Environmental Science
Water Research & Technology

Accepted Manuscript

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View Article Online
DOI: 10.1039/D5EW01049G

Public health practitioners across the globe are increasingly using wastewater-based epidemiology (WBE) to track infectious diseases. We conducted a systematic review on the use of both endogenous and exogenous markers for data normalization. Standardized approaches are lacking, and normalization goals are often unspecified. We emphasize the need for better communication normalization goals.

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DOI: 10.1039/D5EW01049G

Assessing Normalization Methods in Wastewater Based Epidemiology: A Systematic Review

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DOI: 10.1039/D5EW01049G

WBE Normalization Review v2

1. Abstract

Wastewater-based epidemiology (WBE) has emerged as a powerful tool for monitoring community level infectious disease trends. However, there is a lack of consistent normalization and control practices across studies that limits data interpretability and comparability. In this review, we analyzed 247 articles to assess current normalization approaches, focusing on the use of biomarkers and their associated normalization goals. We identified substantial variability in marker selection, application, and reporting, often without clear explanation. Endogenous markers such as pepper mild mottle virus (PMMoV) and CrAssphage were frequently used, primarily to adjust fecal strength, while exogenous controls such as bovine coronavirus (BCoV) and MS2 bacteriophage were commonly applied to evaluate sample recovery efficiency. However, there is no clear distinction between these functions in the literature. Additionally, normalization goals were often unspecified; when reported, they generally were for adjusting flow variability, correlating with clinical data, and accounting for population size. Mapping control types to normalization goals revealed a diverse range of applications but highlighted a lack of standardization. We emphasize the importance of standardized reporting guidelines, including clear documentation of normalization strategies and quality assurance measures, to improve reproducibility, facilitate meta-analyses, and strengthen the role of WBE in public health surveillance.

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2. Introduction

Wastewater-based epidemiology (WBE) has emerged as a valuable tool for public health, enabling the detection of various pathogens, pharmaceuticals, and biomarkers in wastewater. WBE provides early warning signals of disease outbreaks and helps monitor the spread of infections at a population level. For this reason, it has gained significant interest, especially during the COVID-19 pandemic.[1, 2] Beyond SARS-CoV-2, WBE has also been successfully applied to track enteric viruses[3], respiratory pathogens such as influenza virus and respiratory syncytial virus (RSV)[4], antimicrobial resistance genes[5], and illicit drug consumption.[6] Despite its growing use, one of the major challenges in WBE is the accurate quantification and interpretation of target pathogens, particularly due to variations in wastewater composition, environmental conditions, population dynamics, and laboratory and method variability.

Wastewater is inherently heterogeneous and influenced by factors such as dilution from rainfall or snowmelt, fluctuations in wastewater flow, and temporal variations in human activity.[7] If not properly adjusted, these variations can obscure trends in pathogen concentrations over time at a single location and also limit the comparability of results across studies. Consequently, both normalization and process control strategies are essential for improving the reliability and interpretability of WBE data.[8] A variety of approaches are used across studies, including endogenous controls, exogenous controls, and chemical markers. Endogenous controls are naturally occurring viral or bacterial markers in human feces, such as pepper mild mottle virus (PMMoV) and CrAssphage, which help account for human fecal strength.[9] Fecal strength refers to the relative concentration of human feces in a wastewater sample. Exogenous controls

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involve artificially spiked viral surrogates (e.g., MS2 bacteriophage, murine norovirus, or Phi6) that can serve to assess viral recovery efficiency in WBE workflows.[10, 11] Chemical markers include wastewater constituents such as ammonia, biological oxygen demand (BOD), and total nitrogen, which serve as proxies for human fecal strength.[12] Our review included studies that reported the use of these markers regardless of whether they were explicitly used for data normalization, highlighting the need for more consistent terminology and reporting across the literature.

There is an important conceptual distinction that is often overlooked in the literature: when to use endogenous markers or exogenous markers. Endogenous markers, such as PMMoV, are appropriate for normalizing differences in human fecal strength. Endogenous markers can potentially also serve as indirect indicators of recovery following robust validation studies comparing the endogenous control with the target of interest. In contrast, exogenous markers spiked at known concentrations are primarily intended to evaluate sample processing and analytical recovery. Exogenous controls are particularly important when comparing different methods or assessing processes at different labs. Conflating these functions can lead to misinterpretation of data or increased noise in the data, particularly when normalization goals are not clearly defined.

This review is motivated by discussions from a workshop on WBE normalization hosted by a National Science Foundation-funded Research Coordination Network, where researchers identified the need for a structured comparison of existing normalization strategies. While these normalization methods offer promising solutions for addressing variability in WBE data, differences in methodology make it difficult to compare results

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across studies, regions, and surveillance networks. The lack of standardized normalization approaches limits the ability to draw robust conclusions from WBE data, underscoring the need for a systematic evaluation of existing strategies. By systematically assessing normalization methods used in WBE, this review serves to provide insights into their effectiveness and limitations.

3. Materials and Methods

3.1. Scope of Review

The goal of this review is to describe the normalization strategies being carried out to control for inherent variability in the wastewater sampling, concentration, and analysis steps so that the underlying pathogen behavior in the community is more accurate. Studies were included if they were written in English, monitored a human infectious disease by way of wastewater analysis, and sought to quantitatively assess that infectious disease. Papers were excluded if they did not contain primary research, were conference papers, preprints, or had no available abstract or text online. Additional exclusion criteria included research focused solely on: 1) modeling papers, 2) method development/validation/comparison, 3) presence/absence detection, 4) sequencing and/or variant detection, 5) testing in non-wastewater matrices, 6) non-infectious targets, 7) population-level biomarkers that are not human infectious diseases, and 8) papers without primary data collection (i.e., reviews). This review includes literature published through the end of 2023. While additional studies have been published since, the themes, key trends, challenges, and practices in normalization remain applicable to date.

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3.2. Identification of Articles

On October 16, 2023, PubMed and Web of Science were searched using the terms listed in the SI Section. On both Web of Science and PubMed, results were filtered for English language studies only (Figure 1). There were no additional filters or limits applied to the search strategies. All additional article removal was carried out after downloading the citations. There were 1,383 results from PubMed, and 1,814 results from Web of Science for 3,197 total articles. Duplicates were removed using the Endnote "find duplicates" tool (*n* removed = 709), the Zotero "show duplicates" tool (*n* removed = 501), and manual detection by author Sarah E. Philo (SEP, *n* removed = 11). An initial title screening was carried out by SEP, resulting in the removal of 823 articles at this step. The remaining 1,153 articles were screened by SEP using their abstracts only. At this point, abstracts were screened using the inclusion and exclusion criteria listed in Section 3.1. There were 883 articles removed during abstract screening for a final full text review of 270 articles (Table S1). During full text screening, an additional 23 papers were removed that did not meet the inclusion and exclusion criteria. Although it is typical for two or more reviewers to carry out initial inclusion criteria, the authors are confident that papers were more likely to be included than excluded due to the broad inclusion criteria. Additionally, when there was uncertainty about inclusion during initial screening, those papers were maintained in the database for final data collection by multiple individuals.

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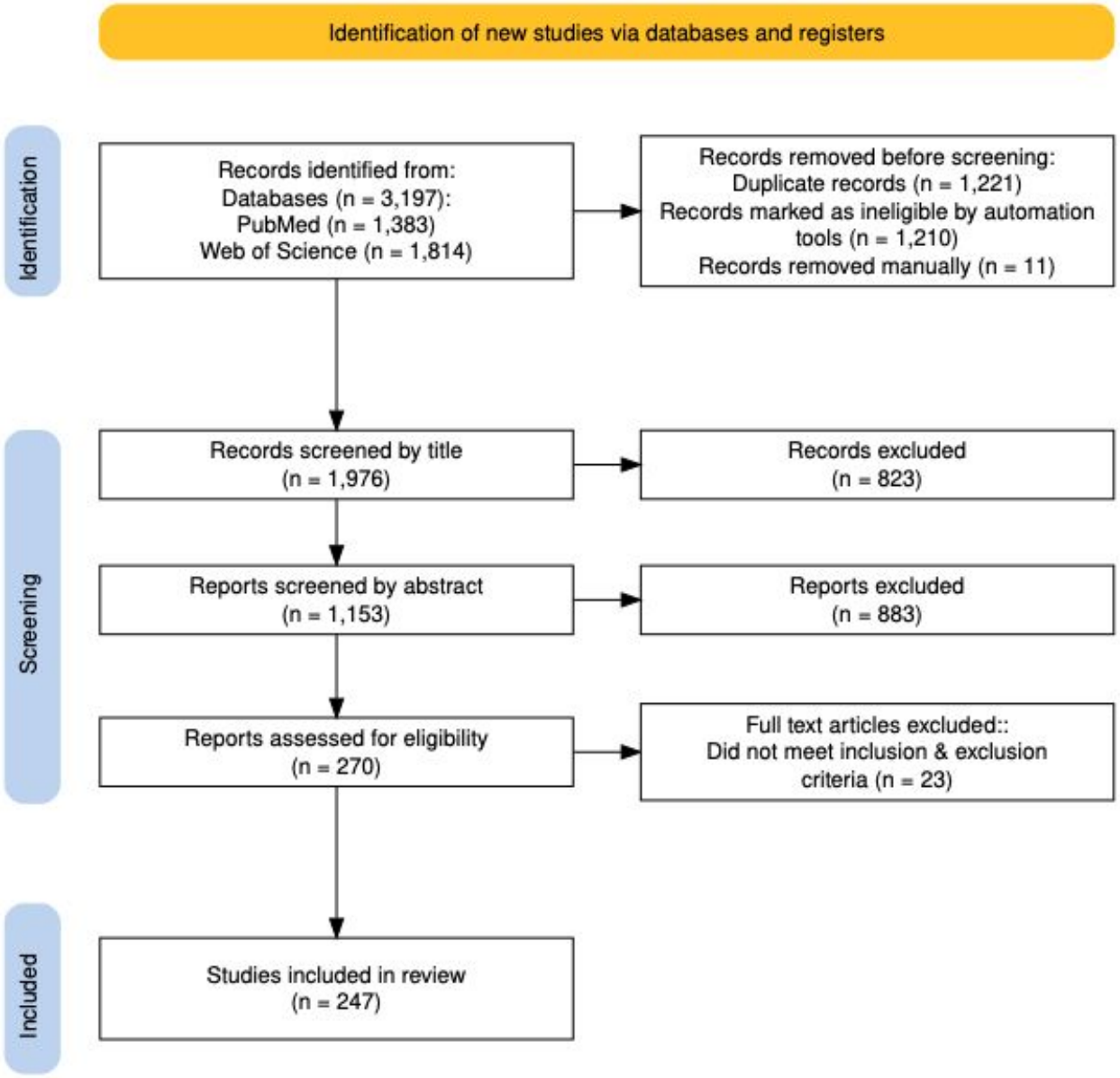

Figure 1. PRISMA 2020 flow diagram[13] showing the identification, screening, and inclusion process for articles.

3.3. Data Collection and Analysis

Authors SEP and Tahmina Ahmed (TA) read through the full text reports to collect information on the samples collected, laboratory methods used, and any normalization calculations carried out (Table 1). If certain criteria were not included in the main text, any supplemental information was read for additional information. If variables collected in this

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review such as methods, controls, etc. were not listed in the primary paper but cited additional published research, those were not consulted to highlight best practices for reporting methods in an article. SEP and TA independently coded information on the exogenous and endogenous controls used and the normalization goal, and discrepancies were resolved by SEP and TA to reach an agreement. All data were maintained in Microsoft Excel (Microsoft Corp., Redmond, WA, USA). Data were analyzed and plots generated using RStudio (R v4.3.3, RStudio v2023.12.1+402) and associated packages. [14] Data were imported using readr, [15] and manipulated using tidyverse and dplyr. [16] Plots were generated using ggplot2, [17] ggpubr, [18] pals, [19] and ggalluvial. [20]

Table 1. Definitions of variables used to systematically collect information from each manuscript included in the structured review.

Variable	Definition
Sampling location	The type of place where the sample included in the study was collected
Sample type	Method of how the sample was collected
Concentration method	Method used to concentrate the collected sample into a smaller volume or mass
Target of interest	The microbial organism that is the main target of the WBE program
Endogenous control	A control originating from or contained within a sample at the time of collection (NIST Joint Task Group Working Group)
Exogenous control	A control not originally present within a sample at the time of collection but added to the sample at some point during a workflow (NIST Joint Task Group Working Group)
Normalization goal	The stated goal provided by the authors for why they normalized their data to

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	account for unmeasurable and/or unknowable variability, i.e. what they are trying to control for. Information on data adjustments made to account for knowable variability (i.e. “adjusting for equivalent sample volume” were not included. There is overlap in some of the stated categories pulled during the review. We were not trying to infer meaning and reported the goals as stated.

4. Results

4.1. Normalization Controls Used in Wastewater-Based Epidemiology

A large majority of the included manuscripts were analyzing wastewater for SARS-CoV-2 (n = 218), and all studies but five were focusing on human viruses. Those five focused on *Candida auris* (n = 1), *Mycobacterium* spp. (n = 1), and antibiotic resistance genes (n = 3). Figure 2 summarizes the use of control types for the normalization process across the reviewed articles. The majority of the studies utilized endogenous controls, particularly PMMoV (*n* = 57) and CrAssphage (*n* = 14), which are frequently cited due to their high prevalence in human feces and relatively stable presence in wastewater. However, there are limitations to their reliability across diverse populations, particularly for PMMoV; measured PMMoV concentrations in wastewater have been found to vary with median income and ethnic background of the captured population, [21] potentially due to differences in dietary preferences. These limitations should be considered when interpreting their use as “stable” normalization markers. A few studies used human-associated markers such as human 18S ribosomal RNA (18S rRNA) and HF183. Other

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endogenous controls used were *Escherichia coli* (*E.coli*), F+ bacteriophage, *Enterococcus* and human polyomavirus. Exogenous controls, such as Bovine Coronavirus (BCoV) (*n* = 36) and other animal viruses non-coronavirus (*n* = 21) were also employed in a substantial number of studies (Table S2). MS2 (*n* = 16) were also used by a few studies primarily to assess viral recovery efficiency. Other exogenous markers included Phi6 bacteriophage (*n* = 10), bovine respiratory syncytial virus (RSV) (*n* = 10), human coronavirus (*n* = 9) and other human non-coronavirus (*n* = 3). Chemical markers (e.g., ammonium and 5-HIAA) were used less frequently and often in combination with biological markers.

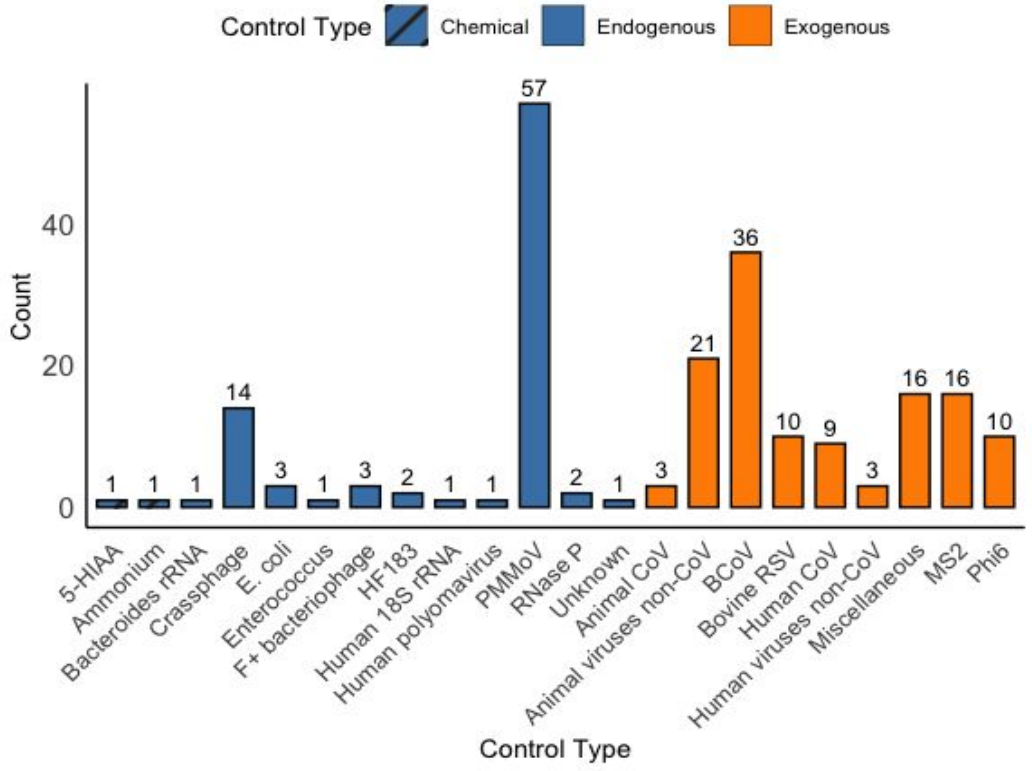

Figure 2. Control types used for normalization in reviewed articles.

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4.2. Normalization Goals

Figure 3 shows that most studies did not clearly state a normalization goal (None/Unknown, *n* = 146). Among those that did, the top goals were to correct for variable flow rate (*n* = 34), correlate with clinical data (*n* = 25), and account for population size variability (*n* = 23). Fewer studies focused on geographic comparability (*n* = 12), variable fecal strength (*n* = 14) and normalization validation (*n* = 12).

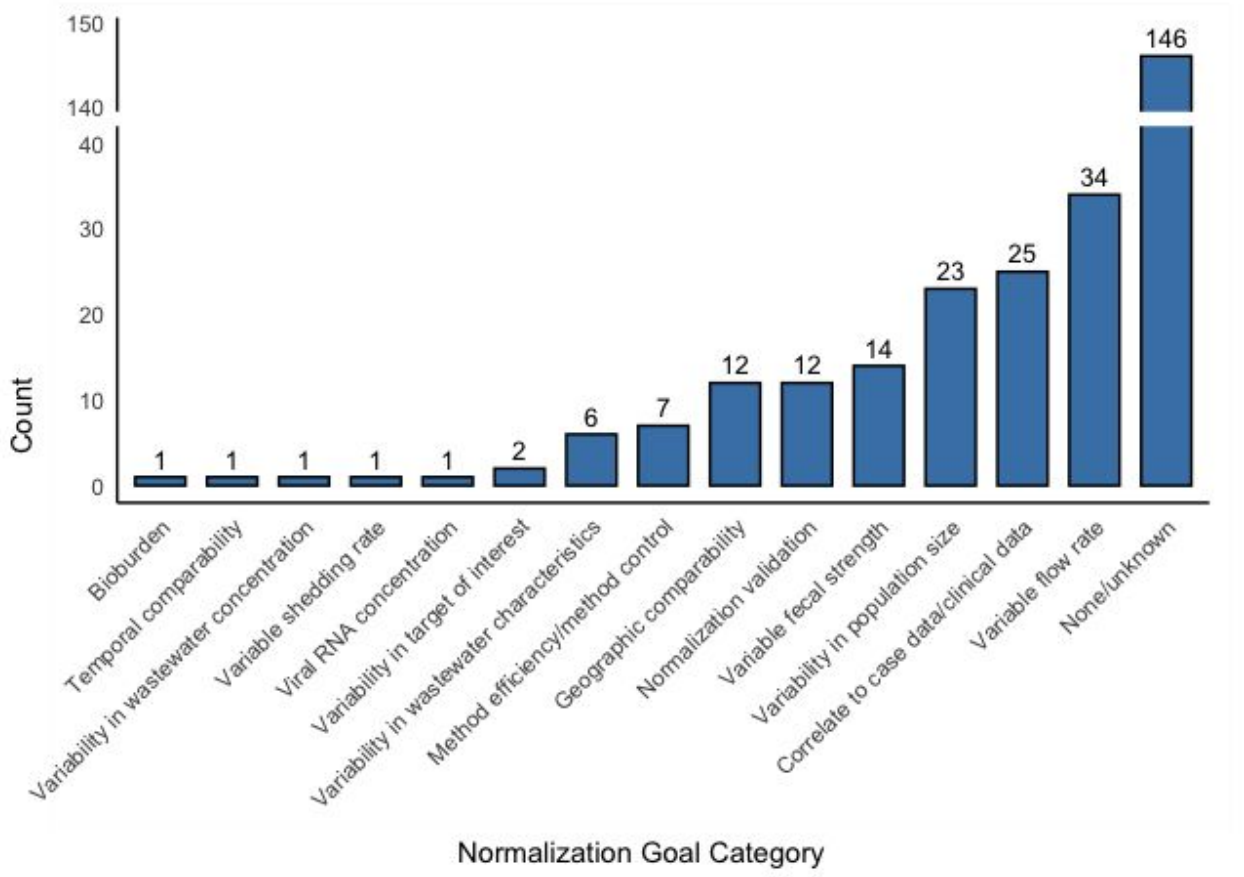

Figure 3. Categories of normalization goals in reviewed articles.

4.3. Mapping Goals to Control Types

The Sankey diagram (Figure 4) visualizes the frequency with which different control types such as chemical, endogenous, and exogenous, were used in relation to various normalization goals. Endogenous controls were most commonly applied,

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especially for goals such as correlating to case data, estimating catchment population, accounting for fecal strength, and validating methods. Exogenous controls were frequently associated with assessing flow rate, method validation, and accounting for variable population size. Chemical markers were used to estimate fecal strength or to account for variable population size, although they were the least frequently employed. The diversity in control use reflects the multifaceted objectives of normalization in WBE studies. However, studies that utilized multiple controls frequently did not clarify what each control was being used to normalize for, emphasizing the need for clear reporting of normalization methods.

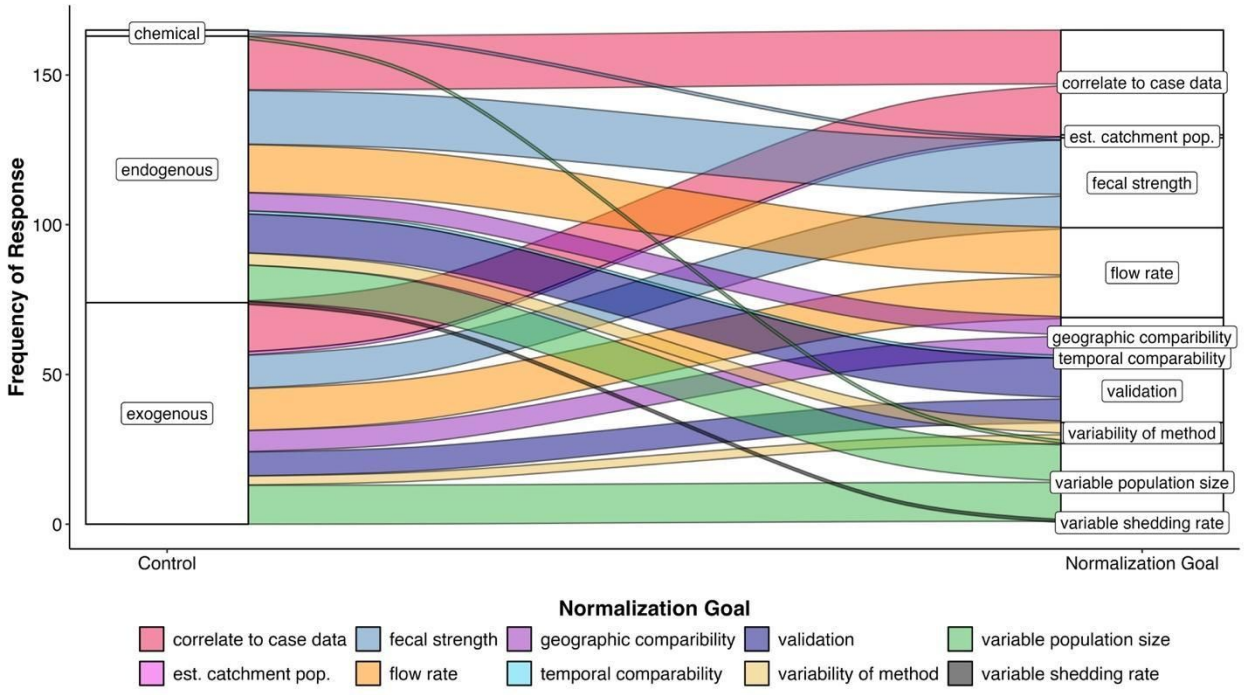

Figure 4. Relationship between control types and normalization goals.

5. Discussion

Normalization in WBE refers to the process of adjusting data on pathogen levels to account for factors that can influence the measured signal, such as fecal load, flow

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rate, or population size, in order to improve interpretability and comparability across time and geographic locations. However, our review highlights substantial variability in how normalization is approached across WBE studies despite the majority of included studies assessing SARS-CoV-2 in wastewater. While normalization can help improve data interpretability and comparability, many studies in this systematic review either did not normalize their data (n = 146) or failed to report normalized results, particularly when normalization did not align with clinical case trends.[10, 22]

WBE data is subject to variability and uncertainty at every stage of the surveillance pipeline. From the moment a pathogen enters the sewage system, challenges such as heterogeneous and inconsistent shedding behavior introduce significant uncertainties.[2, 23] As pathogens travel through sewer networks, their concentration can change due to factors like decay, dilution, and varying infrastructure conditions, which complicates spatial and temporal comparisons.[1, 24] In addition, methodological decisions during sample collection and storage methods (i.e., composite vs. grab sampling, temperature control), along with concentration techniques, may further affect downstream results. To account for methodological variability, spiked exogenous controls are commonly used to assess sample processing and recovery efficiency (Figure 4). However, it is important to distinguish these controls from endogenous markers, which serve a different purpose in addressing population- or fecal-load–related variability.

There is a conceptual distinction that is often overlooked: normalization is a data adjustment strategy, while process controls are quality assurance measures intended to assess sample recovery or method performance. For example, dividing pathogen concentrations by PMMoV or CrAssphage accounts for fecal strength, whereas spiking

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samples with exogenous viruses like BCoV or MS2 evaluates recovery efficiency.[25] Not all studies in our review explicitly stated whether the markers were used for signal correction or methodological validation (Figure 4), and the lack of clarity impairs cross-study comparability and reproducibility. Although these may appear as straightforward points, their inconsistent application across the field underscores the need for systematic evaluation and guidance. Additionally, because the WBE field has grown rapidly since the start of the COVID-19 pandemic, there are no agreed upon standards for normalization.

The majority of the studies utilized endogenous controls such as PMMoV or CrAssphage, presumably as fecal strength or wastewater characteristics, though few explicitly stated this rationale (Figure 4).[26, 27] Similarly, exogenous markers such as BCoV or murine hepatitis virus were commonly spiked into samples, generally to assess methodological performance or recovery efficiency; however, these roles were not always clearly described in the literature.[25] This need for clarity has been emphasized previously, which recommended that normalization markers and process controls be reported when collected and clearly presented to support accurate data interpretation.[28] However, our review suggests that although many studies now report these data, they often fail to explain why or how such markers are used, with more than half of papers not describing a normalization goal for the chosen controls (Figure 3), leaving important gaps in methodological transparency and hindering reproducibility. This highlights the need for field specific reporting standards that mirror existing frameworks such as the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE), digital MIQE (dMIQE), and Environmental Microbiology Minimum Information (EMMI) guidelines, which emphasize clarity in experimental design and the use of internal and

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external controls in various PCR workflows.[29] To improve best practices in WBE, the researchers should explicitly report the intended use of each normalization marker and align their interpretation of results accordingly. Additionally, the equations used to normalize and adjust pathogen data should be clearly stated.

Importantly, each normalization method has context-specific limitations. For example, PMMoV concentrations can vary geographically and seasonally due to differences in human diet, such as pepper consumption, which may reduce its reliability as a consistent normalization marker across diverse populations.[26, 30] CrAssphage abundance is influenced by individual gut microbiota composition and may be less prevalent in children or non-western populations, limiting its global applicability.[31, 32] These limitations must be acknowledged when selecting normalization strategies, if any are used at all, particularly for multi-site or global surveillance efforts. Additionally, given that the bulk of the included studies focused on SARS-CoV-2 and human virus normalization efforts, more work must be carried out to understand normalization behavior with bacterial and non-viral targets. Further, the use of endogenous controls must undergo sufficient validation for the specific target of interest and sampling location. Although normalization is a critical tool to account for unwanted variability, it may not fully correct for all sources of noise in wastewater surveillance data, such as diurnal flow fluctuations, stormwater intrusion, or differences in sewer sheds.[26, 31, 33] Recognizing these constraints is essential, particularly in multi-site or global surveillance efforts.

There is a pressing need for more transparent reporting of normalization objectives in WBE studies in order to facilitate comparisons between studies, geography, and time. First, a working group should be established to prescribe normalization best practices.

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The variability of chosen controls for SARS-CoV-2 WBE in this review suggests guidance is needed. Inherently, the normalization practices will be different based on the category of target of interest. Given the focus on viral targets here, more experimental work is needed to characterize normalization of bacterial targets. In the absence of a normalization best-practices document, we recommend that researchers use two controls: 1) an exogenous control spiked-in to the sample at a known concentration to assess method performance and 2) an endogenous control to quantify matrix performance over time. The justification for choosing these controls must be described in the methods and must be clearly established through either prior in-lab validation work or extensive existing research.

Researchers should clearly define whether normalization is used to adjust for fecal content, to correct for recovery efficiency or both, and indicate how these decisions influence data interpretation. Several groups have emphasized the need for standardized and minimum reporting criteria to enhance the reproducibility and comparability of WBE findings across studies.[28, 34] Both endogenous and exogenous controls were used to adjust for in-sewer effects and differences in populations (Figure 4). Generally, normalizing a target of interest to the chosen exogenous control should not be carried out without extensive validation work showing similar behavior in the chosen sampling, concentration, and detection methods. However, the exogenous control can be used to understand method performance. Normalizing the target of interest to the chosen endogenous control should only be carried out if that endogenous control has been well studied. WBE normalization efforts for SARS-CoV-2 benefitted from the reduced population mobility and well described clinical data during the pandemic. Given that future

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WBE efforts for different targets of interest are unlikely to have well described clinical data and populations like early SARS-CoV-2 efforts, normalizing or adjusting wastewater data in order to improve correlations with clinical cases is improper because it assumes perfect or near-perfect clinical data. However, normalizing to improve correlations with clinical data can be carried out if there is a well described population for whom infections are fully captured. This can be used to inform normalization efforts for similar populations. While this review does not suggest a single universal normalization strategy, existing normalization work can be enhanced by improved reporting and hypothesis driven justifications.

6. Acknowledgments

The graphical abstract was Created in BioRender. Philo, S. (2025) https://BioRender.com/6m3x4h7. The virtual workshop that prompted this work was supported by the National Science Foundation, grant 2202361. We would like to thank the 89 participants at the workshop representing academic researchers, government employees, and members of industry, whose valuable discussion inspired this systematic review.

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The data supporting this article have been included as part of the Supplementary Information.