**Reproducing Research Is Really F#$%ing Hard**

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**Perspective**

XXXX words plus XX references, X figures, and a XXX-word abstract

**Abstract**

The “reproducibility crisis” in science affects microbiology as much as any other area of inquiry, and microbiologists have long struggled to make their research reproducible. In light of this, this Perspective lays out a framework that describes the reproducibility, replicability, robustness, and generalizability of a particular result. It then describes the factors that can threaten this framework and approaches microbiologists can take to overcome the threats. Finally, it provides several exercises for individuals and research groups who wish to gain a better appreciation of their own research practices and what makes them reproducible. Failures to validate previous results have much to teach us about the scientific process and microbial life itself.

**Keywords:** Reproducibility, Microbiome, Scientific method, Research ethics, American Academy of Microbiology

**Introduction**

On first blush, one might argue that any scientist should be able to reproduce another scientist's research with no friction. Yet two anecdotes suffice to describe why this is not the case. The first goes to the roots of microbiology, when Antonie van Leeuwenhoek submitted a letter to the Royal Society, "Concerning little animals" (1) in 1677. This seminal work described novel observations of microorganisms, but the scientific community initially rejected his observations, for various reasons. First, because Leeuwenhoek had little interest in sharing his methods with others, they could not be replicated. Second, he wrote in "low Dutch" and his writing was translated to English and significantly edited. **Add a sentence here saying why this was a problem.** Robert Hooke produced a response to the first objection in **year.** While his compound microscope was inferior to Leeuwenhoek's single lens microscope, he replicated the latter’s findings. In the process, Hooke popularized the compound microscope. This succession of events are illustrative of many of the current problems microbiologists face in reproducing and replicating each other's work. Of course, Leeuwenhoek's work was rigorous, impactful, and robust. It was not sloppy and there was no fraud. But, it was not reproducible or replicable at the time.

The second anecdote takes place far more recently. In 2011 Philip Bourne challenged those attending the [*Beyond the PDF* workshop](https://sites.google.com/site/beyondthepdf/) to reproduce the analysis performed in his group's 2010 study *The* Mycobacterium tuberculosis *Drugome and Its Polypharmacological Implications* (2). The response to that challenge resulted in a unique analysis that challenged concepts critical to understanding reproducible research. It demonstrated that the value of reproducibility, the degree to which research should be reproducible, the amount of effort required to reproduce the research, and who should be able to reproduce the research are not simple questions. Unlike Leeuwenhoek, Bourne's group had been transparent. And like him, they had not been sloppy. Yet the investigators who sought to reproduce their findings estimated that it would take a novice at least 160 hours to decipher the approaches used in the original analysis and an additional 120 hours to implement them.

Both of these anecdotes are at odds with the tone of a recent report by the American Academy for Microbiology's (AAM's) 2015 colloquium, "Promoting Responsible Scientific Research" and its accompanying editorial in *mBio* (3, 4). The report is a useful lens into how microbiologists view the reliability of research in their field. The colloquium identified "(i) sloppy science, (ii) selection and experimental bias, and (iii) misconduct" as the primary contributors to the ongoing problems with insuring the reliability of microbiology research. Although the participants were quick to point out that misconduct was a relatively minor contributor to the problem, the four case studies that accompanied the original report all concern misconduct. None of them reflected the problem that Leeuwenhoek’s case and Bourne’s team case bring to the fore: ensuring that one's research design and methods are sufficiently clear is enormously difficult. Researchers who do good research can still forget a detail from their methodology after a paper is published. This paper argues that while sloppy science, bias, and misconduct exist, they are not the main reason for the reproducibility crisis in microbiology.

The goals of this Perspective are three-fold. First, I hope to give a better framework for thinking about how science is conducted within the microbial sciences. Although I will primarily focus on examples from microbiome research, the principles are generalizable to other areas of microbiology. Second, I provide an overview of various factors that threaten the field’s ability to validate prior results and the tools that we can use to overcome these problems. Third, based on these issues, I suggest several exercises that research groups can use to motivate discussions of these factors.

**Threats to reproducibility**

Science is hard and failure to support an earlier observation does not indicate a failure, but a success, of the scientific method. Yet research that might produce such successes meets a number of barriers. First, there is some disagreement as to what reproducibility and replicability mean. The AAM report used the term reproducibility where others would use replicability (i.e. the ability to generate the same results after repeating the experiment independently of the first) (5). This Perspective will use the most widely used definitions, which describe reproducibility as the ability to regenerate a result given the same dataset and data analysis workflow and replicability as the ability to produce a consistent result with an independent experiment asking the same scientific question (5). Problems with likely lie with

A framework based on the definitions of reproducibility and replicability distinguishes the use of the same or different system or cohort and the same or different methods (Table 1) (8). This framework highlights attempts to determine whether a result is robust to differences in methods or generalizable to different datasets that may have been collected under different conditions. Aside from issues of sloppiness, bias, and fraud, it is scientifically valuable to consider what factors threaten each of the quadrants in this framework.

Factors that threaten both reproducibility and replicability include the fact that having the same result is not just a product of rigorous scientific practice, but also a product of stochastic forces (6). Furthermore, just because a result is reproducible or even generalizable does not guarantee that the result is correct (5). Finally, most research is exploratory and scientists, editors, and funding agencies generally lack the will or ability to confirm previous studies via independent replications or attempts to generalize results in other model systems or human populations (3, 4, 9).

Recently, several research groups, including mine (10), have attempted to validate the result that obese individuals were more likely to have lower bacterial diversity and higher abundances of *Bacteroidetes* and lower abundances of *Firmicutes* in their feces, which engendered much enthusiasm for the role of the microbiome in human health (11, 12). The The Human Microbiome Project, published in 2008, used 16S rRNA gene sequencing (13). Although the original study was performed using poorly reported data curation methods, we and others were able to independently obtain the same results as the original study when using the same dataset. Thus it was reproducible as defined in Table 1. However, when we used the same methods with nine other datasets, we failed to replicate the result. Other groups have also failed to replicate the original result with their own data analysis workflows. This may be due to methodological differences across the replicating studies, differences in study populations, or statistical variation. It is worth noting that those involved in the original Turnbaugh study pursued multiple approaches to better understand the question of whether the microbiota is important in obesity. They initially sought microbiome-based signatures using mouse models (14). They observed stark differences in the microbiota of genetically lean and obese mice and that the microbiota of obese mice could transmit the propensity to gain weight to germ free mice (14). In a human cohort, they generated multiple datasets that each reflected different regions of the 16S rRNA gene. In obese individuals, they observed reduced diversity and relative abundance of *Bacteroidetes* (13). They also used shotgun metagenomic sequencing to postulate the enrichment of carbohydrate processing genes in obese individuals (13). In a smaller cohort study, although the subjects' diversity remained constant, there was the predicted increase in *Bacteroidetes* as subjects lost weight (16). Although each part of their approach had significant weaknesses, including methodological biases and underpowered experimental designs, their results supported the hypothesis that obesity has microbial signatures. Their overall conclusion appears to have been robust within the cohort they studied. The inability to replicate these results in other cohorts, however, indicates their conclusions are not generalizable.

**Threats to Reproducibility.** The poor reporting of the data curation methods in the original study linking obesity to biome is not atypical. Because of word limits in many journals, Materials and Methods sections become a chain of citations to previous work that each cite previous work (9). Improved documentation in supplementary materials or archives such as protocols.io (https://www.protocols.io) for lab-based methods or through GitHub (https://github.com) for data analysis workflows would address these rabbit holes. For data analysis workflows, software such as GNU Make (https://www.gnu.org/software/make/) and the Common Workflow Language (17) make it possible to track data dependencies and automate a workflow. For example, my research group used GNU Make to write a workflow for use in our obesity meta-analysis that produces scripts that are downloadable from the project's GitHub repository such that entering "make write.paper" in the command line will reproduce our analysis. These tools make it possible to trace the provenance of a summary statistic from the manuscript back to the raw data.

The Human Microbiome Project provides an example of another barrier to reproducibility: investigators’ failure to account for confounding variables. In sequence-based analyses, for example, not randomizing samples across sequencing creates this problem. Similar batch effects emerge with many other analytical techniques beyond sequencing as well (18). Human Microbiome Project researchers, for example, recruited 150 people in Houston, TX and 150 in St. Louis, MO (19). DNA extractions for the two sets of subjects were performed at Baylor College of Medicine and Washington University, respectively. Researchers at Baylor College of Medicine, the J. Craig Venter Institute, and the Broad Institute sequenced the DNA from the Houston subjects while researchers at Washington University sequenced the DNA from the St. Louis subjects following the same procedures. Yet the variable with the largest effect size was the subject's city (19, 20). Because the city of origin and the center that did the extractions were perfectly confounded, it was impossible to quantify the impact of regional differences on the microbiome. Instead of being a single study, this became two replicate studies.

Another problem we encountered concerns raw data. As we developed the obesity meta-analysis we were dependent on the original authors to provide the information for two of the ten datasets. Furthermore, the data included the subjects' body mass index (BMI) as categories, but did not provide the actual heights, weights, and BMIs were not available. Our analysis omitted three large datasets from two studies because their data were practically inaccessible due to onerous data sharing agreements (23, 24). Two other datasets required at least a month of effort to obtain (19, 25). Such problems are common (21, 22); although well-established databases exist for sequence data, these data are still often missing, lack the necessary metadata, or are only available upon request from the original authors. Data that goes beyond sequence data can be archived in databases including FigShare (https://figshare.com) and Dryad (<https://datadryad.org>), and more should be.

Changes in sequencing technology, data curation, databases, and statistical techniques presented another complication. The Human Microbiome Project used Roche's 454 platform to sequence the 16S rRNA gene (19). This sequencing platform is no longer commercially available. Data analysis software and databases are also rapidly changing. The mothur software package has had 40 major updates since it was originally released in 2009 (26). The RDP [(27); http://rdp.cme.msu.edu] and SILVA [(28); https://www.arb-silva.de] databases that many use as a reference for aligning and classifying 16S rRNA gene sequences are updated annually and the popular greengenes database files have not been updated since 2013 [(29); http://greengenes.lbl.gov and http://greengenes.secondgenome.com]. With each release, curators expand the number of sequences in the database and make modifications to their taxonomic outline. For software and databases, it is critical that authors report version numbers if there is to be any hope of replicating previous work. Unfortunately, the reliance on web-based workflows like GenBank (https://www.ncbi.nlm.nih.gov/genbank), greengenes, RDP, and SILVA preclude analyzing new data with older versions of the sites. The greengenes website removed their online tools in April 2017, exemplifying the problem with web-based workflows. Their database files are now available through the company, Second Genome, but their tools are not.

“Link rot”—the fact that a web or email address may be deprecated—is a significant problem with accessing data and methods information necessary to reproducing a result Changes in institutional affiliation frequently render email addresses invalidto address email rot, and use it However, the fact that the most important collaborator in reproducing research is the original researcher *at the time,* and it is impossible to email our past selves, remains a significant barrier. Email and link rot can be remedied; memory rot is more difficult.

Other problems with reproducibility reflect the fact that science is not a linear process resembling a pipeline. In reality, questions change and scientists fall into the traps of the "Garden of Many Forking Paths" where they go looking for a desired result (30) or "P-hacking" where large numbers of statistical hypothesis tests are attempted without adequately correcting for performing multiple tests (31). Although it is possible to pre-register data analysis plans (32–34), these are often too stringent for most exploratory research. Alternatives include making research notebooks publicly available using commercial platforms or free tools such as RMarkdown documents (35) and Jupyter notebooks (36). Combined with version control software such as git, these literate programming documents can allow researchers to document the evolution of their analyses.

**Barriers to Replicability.**

Tremendous inter-strain and population variation hinder efforts to replicate results. In microbiome research, it is widely appreciated that the microbiota of research animals from the same litter and breeding facility are largely clonal and distinct from other facilities (15, 38). Mice from two breeding facilities at the same institution may have completely different microbiota. The best example of this phenomenon is the presence of segmented filamentous bacteria in mice purchased from Taconic Farms, but not Jackson Laboratories (39, 40). Thus, the origin of the mice, not the experimental treatment, may explain differences ascribed to the microbiota. This is particularly a problem for genetic models when researchers obtain mutant animals and animals with the wild type background as their control. In such cases using the offspring of heterozygous matings is critical (41). Similarly, comparing the microbiota of obese and lean individuals from a cohort of twins and their mothers in Missouri (13) may have confounding factors that differ from members of Amish communities (25). In these cases, the problem with replicability is not due to the quality of the investigator's experimental practices, but to differences that may be biological, demographic, or anthropological. Thus failure to replicate a study across different cohorts could suggest that other interesting factors play a role in the phenomenon under study.

Uncertain provenance and purity of reagents, organisms, and samples also threaten replicability. Perhaps the best known example is the discovery that HeLa cells contaminate many other cell lines, especially those in the same laboratory (42, 43). Similarly, investigators frequently realize that they are working with bacterial strains that were incorrectly typed or that have evolved during serial passages from the freezer stock (44, 45). Short of resequencing the cells, experimental controls, limiting the number of passages from freezer stocks, and periodic phenotyping of the strains can help to overcome these problems. DNA extraction kits can be contaminated with low levels of bacterial DNA (46). These contaminants have led to the identification of contaminants as being important members of the lung and placental microbiota when mock extractions are not sequenced in parallel (47–49).

A replication may fail because replication is statistical rather than deterministic (6). Every experiment has a margin of error and when the effect size is near that margin of error, it is likely that a statistically significant result in one replicate will not be significant in another. Most researchers use a frequentist null model hypothesis testing approach, meaning they are willing to accept a Type I error of 0.05. Stated more colloquially, they are willing to incorrectly reject a null hypothesis in 5% of the replicates. Further, they rarely quantify the risk they are willing to accept of falsely accepting a null hypothesis (i.e. Type II errors) (50). In our analysis of the microbiota associated with human obesity, we observed that nearly all studies were underpowered to detect 5 or 10% differences in diversity (10). In some cases, an insufficient sample size in the replicate study may explain failure to replicate a study. In other cases, the original study may have been underpowered, rendering it susceptible to an inflated risk of Type I errors (51). Solutions to these problems include authors pre-registering their data analysis plans (32–34), justifying sample sizes based on power calculations (9, 10, 50), and using Bayesian frameworks that allow prior knowledge of the system to influence the interpretation of new results (52, 53).

**Issues with Robustness.** Every method has its own strengths and weaknesses. Therefore, it is important to address a research question from multiple and hopefully orthogonal directions. This strategy combines the strengths of different methods to overcome their individual weaknesses (54). Evaluating the robustness of a result from a single cohort is becoming more common as researchers pursue multiple approaches including 16S rRNA, metagenomics, metatranscriptomics, and metabolomics (55–57). Of course, biases in the underlying cohort design, sample collection and storage, or the nucleic acid processing will propagate through the analyses. The way to remedy this, is to make the methods as independent from each other as possible. For example, sequencing multiple regions of the 16S rRNA gene would not be considered truly independent datasets since the same general method would be applied to the same samples. Layering shotgun metagenomic data onto the 16S rRNA gene sequence results would be marginally more independent, although it uses the same DNA for sequencing, because the method provides information about the genetic diversity and functional potential of a community rather than the taxonomic diversity of a community. Metabolomic data would be even more independent from the DNA-based methods since it requires completely different sample handling and processing steps. Quantitative PCR, cultivation, and microscopy could be similarly layered on these data. Ultimately, it is impossible for the results of each set of methods to be fully independent.

**Barriers to Generalizability.** The gold standard of science is to have a result that is generalizable across populations. Failing to attempt replication studies hinders the ability of researchers to test the generalizability of most results. Scientists often fear being "scooped" (58). In reality, the second researcher who examines the same question has the opportunity to increase the field's confidence that a result is robust or generalizable (59). Generalizability is an important question; model organisms (e.g. *E. coli*) and strains of those organisms (e.g. K-12) have taught us a great deal about the biology of those organisms, but it is not always trivial to generalize that knowledge to related species and strains or from *in vitro* to *in vivo* conditions and on to human subjects (60, 61). Like a failure to replicate or reproduce findings, a failure to generalize is not a failure of science. Rather, for a microbiologist, it is an opportunity to better understand the complex biology of bacteria and how they interact with their environments.

**Need for training.** A key observation from the work of Garijo and colleagues (2) was that the level of detail needed to reproduce an analysis varies depending on the researcher's level of training. An expert in the field understands the nuances and standards of the field, whereas a novice may not know how to install the software. This highlights the need for training. Yet many microbiology training programs focus on laboratory skills while ignoring data analysis skills.

A number of excellent "best practices" documents have emerged in recent years that address the gap (62–67). I have created the Riffomonas project, which expounds on the threats to reproducibility and tools that microbiologists can use to maximize the reproducibility of their analyses (http://www.riffomonas.org). In addition, organizations including Software Carpentry and Data Carpentry offer workshops to introduce researchers to the best practices in reproducible research (68). Massively open online courses have been developed that teach scientists best practices for performing reproducible analyses. The most popular of these is a training program from faculty at the Johns Hopkins Data Science Lab (http://jhudatascience.org). Just as a novice could not reproduce Beethoven's "Für Elise" from sheet music without prior experience playing the piano, novices cannot expect to reproduce a complex experiment and analysis without learning the methods of their discipline.

**Exercises**

The following exercises are meant to motivate conversations within a research group to foster a culture improving reproducibility and replicability and to underscore the threats outlined above.

1. Working away from each other, have two or more people to write instructions on how to fold a piece of paper into an airplane. Have the participants trade instructions, separate, and implement the instructions. How closely did the final airplanes resemble that of the person who developed the instructions? What would have helped to make the reproductions more faithful? How much did the author of the instructions assume about the other person's prior knowledge of paper airplanes, resources, and abilities were assumed? What challenges length limitations place on this exercise? How does this exercise resemble the descriptions in the Materials and Methods section of papers for standard methods (e.g. PCR) and for novel methods (e.g. bioinformatic workflows)?

2. Imagine a graduate student is really excited to see an analysis that you performed in your most recent paper because he or she would like to reproduce it with his or her own data. He or she wants to make sure that the results will reproduce your findings. What steps are likely to cause the student problems? Take a figure from your recent paper and improve the likelihood that a third party would be able to reproduce it. Where are the data and how would another scientist acess them? What calculations were performed to summarize the data? What software was used to generate the figure? Is that software freely available? What steps would the other scientists need to take to generate the figure? When you are confident that you have made the figure as reproducible as you can, give the instructions to a colleague and ask for his or her feedback. Find a figure you find particularly interesting from your favorite paper from a different research group. Can you reproduce the figure? What is standing in your way?

3. Look for threats to reproducibility and replicability that are a product of scientific culture: methods sections are terse or vague, original data are not available, analyses rely on expensive and proprietary software, analysis scripts are available "upon request from the authors," papers are published behind pay-walls. Table 2 provides a rubric that scientists working within the host-associated microbiome field might use to assess their research. Have a discussion within your group about why you do things this way, whether your practices should change, and what would be the easiest to change. For your next paper, work to improve one element within this rubric and commit to an ethic of fostering greater reproducibility.

**Conclusion**

Most research is repeated multiple times within a research group prior to and after publication. Anyone who has done research can attest to how difficult it can be to satisfying their two most important "collaborators." If a scientist does not provide sufficient transparency to allow his or her own lab to reproduce a result, then it is unlikely that any one else can. It is important to see that attempts to guard against threats to reproducibility, replicability, robustness, and generalizability are positive forces that will improve science. They have been considered a form of prevention against false results (5). Before attributing difficulties with reproducibility, replicability, robustness, and generalizability to a dim view of our fellow scientists as sloppy, biased, or untrustworthy, it is worth considering the other factors - biological, statistical, and sociological - that pose a threat. Although there is much room for improvement, we must acknowledge that science is a process of learning and that it is really f#$%ing hard.

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***Table 1.*** Simple grid-based system for defining concepts that can be used to describe the validity of a result. This is a generalization of the approach of Whitaker (8), who used it to describe computational analyses.

***Table 2.*** An aspirational rubric for evaluating the practices host-associated microbiome researchers might use to increase the reproducibility and replicability of their work. Although many of the questions can be thought of as having a yes or no answer, a better approach would be to see the questions as being open ended with the real question being, "What can I do to improve the status of my project on this point?" With this in mind, a researcher is unlikely to have a project that satisfies the "Best" column for each line of the table. Researchers are encouraged to adapt the categories to suit their own needs.

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