that is often used in primers to amplify a specific portion of DNA. During a Polymerase Chain Reaction (PCR), DNA is separated into two different strands from its original double helix. A primer is used to provide a starting point for DNA polymerase, where the DNA is replicated off of the primer onto an original strand. 16S rRNA is a portion of DNA that is commonly chosen to be amplified in archaeal PCR. It takes genetic information from a subunit ribosomal RNA gene that can serve as both a universal primer, and one that is specific for archaea (Yarza et. al, 2014). There are currently more than 4 million entries of species

identified via the 16S rRNA gene, as portions span from life containing eukarya and prokaryotes, to archaea specifically. 16S rRNA is found in all prokaryotes and archaea, and contains portions that are variable, allowing for isolating a specific species' strain of DNA or allowing for sequence mismatches that can yield a new species (Lewis et. al, 2020). However, difficulties come into play when trying to find a primer that is both accurate and also allows for novel discoveries. There is a limited amount of archaeal presence in 16SrRNA databases thanks to their tendencies towards specific environments. In a study comparing various primers efficacy towards archaea, it was found that the generic prokaryotic universal primer performed the worst in terms of archaeal amplification, only yielding Euryarchaeota and Thaumarchaeota, and no other known phyla of archaea (Bahram et. al, 2018). Though an effective primer choice, it does prove to have a bias towards cultured organisms instead of novel discoveries- an emphasis towards proving what is already known instead of what is not. When comparing uncultured genuses of bacteria and archaea utilizing this gene, it was found that 98% of bacteria amplified successfully, compared to 70% of uncultured archaea (Steen et. al, 2019). Though primers utilizing a 16S rRNA gene are one of the most commonly used, there is a push for further studies into developing a primer that targets archaea more effectively.

Figure 2



This figure from the Bahram paper in 2018 shows the areas of DNA. The areas indicated with a V are areas of variable DNA sequencing- this serves as a "barcode" for primers to filter to a specific type of archaea. The gray regions are universal, meaning that amplifying that DNA would not be beneficial to amplifying an archaea specific portion of the DNA. Primers are shown with arrows and letters indicating the name of the primers. Primers aim to adhere to the V1-V9 regions for more particular species identification.

Literature review: How is archaeal diversity measured?

Quantitative PCR

Quantitative PCR (qPCR) is a step further than traditional PCR, as it not only amplifies a specific portion of DNA, but also calculates the concentration of these nucleotide sequences to determine the amount present. Emulsions with fluorescent dye are executed, and samples are monitored via that fluorescence to determine the concentration (Pabinger et. al, 2014). It is an effective method to determine initial amounts of DNA in a sample. However, the efficacy of qPCR is typically assumed to be 100%, when in fact, it is often lower (Ruijter et. al, 2021). Further steps can be taken to determine the efficacy of the assay prior to running qPCR to increase the significance of results. qPCR is also limited to the availability of primers, meaning it is only effective for species that have previously been sequenced and updated into databases.

Functional Gene Analysis

Functional gene analysis differs from typical gene sequencing. In gene sequencing, analysis is done into the specific nucleotide sequence to determine the taxonomy of an organism. Functional gene analysis analyzes the traits an organism has instead of the coding behind it.

Similar to how media is being catered to specific taxonomies to encourage growth of recalcitrant microbes, functional gene analysis is also a resource for separating out species with media. By analyzing metabolic pathways, a specific media can be created according to the needs of the microbe. In ensuring the growth of only specific microbes according to their metabolic profile, no other microbes can grow (Tu et. al, 2014). Eliminating opposing bacteria or archaea pertaining to media can show the diversity within an inoculum sample when placed on different media. Much like how media can be beneficial to identify bacteria based on what grows on it, it can be used similarly for archaea.

16S rRNA

16S rRNA, when used in PCR, also aids in measuring archaeal diversity. In its use in PCR, it serves to amplify only the DNA containing this sub ribosomal unit. Because this is a primer with some areas for variability, it can allow for different taxonomies of archaea to be amplified. Databases are more likely to have various sequencing of 16S rRNA, which means in amplifying and utilizing qPCR, the odds are high there will be a match of the sample in a database. The areas V1 and V2, as shown in figure 2, are most prone to variation within the 16S rRNA gene, which makes them a prime target for primers to be developed to (Bahram et. al, 2018). Taking the genetic sequencing and comparing the similarities and differences to previously updated databases can allow for novel species discoveries. When creating phylogeny of organisms, similarities and differences of genes serve as a reference for determining the order of evolution of organisms. Comparing and contrasting those differences can lead to contextualizing the lineage and phylogeny of a novel species, further broadening the known diversity of archaea.

Metagenomic Sequencing

Metagenomic sequencing differs from utilizing PCR to measure archaeal diversity because it analyzes more DNA present than that of a targeted sample. Samples are taken of areas that are known to contain archaea, such as aquatic environments. DNA of all organisms within this environment is collected, including the bacteria, archaea, and even viruses. Because metagenomic sequencing does not utilize PCR, there are not as many limitations that primers unintentionally impose. The ability to look at DNA variety is not limited to the DNA databases and primer availability, so it can lead to a more comprehensive understanding of an environment. Metagenomic sequencing also eliminates some of the troubles with pure cultures- such as interspecies relationships. Analysis of a community can show the metabolic pathways of an organism because all aspects of the environment it requires to grow are present. Not only are the functional pathways present, but DNA sequencing can also aid in determining taxonomy.

Single Cell Genomics

Single cell genomics first isolates a singular cell of a sample to begin a pure culture. There are various methods to isolate just a single cell, such as the optical tweezers that are mentioned in Figure 1. Optical tweezers focus a laser beam to trap a cell by holding it in the light. This is a method especially of use for cells with specific growth requirements, as it allows for the utmost precision in isolating the cells. Some success has been found utilizing micromanipulators, which allow for extremely delicate movements within a sample. This can be used to choose and isolate a specific cell under a microscope (Prakash et. al, 2012). Manual separation of cells can be done under microscope and with pipette, which is typically most beneficial for cells larger than archaea. Taking a single-cell from an organism and amplifying its

DNA ensures a pure culture and can provide a mass of cells of archaea to analyze individually. Instead of looking at interspecies relationships or archaea within its natural setting, it lets ample analysis of behavior of one specific archaea to provide insight to the roles archaea may play within an ecosystem.

Cultivation Based Approaches

Very similar to functional gene analysis and utilizing genetic identity, cultivation based approaches can measure diversity by limiting growth of some organisms according to growth media. Cultivation based approaches play upon a factor that a specific microbe needs to grow, whether that be a nutritional requirement or temperature. This eliminates some other weed-like microbes growing because the cultivation is chosen for one specific microbe- conditions are deliberately chosen. This is preferable for measuring diversity because it can take the microbes within one sample and with different media and conditions isolate the specific species based on what they can and cannot grow on. This results in pure cultures of the various species and an analysis of some of the diversity.

McCully Case Study

In a novel study published in late 2023, the McCully lab studied the role of a double emulsion for culturing recalcitrant microbes that can be applied to archaeal research. A double emulsion takes a portion of media specific for a target microbe. A cell from said microbe is put into the media, and then is coated in an oil to protect the media from contamination. This method has been previously used for pharmaceuticals and cosmetics development, but 2023 was the first time it was utilized as a method of growth of recalcitrant microbes. Because only a single species

is placed into the specific media, it allows for microbe specific growth. Oil serves as another layer of protection, yielding greater than 99% purity of cultures (McCully et. al, 2023). The method was initially tested with E. Coli as a control, and then other bacteria with specific requirements. *Desulfovibrio ferrophilus* is a recalcitrant bacteria that oxidizes sulfates and was successfully grown into a pure culture. Though this method has not been used yet on archaea since it is new research, it is a method that can be modified to fit specific archaeal needs and produce a pure culture.

Conclusion

Archaeal research is something that continues to grow and build off of previous research- the story is constantly developing. Why some microbes are recalcitrant to growth is the first step of understanding the complex nature behind archaeal research. Taking the findings from the DeLong paper that archaea are found all around, not just in extreme environments, drastically expanded the field of microbiology since archaea were now easier to access. Analysis of genetic identity and insight into the time of experiments can lead to more successful archaea cultivation, ultimately resulting in pure cultures. Archaeal primers are chosen based on availability in databases, which is continuing to evolve based off of the uncultured becoming the cultured. Genetic sequencing of these previously uncultured microbes and updates to known sequencing leads back to question one: perhaps microbes are not recalcitrant to growth, there just are not yet the correct tools to sequence and identify them. Lastly, archaeal diversity also leads back to primer availability. Documenting the pure cultures and increasing inoculum sources adds necessary updates of genealogy for updating the potentials of diversity in a singular inoculum source. Analysis of the environments of archaea provides insight to the interspecies relationships and behaviors of microbes that might aid in culturing the recalcitrant microbes.

Further research from this project can be taken and applied in a laboratory setting. There are so many methods being studied in attempts to culture previously undocumented microbes that future research possibilities are endless. This field is ever developing, with successful methods used within the last year, such as the double emulsions technique used in the McCully paper (2023). The vast majority of life on earth is microbial, and of that microbes full of untapped potential. Archaea in particular have unknown potential- whether they are used for

bioremediation of polluted environments or play a crucial role within larger organisms is something that is yet to be discovered. Archaea, especially Thaumarcheota, are particularly special because of the potential significance they can play in cleaning up polluted ecosystems. In Iowa specifically, nitrogen-based fertilizers are commonly used on the farmland, contributing to dead zones within the Gulf of Mexico or toxins invading water sources within the state. Understanding the uses of Thaumarchaeota can be key to fixing the impending crisis of unintentional pollution. Deliberate emphasis on expanding archaeal research is the next era of microbiology, and it is happening now.

Works Cited

Bahram, M., Anslan, S., Hildebrand, F., Bork, P., & Tedersoo, L. (2018). Newly designed 16S rRNA metabarcoding primers amplify diverse and novel archaeal taxa from the environment. *Environmental Microbiology Reports*, *11*(4), 487–494. https://doi.org/10.1111/1758-2229.12684

DeLong, E. F. (1992). Archaea in coastal marine environments. *Proceedings of the National Academy of Sciences of the United States of America*, *89*(12), 5685–5689.

Distaso, M. A., Bargiela, R., Brailsford, F. L., Williams, G. B., Wright, S., Lunev, E. A., Toshchakov, S. V., Yakimov, M. M., Jones, D. L., Golyshin, P. N., & Golyshina, O. V. (2020). High Representation of Archaea Across All Depths in Oxic and Low-pH Sediment Layers Underlying an Acidic Stream. *Frontiers in Microbiology*, *11*, 576520. https://doi.org/10.3389/fmicb.2020.576520

Guerra, C. A., Bardgett, R. D., Caon, L., Crowther, T. W., Delgado-Baquerizo, M., Montanarella, L., Navarro, L. M., Orgiazzi, A., Singh, B. K., Tedersoo, L., Vargas-Rojas, R., Briones, M. J. I., Buscot, F., Cameron, E. K., Cesarz, S., Chatzinotas, A., Cowan, D. A., Djukic, I., van den Hoogen, J., … Eisenhauer, N. (2021). Tracking, targeting, and conserving soil biodiversity. *Science*, *371*(6526), 239–241. https://doi.org/10.1126/science.abd7926

Leadbetter, J. R. (2003). Cultivation of recalcitrant microbes: Cells are alive, well and revealing their secrets in the 21st century laboratory. *Current Opinion in Microbiology*, *6*(3), 274–281. https://doi.org/10.1016/S1369-5274(03)00041-9

Lewis, W. H., Tahon, G., Geesink, P., Sousa, D. Z., & Ettema, T. J. G. (2021). Innovations to culturing the uncultured microbial majority. *Nature Reviews Microbiology*, *19*(4), 225–240. https://doi.org/10.1038/s41579-020-00458-8

McCully, A. L., Loop Yao, M., Brower, K. K., Fordyce, P. M., & Spormann, A. M. (2023). Double emulsions as a high-throughput enrichment and isolation platform for slower-growing microbes. *ISME Communications*, *3*(1), 47. https://doi.org/10.1038/s43705-023-00241-9

Pabinger, S., Rödiger, S., Kriegner, A., Vierlinger, K., & Weinhäusel, A. (2014). A survey of tools for the analysis of quantitative PCR (qPCR) data. *Biomolecular Detection and Quantification*, *1*(1), 23–33. https://doi.org/10.1016/j.bdq.2014.08.002

Prakash, O., Shouche, Y., Jangid, K., & Kostka, J. E. (2013). Microbial cultivation and the role of microbial resource centers in the omics era. *Applied Microbiology and Biotechnology*, *97*(1), 51–62. https://doi.org/10.1007/s00253-012-4533-y

Ruijter, J. M., Barnewall, R. J., Marsh, I. B., Szentirmay, A. N., Quinn, J. C., van Houdt, R., Gunst, Q. D., & van den Hoff, M. J. B. (2021). Efficiency Correction Is Required for Accurate Quantitative PCR Analysis and Reporting. *Clinical Chemistry*, *67*(6), 829–842. https://doi.org/10.1093/clinchem/hvab052

Steen, A. D., Crits-Christoph, A., Carini, P., DeAngelis, K. M., Fierer, N., Lloyd, K. G., & Thrash, J. C. (2019). High proportions of bacteria and archaea across most biomes remain uncultured. *The ISME Journal*, *13*(12), 3126–3130. https://doi.org/10.1038/s41396-019-0484-y

Tu, Q., Yu, H., He, Z., Deng, Y., Wu, L., Van Nostrand, J. D., Zhou, A., Voordeckers, J., Lee, Y.-J., Qin, Y., Hemme, C. L., Shi, Z., Xue, K., Yuan, T., Wang, A., & Zhou, J.

(2014). GeoChip 4: A functional gene-array-based high-throughput environmental technology for microbial community analysis. *Molecular Ecology Resources*, *14*(5), 914–928. https://doi.org/10.1111/1755-0998.12239

Yarza, P., Yilmaz, P., Pruesse, E., Glöckner, F. O., Ludwig, W., Schleifer, K.-H., Whitman, W. B., Euzéby, J., Amann, R., & Rosselló-Móra, R. (2014). Uniting the classification of cultured and uncultured bacteria and archaea using 16S rRNA gene sequences. *Nature Reviews Microbiology*, *12*(9), Article 9. https://doi.org/10.1038/nrmicro3330

(N.d.).