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The Great Microbiome Extraction Anomaly: How Extraction Bias Distorts Microbial Community Profiles

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ABSTRACT

Research on the human microbiome has become one of the most frequently published and highly cited areas in science. In parallel, environmental DNA (eDNA) and RNA (eRNA) microbiome studies have expanded rapidly, often using similar technical workflows. Numerous studies optimizing these workflows have demonstrated that nucleic acid purification can significantly influence bacterial metagenomic outcomes. Although this issue is widely acknowledged and efforts are often made to ensure workflow consistency, such protocol standardization may obscure a deeper problem. Specifically, even when protocols are consistent, the resulting data is unlikely to reflect the true microbial community. This systemic bias, herein described as *The Great Microbiome Extraction Anomaly*, refers to the widespread distortion of observed microbial community composition caused by variation in nucleic acid extraction efficiencies across microbial nucleic acid states, species biology, and specimen composition. The term draws inspiration from *The Great Plate Count Anomaly*, a long-recognized discrepancy between observable and cultivable microbial diversity. *The Great Microbiome Extraction Anomaly* presents a parallel methodological challenge which, like the original, will likely require novel technological innovation to resolve, as to date, no current protocol has achieved truly unbiased microbial DNA recovery. Currently, this limitation can only be addressed with carefully considered analytical controls that enable transparent reporting of microbial DNA recovery biases. However, the rate of control use in microbiome research remains very low. This review examines the evidence for nucleic acid state, microbe biology, and specimen-specific nucleic acid extraction efficiency biases specifically within eDNA and eRNA microbiome workflows, while citing evidence of extraction bias from human microbiome and molecular diagnostic research to demonstrate the broader constraints underlying differential microbial nucleic acid recovery. In addition, this review evaluates the extent to which controls are implemented in microbiome research, outlines explicit examples of analytical controls that are essential for inclusion in microbiome research, and argues that implementing these robust analytical control strategies, especially the use of positive controls, is essential to detect and mitigate the biases of *The Great Microbiome Extraction Anomaly*.

1 | Introduction

Communities of microorganisms inhabit virtually every surface on Earth and play a critical role in shaping the health and ecological function of their respective environments (Cockell 2025; Kristjánsson and Hreggvidsson 1995; Vreeland et al. 2023). The composition of the human microbiome has been linked to an astonishingly wide array of health outcomes, including influencing patient mortality following bone marrow transplantation (Peled et al. 2020), contributing to the development of mental health (Radjabzadeh et al. 2022) and neurodegenerative (Loh et al. 2024) disorders, and influencing tumor development and metastasis (Sun et al. 2025; Cao et al. 2024). In parallel, assessments of environmental microbiomes have provided fundamental insight into

biodiversity (Zhang et al. 2020; Thompson et al. 2017; Herrera and Cockell 2007), microbial niche characterization (Compant et al. 2021; Feng et al. 2025; Yuan et al. 2025), pathogen surveillance (Bass et al. 2023; Nousias et al. 2025), industrial (Grisoli et al. 2009; Lea-Smith et al. 2025; Liddicoat et al. 2022; De Filippis et al. 2021) and agricultural (Orr et al. 2022; Stenger et al. 2025; Xing et al. 2024) planning, and evaluating anthropogenic impacts (Abreu et al. 2022; Vecherskii et al. 2023; Lin et al. 2023). Given their broad and influential role in both health and ecology, understanding the composition of microbiomes has been a major focus of biomedical and ecological research for decades.

Historically, microbial research relied on culturing cells on non-selective media such as nutrient agar to form colonies. However, this approach was inherently limited, as most microorganisms cannot be grown under standard laboratory conditions (Curtis and Sloan 2004; Lagier et al. 2015). This discrepancy, termed the '*Great Plate Count Anomaly*' (Staley and Konopka 1985), refers to the long-standing observation that the number of cells observed microscopically in a specimen often far exceeds the number of colonies that can be recovered through standard culture. To address this limitation, researchers developed specialized culture techniques, such as anaerobic culture (Lewis et al. 2021), or employed culture-independent methods, such as fluorescence microscopy (Caviedes et al. 2000) and flow cytometry (Monger and Landry 1993) to directly detect microbial cells. The introduction of commercially available polymerase chain reaction (PCR) in the late 1980s represented a significant breakthrough in microbial analysis, as PCR enabled the detection of microbial DNA with extraordinary sensitivity, potentially as low as a single genome copy (Forootan et al. 2017). This allowed researchers to isolate DNA from complex samples and assess the presence, and later with quantitative PCR (qPCR), estimate the abundance (Bustin et al. 2025), of specific microbial species (Jian et al. 2020; Tkacz et al. 2018). Nevertheless, PCR and qPCR remain limited as low-throughput techniques that require pre-designed specific primers. Advances in molecular biology, particularly high-throughput untargeted DNA sequencing, along with the increasing availability of microbial reference genomes, gave rise to the culture-independent screening technique of microbial metagenomics (Kim et al. 2024; Alves et al. 2018). Since the early 2000s, microbial metagenomics and other high-throughput sequencing approaches have driven a dramatic expansion in microbiome research (Figure 1).

As high-throughput sequencing became central to microbiome research, methodological advances developed unevenly across the analysis pipeline. Although no standardized microbiome protocol has been universally adopted (Berg et al. 2020), a typical microbiome study generally includes three protocol stages: (i) specimen collection, (ii) nucleic acid purification, and (iii) molecular analysis (Figure 2). The greatest innovation has occurred at the molecular analysis stage, where microbial DNA and RNA are detected and quantified. Advances in sequencing platforms and bioinformatic tools, which generally remain dependent on PCR amplification, have enabled high-speed, high-resolution, and cost-effective characterization of microbial nucleic acids (Hiraoka et al. 2016; Navgire et al. 2022). Specimen collection has also seen considerable innovation, particularly in the development of eDNA or eRNA sampling (Métris and Métris 2023; Alexander et al. 2023). In contrast, nucleic acid purification, despite some innovation in techniques and automation (Lee et al. 2010), has remained relatively unchanged over the past 25 years. Nucleic acid purification typically includes four stages: (i) lysis of microbial structures (e.g., cells, cell walls, spores), (ii) separation of nucleic acids from other biomolecules (e.g., nuclease enzymes, fats, sugars), (iii) removal of PCR inhibitory substances (e.g., salts, ethanol, tannins), and (iv) elution or concentration of nucleic acids (Rieder et al. 2024). Although commercial kit-based nucleic acid purification methods are widely used (Thompson et al. 2017), relatively little attention has been paid to whether current protocols consistently recover nucleic acids from all microbial species within a specimen.

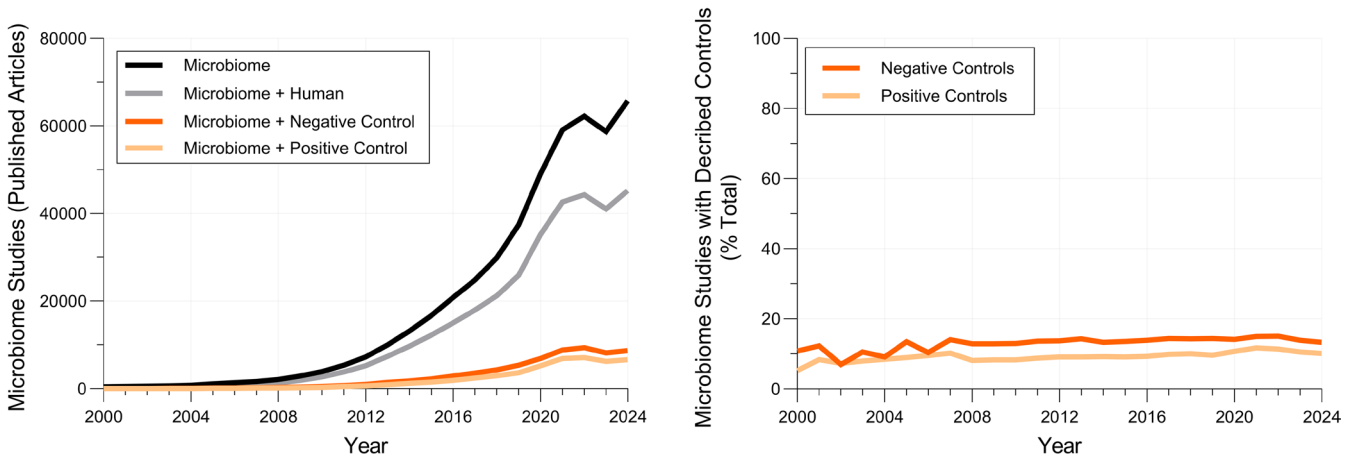

FIGURE 1 | Trends in microbiome publications and reporting of experimental controls (2000–2024). (*Left*) Annual number of microbiome-related publications retrieved from Lens.org (Jefferson et al. 2019), including: all microbiome studies (black line), studies mentioning both microbiome and "human" (gray line), studies including a described negative control (dark orange line), and studies including a described positive control (light orange line). (*Right*) Relative microbiome studies per year that described a negative (dark orange) or positive (light orange) control. Human microbiome publications are shown separately to illustrate that control implementation trends are similarly low across both human and environmental microbiome research.

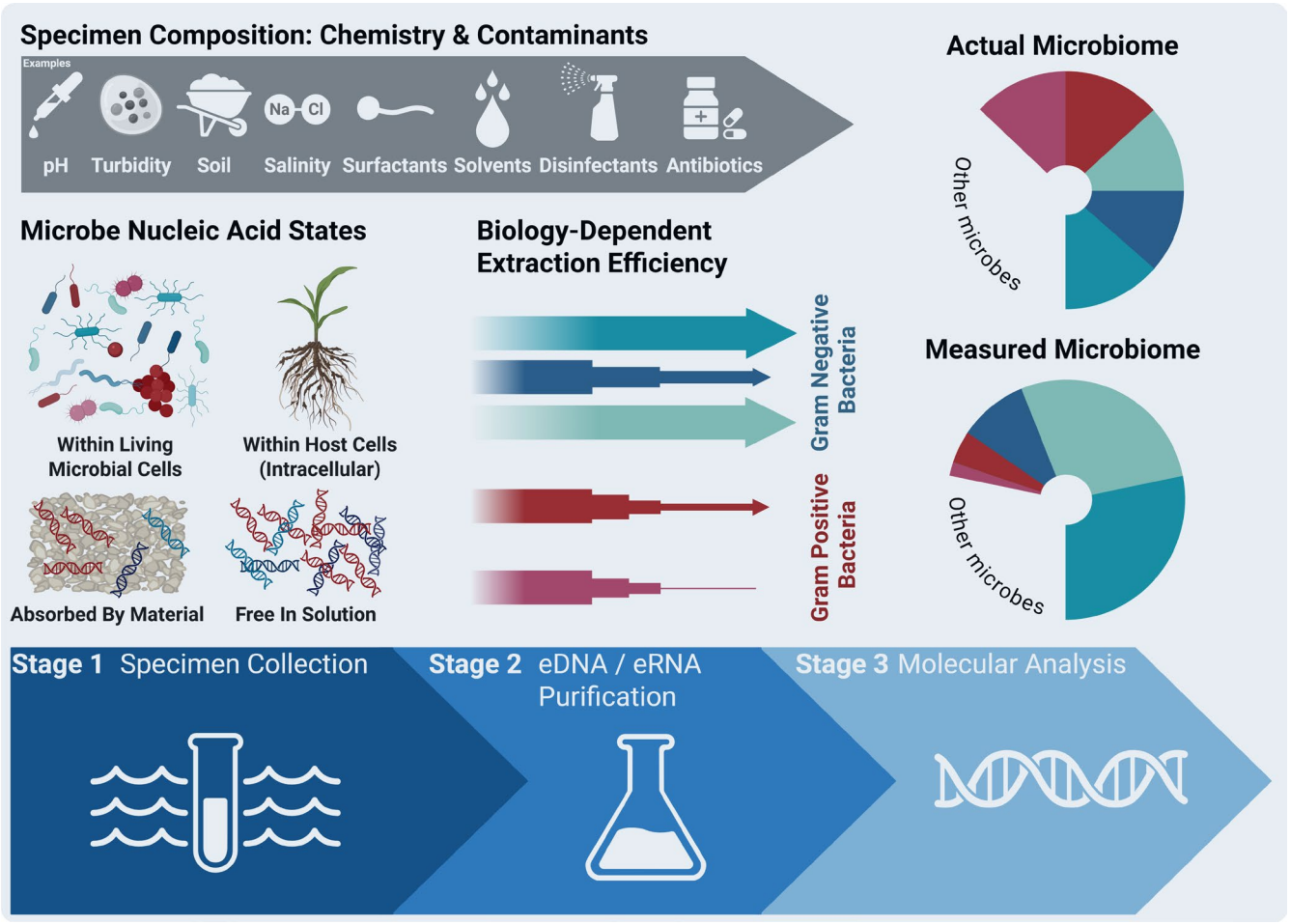

FIGURE 2 | The Great Microbiome Extraction Anomaly: The influence of specimen composition, nucleic acid state, and biology-dependent extraction efficiency on measured microbiome composition. (Stage 1) At the point of specimen collection, nucleic acid recovery can be affected by the physical, chemical, or biological properties of the specimen, including pH, turbidity, salinity, soil type, surfactants, solvents, disinfectants, antibiotics, and heavy metals. Microbial nucleic acids can exist in various physical states, including: (i) within intact microbial cells, (ii) within host cells (intracellular), (iii) adsorbed by particulate material, or (iv) free in solution. (Stage 2) The efficiency with which microbial environmental DNA (eDNA) and RNA (eRNA) are recovered during extraction may also depend on the biology of the microorganism. For example, living Gram-positive bacteria often exhibit markedly less recovery than living Gram-negative bacteria. (Stage 3) Even under a consistent extraction protocol and molecular analysis, these biases can cause divergence between the actual microbial community present, and the measured community composition. Adapted from Browne et al. (2024).

2 | Evidence Supporting Species-Specific Protocol-Dependent Microbial Nucleic Acid Recovery

Over many years, numerous studies optimizing microbial nucleic acid extraction protocols have reported that the extraction method can significantly alter the apparent composition of the human microbiome (Costea et al. 2017; Wesolowska-Andersen et al. 2014; Teng et al. 2018; Yuan et al. 2012; Willner et al. 2012; Wu et al. 2010; Morgan et al. 2010; Thiruppathy et al. 2025; Toto et al. 2025). Although much of the optimization work on nucleic acid extraction has occurred in human-associated microbiome studies, this protocol-dependent distortion has also been reported in environmental microbiome studies (Table 1). This variability is not stochastic error, but rather reflects a systematic, protocol-dependent distortion of the detectable microbial community induced by variation in: (i) nucleic acid state, (ii) species-specific cell biology, and (iii) specimen chemistry and composition. There has not yet been a comprehensive and integrated systematic assessment of how nucleic acid states, microbial biology, and specimen-specific factors influence species-specific microbial extraction efficiencies.

Nucleic acid extraction protocols do not consistently recover nucleic acids from all microbial species or across all nucleic acid states. Within a specimen, nucleic acids can occur in at least four states: (i) enclosed within intact microbial cells, (ii) associated with host or other cellular material (e.g., intracellular bacteria), (iii) adsorbed onto material within the specimen (e.g., within sediments or organic matter), and (iv) free extracellular DNA or RNA in solution (Mauvisseau et al. 2022) (Figure 2: Stage 1). Nucleic acids that are not enclosed within living microbial cells are referred to as 'relic' DNA or RNA, which may persist for remarkably long periods in the environment and do not accurately represent the current living microbiome (Carini et al. 2016). Relic nucleic acids can be selectively removed by chemical or enzymatic degradation, most commonly through the application of membrane-impermeable propidium monoazide

TABLE 1 | Selected studies demonstrating differential nucleic acid extraction efficiencies across microbial species, nucleic acid states, and specimen types.

Study	Specimen	Experiment	Findings
Albertsen et al. (2015)	Activated Sludge (Sewage)	Evaluated several commercially available and optimized DNA extraction protocols	An effect on the observed community was found on all parameters tested
Browne et al. (2024)	Fresh and Marine water	Bacterial eDNA and eRNA degradation were tracked over time	Species-specific degradation was observed
Child et al. (2024)	Soil	Compared five commercial DNA extraction kits, across soil types	The bacterial community composition varied greatly between kits and soil types
Cronin et al. (2024)	Peat soil	Three preservation and five commercial extraction kits were compared across sites	Preservation and extraction method influenced alpha and beta diversity
Du et al. (2025)	Soil	Compared five methods including: DNase and PMA pretreatment to extract DNA	The bacterial community composition varied greatly between methods
Finn et al. (2023)	Cropland and forest soils	Tested six pre-treatments, including: freezing, drying, fresh analyses	Pre-treatment affected community composition, more in fungi
Galla et al. (2024)	Soil, rhizosphere, invertebrates, feces	Tested five commercial DNA extraction kits	Microbiota alpha and beta diversity estimates varied greatly by kit
Iturbe-Espinoza et al. (2021)	Soil	Compared commercial extraction kits	Kit-specific underrepresentation of Gram-positive bacteria were observed
McCarthy et al. (2015)	Freshwater	Evaluated several commercially available and optimized protocols	Strong extraction protocol-dependent biases observed for specific phyla
Reynolds and Cadillo-Quiroz (2024)	Peatlands	Specimen preservation buffers were compared	Preservation buffers caused bacterial community shifts after 3 days
Shi et al. (2022)	Freshwater sediments	Tested three commercial extraction kits	Kit choice impacted eukaryotic diversity
Sirois and Buckley (2019)	Soil	Synthetic eDNA decay was tracked over time	Relic DNA skewed the microbial community profiles

(PMA) (Faulwetter et al. 2013) or nucleases (Wang et al. 2024). However, this approach is limited by the incomplete efficiency of the treatment, particularly in inhibitor-rich matrices or when substantial amounts of nucleic acids are bound to materials within the specimen (Seinige et al. 2014; Taylor et al. 2014; Kaur et al. 2025). Further complicating recovery, interspecies nucleic acid structural differences affect their relative stability and binding affinities (Weiß et al. 2016; Wang and Liu 2022; Bhoyar et al. 2024), and nucleic acids in different states exhibit species- and specimen-specific degradation kinetics (Browne et al. 2024).

Microbial cell biology strongly influences extraction efficiency when recovering nucleic acids from intact cells. Biology dependent extraction efficiency (Figure 2: Stage 2) generally arises from the inherent structural differences between microbial species that determine the species susceptibility to lysis (de Bruin and Birnboim 2016), including membrane composition (e.g.,

sterol content (Shehadul Islam et al. 2017), cross-linked proteins (Sun et al. 2022)), cell wall structure (e.g., peptidoglycan thickness (Kim et al. 2015)), and extracellular protective layers (e.g., biofilms (Mandakhalikar et al. 2018), spores or capsules (Encheva et al. 2006)). For example, the thick peptidoglycan layers of Gram-positive bacteria (Li et al. 2020), fungal cell walls (Karakousis et al. 2006), and helminth eggs (Doyle et al. 2019) are well-recognized barriers to nucleic acid extraction protocols. In parallel, specimen-specific factors influence nucleic acid recovery when the chemistry or contaminants of the specimen alter extraction performance. Environmental microbiome specimens may include water, soil, sediment, sand, air, or other substrates. Influential conditions include pH (Uyttendaele et al. 1996), turbidity (Williams et al. 2017), salinity (Browne et al. 2024), soil-type (Zhou et al. 1996) or other variable sample matrices (Galla et al. 2024), and the presence of inhibitory or facilitating compounds such as solvents (Arcella et al. 2014), disinfectants (Acharya et al. 2020), antibiotics (Larsson and Flach 2022), heavy metals (Huang et al. 2021), and thermal stressors (Foysal and Salgar-Chaparro 2024) (Figure 2: Stage 1 and 2). These specimen-specific conditions interact with microbial biology to modify lysis effectiveness, DNA persistence, and nucleic acid binding, thereby altering the apparent composition of the recovered microbial community. The combined influence of nucleic acid state, microbial biology, and specimen-specific conditions generates a protocol-dependent extraction efficiency for each species, resulting in a systematic distortion of the measured microbiome.

3 | The Great Microbiome Extraction Anomaly

To address this challenge, researchers commonly adopt a standardized protocol within a given study and report methods in accordance with reporting guidelines (Thompson et al. 2017; Mirzayi et al. 2021) which are intended to improve transparency and reproducibility rather than enforce uniform methodological standardization across the field. However, this presents a paradox: while standardization within a study improves comparability across samples, it may introduce protocol-specific systematic bias by favoring the recovery of nucleic acids from specific nucleic acid states, microbes with a certain cell biology, or specimens of a certain chemistry and composition that are more amenable to the selected protocol. This review defines this phenomenon as *The Great Microbiome Extraction Anomaly*: a systemic and pervasive distortion of observed microbial community profiles caused by differential nucleic acid extraction efficiencies. Like other bias-inducing phenomena, such as PCR amplification efficiency, this bias can be reduced through optimization but cannot be entirely eliminated. It mirrors the earlier *Great Plate Count Anomaly* by revealing a persistent methodological bias in microbial observation.

The human molecular diagnostic field provides one of the most intensively studied models of microbial nucleic acid extraction optimization and therefore serves as a useful case study for understanding the technical limits of unbiased DNA recovery. Although this review focuses on environmental eDNA and eRNA workflows, these studies provide strong evidence that the challenge of uniformly extracting nucleic acids across nucleic acid states, species, and specimen types is unlikely to be overcome with currently available technologies. Numerous studies, particularly within human molecular diagnostics, have sought to optimize extraction protocols for unbiased microbial DNA recovery (Na et al. 2025; Dalla-Costa et al. 2017; Loonen et al. 2013). Much of this research has focused on replacing traditional blood culture for sepsis diagnosis with molecular tools, which, in theory, offer the sensitivity and specificity required for the rapid and accurate detection of a broad range of pathogenic microorganisms (Peri et al. 2022). However, whole-blood specimens pose a major technical barrier due to the overwhelming abundance of host DNA (Islam Sajib et al. 2024), and the presence of potent PCR inhibitors such as heme and immunoglobulins (Sidstedt et al. 2018), which interfere with downstream molecular workflows. Another significant hurdle for human molecular diagnostics lies in achieving equal DNA recovery, and therefore equivalent diagnostic sensitivity from all potential microbial pathogens (Gosiewski, Flis, et al. 2014; Gosiewski, Szała, et al. 2014; Gosiewski et al. 2017). In response, some researchers have developed highly optimized sepsis diagnostic extraction protocols that systematically refine each step to improve recovery across the various pathogenic microbial species (Dalla-Costa et al. 2017; Loonen et al. 2013; Liborio et al. 2024). While these efforts have contributed important advances, they have focused on a narrow set of clinically relevant species, and no single protocol has demonstrated consistent recovery from all microbial pathogens (Gosiewski et al. 2017; Sinha et al. 2018). Consequently, no molecular diagnostic has demonstrated the diagnostic sensitivity or specificity required to replace traditional blood culture (Moragues-Solanas et al. 2021; Browne et al. 2022; Kumar and Srivastava 2025). In contrast, microbiome studies often aim to recover nucleic acids from potentially thousands of microbial species across a range of variable specimen types. Collectively, these studies from sepsis diagnostics highlight that, due to the vast biological and structural variety of target organisms, it remains technically unfeasible to achieve uniform nucleic acid extraction using a single method.

4 | Analytical Controls as Essential Tools for Reliable Microbiome Data

As no optimization strategy using current technology is likely to fully mitigate the bias of *The Great Microbiome Extraction Anomaly* or other sources of error, microbiome research must incorporate comprehensive analytical controls at every stage of the workflow (Table 2). During specimen collection, culture-independent profiling partially addresses *The Great Plate Count Anomaly* by enabling detection of non-cultivable species; however, taxonomic annotation remains constrained by reference databases that are biased toward cultured and genomically characterized organisms, while DNA-free negative controls detect contamination by exogenous DNA sequences. Because microorganisms are ubiquitous, even DNA-free negative controls are likely to contain trace levels of background contamination (Salter et al. 2014; Eisenhofer et al. 2019; Liang et al. 2018). During nucleic acid purification, extraction positive controls are essential for identifying protocol-dependent distortions caused by *The Great Microbiome Extraction Anomaly*. These positive controls should be absent from the native sample, representative of the study's target organisms, account for all relevant nucleic acid states and specimen compositions and be transparently

reported. Importantly, transparent reporting should emphasize that every protocol introduces some degree of bias. Researchers should not be discouraged from presenting microbiome data when analytical positive controls reveal substantial distortion. Peer review should recognize that such biases are inevitable and evaluate studies on the basis of methodological transparency and rigor. In the molecular analysis stage PCR or sequencing positive controls enable detection of platform- or chemistry-specific biases, and in the bioinformatics stage, standardized pipelines, shared code, and full disclosure of control-derived data minimize algorithmic misclassification and database-related bias. Without this suite of negative and positive analytical controls, microbiome data cannot be meaningfully interpreted, as true biological variation cannot be distinguished from artifacts introduced at any stage of the analysis.

The actual composition of analytical controls will vary according to the strategic goals of each study. Importantly, a distinction should be made between standalone mock community controls processed in a DNA-free matrix and mock community spike-in controls added directly to environmental specimens, as these approaches address different methodological questions. Standalone mock communities assess protocol-dependent extraction efficiency, whereas spike-in controls additionally evaluate specimen-specific effects on microbial recovery. As an illustrative example, consider a metabarcoding survey of the viable microbiome in freshwater eDNA comparing two sites along a river system: one upstream of anthropogenic activity and one downstream of an industrial discharge point (Figure 3A). Water specimens, collected in bleach-treated, DNA-free Scott bottles, would be vacuum-filtered through sterile 0.22 μm mixed cellulose ester membranes. To remove relic DNA, filters could be subjected to PMA treatment applied directly to the membrane surface (on-filter method). DNA would then be isolated using silica-column-based DNA isolation following established protocols (Browne et al. 2024). Whole genome shotgun metagenomic sequencing would subsequently characterize both bacterial and fungal communities present in the viable fraction of the microbiome. In this example, the analytical control set would comprise: (i) DNA-free water (negative control), (ii) DNA-free water with a mock community (positive control without PMA), (iii) DNA-free water with a mock community and PMA treatment (positive control with PMA), and (iv) an environmental specimen with a mock community and PMA treatment (Figure 3). In this example, controls ii and iii represent standalone extraction positive controls, and control iv represents a specimen spike-in positive control.

The concentration of specimen-spiked mock community controls in microbiome metagenomic workflows should be sufficient to ensure reliable detection while remaining low enough to avoid overwhelming the native microbial signal. Analysis of the commercially available ZymoBIOMICS Microbial Community Standard, which includes three easy-to-lyse Gram-negative bacteria (e.g., *Escherichia coli*), five tough-to-lyse Gram-positive bacteria (e.g., *Listeria monocytogenes*), and two tough-to-lyse yeasts (e.g., *Saccharomyces cerevisiae*), found the mock community had no impact on host alpha and beta diversity estimates when its contribution to total sequencing reads remained below 10% (Galla et al. 2023). If the expected sequencing depth of a specimen is unknown, an initial uncontrolled investigation

TABLE 2 | Analytical controls as essential tools for reliable microbiome data.

Bias	Primary affected stage	Core mechanism	Typical signature	Primary mitigation
Great Plate Count Anomaly	Specimen Collection	Most species are adapted to specific environmental niches	Cultivable fraction is far less than the microscopic or sequence diversity	Culture-independent profiling
Contamination	Specimen Collection	Inadvertently adding exogenous DNA	Negative controls show species present	*Negative controls* (DNA-Free controls)
Extraction Efficiency	Nucleic Acid Purification	Differential recovery across DNA states, species biology, and specimen chemistry	DNA isolation protocol influences species abundance	*Extraction Positive Controls* (DNA state, species and specimen appropriate controls)
PCR and Sequencing bias	Molecular Analysis	The PCR or platform-specific physics or chemistry favors specific sequence contents	PCR conditions or sequencing platforms influence species relative abundance	*PCR or Sequencing Positive Controls*; Standardized library preparation
Bioinformatic bias	Molecular Analysis	Algorithm or reference databases misclassification	Analysis pipelines influence species relative abundance	Standardize pipelines; share code and raw data including controls

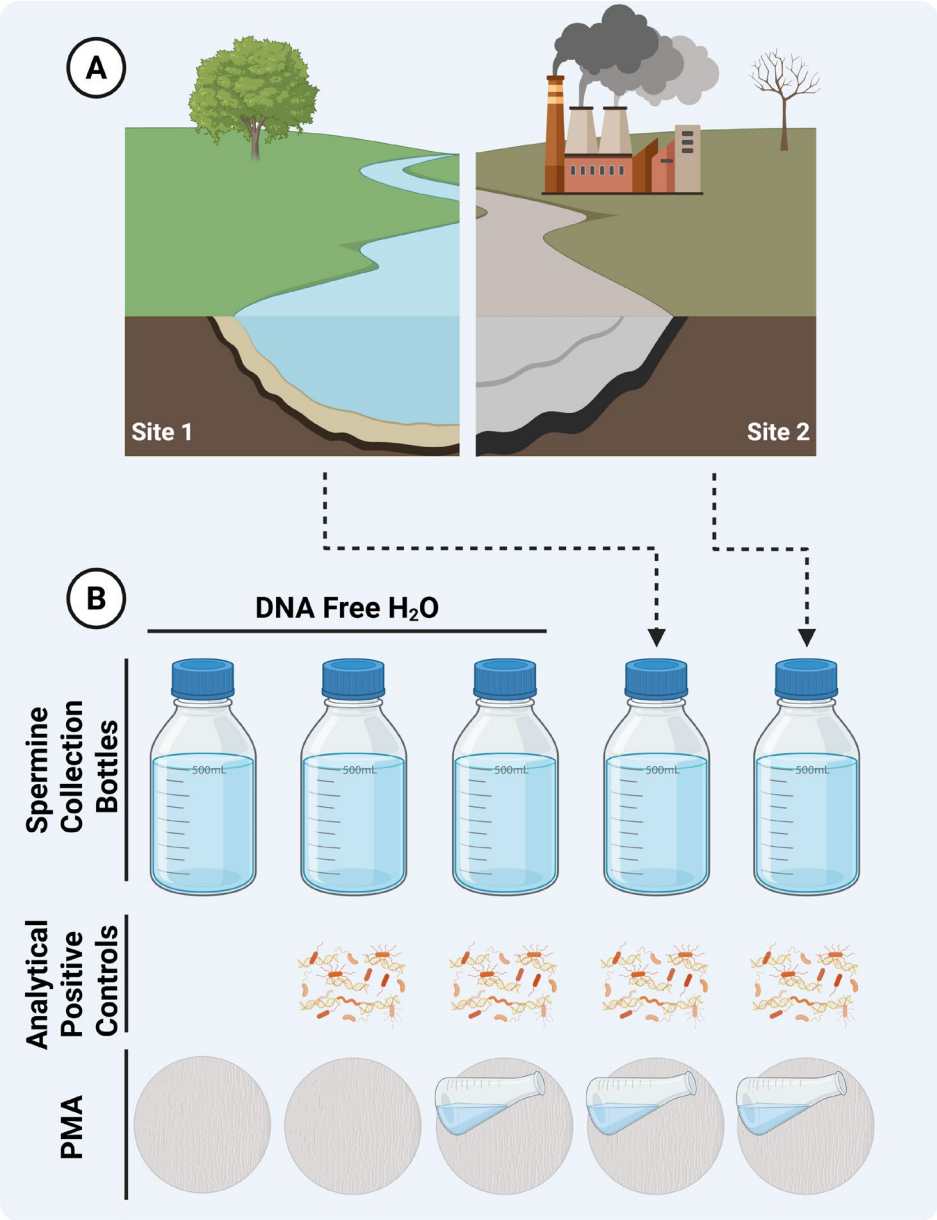

FIGURE 3 | Illustrative experimental design demonstrating the analytical controls required for a metabarcoding survey of the viable microbiome in freshwater. (A) Schematic of specimen collection sites, including a relatively pristine upstream environment (Site 1) and a downstream site impacted by industrial activity (Site 2). (B) Workflow for analytical controls, with specimens collected in 500 mL Scott bottles (Specimen Collection Bottles) with a mock microbial community inclusive of microbes and gDNA (Analytical Positive Controls), then filtered through 0.22 μm mixed cellulose ester membranes (circle filters). Pre-filtration propidium monoazide (PMA) treatment is represented in the figure as an Erlenmeyer flask over the circle filters. DNA-free water collected in sterile 500 mL bottles is used to establish the following control set: (i) DNA-free water (negative control), (ii) DNA-free water with a mock community (positive control without PMA) and (iii) DNA-free water with a mock community and PMA treatment (positive control with PMA). Environmental specimens from each site are spiked with a mock community and subjected to PMA treatment.

should be performed. Commercial mock microbial community standards are limited in scope by fixed species lists and may not be appropriate for all microbiome studies. Therefore, the presence or absence of their constituent species should be verified by PCR prior to use. In the illustrative study above, the mock community would ideally include several Gram-positive and Gram-negative bacterial species, together with a fungal mock microbiome (Table 3). Species should be incorporated both cell-based mock communities (colony-forming units; CFU/500 mL) derived from cultured isolates and DNA-based mock communities (purified genomic DNA spikes; gDNA copies/500 mL), comprising different species in each format to permit inclusion within a single composite spike-in control, thereby enabling quantification of both cell-associated and free-DNA recovery, and the efficiency of PMA treatment.

The inclusion of controls is recommended by major human (Proctor et al. 2019; Qin et al. 2010; Sinha et al. 2015) and environmental (Thompson et al. 2017) microbiome consortia as part of their standardized protocols. However, despite the recognized importance of controls, their use remains rarely reported in microbiome research (Sinha et al. 2015; Welsh and Eisenhofer 2024;

Hornung et al. 2019). Based on a Lens.org (Jefferson et al. 2019) keyword search, of the 476,140 primary research publications between 2000 and 2024 which contained a reference to the microbiome, only 67,194 (14.11%) and 49,211 (10.33%) referenced negative and positive controls respectively. This rate of describing microbiome negative and positive controls has remained relatively consistent since 2000 (Figure 1; Right Panel). These figures reflect keyword presence and do not capture all instances where control strategies were used but not explicitly described, or where controls were discussed but not implemented. Nevertheless, it is clear that as microbiome research output grew the relative reporting of controls in both general and environmental microbiome research has remained consistently low. As the microbiome field continues to expand, especially in environmental applications, the routine implementation of well-designed analytical controls will be critical to improving data reliability, reproducibility, and interpretability.

5 | Overcoming the Bias of The Great Microbiome Extraction Anomaly

Current research to overcome the bias of *The Great Microbiome Extraction Anomaly* is largely focused on improving nucleic

TABLE 3 | Candidate analytical positive control species for a freshwater mock community or spike-in controls.

Species	ATCC number	Natural habitat	Growth strategy in a standard[a] PC2 Lab	Why absent from freshwater
Gram positive bacteria				
Staphylococcus epidermidis	14,990	Human skin microbiota	Aerobic, 37°C, Tryptic Soy Agar/Broth	Human commensal; not free-living in freshwater
Bacillus subtilis	6051	Soil	Aerobic, 30°C, Standard Nutrient Broth	Terrestrial; survives in water only transiently
Geobacillus stearothermophilus	7953	Geothermal Springs	Aerobic, 55°C, Standard Nutrient Agar/Broth	Thermophilic; cannot survive ambient stream temps
Listeria innocua	33,090	Soil	Aerobic, 37°C, Brain Heart Infusion Agar/Broth	Not adapted to freshwater ecosystems
Micrococcus luteus	4698	Skin, dust, air	Aerobic, 30°C, Tryptic Soy Agar/Broth	Primarily airborne or surface-associated, not aquatic
Gram negative bacteria				
Thermus thermophilus	27,634	Geothermal springs	Aerobic, 70°C, Thermus medium	Thermophilic; can't survive ambient stream temps
Halomonas elongata	33,173	Marine	Aerobic, 30°C, Halomonas Medium	Requires elevated salinity; freshwater lethal
Vibrio natriegens	14,048	Marine	Aerobic, 26°C, Nutrient Agar with 1.5% added NaCl	Requires elevated salinity; freshwater lethal
Pseudoalteromonas haloplanktis	14,393	Marine	Aerobic, 26°C, Marine Medium 2216	Requires elevated salinity; freshwater lethal
Aliivibrio fischeri	7744	Marine	Aerobic, 26°C, Photobacterium Broth	Requires elevated salinity; freshwater lethal
Fungi				
Saccharomyces cerevisiae	9763	Baker's yeast	Aerobic, 30°C, YPD Agar/Broth	Domesticated yeast; non-aquatic
Schizosaccharomyces pombe	24,843	Fermented beverages	Aerobic, 30°C, YPD Agar/Broth	Found in fermentations; not a natural freshwater species
Aspergillus niger	6275	Soil	Aerobic, 24°C–26°C, Potato Dextrose Agar/Broth	Primarily terrestrial; survives poorly in freshwater
Neurospora crassa	10,815	Soil	Aerobic, 24°C–26°C, Potato Dextrose Agar/Broth	Terrestrial; requires plant debris, not aquatic
Candida utilis	9226	Industrial	Aerobic, 24°C, YM Agar/Broth	Domesticated, industrial strain; not freshwater-associated

Abbreviations: ATCC, American Type Culture Collection; PC2, Physical Containment Level 2; YM, yeast and mold; YPD, yeast extract-peptone-dextrose.
[a]Standard: without specialist microbiological containment and growing equipment.
Source: www.atcc.org.

acid extraction efficiency through incremental methodological refinements. These include strategies such as positive-selection of microbes from specimens using magnetic nanoparticles (Cai et al. 2023; Xu et al. 2025; Quintana-Sánchez et al. 2022), high-efficiency colloid flocculation (Pinaev et al. 2022) or filtration (Turner et al. 2014), blocking DNA adsorption to particles (Lever et al. 2015; Sakai 2021), various combinations of enzymatic and mechanical lysis (Govil et al. 2019), or DNA stabilization (Browne et al. 2024; Matange et al. 2021). While it remains possible that synergistic combinations of these approaches could achieve substantially improved recovery efficiencies, developing a protocol with truly microbiome-wide, near-uniform extraction efficiency will likely require novel technological innovation. In principle, extraction-associated bias may be circumvented by employing direct non-nucleic-acid-based detection platforms that identify intact microorganisms such as colorimetric sensors (Yang et al. 2023), bacteriophage and antibody-based immunodetection (Roth et al. 2021), or laser-based fingerprinting spectroscopy (Su et al. 2024). However, these PCR- and nucleic-acid-free technologies each introduce distinct biases and are particularly limited for eDNA microbiome analysis where the composition of species is generally highly heterogeneous and largely unknown. Bioinformatic mitigation of bias, wherein measured abundance is corrected to actual abundance as the product of the protocol-dependent detection efficiency, is another strategy which has received considerable attention (Nearing et al. 2021; Przymus et al. 2024). These approaches assume detection bias is systematic and reproducible within a defined protocol and specimen (McLaren et al. 2019), allowing computational correction, particularly when supported by appropriate spike-in controls (Brooks et al. 2015; Rauer et al. 2025). However, substantial uncertainty remains regarding how species-specific biology interacts with specimen composition, nucleic acid states, and downstream amplification or sequencing biases. Future research providing careful curation of reference databases containing such information could enhance the interpretation of these models. Regardless of the technological advances, internal positive analytical controls will remain essential to directly quantify recovery efficiency and transparently report residual bias.

6 | Conclusions

This review defines *The Great Microbiome Extraction Anomaly* as an under-recognized source of bias in microbiome research resulting from variable species-specific nucleic acid extraction efficiencies favoring the recovery of nucleic acids from specific nucleic acid states, microbes with a certain cell biology, or specimens of a certain chemistry and composition. Drawing primarily on evidence from human molecular diagnostic studies, where substantial efforts have been made to optimize extraction protocols, this review has demonstrated that no existing method achieves complete and uniform recovery of nucleic acids across all pathogenic microbial species. Given this limitation, it is unlikely that current technologies can produce an unbiased representation of environmental microbial communities from a complex specimen. While *The Great Microbiome Extraction Anomaly* focuses on biases introduced during nucleic acid extraction, each stage of the microbiome workflow including specimen collection and molecular analysis can contribute to systemic bias. Therefore, bias from *The Great Microbiome Extraction Anomaly* is likely to often be convoluted with further biases arising from specimen collection and molecular analysis and must be appropriately controlled. Based on keyword trends in the published literature, the adoption of negative and positive controls remains minimal across microbiome studies. Broader and more consistent implementation of well-designed analytical positive controls in eDNA and eRNA workflows will be critical to ensuring the continued advancement and credibility of environmental microbiome research.

7 | Methods

Publication data were retrieved from The Lens.org platform consistent with the metadata framework as previously described (Jefferson et al. 2019), using the Scholarly Works search interface for the period from January 1, 2000, to December 31, 2024. Four search queries were applied: (i) “microbiome” OR “microbiota” (476,140 publications); (ii) (“microbiome” OR “microbiota”) AND “human” (336,517 publications); (iii) (“microbiome” OR “microbiota”) AND (“negative control” OR “blank control” OR “no template control*” OR “no-template control*” OR “no-template control*” OR NTC OR “N T C” OR NC)** (67,194 publications); and (iv) (“microbiome” OR “microbiota”) AND (“positive control” OR “mock community” OR “mock sample” OR “spike-in*” OR “reference standard*”)** (49,211 publications). Searches were refined to original research articles only. Publication counts were grouped by year and visualized using GraphPad Prism version 10.4.1.

Author Contributions

D.J.B. conceived, researched, and wrote the manuscript.

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Conflicts of Interest

The author declares no conflicts of interest.

Data Availability Statement

All datasets for this study are presented in this publication.

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