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Pathogen Removal Credits for Wastewater Treatment: Guidance for Study Plans and Reporting

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Pathogen Removal Credits for Wastewater Treatment: Guidance for Study Plans and Reporting

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Abstract and Benefits

Abstract:
The objective of this project was to provide guidance on the development and implementation of study plans to validate pathogen $\log_{10}$ reduction values (LRVs) to assign credits for secondary and tertiary wastewater treatment processes in water reuse systems. Using systematic literature review, consultation with industry experts, meta-analysis, and in-silico analysis, the following findings and recommendations were made: 12 to 24 wastewater samples collected from at least one influent and one effluent location for at least one year generally provides enough certainty to demonstrate LRV credits with a precision of 0.1 or 0.5 $\log_{10}$ units. In addition to pathogen concentrations, studies should report concentrations of surrogates, performance monitoring parameters, and design or operational factors that influence pathogen LRVs. Pathogen concentrations generally follow a left-skewed distribution and pathogen LRVs generally follow a symmetrical, bell-shaped distribution. The calculation of LRV statistics should account for correlation between influent and effluent concentrations, and appropriate statistical methods should be used when there are non-detects in the data set.

Benefits:

- Provides a justification for sample size and LRV credit precision
- Cost data presented helps subscribers perform cost/benefit analysis of LRV validation studies
- Provides recommendations for appropriate ways to calculate mean and percentiles of the LRV
- Provides recommendations for how to calculate LRVs from data sets with non-detects

Keywords: pathogen removal, LRV credits, wastewater treatment, data analysis, study plan design

Contents

Tables

Figures

Acronyms and Abbreviations

AWWA	American Water Works Association
BOD_5	Biochemical oxygen demand (five day)
BOD-T	Biochemical oxygen demand (total)
CCL	Critical control limit
CCR	California's Code of Regulations
COD	Chemical oxygen demand
CoV	Coefficient of variation, equal to the mean divided by the standard deviation
COVID-19	Coronavirus disease 2019
dPCR	Digital polymerase chain reaction
DPR	Direct potable reuse
DOC	Dissolved organic carbon
FIB	Fecal indicator bacteria
g	Gravitational force (g-force) equivalents
gc	Gene copies
GROS	Gamma regression on order statistics
HAdV	Human adenovirus
HLR	Hydraulic loading rate
HRT	Hydraulic retention time
KM	Kaplan and Meier
LRV	Log_{10} removal value
MBR	Membrane bioreactor
MCMC	Markov Chain Monte Carlo
MLE	Maximum likelihood estimation
MLSS	Mixed liquor suspended solids
MSR	Matrix spike recovery
NTU	Nephelometric turbidity unit
NWRI	National Water Research Institute
OC San	Orange County Sanitation District
OCWD	Orange County Water District
OMP	Operational monitoring parameter
ORV	Operating range value
PAC	Project advisory committee
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMMoV	Pepper mild mottle virus
Q&A	Question and answer

QA/QC	Quality assurance and quality control
QAPP	Quality assurance project plan
QMRA	Quantitative microbial risk assessment
qPCR	Quantitative polymerase chain reaction
RMSE	Root mean square error
ROS	Regression on order statistics
RT-qPCR	Quantitative polymerase chain reaction with reverse transcription
SARS	Severe acute respiratory syndrome
SARS-CoV-2	SARS coronavirus 2, the strain that causes COVID-19
SD	Standard deviation
SRC	Sulphite-reducing clostridia
SVI	Sludge volume index
SWRCB	The California State Water Resources Control Board
TAC	Technical advisory committee
TOC	Total organic carbon
TSS	Total suspended solids
SRT	Solids retention time (same as mean cell residence time)
US EPA	United States Environmental Protection Agency
WEF	The Water Environment Federation
WRF	The Water Research Foundation
WWTP	Wastewater water treatment plant

Executive Summary

The overall goal of this project was to provide guidance on the development and implementation of study plans to validate pathogen $\log_{10}$ reduction values (LRVs) to assign credits for secondary and tertiary wastewater treatment processes in water reuse systems. Specifically, there were three objectives. First, there was a need to provide recommendations for **study plan design**, such as choosing an appropriate sample size, sampling frequency, sampling locations; selecting which microbial groups to analyze; and what other types of data to collect. There was also a need to **standardize laboratory methods** and make specific recommendations about sample collection, preparation, storage, quality assurance and quality control (QA/QC), and processing. Finally, there was a need to standardize **data management and data analysis**—specifically, methods for calculating LRV statistics (e.g., mean, standard deviation, quantiles), including from datasets with "non-detect" results as well as assumptions about data distributions.

A systematic literature review, consultations with experts, meta-analysis, and in-silico analyses (computational simulations) were used to collect qualitative and quantitative data to address the objectives outlined above. The following are the major findings associated with the first research objective, which was to provide recommendations for a study plan design:

- **Sample sizes of 12 to 24 are sufficient to credit LRVs, sometimes with precision of 0.1 $\log_{10}$ units.** The precision of the LRV credit being sought should be proportional to the width of the 95% confidence interval of the statistic used to claim LRV credits (e.g., the 5th percentile). For example, if the width of the 95% confidence interval is less than 0.5 $\log_{10}$ units, then a precision of 0.1 $\log_{10}$ units may be justified (e.g., granting a credit of 0.6 or 0.7 or 0.8 $\log_{10}$ units). However, if the width of the confidence interval is greater, it might not make sense to grant credits below 1.0 $\log_{10}$ units at a precision of 0.1 $\log_{10}$ units. The width of the confidence interval on a 5th percentile is proportional to the mean and standard deviation of the data set as well as to the number of samples. Computer simulations indicated that the additional precision gained from collecting 48 samples instead of 20 or 24 samples may not be worth the additional cost. The use of 12 samples could be sufficient in some cases if the width of the 95% confidence interval is sufficiently small. If data from validation studies is analyzed as it gets collected, the sample size could be considered sufficient once a certain level of precision in the percentile estimate is reached (which may happen before 20 samples are collected). Wastewater processes that might fall into this category include biological treatment systems, especially in regions where the climate, the pathogen loads, and the treatment plant operating conditions do not change drastically throughout the year. The recommended sampling frequency is once or twice monthly, to include all seasons of the year and to capture a wide range of operating conditions. It is important to capture all characteristics of treatment plant operation, including but not limited to variability in source water quality, operating conditions, and factors that influence pathogen removal in all processes being studied.
- **It is important to measure concentrations of pathogen surrogates and LRV "influencing factors.**" Hourly variations in pathogen concentrations in untreated wastewater can span

orders of magnitude. This variability might be a result of the prevalence of shedding within the community and/or the limitations associated with grab samples. For pathogen surrogates and microbial indicators ubiquitously shed by most of the population, hourly variability is much lower. If the reduction of surrogates is comparable to the reduction of pathogens, then the exclusive use of surrogates in future validation studies may be warranted. The collection of information related to design, operational, and environmental factors known to directly influence pathogen reduction should be standardized to compile a large data set that can be used for future meta-analysis and pathogen LRV modeling. This could potentially augment validation studies in the future.

- **Composite sampling may be necessary, especially in raw wastewater without flow equalization.** Pathogen concentrations and LRVs have temporal variability, with seasonal trends being the most noticeable. There is consensus in the literature that composite samples are more representative than grab samples, but there are tradeoffs with cost (e.g., equipment and time) and quality control (e.g., storing a sample for up to 24 hours). Nevertheless, in wastewater treatment systems that lack flow equalization, the use of composite sampling strategies is likely worth the additional investment, especially for raw wastewater samples. Composite samples may also provide more representative estimates of daily average concentrations in small systems for pathogens with low prevalence and high diurnal variability. Grab samples may suffice otherwise.

The major findings associated with the second research objective, to standardize laboratory methods, are summarized below:

- **Studies can cost up to $500,000, but costs could be reduced if approaches are standardized.** Laboratory fees associated with analyzing wastewater samples for coliphages, *E. coli*, *Giardia*, *Cryptosporidium,* and enteric viruses (including the quantification of cultivable viruses and the quantification of viral gene copies [gc] using quantitative polymerase chain reaction [qPCR], or qPCR with reverse transcription [RT-qPCR]) were found to be approximately $3,000 per sample. In addition, the analysis of the cost data revealed that typical non-laboratory costs ranged from approximately $100,000 to $300,000 per study, with approximately 10% of that used for study planning, 25% for sample collection and shipping, 25% for data analysis, 15% for report writing, and 25% for time spent working with regulators and external expert advisory panels. Based on this cost analysis, a pathogen LRV validation study with 24 samples collected at two locations (i.e., one influent and one effluent point) analyzed for the parameters mentioned above would typically cost more than $300,000 and could cost nearly $500,000. The costs of studies could be reduced by ~$50,000 if study planning, data management, and data analysis approaches are standardized.
- **QA/QC tasks and expediting analyses can more than double the cost of LRV validation studies.** Laboratory fees for waterborne pathogen analyses can vary if additional QA/QC measures are requested, if modifications to the method are requested, or if faster turnaround times are requested. The cost can also vary based on the sample matrix. For example, analyzing raw sewage samples for *Giardia* and *Cryptosporidium* can cost more than twice as much as analyzing treated wastewater samples. The inclusion of QA/QC measures such as matrix spikes, method blanks, ongoing precision and recovery controls,

and the analysis of larger effective sample volumes can more than triple the cost of analysis per sample. For virus analysis, adding matrix spikes and other process controls can potentially increase the cost per sample by $250 to $500. Some labs also charge 20% to 50% extra for expedited turnaround times.

- **Lab methods are mostly standardized; several virus concentration methods perform similarly.** Most laboratory methods used for pathogen LRV validation studies are standardized—apart from the methods used to concentrate waterborne viruses (e.g., via qPCR or RT-qPCR). There are dozens of different virus concentration methods described in the literature, each with different recovery efficiencies. Based on a systematic literature review and meta-analysis, the adsorption-extraction, ultrafiltration, ultracentrifugation, polyethylene glycol (PEG) precipitation, and skimmed milk flocculation methods are the most suitable for concentrating viruses from wastewater. The use of Sterivex™ filters, aluminum hydroxide precipitation, and centrifugation at speeds below 100,000 gravitational force equivalents (i.e., 100,000g) should be avoided. The best method for concentrating viruses is different for different viruses, and this should be considered when choosing a proxy virus for matrix spikes.
- **Concentrations should be corrected for recovery if there are large differences in efficiency.** LRVs will be underestimated if the recovery is more efficient for effluent samples than it is for influent samples and overestimated if recovery is more efficient for influent samples than for effluent samples. Given the recommended precision for LRV credits, matrix spike recoveries shall be used to correct the pathogen concentrations only if the difference between the LRV based on recovery-corrected concentrations and the LRV based on uncorrected concentrations is greater than the level of precision used to issue the LRV credit (e.g., 0.1).

Finally, the following are the most important findings associated with the third research objective, which was to standardize the approach used for data management and data analysis:

- **Pathogen concentration distributions are skewed; LRVs have symmetrical bell-shaped distributions.** While many authors of past studies have assumed that pathogen concentrations are lognormally distributed, very few authors have tested this assumption rigorously. The few studies that performed statistical analyses have concluded that pathogen concentration distributions were generally lognormal, or that $\log_{10}$ differences between influent and effluent concentrations had symmetrical bell-shaped distributions. Normality tests should be a standard part of the statistical methods used to evaluate data from pathogen LRV studies.
- **More robust data analysis methods may be needed to calculate statistics of censored datasets.** Pathogen LRV validation studies should be carefully designed to avoid non-detect results. This can be done by using larger sample volumes, processing larger portions of concentrated samples, and analyzing sample replicates. Nevertheless, despite these efforts, non-detect results may still occur from time to time. When datasets are censored (i.e., when some of the samples are “non-detects”), it is important to use appropriate statistical methods to analyze the data. When working with censored datasets, statistical modeling always produces better estimates of parameters than the substitution method. The maximum likelihood estimation (MLE) and regression on order statistics (ROS) methods are

recommended for analyzing censored pathogen concentration datasets when the data follow a log-normal distribution. The non-parametric Kaplan-Meier (KM) method can be used when the distribution is not log-normal.

- **The calculation of LRV percentiles should consider the correlation between concentrations.** Influent and effluent pathogen concentrations often fluctuate up and down with each other throughout the year. The three approaches that have been most commonly used in the literature and in practice either: 1) assume complete independence between samples; 2) incorporate the covariance between samples in the estimation of standard deviations and percentiles; or 3) treat samples as paired. The first two approaches require the assumption that concentration data are lognormally distributed while the third approach is only appropriate for batch reactors or plug flow systems. When calculating LRV statistics from a data set for a wastewater treatment plant with flow-through reactors that have more mixing, the covariance approach is recommended. Bayesian models, which do not assume a distribution for pathogen concentrations or LRVs, can also be used, but the team analyzing the data must be experienced in Bayesian statistics.

Based on the recommendations resulting from this study, web-based guidance materials have been developed to help standardize the process of LRV validation study planning, data management, and data analysis. These online materials are described in this report.

Related WRF Research

- Indicator Viruses for Advanced Physical Treatment Process Performance Confirmation (4955)
- Demonstrating Virus Log Removal Credit for Wastewater Treatment and Reverse Osmosis for Potable Reuse at OCWD (5041)
- Advancing Safety and Reliability to Protect Public Health: Identifying Quantitative Reductions of Viral Pathogens and Surrogates for Water Reuse Applications (5126)

CHAPTER 1

Introduction and Background

1.1 Historical Context of Water Reuse in the United States

The need to include recycled water into the water management portfolio in the United States has been recognized for more than half a century to sustainably alleviate water stress caused by population growth, urbanization, and climate change (Leverenz et al., 2011). Planned potable reuse operations in California started as early as the 1960s and the 1970s, with Los Angeles County Sanitation District's Montebello Forebay project and Orange County Water District's groundwater injection system (US EPA and CDM Smith, 2018). One of the potential risks associated with wastewater reuse is the transmission of diseases caused by bacterial, viral, or protozoan pathogens, which are shed in high numbers in the excreta of infected humans, and therefore can be present in high concentrations in municipal wastewater (Rose and Jiménez-Cisneros, 2019). Many waterborne pathogens have very low infective doses, so the overall reduction for potable reuse systems must be very high, often more than 10 $\log_{10}$ units (Rose et al. 2019).

1.2 Regulating Pathogen Removal in Water Reuse Systems

The indirect reuse of recycled wastewater via groundwater augmentation has been regulated in California since 1978. These regulations went through several improvements over the years. In 2012, the National Water Research Institute (NWRI) commissioned an advisory panel sponsored by the WateReuse Foundation, which led to a report that examined criteria for direct potable reuse (DPR) (NWRI, 2012). In 2014, another revision was made to the California regulations governing the indirect potable reuse of recycled water via groundwater augmentation, which stands to this day. A year later, the WateReuse Foundation in collaboration with NWRI, the American Water Works Association (AWWA), and the Water Environment Federation (WEF) published a Framework for Direct Potable Reuse (NWRI et al., 2015). In 2018, the California State Water Resources Control Board (SWRCB) adopted regulations for indirect potable reuse via surface water augmentation. Regulations for direct potable reuse in California were still forthcoming at the time of this report, with plans to be adopted on or before December 31, 2023 (NWRI, 2022).

California's Code of Regulations (CCR) Chapter 3 of Division 4 from Title 22 (CCR 2023) stipulates how recycled water can be indirectly reused for potable water supply via groundwater replenishment, and as of October 2018, via surface water augmentation. Currently, 22 CCR §60320.108 (CCR 2014a) and §60320.208 (CCR 2014b), which cover indirect potable reuse applications via groundwater replenishment, stipulate the regulation of water resource recovery facilities based on a $\log_{10}$ reduction credit system. The treatment facility must provide 12-$\log_{10}$ enteric virus reduction, 10-$\log_{10}$ *Giardia* cyst reduction, and 10-$\log_{10}$ *Cryptosporidium* oocyst reduction (i.e., the so-called 12-10-10 rule, which was based on a risk goal of 1 infection per 10,000 people per year (Soller et al. 2017)). This regulation requires treatment trains to have at least three processes each credited with at least 1.0-$\log_{10}$ reduction,

and no single unit process can be credited with more than 6-$\log_{10}$ reduction. The most recent addition to California's indirect potable reuse regulations, 22 CCR §60320.308 (CCR 2018), covers indirect potable reuse via surface water augmentation. This regulation requires facilities to provide at least 9-$\log_{10}$ enteric virus reduction, 8-$\log_{10}$ *Giardia* cyst reduction, and 9-$\log_{10}$ *Cryptosporidium* oocyst reduction (i.e., 9-8-9), as long as any 24-hour delivery of recycled municipal wastewater does not constitute more than 10% of the water withdrawn from the augmented reservoir. The regulation also includes a "V/Q" requirement, which impacts LRV requirements. If the withdrawn water contains less than 1% of any 24-hour delivery of recycled water, then the reduction requirements are reduced by an order of magnitude (e.g., 8-7-8 instead of 9-8-9).

The LRV crediting approach is also described in two documents published by the California SWRCB, which proposed frameworks for regulating direct potable reuse (California SWRCB, 2019; 2018). Several studies have been completed to support the development of reuse regulations (Pecson et al. 2021a; 2021b). The direct potable reuse framework for the State of California, which was still under development at the time of this report, stipulated draft criteria suggesting that "the sum of the treatment process validated log reductions for the treatment train shall be at least 20 log for enteric virus, 14 log for Giardia cysts, and 15 log for Cryptosporidium oocysts" (California SWRCB, 2021). However, the expert panel assigned to review the SWRCB's draft direct potable reuse criteria published a memo arguing that these draft criteria "chose the most conservative assumptions" for several variables, but that "layering the most conservative assumptions upon each other results in unrealistic and impracticable processes that offer no additional significant positive effects on public health" (NWRI, 2022). California is not the only state to use the $\log_{10}$ reduction credit system to regulate potable reuse activities—Arizona, Nevada, and Texas state regulations utilize a similar approach.

1.3 LRV Validation Studies

There are generally three types of data collection efforts when it comes to characterizing and/or complying with pathogen removal requirements in secondary and tertiary wastewater treatment processes in a water reuse system: 1) technology validation (e.g., for pathogen LRV crediting); 2) ongoing operational performance monitoring (e.g., for compliance); and 3) research purposes. Technology validation studies for some secondary and tertiary wastewater treatment processes may include the direct measurement of pathogens in water samples but also include measurements of operational monitoring parameters (OMPs) or other constituents that may *indicate* pathogen removal efficiency, or measurements of design, operational, or environmental factors that are known to *influence* pathogen removal efficiency. Not all technology LRV validation studies include the direct measurement of pathogens in water samples; for advanced treatment processes, the direct measurement of pathogens is often not conducted for various reasons, including the cost of analysis and the difficulty in detecting pathogens at the levels needed to demonstrate the desired LRV. Ongoing OMP data collection efforts include measurements of the performance monitoring parameters related to pathogen removal, but would typically not include measurements of pathogens or pathogen surrogates. Projects done for research purposes might include measurements of pathogens, surrogates, performance monitoring parameters, and other parameters, usually with the objective of

better understanding the efficiency of removal, the mechanisms associated with pathogen removal, or the appropriateness of a pathogen surrogate.

For many treatment processes, including second stage filtration, slow sand filters, chlorination, ozonation, microfiltration, ultraviolet processes, and processes that use bag or cartridge filters, performance criteria can be established based on US EPA guidance documents (US EPA, 1991; 1992; 1999a; 1999b; 2003; 2004; 2006a; 2006b; 2006c; 2010; 2012a). However, there are no guidance documents for the provision of pathogen reduction credits for secondary and tertiary wastewater treatment processes. In the past, potable reuse facilities in California have sought the provision of these credits based on historical pathogen datasets, particularly the one published by Rose et al. (2004). Current and proposed regulatory frameworks rely on the ability of reuse projects to demonstrate pathogen LRVs in secondary and tertiary wastewater treatment processes through site-specific LRV validation studies. The regulatory agencies review pathogen LRV validation studies to approve LRV credits requested based on data collected by the reuse project. However, pathogen reduction in a treatment unit process is highly dependent on design, operational, and environmental factors of the treatment technologies used (Rose and Jiménez-Cisneros, 2019). Thus, it is sensible that many regulators require site-specific studies to assess pathogen reduction in secondary and tertiary treatment operations. Nevertheless, standard practices for generating study plans for this type of assessment are lacking. Thus, there is a need to provide specific recommendations for conducting these types of studies, especially on the topics summarized in Table 1-1.

Table 1-1. Knowledge Gaps For Studies of Pathogen Reduction in Wastewater Treatment Facilities.

Topic	Knowledge Gaps
Experimental study plan design	• Sample size determination • Sampling frequency and location • Cost of study plans • Ongoing performance monitoring • Selection of microorganisms (e.g., appropriate and representative pathogens, non-pathogenic microbial process indicators/surrogates, and other process indicators)
Use of standardized laboratory methods	• Sample collection, preparation, and processing • Quantification of pathogens/process indicators • Quality assurance and quality control (QA/QC) • Criteria to define and select capable laboratories
Statistical approaches for data analysis	• Data distributions and transformations • Handling left-censored data (non-detects) • Calculation of the $\log_{10}$ reduction value • Interpretation of temporal variations

Experimental study plans require determination of an appropriate sample size, sampling frequency, sampling locations, as well as selection of the appropriate groups of microorganisms to quantify, and other types of ongoing performance monitoring requirements. While there are well-developed statistical approaches to determine the sample size necessary to obtain a desired level of precision, they often depend on assumptions regarding the distribution of the

data, the independence of samples collected, and the type of statistical analysis that will be performed using the data.

The standardization of laboratory methods for detecting and quantifying pathogens in wastewater and recycled water systems are likewise complicated. First, very high $\log_{10}$ reductions are required to protect public health (e.g., 10 – 12 $\log_{10}$ units in some cases, as illustrated above) because infected individuals excrete pathogens in very high numbers, and because some pathogens have extremely low infectious doses. Therefore, to demonstrate the level of pathogen removal required, the pathogen concentrations that must be quantified in treated water are extremely low, and in many cases, impossible to detect (*i.e.*, would require filtering thousands of liters of water or more). Secondly, there are hundreds of different types of pathogen strains, and it is not practical to measure each one. It is also difficult to identify the best methods to use (Gerba et al., 2018). Very few methods are standardized for pathogen detection. Furthermore, multiple variations of methods are reported in the scientific literature, and they all have different efficiencies. There is a need to provide clear guidelines for sample collection, sample preparation, and sample storage. There is also a need to provide guidance about which methods should be used for pathogen concentration, detection, and quantification. Finally, there is a need to provide clear guidelines about how data should be analyzed. QA/QC procedures must be specified and standardized for pathogen LRV validation studies to ensure that data collection and analyses are conducted in a way that is consistent with the state-of-the-art science. State regulators also need a way to determine which laboratories can complete the analysis with the recommended QA/QC procedures.

Finally, pathogen data analysis is riddled with complications that cause considerable confusion (von Sperling et al., 2020). First, given the limitations described above with laboratory methods and the low concentrations required by regulations to protect public health, it is very common to experience data sets with non-detect results. A non-detect result does not necessarily mean that the water does not contain any pathogens, rather it may indicate that the concentrations are too low to quantify, given the limitations associated with sampling volumes and analytical methods. Another complicating characteristic of pathogen data analysis is that data distributions are often skewed, potentially requiring data transformations to complete statistical analyses without violating the underlying assumptions of the tests. Lastly, pathogen concentrations are known to have high temporal variations, which can occur diurnally, weekly, or seasonally, depending on the pathogen or indicator. The omission of high spikes in pathogen concentrations, or the inappropriate averaging of pathogen concentrations or LRVs across unit processes operating in parallel, can lead to characteristic overstatements of the average performance of the system (Schmidt et al., 2020). As such, it is necessary to provide guidance on appropriate statistical approaches for analyzing data from site-specific pathogen reduction studies.

1.4 Overall Goal and Objectives

The goal of this report is to provide guidance on the development and implementation of study plans to estimate pathogen LRVs in secondary and tertiary wastewater treatment processes for the purposes of assigning credits for water reuse systems. To do this, a systematic literature review was conducted on topics associated with the knowledge gaps identified in Table 1-1

above. Also, stakeholders from water and wastewater authorities, consulting firms, laboratories, and water reuse regulators offered advice about what types of guidance materials provide the greatest value for pathogen LRV studies. This report summarizes the findings from the literature review and these stakeholder meetings.

CHAPTER 2

Methodology

2.1 Literature Review

Standard guidelines and methodologies for conducting systematic literature reviews were followed (Henderson et al., 2010; Khan et al., 2003; Moher et al., 2015; Pullin and Stewart, 2006) by defining clear goals, conducting reproducible searches, minimizing bias in the screening process, performing a quality assessment of screened studies, and synthesizing findings in a systematic fashion. The research questions that guided the literature review are listed below, grouped by topic. Earlier versions of these questions were shared with the project advisory committee (PAC) and the technical advisory committee (TAC) and were refined based on their feedback. The questions below represent the final research questions that guided this study.

1. How should experimental study plans be designed to estimate pathogen LRVs to assign credits?
    a. What precision is needed to estimate LRV credits, and what is an appropriate sample size?
    b. What microbial groups or process indicators should be measured?
    c. Where, how, when, and how frequently should samples be collected for site specific studies, based on temporal trends in pathogen concentrations and LRVs?
    d. What type of ongoing performance monitoring should be required?
2. What standard laboratory methods should be used to quantify pathogen concentrations?
    a. What are the current practices for sample collection, concentration, extraction, and quantification of pathogens and other microbial process indicators (viruses in particular)?
    b. What are the costs of analyzing samples?
    c. What QA/QC protocols should be considered and what are the implications on cost?
3. What statistical approaches for data analysis should be used to estimate LRVs?
    a. How are concentration and LRV data distributed and what data transformations are necessary?
    b. What statistical methods should be used to handle left-censored data (non-detects)?
    c. What LRVs have been previously reported for secondary and tertiary wastewater treatment processes, and how were they calculated? How should LRVs be calculated?

Information was gathered from the scientific literature using a systematic review process to address all research questions, apart from questions 1a, 2b, 2c, and 3c. Alternative methods were used to address those four questions for the reasons summarized below:

- Question 1a is about sample size and the precision of statistics on the estimated LRV—this topic is better addressed by applying statistical theory about uncertainty (*e.g.*, methods for calculating confidence intervals for quantiles) rather than systematic literature review. As

such, an *in-silico* analysis was conducted to characterize the uncertainty associated with the estimation of LRV quantiles for different scenarios.

- Questions 2b and 2c are about laboratory costs, QA/QC procedures, and the implications of these QA/QC procedures on laboratory costs. This type of cost data is not available in the peer-reviewed literature. As such, primary data on costs were collected by consulting commercial laboratories and industry experts. Since
- Question 3c is about the way LRVs have been calculated and reported for wastewater treatment processes in LRV validation studies and how they should be reported moving forward. Information about how LRVs have previously been calculated and reported can only be found in the "grey literature" (e.g., California Title 22 engineering reports) and by consulting with experts who have been involved in LRV validation studies. To identify these reports, the internet was searched (non-systematically) using Google to identify existing Title 22 reports and other “grey literature” reports from pathogen LRV validation studies. Regarding the second part of Question 3c (how LRVs should be calculated), a preliminary assessment of Boolean strings for systematic review of the scientific literature revealed few relevant articles on the topic (and many non-relevant articles). Globally, there are very few researchers who have expertise in the areas of microbiology, engineering, and statistics, and who have published on this topic. The few researchers who have published in this area are well known in the scientific community. As such, this question was addressed by applying statistical theory, consulting with industry experts, and augmenting observations from those consultations with non-systematic review of the scientific literature (specifically reviewing the literature of the few researchers who are known to have published on this topic).

For the rest of the research questions, studies from the peer-reviewed literature were identified using a systematic literature review methodology (using the process depicted in Figure 2-1). Boolean search strings were designed and tested to optimize their performance. Results were restricted to English language and peer-reviewed publications. Tables 2-1 through 2-3 show the Boolean strings used, the databases searched, and the number of papers identified. The following protocols were used to identify and select studies to include in the review: 1) first, titles and abstracts were reviewed and articles with titles or abstracts that did not meet the criteria specified in Table 2-4 were eliminated; 2) then, the full text of qualifying articles was reviewed; 3) finally, bibliographies of the included studies were also reviewed to identify other relevant articles that may have been missed in the initial search. In some cases, other relevant articles that were not found through the systematic search were included in the review, because these articles were either already familiar to the authors or they were recommended by members of the TAC or the PAC.

The Topic 1 search strategy (Table 2-1) yielded 121 total results. After initial screening, 32 papers met the criteria for full text review. The Topic 2 search strategy (Table 2-2) yielded 64 total results. After initial screening, 21 of those papers met the criteria for full text review. The Topic 2 search strategy (Table 2-3) yielded 84 total results. After initial screening, 15 of those papers met the criteria for full text review. The full list of references that remained after screening, which were used for the literature survey, can be found in Appendix A.

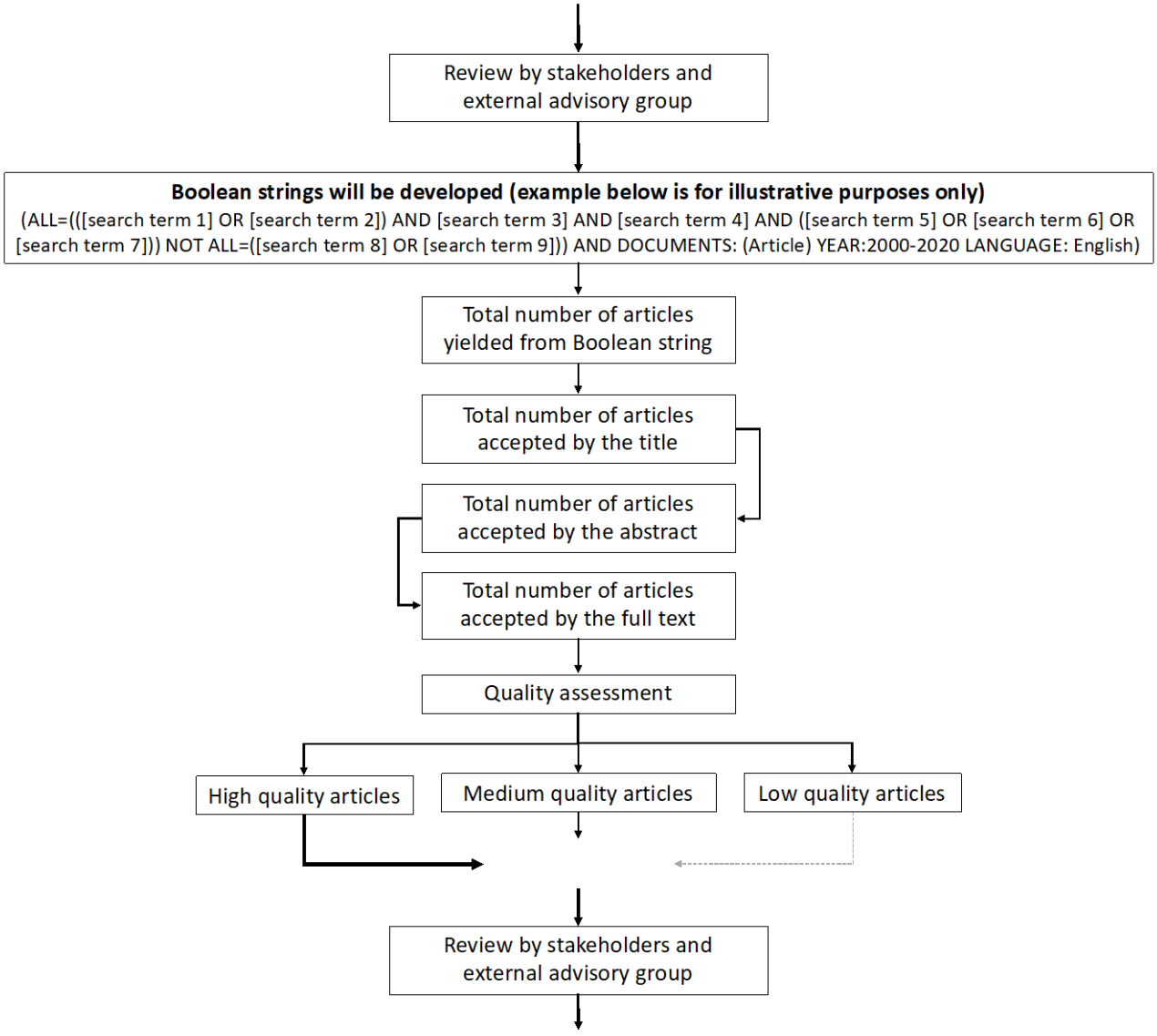

Figure 2-1. Process for Ensuring QA/QC during the Systematic Literature Review Process.

After reviewing the full text of all qualifying articles, the findings were synthesized in this report. The quality of the synthesis of findings from the literature review was controlled by going through several rounds of review from a TAC consisting of external experts representing different sectors and disciplines (e.g., public and private, scientific and practitioner, microbiology and engineering).

Table 2-1. Summary of Strategy for Topic 1 of the Literature Review

Research Question	Strategy	Boolean String Used for Systematic Review	Database(s) Searched
1a. What precision is needed to estimate LRV credits, and what is an appropriate sample size?	Non-systematic literature review and *in silico* analysis (data simulations)	n/a	n/a
1b. What microbial groups or process indicators should be measured?	Systematic literature review	Title/Abstract/Keywords: (“wastewater” OR “sewage”) AND (“pathogen” OR “protozoa” OR “virus”) AND “removal” AND (“correlation” OR “process indicator” OR “performance indicator”)	Science Direct
1c. Where, how, when, and how frequently should samples be collected for site specific studies, based on temporal trends in pathogen concentrations and LRVs?	Systematic literature review	Title/Abstract/Keywords: ("temporal" OR "season" OR "seasonality") AND ("variability" OR "variation") AND "wastewater" AND ("pathogen" OR "virus")	Science Direct
1d. What type of ongoing performance monitoring should be required?	Systematic literature review	Title/Abstract/Keywords: (“wastewater” OR “sewage”) AND (“pathogen” OR “protozoa” OR “virus”) AND “removal” AND (“influence” OR “affect”) AND “factor”	Science Direct

Table 2-2. Summary of Strategy for Topic 2 of the Literature Review

Research Question	Strategy	Boolean String Used for Systematic Review	Database(s) Searched
2a. What are the current practices for sample processing and quantification of pathogens and other microbial process indicators (viruses in particular)?	Systematic literature review	All Fields: (“wastewater” OR “sewage”) AND “pathogen” AND (“compare” OR “comparison”) Title/Abstract/Keywords: (“concentration method”) AND (“virus” OR “coliphage” OR “bacteriophage”)	Science Direct
2b. What are the costs of analyzing samples?	Collection of primary cost data and consultations with industry experts	n/a	n/a
2c. What QA/QC protocols should be considered and what are the implications on cost?	Review of quality assurance project plan (QAPP); collection of primary cost data and consultations with industry experts	n/a	n/a

Table 2-3. Summary of Strategy for Topic 3 of the Literature Review

Research Question	Strategy	Boolean String Used for Systematic Review	Database(s) Searched
3a. How are concentration and LRV data distributed and what data transformations are necessary?	Systematic literature review	Title/Abstract/Keywords: “wastewater” AND “distribution” AND (“pathogen” OR “indicator”) AND (“density” OR “concentration”) AND ("systematic review" OR "meta-analysis")	Science Direct
3b. What statistical methods should be used to handle left-censored data (non-detects)?	Systematic literature review	All Fields: (("pathogen" AND “distribution”) AND ("sewage" OR "wastewater" OR "waste water") AND "censored" AND "data") NOT ("SARS-CoV-2" OR "coronavirus")	PubMed Central
3c. What LRVs have been previously reported for secondary and tertiary wastewater treatment processes, and how were they calculated? How should LRVs be calculated?	Internet search, consultations with industry experts, and non-systematic literature review	Google was searched non-systematically to identify reports from pathogen LRV validation studies (e.g., Title 22 engineering reports); the literature of certain authors who are known to have published in this area was searched non-systematically	n/a

Table 2-4. Criteria for Screening Articles for the Systematic Literature Reviews

Research Question	Initial Number of Results	Criteria Used to Screen by Title/Abstract	Number of Papers Full Text Reviewed
1b. What microbial groups or process indicators should be measured?	47	Eliminate all papers with titles/abstracts that did not focus on the use of a pathogen and a process indicator or surrogate in wastewater	13
1c. Where, how, when, and how frequently should samples be collected for site specific studies, based on temporal trends in pathogen concentrations and LRVs?	42	Eliminate all papers with titles/abstracts that did not indicate a study about wastewater, that were not about pathogens, or that were more about QMRA or about correlating SARS-CoV-2 temporal trends to COVID rates	13
1d. What type of ongoing performance monitoring should be required?	32	Eliminate all papers that were not explicitly related to design and operational factors that influence pathogen LRVs in wastewater treatment processes	6
2a. What are the current practices for sample collection, concentration, extraction, and quantification of pathogens and other microbial process indicators?	64	Eliminate all papers that did not quantitatively measure the efficiency of recovery for virus concentration methods from untreated wastewater samples, or that used methods that were too obscure, not well described, or experimental	21
3a. How are concentration and LRV data distributed and what data transformations are necessary?	4	Eliminate all papers that did not assess the distribution of pathogens in wastewater from a large dataset	4
3b. What statistical methods should be used to handle left-censored data (non-detects)?	80	Eliminate all papers that do not report the use of censored data, that use data sources that are not quantitative, that report data from microorganisms that are not pathogens or indicators, or in samples that are not sewage or wastewater	11
TOTAL	**269**	**TOTAL**	**68**

2.2 Stakeholder Meetings

Three workshops were held via Zoom with TAC and PAC members who represented: 1) water and wastewater authorities/districts; 2) consulting firms that have done pathogen LRV studies; 3) laboratories that analyze pathogen concentrations in water and wastewater samples; and 4) academics with expertise in the area, including those who have served on expert panels. In addition, separate meetings were held with state regulators of water reuse projects, and they were invited to the second workshop. During each of the workshops, the project team provided a brief presentation about the preliminary findings from the literature review or the proposed approach for data reporting and LRV calculations. Then, there was a semi-structured question and answer (Q&A) session. For Workshop #1, the Q&A session was guided by the following questions:

1. What methods should be used to calculate pathogen LRVs from a wastewater treatment system?
2. What statistics should be calculated (mean, median, percentiles, etc.) for determining credits?
3. What supporting data (surrogate monitoring and operational parameters) should be collected?
4. What features should be included in web-based guidance materials for pathogen LRV validation studies?
5. What data standards are important for pathogen LRV credit studies?

After the first workshop, based on feedback received from the TAC, the PAC, and the state regulators, concepts for web-based guidance materials were developed, including a study planning tool, a data management system, and an LRV calculator. The following questions guided Q&A for Workshop #2:

1. Which functionalities would provide the greatest and least value to the guidance materials?
2. What changes should be made to the concepts of the proposed web-based guidance materials?
3. Are there other functionalities that would provide greater value than the ones proposed?

Following the second workshop, feedback was gathered from the TAC members, the PAC members, and the state regulators. In addition, a separate meeting was held with some new potential users of the web-based guidance materials (e.g., an additional wastewater authority and their consulting partners who were in the process of planning an LRV study for their reuse project). The design of the web-based guidance materials was modified based on this feedback, then the materials were developed with support from Venthic Technologies (Venthic, 2023). A third workshop was held individually with each of the TAC members between September and November of 2022, once the beta version of the guidance materials were ready to be pilot tested. Prior to this workshop, TAC members were shown a demonstration of the functionalities of the online guidance materials, then they were asked to provide feedback about the beta version of these online materials, based on the following Q&A topic questions:

1. What feature of the online guidance materials did you find most useful? Least useful?

2. What are the most confusing parts about the guidance materials ?
3. Is there anything about the guidance materials that did not work as expected?
4. What situation or scenario would make you consider using the guidance materials?
5. Would your organization feel compelled to pay a minimal fee for the use of these materials? If so, how much (approximately)? If not, then why wouldn't you feel compelled to use them?
6. Would you recommend the use of these guidance materials to anyone else?

In addition to the workshops, separate interactions with stakeholders were made via email, where additional information and feedback was gathered about the draft report and the web-based guidance materials. Feedback obtained during the workshops and from these email interactions was used to revise the online guidance materials.

CHAPTER 3

Findings and Results

3.1 Experimental Study Plan Design

3.1.1 Precision and Sample Size (Research Question 1a)

When planning an experimental site-specific validation study for pathogen reduction, one of the first questions to be determined is the sample size. The number of samples collected and analyzed has a direct influence on the cost of a study and also on the precision of LRV statistics that will be calculated using the data from the study. Statistical power analysis can be used to determine the minimum required sample size when estimating the mean value of a population (for a desired significance level and effect size). However, when assigning an LRV credit, regulators more commonly look for a quantile of the LRV distribution instead of the mean (Carollo, 2017; City of San Diego, 2018; Nellor Environment Associates et al., 2019; California SWRCB, 2019; Woodard & Curran, 2020). In California, LRV credits for secondary and tertiary wastewater treatment processes have been sought based on the 5th percentile of data from an LRV validation study (personal communication with TAC members). The California SWRCB has suggested that for the purpose of LRV crediting, validating individual treatment processes to achieve an LRV at the 5th percentile "can be done using at least 20 data points" (California SWRCB, 2019). If quantiles or percentiles are used to seek LRV credits, then the determination of an appropriate sample size for LRV validation studies should consider *the uncertainty associated with the estimate of that quantile*. The temporal variability in performance (*e.g.*, throughout the year, with respect to different operational conditions, etc.) must also be considered when designing the LRV validation study plan. For example, samples must be spread out temporally to capture typical variability in treatment process operation, source water quality, and other environmental factors that may affect the treatment processes' ability to remove pathogens.

3.1.1.1 Methodology to Simulate the Precision of Quantile Estimates

To assess the uncertainty associated with estimating a percentile of the LRV, Monte Carlo simulations were conducted (each with N = 10,000 iterations) of hypothetical LRV data sets with different sample sizes. Here, sample size refers to the number of sample *sets* (*i.e.*, the number of times wastewater samples are collected at the influent and the effluent). To simulate the data sets, either 12, 20, 24, or 48 samples were randomly selected from a normal distribution representing the LRV, with a mean of either 1.0 or 3.0, and a coefficient of variation (CoV) of either 10%, 30%, or 50%, resulting in a total of 24 simulations (see Table 3-1). When data are normally distributed, the CoV is defined as the standard deviation (SD) divided by the mean (Equation 3-1).

$$\mathrm{CoV} = \frac{\sigma}{\mu} \quad \text{(Equation 3-1)}$$

where σ is the SD
μ is the mean

When data (X) are log-normally distributed such that Y = $\log_{10}$(X) and Y ~ N(μ_L,σ_L), then the CoV is defined using Equation 3-2 (Canchola et al. 2017).

$$\mathrm{CoV} = \sqrt{e^{\ln(10)^2\sigma_L^2} - 1} \quad \text{(Equation 3-2)}$$

where σ_L is the standard deviation of the $\log_{10}$-transformed data

For each of the 24 simulations, the 95% confidence interval around the 5th percentile was parametrically estimated using the V-statistic (Ialongo, 2019) (see Appendix B). The simulated mean LRVs and CoVs are within range of what has been reported in previous LRV validation studies of wastewater treatment processes. For example, for the LRV validation study done for the City of San Diego's Pure Water Project, the mean LRVs for secondary biological treatment processes were between 2 and 3 for *Giardia* and *Cryptosporidium*, with a CoV of 14% for *Giardia* and a CoV of 36% for *Cryptosporidium* (calculated based on data provided by Trussell Technologies). Somatic coliphage data from the LRV validation study at Orange County Sanitation District's Wastewater Treatment Plants No. 1 and 2 (Polanco et al. 2022) yielded mean LRVs that ranged from less than 1-log less than 2-$\log_{10}$ with CoVs of ranging from 27% to 57% for trickling filters; mean LRVs for somatic coliphages in activated sludge systems were slightly greater than 2 with CoVs ranging from 14% to 21% (calculated based on data provided by OCWD).

3.1.1.2 Results of the Monte Carlo Simulations

The results (Table 3-1) show that the width of the 95% confidence interval around the estimate of the 5th percentile of the LRV is more affected by the mean LRV and the CoV than it is by doubling the sample size from 24 to 48. When the sample size was 12, the width of the confidence interval around the 5th percentile was generally greater than 0.5 $\log_{10}$ units, except when LRV was 1.0 and the CoV was 10% (i.e., low LRV process with low variability). When the sample size was increased to 24, the width of the confidence interval around the 5th percentile ranged from 0.13 to 0.66 for a true mean LRV of 1.0, when the coefficient of variation equaled 10% and 50%, respectively. When the sample size was increased to 48, the width of the confidence interval around the 5th percentile decreased by approximately 33% but was still as high as 0.45 for a true mean LRV of 1.0 when the coefficient of variation was 50%. The confidence interval widths were even larger when the true mean LRV was 3.0. These results revealed that the uncertainty associated with estimating a quantile of a dataset (*e.g.*, to estimate the 5th percentile of an LRV distribution) is impacted not only by the number of samples analyzed, but also by the mean and the SD of the data.

3.1.1.3 Illustrative Examples

Consider the following two hypothetical studies based on the simulation results:

- Study A, where 12 samples are collected at each point (influent and effluent), and where the mean LRV is equal to 1.0 with a SD of 0.5, so the CoV equals 50% (see Simulation No. 3 in Table 3-1)
- Study B, where 24 samples are collected at each point (influent and effluent), and where the mean LRV is equal to 3.0 with a SD of 0.9, so the CoV equals 30% (see Simulation No. 17 in Table 3-1)

For Study A, the 95% confidence interval around the estimate of the 5th percentile has a width of 1.00. For Study B, the width is 1.18. Even though the CoV is lower for Study B, the uncertainty is higher. This result illustrates that the uncertainty in quantile estimates becomes larger with higher CoVs. As such, *if treatment processes with higher mean LRVs also have more variation in the LRV performance than processes with lower mean LRVs (i.e., if the CoV is consistent for low and high LRV processes), this would mean that smaller sample sizes would be acceptable for treatment processes seeking lower LRV credits.* The relationship between mean LRVs and CoVs has not yet been well established for different treatment processes, so the only way to know the precision of an LRV 5th percentile is by calculating the confidence interval based on the data collected.

Table 3-1. Summary of Sensitivity Analysis for the Estimation of the 5th Percentile of the LRV

Simulation No.	Sample size	Mean LRV	Standard Deviation	Coefficient of Variation (CoV)	Width of the 95% confidence interval on the estimated 5th percentile of the LRV
1	12	1.0	0.1	10%	0.20
2	12	1.0	0.3	30%	0.60
3	**12**	**1.0**	**0.5**	**50%**	**1.00**
4	12	3.0	0.3	10%	0.60
5	12	3.0	0.9	30%	1.80
6	12	3.0	1.5	50%	3.00
7	20	1.0	0.1	10%	0.15
8	20	1.0	0.3	30%	0.44
9	20	1.0	0.5	50%	0.73
10	20	3.0	0.3	10%	0.44
11	20	3.0	0.9	30%	1.31
12	20	3.0	1.5	50%	2.19
13	24	1.0	0.1	10%	0.13
14	24	1.0	0.3	30%	0.39
15	24	1.0	0.5	50%	0.66
16	24	3.0	0.3	10%	0.39
17	**24**	**3.0**	**0.9**	**30%**	**1.18**
18	24	3.0	1.5	50%	1.97
19	48	1.0	0.1	10%	0.09
20	48	1.0	0.3	30%	0.27
21	48	1.0	0.5	50%	0.45
22	48	3.0	0.3	10%	0.27
23	48	3.0	0.9	30%	0.81
24	48	3.0	1.5	50%	1.35

Not all reuse projects will need to claim LRV credits with a precision of 0.1 $\log_{10}$ units. As another example, suppose a state regulator requires the reuse project to claim credits based on the 5th percentile of the LRV. Suppose two validation studies are conducted for *Cryptosporidium* on two different biological wastewater treatment systems. During the first year of the studies, 12 samples of raw wastewater and 12 samples of treated effluent were collected monthly from each treatment plant and analyzed for *Cryptosporidium*. Based on the data from these samples, the mean LRV was 3.4 with a SD of 0.5 (CoV = 15%) in Study #1 and the mean LRV was 1.4 with

a SD of 0.1 (CoV = 9%) in Study #2. The point estimates (and 95% confidence intervals) of the 5th percentiles of the LRVs were found to be 2.6 (95% CI: 1.8, 2.9) for Study #1 and 1.2 (95% CI: 1.1, 1.3) for Study #2. In this case, Study #2 should conclude the study, due to the higher precision of the 5th percentile estimate and claim an LRV credit of 1.2 (with a precision of 0.1 $\log_{10}$ units). Study #1 on the other hand, due to the lower precision of the 5th percentile estimate, has a choice. One option is for them to conclude the study now, and settle for an LRV credit of 2 (rounding down to the nearest integer) instead of 2.6.

Another option for Study #1 is to continue the study for another year, collecting another 12 samples, to attempt to increase the precision of their estimate of the 5th percentile, to potentially claim an LRV credit of 2.6 (with a precision of 0.1 $\log_{10}$ units). Suppose Study #1 decides to continue, and after collecting a total of 24 samples, the mean LRV is now 3.5 with an SD of 0.4 (CoV = 11%) and a 5th percentile of 2.5 (95% CI: 2.2, 2.8). The confidence interval around the 5th percentile is smaller, so these results might justify an LRV credit of 2.5 (with a precision of 0.1 $\log_{10}$ units).

3.1.1.4 Implications for Practice

Based on the results of the simulations described above, it may be futile to assign LRV credits for a wastewater treatment process with a precision of 0.1 $\log_{10}$ credits, unless the width of the confidence interval is close to that precision. Otherwise, the uncertainty associated with the estimate of the LRV quantile overshadows the precision of the LRV credit being sought. For the City of San Diego LRV study, the Independent Advisory Panel decided that this level of accuracy on the 5th percentile estimate was not warranted because the use of the 5th percentile for LRV crediting already incorporates uncertainty and variability associated with the LRV estimates (personal communication with members of the TAC). However, there is a distinction between uncertainty and variability. The true LRV may be ***variable*** throughout the year due to a variety of influencing factors that affect pathogen reduction efficiency (see Section 3.1.4) and variability associated with laboratory measurements. However, the statistical ***uncertainty*** associated with estimates of statistics (such as the mean or the 5th percentile) is different, and is largely driven by the sample size and the characteristics of the data distribution, as illustrated above.

It is important to note that the method described by Ialongo (2019) and illustrated in Appendix B requires the underlying distribution of the variable to be Gaussian. Although several researchers have reported lognormally distributed pathogen concentrations and normally distributed LRVs (see Section 3.3.1), if the underlying distribution of the LRV is not normal, then the method by Ialongo (2019) should not be used. Alternative nonparametric methods to estimate 95% confidence intervals on quantiles, described by Beran and Hall (1993), are available. A second set of simulations was conducted using this nonparametric method (see Appendix C) to demonstrate that even when nonparametric methods are used, the same trends apply regarding the relationship between the mean and CoV of the dataset, the sample size, and the width of the confidence interval around a quantile estimate.

3.1.1.5 Recommendations for LRV Validation Studies

Based on the results from this study, the recommended sample size is between 12 and 24 samples and depends on the precision of the LRV credit to be claimed. Monthly sampling for at least one year is recommended. If, after one year, either of the following is true, then the study can be concluded: 1) the quantile can be estimated with low precision (e.g., the confidence interval on the quantile is less than 0.5 $\log_{10}$ units); or 2) if the LRV credit to be claimed is greater than 1, and the precision does not need to be so high, so the LRV credit can be rounded down to the nearest integer. In some cases, it might make sense to continue the study for another year to achieve a total sample size of N = 20 or 24 to attempt to claim an LRV credit with a precision of 0.1 $\log_{10}$ units. The additional precision associated with 48 samples compared to 24 samples does not appear to be worth the added cost of doubling the amount spent on sample collection, shipping, and laboratory fees. Some state regulators already limit the precision for LRV credits. For instance, the minimum LRV credit that can be claimed for a process in the State of Nevada is 1.0 (personal communication with members of the TAC). However, the simulations conducted here suggest that LRV credits below 1.0 (with a precision of 0.1 $\log_{10}$ units) could be justified if the width of the confidence interval of the LRV quantile is sufficiently small.

The primary basis for this sample size recommendation is the results of the simulations of confidence intervals on the 5th percentile. However, there are other considerations to determine the number of samples that needs to be collected to capture all the characteristics of the treatment, source water quality variability, variability in the influencing factors in all the processes being studied. These recommended sample sizes are consistent with recommendations made by Sidhu et al. (2017), who suggested that a minimum of 10, and preferably 20 samples, are required to fully capture variability in the removal of viruses from wastewater during the activated sludge process.

3.1.2 Microbial Groups and Process Indicators (Research Question 1b)

There are hundreds of different species of waterborne pathogens that can be transmitted by recycled water (Rose and Jiménez-Cisneros, 2019), and currently it is not practical to quantify all of them. Furthermore, even if select groups of human pathogens are monitored in a study to determine LRV credits, it is impractical to quantify them on an ongoing basis after the study is completed for continued performance monitoring. The use of microbial process indicators (sometimes also referred to as surrogate organisms) is common in research and in practice (Momba et al., 2019). For example, bacteriophages are thought to be good surrogates for the removal of enteric viruses in wastewater and advanced treatment systems (Amarasiri et al., 2017; McMinn et al., 2017). Bacterial spores are used as surrogates for the reduction of environmentally stable pathogens such as protozoan (oo)cysts during treatment (Stelma, 2018). *E. coli* and enterococci are commonly used as indicators of fecal pollution to water bodies, but they may also be good process indicators for the removal of bacterial pathogens during treatment processes. Still, in several different studies, authors have pointed out a lack of strong correlation between any single indicator-pathogen combination, noting instead that the simultaneous use of multiple microbial indicators is best for predicting pathogen reduction (Agulló-Barceló et al., 2013; Harwood et al., 2005).

3.1.2.1 Target Pathogen Groups

According to the US EPA Surface Water Treatment Rules, pathogen LRVs must be demonstrated for viruses, *Giardia*, and *Cryptosporidium*. In a site-specific pathogen LRV validation study, the choice of which pathogen groups to target for LRV credits should be strategic. For example, if the facility already has sufficient credits for *Giardia* and *Cryptosporidium* from other unit processes in the treatment train, then it might be more cost-effective to focus the site-specific study of the wastewater treatment processes on viruses. Water and wastewater utilities and their industry partners should compare the costs associated with implementing a site-specific pathogen LRV validation study with the costs associated with obtaining LRV credits elsewhere in the treatment train. See Section 3.2 for a detailed analysis of the cost of these types of studies.

3.1.2.2 Laboratory Methods

In general, the methods used to quantify pathogens and other microorganisms in water and wastewater samples can be broadly categorized as cultivation (culture-based), molecular, and microscopy-based (cell counting) methods. Cultivation methods measure the ability of microorganisms to multiply under laboratory-controlled conditions, express certain enzymes in the presence of selective or differential growth media, or infect host cells *in vitro*. The concentrations measured from cultivation methods correspond to microorganisms that have not lost their viability. Some cultivation methods rely on direct counts (e.g., membrane filtration, plaque assays), while others rely on statistical models to estimate quantities based on a series of positive or negative results (e.g., most probable number method, median tissue culture infectious dose). Both direct count and statistical-based cultivation methods may involve direct analysis of a water sample (e.g., direct plating) or may require concentration steps prior to analysis (e.g., membrane filtration).

Molecular methods refer to protocols used to detect targets from specific segments of genetic material (DNA or RNA) or proteins that originate from a particular organism. Protocols typically include a biological sample collection step followed by molecule isolation and characterization. These methods are very specific to the pathogen species/strain, but do not provide information about viability. Molecular methods are commonly used to quantify viral pathogens, given the limitations associated with cell-culture methods (Gerba et al., 2017). Molecular methods commonly used in the scientific literature to study pathogen reduction in wastewater treatment processes, such as qPCR and digital PCR (dPCR), detect a portion of the microorganism's genome, and may not account for the loss of pathogen viability caused by damage to the cell wall, membrane, or virus capsid (Wigginton et al., 2012), or even due to damage to the genome, as long as the PCR-targeted region is still intact (Pecson et al., 2009).

Microscopy (cell counting) methods are more commonly used for protozoan and helminth pathogens, and are less automated, requiring trained microbiologists in some cases. For the most part, microscopy-based methods for detecting pathogens do not provide information about viability. Immunomagnetic separation and immunofluorescence staining (e.g., as described in the standard US EPA method for *Giardia* and *Cryptosporidium* [US EPA, 2005]) help increase the specificity of microscopy methods, but do not provide information about viability.

Many US regulators consider the methods described in US EPA standard 1615 for the quantification of cultivable enteric viruses to be the "gold standard" for providing LRV credits for viruses. For *Giardia* and *Cryptosporidium*, standardized EPA methods based on the use of microscopy are considered the "gold standards" for validating wastewater treatment processes for LRV credits. However, it may be helpful to include the analysis of viral, protozoan, or even bacterial process indicators or surrogates. The use of different process indicators should be strategic based on the types of treatment processes being studied.

3.1.2.3 Viral Process Indicators (Surrogates)

Generally, qPCR or dPCR methods to detect specific enteric viruses can provide value in LRV validation studies for treatment processes such as membrane bioreactors, where physical removal (not inactivation) is the primary mechanism (Verbyla and Rousselot, 2018). Bacteriophages have been reported in some studies to be good surrogates for enteric virus removal in biological wastewater treatment processes (Dias et al., 2018; McMinn et al., 2017). Other studies have reported that phages are not always suitable indicators for the presence of human enteric viruses (Truchado et al., 2021). However, many of these studies have quantified coliphages using culture-based methods and enteric viruses using qPCR-based methods. The latter method does not account for potential loss of viability due to capsid damage. Sidhu et al. (2018) noted similar removal of somatic coliphages, human polyomavirus, and human torque teno virus in an activated sludge system when qPCR was used to detect all groups but found a significantly lower removal of human adenovirus. The authors suggested that human adenovirus can be used as a model microorganism in activated sludge processes on the basis that its removal was lower than that of other virus groups. Verbyla and Mihelcic (2015) found similar removal of coliphages and enteric viruses in wastewater treatment ponds based on a literature review and meta-analysis, although removal was highly variable in general. Wu et al. (2020) reported a strong correlation between crAssphage, human adenovirus, and human polyomavirus throughout an activated sludge treatment process, indicating the potential for crAssphage to be used as a viral process indicator during wastewater treatment. Correlations between crAssphage and somatic coliphage concentrations were also strong in that study, but somatic coliphage did not correlate as well with adenovirus and polyomavirus.

3.1.2.4 Bacterial Process Indicators (Surrogates)

E. coli, enterococci, and fecal coliforms are the most common bacterial indicator groups used in practice. However, they do not make very good process indicators or surrogates for LRV crediting studies. Their removal has been reported to be slightly greater than bacterial pathogens in activated sludge systems (Naughton and Rousselot, 2017) and membrane bioreactors (Verbyla and Rousselot, 2018), but more importantly, compared to protozoa and viruses, the removal of bacterial pathogens and bacterial fecal indicators generally tends to be greater. Furthermore, there are no bacterial LRV requirements stipulated in Title 22 of the California Code of Regulations. As such, there is limited utility in measuring the concentrations of *E. coli*, enterococci, or coliforms for site-specific studies of pathogen LRVs for the purpose of crediting.

3.1.2.5 Protozoan Process Indicators (Surrogates)

Surrogates for *Giardia* and *Cryptosporidium* removal in wastewater treatment processes have not been studied as extensively in the literature compared to virus surrogates. Two potential options for protozoan process indicators include fluorescent beads and bacterial spores such as *Clostridium perfringens* or sulphite-reducing clostridia (SRC). Agulló-Barceló et al. (2013) reported a high correlation between the reduction of SRC spores and the reduction of total *Cryptosporidium* oocysts. However, Wen et al. (2009) found that that the removal of SRC spores was lower than the removal of *Giardia* and *Cryptosporidium* (oo)cysts, and that the use of particles as surrogates for protozoan parasite removal in wastewater treatment plants was inaccurate. More research is needed to determine the suitability of these surrogates for making inference on the removal of *Giardia* cysts and *Cryptosporidium* oocysts in wastewater treatment processes, specifically for the purposes of LRV crediting.

3.1.3 Representative Sample Collection (Research Question 1c)

Wastewater flow rates, pathogen concentrations, and corresponding pathogen loadings in raw wastewater display a variety of temporal variations: 1) hourly variations within a 24-hour day; 2) daily variations within a typical week; and 3) seasonal variations within a typical year. Hourly variations are likely similar for all excreted pathogens in each wastewater treatment system, based on typical flow rate variations and patterns associated with the time of the day people tend to use the bathroom. Pathogen concentrations in raw wastewater may also vary greatly from one day to another, especially in sewer sheds with combined sewer systems, but also in separate sanitary sewer systems with high levels of inflow and infiltration. Sewer sheds with large populations present during weekdays and lower populations present during weekends (or vice versa), might see large fluctuations in pathogen concentrations from day to day. Finally, pathogen concentrations in sewage vary considerably between seasons, based on disease dynamics, differences in per capita water usage rates, and the influence of weather events such as precipitation. Such variations are important to consider when designing a pathogen LRV validation study sampling plan.

3.1.3.1 Seasonal Variations in Concentrations

Average *E. coli* concentrations in sewage were found to be generally lower and much more variable during combined sewer overflows than they were during dry weather conditions in the summer in two Canadian sewer sheds (Madoux-Humery et al., 2013). In North America, morbidity and hospitalization rates associated with some viral diseases (e.g., rotavirus and norovirus) exhibit strong seasonal trends, with peaks in the winter and early spring months (Morton et al., 2015). However, year-round wastewater surveillance has shown that even viruses with seasonal disease trends are still detected in wastewater during off-peak months (Barril et al., 2015). Leptospira was detected more frequently and at significantly higher concentrations in the rainy season compared to the dry season, and when grab samples were collected in the morning rather than the afternoon (Casanovas-Massana et al., 2018). The combination of high pathogen concentrations that coincide with low flowrates can be considered the worst-case scenario, as it would result in the highest concentrations. However, this may not be common—Tolouei et al. (2019) found that *Giardia*, *E. coli*, and *C. perfringens* concentrations in raw wastewater were positively correlated with sewage flow rate. The authors reported that the concentrations of these three microbial groups displayed orders-of-

magnitude hourly variations within a single storm event, and that their variations were more drastic than wastewater micropollutants. They also found that fecal indicator bacteria concentrations in sewage reached their peak values in the early afternoon for four different storm events, but that the peaks were later in the evening during trace precipitation events, suggesting the influence of diurnal defecation patterns.

3.1.3.2 Diurnal and Hourly Variations in Concentrations

The abundance of publications on wastewater surveillance during the COVID-19 pandemic has offered insights about both day-to-day and diurnal variations in the concentrations of excreted pathogens. Day-to-day variations in pathogen concentrations can span orders of magnitude. Li et al. (2021) reported that the concentrations of SARS-CoV-2 RNA in the liquid and solid fractions of raw wastewater changed by 1 and 2 orders of magnitude, respectively, within only a few days, despite their use of daily flow-weighted composite samples.

Diurnal (hourly) variations in pathogen concentrations can also span orders of magnitude, but the variability appears to depend on the prevalence of shedding within the community. For some surrogates such as bacteriophages, which are ubiquitously shed in the feces of practically every person, the variability in diurnal concentrations is much less. Ahmed et al. (2021a) studied the concentrations of crAssphage, HAdV, and pepper mild mottle virus (PMMoV) concentrations in hourly grab samples and time-based 24-hour composite samples collected during three days. They found remarkably stable concentrations of crAssphage in the grab samples, with less than 0.5-$\log_{10}$ fluctuations, but much higher fluctuations in the hourly concentrations of PMMoV and HadV, with PMMoV concentrations fluctuating approximately one order of magnitude within the same day, and HadV concentrations fluctuating nearly two orders of magnitude within the same day. Curtis et al. (2020) performed a similar study, focused on the concentrations of SARS-CoV-2 in grab samples collected from the raw wastewater at a large wastewater treatment plant every 2 hours for 72 hours during the beginning of the pandemic. They reported relatively low variability and good agreement with 24-hour flow-weighted composite samples collected during the same three days. Nevertheless, the within-day fluctuations of SARS-CoV-2 RNA concentrations spanned an order of magnitude. Guo et al. (2019) also reported hourly variations in the microbial community of raw wastewater based on 16S rRNA gene amplicon sequencing at 4-hour intervals for two days, with *Proteobacteria* relative abundance ranging from 44 to 63%.

For human pathogens causing diseases with relatively low prevalence, grab samples may be more susceptible to higher variability than composite samples, leading to higher standard deviations in the LRV measurements and less certainty in the estimates of LRV statistics such as the 5th percentile. The LRV for rotavirus in an activated sludge system, based on concentrations measured in 24-hour time-based composite samples, was found to be 0.82-$\log_{10}$—however, when grab samples were collected during the same 24-hour time period, one set of paired grab samples only estimated a removal of 6% (Chalapati Rao et al., 1987). This is likely related to the authors' calculation of the LRV from paired influent and effluent samples (collected at the same time). The collection of a sample at the effluent does not represent the same plug of water as the sample collected at the influent, even if the timing of sample collection at the influent and effluent locations is offset by the theoretical hydraulic retention time (HRT) (Sidhu et al., 2017).

This has important implications about how LRV statistics should be calculated from a data set of influent and effluent pathogen concentrations. See Section 3.3.1 for more details.

3.1.3.3 Recommendations for Sample Collection

Based on the evidence presented above, wastewater samples should be collected over the course of at least one year to capture seasonal trends and trends in operational conditions at the treatment plant. These samples should also be collected on different days of the week (including the weekends) to account for potential patterns that may exist from one day to another. Finally, the collection of composite samples instead of grab samples might help reduce the variability associated with hourly fluctuations in the concentrations of pathogens in wastewater samples, especially in raw wastewater samples from systems that lack flow equalization. Holding times and storage temperatures must be considered if composite sampling is used, since the first subsample would be 24 hours old by the time sampling is complete (if 24-hour composite samples are collected). If surrogates that are ubiquitously excreted by the population are targeted (e.g., crAssphage, coliphage, or other bacteriophages), then grab samples may be sufficient since the hourly fluctuations in the concentrations of ubiquitous surrogates are minimal. More research is needed to better understand the impact of hourly fluctuations in the concentration of less-prevalent pathogenic microorganisms vs. more ubiquitous microorganisms (e.g., microbial process indicators), especially for systems serving small populations.

3.1.4 Influencing Factors and OMPs (Research Question 1d)

The treatment processes used to comply with pathogen reduction requirements in a reuse system must be validated by the water utility and approved by the state regulator to demonstrate that the treatment process will "reliably achieve the credited LRV" (California SWRCB, 2019). In California, the LRV must also be "correlated with a parameter that is routinely measured and indicates ongoing attainment of the LRV" (California SWRCB, 2019). Validation plans for LRV crediting studies, which are proposals that describes how pathogen LRVs in treatment process will be validated, must describe how the study will be conducted, what data will be collected, and how the data will be analyzed. The Australian Water Recycling Centre of Excellence protocol template is one resource that has been used by California regulators to help develop validation plans (Australian Water Recycling Centre of Excellence, 2016). These validation plans should specify which additional parameters will be measured (in addition to pathogen/microbial concentrations). Validation plans should demonstrate an understanding of the mechanisms responsible for pathogen inactivation or removal from the wastewater in the process(es) being studied and should provide a list of the relevant factors that influence pathogen removal efficiency (such as HRT, SRT, water temperature, etc.). Additional parameters to be measured in LRV crediting studies can be described as influencing factors or OMPs.

Influencing factors can be defined as design, operational, or environmental characteristics that influence pathogen reduction efficiency in a given treatment process, based on what is known about the mechanisms for pathogen removal or inactivation in that process. OMPs are parameters that correlate with pathogen LRVs and can be reliably used as indicators of

pathogen LRV efficiency. The difference between influencing factors and OMPs are that *influencing factors affect pathogen reduction* while *OMPs indicate treatment efficiency*.

For OMPs to be effective indicators of pathogen LRVs, they should be affected by influencing factors the same way pathogen LRVs are affected by the influencing factors. The challenge with OMPs is that they may also be influenced by other factors that do not influence pathogen removal (Figure 3-1). Likewise, pathogen removal may be influenced by other factors that do not affect OMPs. For example, Koivunen et al. (2003) found that lower BOD, COD, TSS, and phosphorus concentrations in the effluent of a tertiary filter corresponded with greater pathogen removal. However, there are other factors that could affect the concentrations of these water quality parameters (such as higher or lower concentrations in the raw wastewater) that might not necessarily have an impact on pathogen LRVs. Pathogen surrogates might be effective OMPs to demonstrate pathogen LRVs, as the removal of pathogens and surrogates *should* be affected by influencing factors in a similar way.

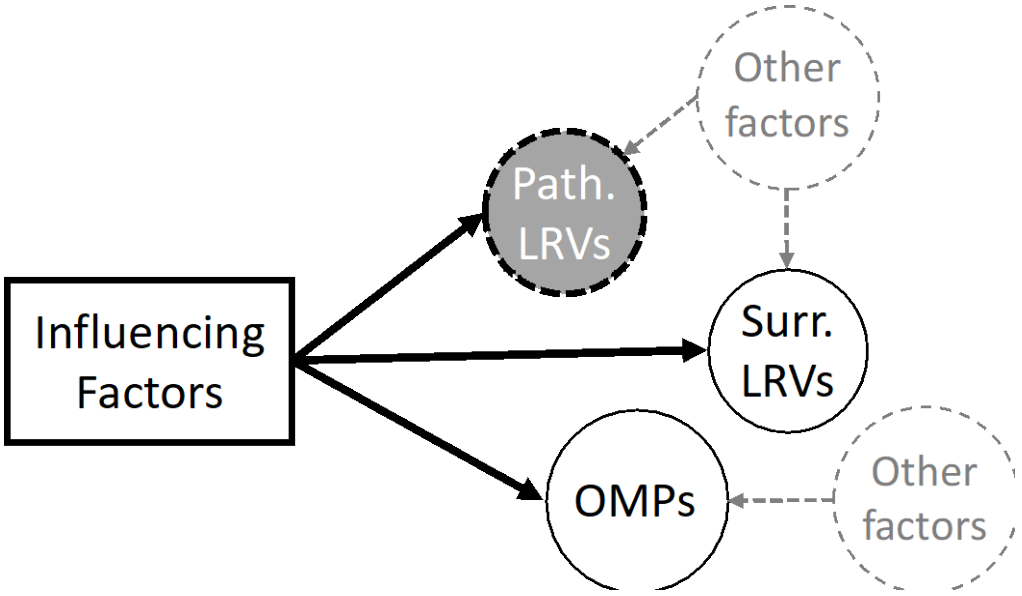

Figure 3-1. Depiction of the Relationship Between Influencing Factors, the Reduction of Pathogens and Surrogates, and the Concentrations of OMPs.

Some examples of influencing factors for pathogen removal during biological treatment (e.g., due to the mechanisms of predation or adsorption) are water temperature, HRT, solids retention time (SRT), and sludge recirculation rates. Some examples of influencing factors for pathogen removal in media filters (e.g., due to the mechanisms of physical straining and adsorption) are the type of pathogen (e.g., virus, bacteria, protozoa) and the grain size and surface properties of the media used in the filter (Oakley and von Sperling, 2017). In membrane bioreactors (MBRs), Branch et al. (2021) reported that influencing factors such as SRT, HRT, mixed liquor suspended solids (MLSS) concentrations, flux, membrane properties, and transmembrane pressure affected the $\log_{10}$ removal of viruses, protozoan pathogens, and bacterial indicators. The authors also found strong correlations between pathogen LRVs and OMPs such as pH, temperature, and dissolved organic carbon (DOC) concentrations in the filtrate.

3.1.4.1 Critical Control Point Approach

The concept of a hazard analysis with critical control points was first developed in the 1960s by NASA and Pillsbury to produce food that was free of pathogens and that had a long shelf-life for space travel (Weinroth et al. 2018). The food safety literature has then been applied to describe pathogen control in wastewater reuse systems. Critical control points are defined as a point in a treatment train at which a control is applied to reduce the pathogen concentrations to an acceptable level (e.g., a treatment unit process). Based on project experience in California, LRV credit for pathogens may be awarded if (after completing a site-specific validation study) the plant performance is maintained in a normal operating envelope generally consistent with how the plant was operated and performed during the pathogen LRV study.

3.1.4.2 Critical Control Limits / Operating Range Values

In the case of the North City Pure Water Project (City of San Diego, 2018), parameters and values (limits) for these parameters at the wastewater treatment facility were selected and referred to as critical control limits (CCLs). In the case of the Orange County Water District (OCWD) Groundwater Replenishment System, in the pathogen LRV validation study for the Orange County Sanitation District wastewater treatment plants, these parameters were referred to as Operating Range Values (ORVs). These CCLs or ORVs are maximum or minimum values of biological, chemical, or physical parameters that are consistent with the normal performance of the treatment process (e.g., with respect to pathogen removal) and are used by regulators to assess whether the process performance is within the operating envelope observed during the pathogen LRV study, which is a conservative way of ensuring that the process is meeting its performance goals with respect to pathogen removal.

In the case of the North City Pure Water Project (City of San Diego, 2018), raw wastewater, secondary effluent, and tertiary filter effluent were selected as the monitoring locations to assess performance of the activated sludge treatment process and tertiary filters as critical control points. As an example, the critical control limits described by the City of San Diego (2018) for secondary treatment processes at the North City Water Reclamation Plant include daily monitoring of SRT to ensure a 30-day average of at least 9 days, and the continuous online monitoring of ammonia at the end of the aeration basin to ensure daily average concentrations do not exceed 1 mg/L as N. Truckee Meadows Water Reclamation Facility adopted the same criteria based on the San Diego study. Critical control limits for tertiary treatment processes include continuous online monitoring of turbidity and total organic carbon (TOC) in tertiary treated effluent, to ensure a daily average TOC less than 11 mg/L, a 24-hour average turbidity of 1.5 NTU, no more than 2.5 NTU in 5% of continuous samples within a 24-hour period, and no single reading exceeding 5 NTU. For the Orange County Sanitation District (OC San) facilities, limits based on SRT and total biochemical oxygen demand (BOD-T) were proposed for different unit processes, as well as turbidity for the blended effluent after exiting OC San facilities and undergoing microfiltration at a facility operated by the OCWD (Polanco et al. 2022).

3.1.4.3 Influencing Factors and OMPs

In summary, the critical control point approach is conservative because it means that LRV credits would only be granted if the treatment plant is operated under the same range of conditions experienced during the period that the pathogen validation study was underway. It

is possible that pathogen reduction might still be effective even when the treatment plant operates outside of the range of the CCLs or ORVs. In California, a project operating outside of the approved window of ORVs may continue to operate but cannot claim any LRV credits for that process during that time. To avoid the loss of LRV credits due to operating windows, LRV validation studies should plan to collect samples during extreme operating conditions—in other words, this can be built into their proposed LRV validation study sampling plan.

An alternative to the critical control point approach would be to utilize a combination of modeling together with periodic collection of validation and test data. For example, when a large enough database of LRVs from previous validation studies is compiled, the database can be used to build a model that predicts the LRV for a particular pathogen in a particular treatment process operating under particular environmental and operational conditions. Then, an LRV validation study could be designed with a smaller sample size, with the purpose of confirming that the model's predictions are true. Additional samples could be periodically collected over a longer period, specifically on days when the treatment plant has to operate outside of the range of CCLs or ORVs, to help update or validate the process LRV model. This would contribute to a more robust overall data set of studies performed at multiple facilities, under a wider range of design and operating conditions. However, to develop a robust training data set for such a model, data about the relevant pathogen concentrations, influencing factors, surrogate concentrations, and performance monitoring parameters would have to be collected and compiled from multiple studies, all using the same consistent data management approach. Tables 3-2 and 3-3 show the relevant influencing factors and OMPs that are recommended to validate pathogen LRVs in a variety of common secondary and tertiary wastewater treatment processes.

Table 3-2. Influencing Factors for Primary, Secondary, and Tertiary Treatment Processes

Treatment Unit Process	Influencing Factor	Relation to Pathogen Removal	References
Clarifier	Hydraulic overflow rate	Lower overflow rates cause greater pathogen removal	(Oakley, 2018)
Clarifier	Sludge removal	Excessive sludge accumulation decreases pathogen removal	(Oakley, 2018)
Clarifier	Sludge volume index (SVI)	Sludge bulking and foam may decrease pathogen removal	(Oakley, 2018)
Chemically enhanced primary clarifier	Coagulant dose	Correct coagulant dose causes greater pathogen removal	(Oakley, 2018)
Activated sludge	Solids retention time (SRT)[1]	Shorter solids retention times decrease pathogen removal	(Naughton and Rousselot, 2017)
Activated sludge	Hydraulic retention time (HRT)	Higher retention times may increase pathogen removal	(Naughton and Rousselot, 2017)
Membrane bioreactor	Membrane pore size (and pathogen diameter)	Larger pathogen/pore size ratio increases pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)

[1] Also known as mean cell residence time (MCRT)

Treatment Unit Process	Influencing Factor	Relation to Pathogen Removal	References
Membrane bioreactor	Salinity of feed water	Short-term salinity shock may decrease pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)
Membrane bioreactor	Integrity testing	Increases in pressure decay rate indicates less pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)
Trickling filter	Hydraulic loading rate (HLR)	Low hydraulic loading rates increase pathogen removal	(Oakley and von Sperling, 2017)
Trickling filter	Recirculation rate	Recirculation of effluent increases pathogen removal	(Oakley and von Sperling, 2017)
Rapid sand filter (tertiary treatment)	Hydraulic loading rate (HLR)	*E. coli* removal reached 2-log at HLR of 100 – 150 L/m^2/d, but only 1-log at HLR of 200 – 800 L/m^2/d	(Tonon et al., 2015)
Rapid sand filter (tertiary treatment)	Filter depth	Greater filter depth results in increased pathogen removal	(Bali et al., 2011)
Rapid sand filter (with coagulant added)	Effluent biochemical oxygen demand (BOD), chemical oxygen demand (COD), total suspended solids (TSS), and/or total phosphorus	Lower BOD/COD, TSS, and P concentrations corresponded with greater pathogen removal	(Koivunen et al., 2003)

Table 3-3. Summary of OMPs for Primary, Secondary, and Tertiary Treatment Processes

Treatment Unit Process	OMP	Relation to Pathogen Removal	References
Clarifier	TSS	Higher TSS concentrations may indicate lower pathogen removal	(Oakley, 2018)
Activated sludge	Ammonia concentrations at the end of aeration	High ammonia concentrations indicate low pathogen removal	(City of San Diego, 2018)
Membrane bioreactor	Ammonia concentrations in feed water	Short-term ammonia shock may indicate lower pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)
Membrane bioreactor	Permeate turbidity	Higher permeate turbidity indicates less pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)
Membrane bioreactor	Permeate TOC	Higher permeate TOC indicates less pathogen removal	(Hai et al., 2014; Verbyla and Rousselot, 2018)
Trickling filter	Total biochemical oxygen demand (BOD-T, 30-d avg.)	Higher BOD-T in effluent indicates abnormal performance of trickling filter; suggests less virus removal	(Polanco et al., 2022)
Rapid sand filter (tertiary treatment)	Effluent BOD/COD, TSS, and total phosphorus	Lower BOD/COD, TSS, and P concentrations corresponded with greater pathogen removal	(Koivunen et al., 2003)

3.2 Laboratory Methods and Costs

3.2.1 Sample Processing and Quantification (Research Question 2a)

3.2.1.1 Concentration of Bacteria from Wastewater

The concentration of bacteria via membrane filtration is relatively straightforward, as the cells of bacteria and protozoa will be retained on a 0.45-µm membrane, which is porous enough for wastewater and treated water to pass through. The membrane can be transferred to a petri dish with media for growth of colony-forming units. However, depending on the target species, growth on differential or selective media may not be sufficient, and further analyses may be needed for confirmation.

3.2.1.2 Concentration of *Giardia* and *Cryptosporidium* from Wastewater

Protozoan pathogens such as *Giardia* and *Cryptosporidium* are larger than most bacterial cells, and can also be concentrated using membrane filtration, but also using other methods such as centrifugation and flocculation (Falk et al., 1998; Shepherd and Wyn-Jones, 1996). The standard US EPA methods for the quantification of *Giardia* and *Cryptosporidium* require the concentration of cysts and oocysts by filtration, concentration of filter eluent by centrifugation, and subsequent immunomagnetic separation, prior to identification and enumeration via microscopy (US EPA, 2012b, 2005). Methods for concentrating protozoan pathogens (*Giardia* and *Cryptosporidium*) from raw sewage and from treated wastewater are standardized in EPA Method 1693 (US EPA, 2014), and involve the use of immunomagnetic separation beads, along with kaolin to remove fats, oils, organics, and heavy particulates and concentrate (oo)cysts from wastewater samples. The recovery efficiency of Method 1693 is measured using an internal standard (e.g., ColorSeed™) consisting of inactivated *Cryptosporidium* oocysts and *Giardia* cysts that are labelled with a red florescent dye (which allows them to be distinguished from normal oocysts and cysts, which only show green fluorescence). Recoveries between ~10% and 100% are considered acceptable for *Giardia* and *Cryptosporidium* (based on matrix spikes with ColorSeed™ beads).

3.2.1.3 Concentration of Viruses from Wastewater

The concentration of viruses from sewage samples is less straightforward, as their diameters are often smaller than the pore sizes of most membranes, and they may only be present in very dilute concentrations, especially in tertiary treated wastewater. For the standardized EPA Method 1615, which has been commonly used as the “gold standard” for most site-specific LRV validation studies of wastewater treatment processes, ultrafiltration methods are required to concentrate viruses from very large volumes (up to 600 L for tertiary wastewater). The filtration apparatus set up and protocol for filtration, including important details such as flushing the filtration apparatus and removing residual chlorine with sodium thiosulfate, are fully described in EPA Method 1615 (Fout et al. 2014). For raw sewage samples, polyethylene glycol (PEG) precipitation is also used (Lewis and Metcalf, 1988).

The use of methods with the highest recoveries may help avoid “non-detect” results, especially in treated wastewater samples. Matrix spikes are used to measure percent recovery of viruses from wastewater samples. Results from matrix spike recoveries have been used to “correct” for

the loss of pathogens during the concentration process in previous LRV studies (Pecson et al. 2021a). Discussions with members of the TAC and PAC who have been involved with pathogen LRV validation studies confirmed reports in the literature that matrix spike recovery (MSR) efficiency can vary from sample to sample. This has prompted some practitioners to include matrix spike recoveries for every sample in pathogen LRV validation studies, correcting for recovery efficiency based on the process controls, which essentially doubles the laboratory costs. In other studies, MSRs have been performed for 1 out of every 3 or 1 out of every 5 samples (Polanco et al., 2022). Recoveries between 5% and 200% (based on matrix spikes with MS2 or PhiX174 phage) are generally considered to be acceptable for viruses. Detailed descriptions of acceptable MSRs for various laboratory methods are provided in the QAPP produced under WRF Project #4952 (Cel Analytical, 2021). This document specifies the use of PEG precipitation to concentrate viruses from wastewater samples as described in the revised EPA Method 1615, v1.1 (Fout et al. 2014).

Our review of the scientific literature revealed a very large number of methods that have been used to concentrate pathogens (and viruses in particular) from sewage samples (Figure 3-2). Most comparative studies have only focused on 3 or 4 alternative methods at a time. A meta-analysis of virus recovery data extracted from papers identified through the literature review (Ahmed et al., 2021b, 2020; Crank et al., 2020; Haramoto et al., 2020; Hata et al., 2021; Jafferali et al., 2021; Kaya et al., 2022; Kevill et al., 2022; Lu et al., 2020; McMinn et al., 2021; Medema et al., 2020; Oh et al., 2022; Randazzo et al., 2020; Rusiñol et al., 2020; Ye et al., 2016) revealed that with the exception of methods using Sterivex filters (e.g., with cutoffs of 0.22 µm), the use of filtration-based methods, especially adsorption-extraction methods, but also ultrafiltration methods (e.g., centrifugal devices, concentrating pipettes) generally performed similar or better than PEG precipitation and other centrifugation-based methods regarding the recovery of process controls (matrix spikes) (Table 3-4). The mean efficiency of adsorption-extraction methods for the concentration of viruses from sewage, based on 13 experiments, was found to be 40% (Table 3-4). Generally, the adsorption-extraction method requires the addition of $MgCl_2$ and the acidification of samples to a pH of 3.5 – 4.0 prior to filtration, however Ahmed et al. (2020) found that the recovery of murine hepatitis virus using adsorption-extraction was more efficient when samples were not acidified.

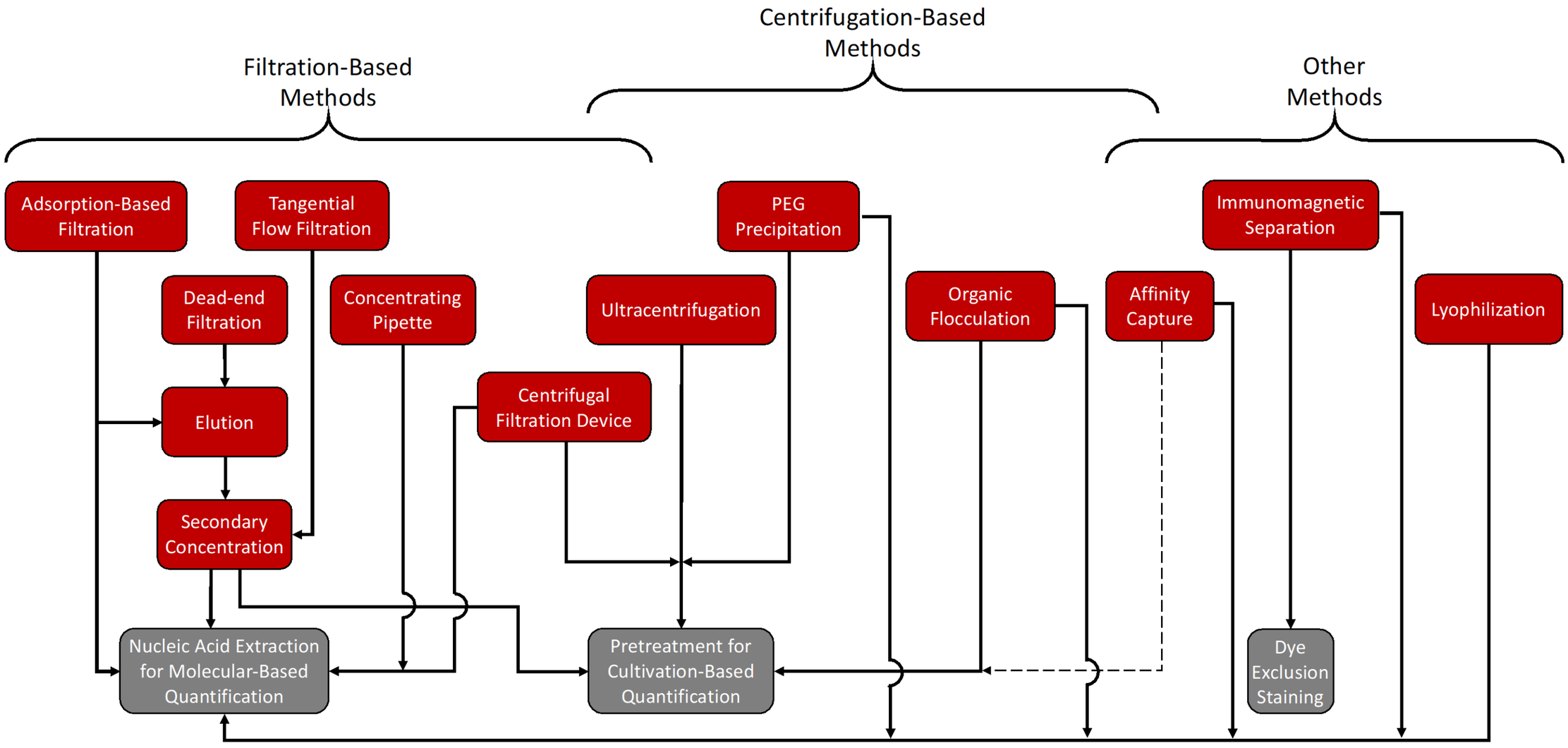

Figure 3-2. Methods Used to Concentrate Viruses and Protozoa from Wastewater Samples.

In comparative studies (Ahmed et al., 2021b; Calgua et al., 2013; Crank et al., 2020; Kevill et al., 2022; Rusiñol et al., 2020), where the concentrations of indigenous viruses from sewage were compared in split samples using different concentration methods, adsorption-extraction, PEG precipitation, ultrafiltration (including concentrating pipettes), and ammonium sulfate precipitation also generally performed well. The adsorption-extraction method was not always the most efficient method for all virus groups in these comparative studies, and the best method was often different for different viruses. For example, Ahmed et al. (2021b) found that wastewater samples processed using a concentrating pipette yielded higher concentrations of indigenous enteroviruses compared with samples where viruses were concentrated using the adsorption-extraction method. However, the opposite was true for human adenovirus, which showed higher concentrations when the adsorption-extraction method was used (Ahmed et al., 2021b). Also, Crank et al. (2020) found higher concentrations of indigenous crAssphage and human polyomaviruses in wastewater samples that were concentrated using PEG precipitation compared to ultracentrifugation, and Kevill et al. (2022) found that the use of PEG precipitation produced higher concentrations of crAssphage and SARS-CoV-2 than the use of concentrating pipettes and an ammonium sulfate precipitation method. Based on the literature, it appears that adsorption-extraction, ultrafiltration, PEG precipitation, skimmed milk flocculation, and ultracentrifugation-based methods are all suitable for concentrating viruses from wastewater. The use of Sterivex filters, aluminum hydroxide precipitation, and centrifugation (at speeds below 100,000*g*) should generally be avoided. The best method for concentrating viruses is also likely dependent on which viruses are being analyzed.

Process controls (matrix spikes) should be analyzed for QA/QC in pathogen LRV validation studies, but there is still some debate in the literature about whether process control recoveries should be used to correct for the concentration of native viruses. Virus recovery efficiencies are different for different viruses, as indicated above (Ahmed et al., 2021b), so the type of proxy virus used to assess recovery efficiency can impact the apparent recovery of native virus of interest (Kantor et al., 2021). Polanco et al. (2022) used matrix spike recovery samples (approximately one per every three raw wastewater samples and approximately one per every five treated wastewater samples), calculated the recovery efficiency based on the average recovery of average recovery of spiked coliphages and poliovirus, and used this average recovery to correct for the concentrations of coliphage concentrations and cultivable enteric virus concentrations. Pecson et al. (2022) also corrected virus, *Giardia*, and *Cryptosporidium* concentrations for measured recovery efficiencies, using MS2 and PhiX174 coliphage for virus recovery, and ColorSeed™ for protozoan pathogens.

Kantor et al. (2021), on the other hand, argued that virus recovery efficiencies assessed with matrix spikes (process controls) should not be used to correct for the concentrations of native viruses. However, their argument was in the context of quantifying SARS-CoV-2 in wastewater for public health surveillance, based on a hypothesis that the ratio of the RNA in intact SARS-CoV-2 particles to the RNA in nonintact SARS-CoV-2 particles will decrease as wastewater travels through the sewer system and during sample collection and analysis (Bivins et al., 2020; Wurtzer et al., 2021), and that the efficiency of recovering RNA from intact vs. nonintact viruses may be different. This situation may be unique to SARS-CoV-2 because its persistence in wastewater is less than the persistence of most waterborne pathogenic viruses (de Oliveira et

al., 2021). Most of the virus recovery studies used in the meta-analysis were published after March 2020, with the purpose of drawing analogies to SARS-CoV-2, to inform wastewater surveillance methods in the context of the pandemic. SARS-CoV-2, an enveloped virus transmitted via the respiratory route, is phenotypically very different from most waterborne pathogenic viruses, and as such, their concentration efficiencies may be very different from each other. The goals of wastewater surveillance are also very different than the goals of pathogen LRV validation studies.

In the context of a pathogen LRV validation study, differences in the recovery efficiency between the influent and effluent sample points can produce misleading estimates of the LRV. If the recovery efficiency at the effluent is greater than it is at the influent, then the LRV might appear to be lower than they actually are. But, if the recovery is better in the treated wastewater than it is in the untreated samples, then the LRV might be overestimated. For LRV credits sought with a precision is 0.1 $\log_{10}$ units (see Section 3.1.1), pathogen concentrations should be corrected for recovery only if the LRV based on recovery-corrected concentrations is 0.1 units greater than or less than the LRV based on uncorrected concentrations. For LRV credits with lower precision, concentrations should be corrected for recovery only if it would change the LRV by more than 0.5 $\log_{10}$ units, for a maximum correction factor of 1.0 $\log_{10}$ units. If correcting for the recovery would change the LRV by more than 1.0 $\log_{10}$ units, then something should be done in the laboratory to try and equalize the recovery efficiencies between the influent and effluent sample locations.

Table 3-4. Summary of Recovery Efficiencies Calculated Through Meta-Analysis Of Methods Used To Concentrate Viruses From Wastewater Samples

Method	Recovery Efficiency[1] (%) Mean	Recovery Efficiency[1] (%) SD	Number of experiments	References[4]
Filtration-Based Methods				
Adsorption-Extraction	40.0%	23.6%	13	(a) (f) (j) (k)
Sterivex-GP filtration	1.0%	1.8%	8	(d)
Ultrafiltration	23.6%	23.3%	31	(c) (d) (f) (i) (j) (m) (o) (l)
Ultrafiltration & PEG precipitation	12.2%	8.6%	4	(d)
Concentrating pipette	27.1%	20.3%	29	(a) (e) (i) (g)
Centrifugation-Based Methods				
Aluminum hydroxide precipitation	11.0%[3]	3.8%	4	(f) (n)
Ammonium sulfate precipitation	18.3%	12.4%	7	(e)
PEG precipitation	23.3%	17.3%	11	(b) (d) (e) (f) (j)
Skimmed milk flocculation	29.0%	6.7%	4	(i) (l)
Centrifugation (analysis of pellet)	5.6%	4.6%	8	(a) (d)
Ultracentrifugation	33.5%	n/a	2	(b) (f) (j) (l)
Other Methods				
Lyophilization-based method	No data[2]	n/a	0	
Affinity-capture method	20.6%	24.8%	5	(h)

[1] The reported recovery efficiencies based on process controls (matrix spikes), which differed from study to study, but included bovine respiratory syncytial virus (14 experiments), heat-inactivated SARS-CoV-2 (11 experiments), non-SARS human coronaviruses (5 experiments), bovine or porcine coronaviruses (6 experiments), MS2 coliphage (5 experiments), other F-specific RNA phages (2 experiments), DNA phages (2 experiments) murine hepatitis virus (2 experiments), and several others with one experiment each, including mengovirus, murine norovirus, adenovirus, rotavirus, and Tulane virus.

[2] There were no studies on recovery for this method using process controls (matrix spikes), but in a comparative study, its recovery was less than half of the recovery using skimmed milk flocculation (Calgua et al. 2013).

[3] In untreated wastewater; in secondary and tertiary treated wastewater, recovery was lower (average of 4.8%).

[4] a) Ahmed et al. 2021b; b) Crank et al. 2020; c) Jafferali et al. 2021; d) Kaya et al. 2022; e) Kevill et al. 2022; f) Lu et al. 2020; g) McMinn et al. 2021; h) Oh et al. 2022; i) Rusiñol et al. 2020; j) Ahmed et al. 2020; k) Haramoto et al. 2020; l) Calgua et al. 2013; m) Medema et al. 2020; n) Randazzo et al. 2020; o) Ye et al. 2016

3.2.2 Laboratory Costs (Research Question 2b)

Laboratory costs associated with the following standardized methods for quantifying coliphages, enteric viruses, *E. coli*, and *Cryptosporidium*/*Giardia* in wastewater samples were gathered from commercial laboratories in the United States: EPA Methods 1602/1642 (coliphage), 1615 (enteric viruses by cultivable method or molecular method), 1623/1623.1/1693 (*Cryptosporidium*/*Giardia*), and Standard Methods protocols SM 9223B (for *E. coli*), SM 9510 (for enteric viruses), and SM 9711B (for *Giardia* and *Cryptosporidium*). Costs were also gathered for some non-standardized PCR-based methods for quantifying enteric viruses in wastewater samples. It is important to note the limited number of laboratories that offer certain testing services. The availability of testing varies greatly by pathogen. *Cryptosporidium*/*Giardia* and *E. coli* testing capabilities are readily available at several labs across the country. Cost data was collected from eight laboratories for *E. coli* analysis, five laboratories for *Cryptosporidium*/*Giardia* analysis, four laboratories for (culture-based) enteric virus analysis, and two laboratories for coliphage analysis. Cost varied for all methods, but especially for *Cryptosporidium*/*Giardia* and enteric virus analysis. The means and CoVs of the cost values were calculated for each microbial target on a per-sample basis (Table 3-5).

Table 3-5. Summary of Average Laboratory Fees for Sample Analysis.

Statistic	Enteric Viruses via EPA 1615 (culture and RT-qPCR) [2]	Enteric Viruses (culture-based only)	Enteric Viruses (RT-qPCR only)	Coliphage	*E. coli*	*Giardia* and *Cryptosporidium* [3]
Average[1]	$ 1,995	$ 915	$ 358	$ 226	$ 56	$ 618
Coefficient of Variation	29%	44%	35%	11%	39%	43%
Number of quotes	2	2	3	5	11	8

[1] Arithmetic mean of the cost per sample

[2] Based on EPA Method 1615 or the EPA ICR Microbial Laboratory Manual (EPA/600/R-95/178 with updated EPA/600/4-84/013); does not account for any additional cost of any additional controls not included in those protocols

[3] Based on EPA Methods 1623, 1623.1, or 1693; assumes no extra slides; in some studies, 8 additional slides were needed per sample at a cost of $95 per additional slide

At an average cost of $1,995 per sample, the most expensive analysis to perform is EPA Method 1615, which includes the detection of multiple groups of enteric viruses using both culture-based methods and RT-qPCR methods. The second most expensive type of analysis was the quantification of enteric viruses using culture-based methods only, at an average cost of $915 per sample. The third most expensive type of analysis was the quantification of *Giardia* and *Cryptosporidium* using either EPA Methods 1623/1623.1 or SM 9711B, at an average cost of $667 per sample. The fourth most expensive method offered was the quantification of enteric viruses using non-standardized RT-qPCR or RT-ddPCR methods (protocols based on the scientific literature), at an average cost of $358 per sample (includes the cost of sample extraction). The average costs for coliphage and *E. coli* analyses, respectively, were $226 and

$56 per sample. Therefore, to analyze samples for enteric viruses (cultivable and RT-qPCR), coliphages, *E. coli*, *Giardia*, and *Cryptosporidium* (which, as described in Section 3.1.3, is a typical panel of analytes for this type of study), the average cost would be approximately $3,000 per sample for laboratory fees alone. The use of surrogates for *Giardia*, *Cryptosporidium* and enteric viruses can reduce the overall cost of sample analysis. A discussion about the use of surrogates can be found in Section 3.1.2 of this report. The overnight shipping costs to send samples to laboratories for analysis will vary depending on where the laboratory is located compared to the treatment plant, but the average cost to ship samples from a handful of surveyed studies in the western United States was found to be approximately $100 per sample, assuming one overnight shipment per sampling event that includes two samples (influent and effluent) as appropriate for each method (target analyte) included in the study.

The cost of laboratory analyses can vary greatly for multiple reasons, including if additional QA/QC measures are requested, if a faster turn-around time is requested to provide the results, and if modifications to the method are requested (e.g., to bring down the limit of detection). As an example, one laboratory informed us that the cost to analyze one sample for *Giardia* and *Cryptosporidium* using EPA Method 1693 would be approximately $360, assuming no method blanks, no use of internal standard (e.g., ColorSeed™), no precision and recovery measurements, and the examination of only one slide (hemacytometer) per sample (many hemacytometers or well slides used in this procedure can only accommodate small volumes, e.g. 10 µL, which means that only a portion of the sample is analyzed under the microscope, unless multiple slides are prepared per sample). The cost could increase to more than $1,500 per sample if requests are made for ColorSeeding (additional $100/sample), extra slides (additional $95/slide), ongoing precision and recovery (additional $230 per sample batch), and method blanks (additional $230/blank). The cost can also vary based on the sample matrix. For example, for EPA Method 1693, one lab charged $350 per sample of secondary effluent, but $850 per sample of raw sewage. In one project, the lab fees for *Cryptosporidium* and *Giardia* analysis were $1,250 per sample, which included the cost of field matrix spikes performed for every sample. For virus analysis via RT-qPCR, most labs generally charged around $250 for each additional assay performed on an extracted sample, so adding a matrix spike (process control) to measure recovery during sample concentration (e.g., mengovirus, murine norovirus) or the efficiency of nucleic acid extraction (e.g., salmon sperm DNA) would increase the cost per sample by approximately $250 to $500, depending on how many additional controls are added. Some labs also marked up the analysis cost per sample by 20% to 50% for expedited turnaround times.

In addition to laboratory costs, the completion of a pathogen removal study incurs a considerable effort from personnel time dedicated to planning the study, setting up sampling equipment, collecting samples, concentrating samples, shipping samples with chains of custody, coordination with laboratories, analyzing laboratory data, report writing, etc., as well as the cost of materials and supplies associated with field work and sample collection (e.g., personal protective equipment, etc.). Some water utilities and water districts have been able to complete this work in house, while others contracted the work out to consulting firms. Analysis of the cost data collected revealed that the average cost associated with everything other than laboratory costs was reported to be anywhere from approximately $100,000 to approximately

$300,000 per study (approximately $3,000 per sample on average), with high variability from one study to another (potentially based on variations in the cost of labor from state to state). On average, 10% of these "soft costs" were spent on study planning, 25% on sample collection and shipping fees, 25% on personnel time for data analysis, 15% on report writing, and 25% on working with regulators and an expert external advisory panel. Based on this overall cost analysis, a pathogen LRV validation study with 24 samples collected at two different locations (influent and effluent), analyzed for enteric viruses (cultivable and RT-qPCR), coliphages, *E. coli*, *Giardia*, and *Cryptosporidium* would likely end up costing approximately $300,000 on average.

Based on recommendations made by the TAC, a web-based decision support tool was created to help practitioners perform cost estimates of pathogen LRV validation studies, based on the number of sample locations (e.g., influent point[s], effluent point[s], and any interior points), the sample size (i.e., number of samples to be collected at each location), the microbial parameters to be analyzed, and an assumption about the cost of labor (i.e., soft costs) associated with a study. This tool is freely available at the following website: https://credit.waterpathogens.org/form/calculator. A user manual can be found in Appendix D.

3.2.3 Quality Assurance and Quality Control (Research Question 2c)

QA/QC protocols for the quantification of pathogens and surrogates in wastewater were developed through WRF Project 4952 (in process), "Pathogen Monitoring in Raw Wastewater" and are described in detail in the Quality Assurance Project Plan (QAPP) (Cel Analytical, 2021). The primary objectives for this project were to collect data on the occurrence of norovirus, adenovirus, enterovirus, SARS-CoV-2, bacteriophage, *Cryptosporidium* and *Giardia* from five public wastewater water treatment plants (WWTPs), and to modify and optimize methods for the collection and analysis of pathogen data in raw wastewater. The EPA methods 1615 v1.1, 1693, and 1602 (all modified by WRF Project 4988 (in process)) were used to quantify enteric viruses, *Giardia*, *Cryptosporidium*, and bacteriophages, respectively. In addition, RT-qPCR methods were used to quantify SARS-CoV-2 RNA.

The quality objectives and criteria specific in this QAPP included data quality indicators for precision, accuracy, sensitivity, completeness, bias, and representativeness. Specifically, quality control samples for *Giardia* and *Cryptosporidium* analysis in accordance with EPA Method 1693 (with modifications) included initial demonstration of capability, ongoing precision and recovery, matrix spikes, as well as positive and negative staining controls. Quality control samples for bacteriophage analysis included negative controls (method blanks), positive controls (with MS2 and PhiX174), and laboratory fortified sample matrix spikes, all of which are described in EPA Method 1602. For enteric virus analysis, in accordance with EPA Method 1615, quality control samples included initial demonstration of capability, ongoing precision and recovery (including positive and negative controls), and matrix spikes, for both enveloped and non-enveloped viruses. Positive and negative controls for adenovirus and enterovirus on two different host cell lines were also included.

The results from the quality control checks must be used to decide whether to keep, discard, or correct results from a sample. For example, MSR data may be used to correct the concentrations to account for loss during sample processing, as described above. Any samples

or sample batches that fail the negative control check would be suspected of contamination, and the results might not be used, unless the extent of contamination was negligible compared to the quantity detected in the sample.

3.3 Statistical Methods for Data Analysis

3.3.1 Distributions of Pathogen Concentrations and LRVs (Research Question 3a)

In the scientific literature (especially from the fields of environmental microbiology), concentrations of pathogens in wastewater are often discussed with respect to their base 10 logarithmic order of magnitude (von Sperling et al., 2018). The mean of these $\log_{10}$-transformed concentrations is equivalent to the geometric mean of the non-$\log_{10}$-transformed concentrations. Although it is generally acknowledged in the scientific community that pathogen concentrations in wastewater are skewed, there is still some debate as to which measure of central tendency is the most appropriate to use (Benke and Hamilton, 2008; Haas, 1996; Karavarsamis and Hamilton, 2010). Pathogen distributions are commonly assumed to be lognormal, however, other distributions such as the "hockey stick" distribution (McBride et al., 2013; Schoen et al., 2017) have also been used.

Results from the literature review identified five studies that rigorously characterized the distribution of pathogen concentrations in wastewater (Table 3-6) (Nappier et al. 2019; Pouillot et al. 2015; Eftim et al. 2017; Pecson et al. 2021; 2022; Jones et al. 2022). In all cases, the pathogen concentration distributions were bell shaped and symmetrical on a log-scale, with the exception of some of the distributions reported by Jones et al. (2022), which were skewed. In some studies (Nappier et al. 2019; Pecson et al. 2021; 2022; Jones et al. 2022), the Shapiro-Wilk test revealed that pathogen concentrations were lognormally distributed. For other studies (Pouillot et al. 2015; Eftim et al. 2017), a comparison of the mean and median values of the distributions, as well as the differences between the medians and upper and lower quantiles (if these values were presented), confirmed that most distributions of $\log_{10}$-transformed concentrations were fairly symmetrical. Most studies focused only on viruses, but Pecson et al. (2021; 2022) rigorously analyzed distributions of the concentrations of *Cryptosporidium* and *Giardia*, in addition to a variety of enteric viruses, in five different wastewater treatment plants, and reported that nearly all concentrations followed lognormal distributions based on Shapiro-Wilk tests.

Table 3-6. Pathogen Concentration and LRV Distributions in Wastewater Treatment Systems

Reference	Microbial Groups	Data Source(s)	Sample Size	Influencing Factors Considered	Matrix	Concentration Distribution	Method for Assessing Distribution	How Non-Detects Were Handled
Nappier et al. (2019)	F-specific, somatic coliphage	Peer-reviewed literature	730	Seasonality Region	Untreated Wastewater	Lognormal	Shapiro-Wilk test	Kaplan-Meier or Maximum Likelihood
Pouillot et al. (2015)	Norovirus, F-specific coliphage	Peer-reviewed literature and government data	2,943	Seasonality Genogroup WWTP	Untreated Wastewater	Log-Symmetrical[1]	Comparison of median, mean, and quantiles of log-transformed concentrations	Turnbull Expectation-Maximization
Eftim et al. (2017)	Norovirus	Peer-reviewed literature and government data	850	Seasonality Region	Untreated Wastewater	Log-Symmetrical[2]	Comparison of median and mean of log-transformed concentrations	Substituted with the LOD
Pecson et al. (2021; 2022)	*Cryptosporidium*, *Giardia*, enteric virus (culture), HAdV (culture), enterovirus (RT-qPCR), HAdV	Laboratory analyses	120	Pathogen Group, Genogroup WWTP	Untreated Wastewater	Lognormal[3]	Shapiro-Wilk test	Maximum Likelihood
Jones et al. (2022)	F-specific, somatic coliphage	Peer-reviewed literature	1,140	Seasonality Region Treatment Level	Untreated Wastewater, Treated Wastewater	Lognormal and other[4]	Shapiro-Wilk test	Not disclosed

[1] The differences between the median and 0.025 quantile were compared with the differences between the 0.975 quantile and the median, and on average the two differences were within 0.1 $\log_{10}$ unit, both for the log-transformed concentrations and for LRVs in biological secondary wastewater treatment processes, indicating symmetrical distributions with minimal skew

[2] Medians were within 0.1 $\log_{10}$ unit from the means for most distributions in this study (average difference between median and mean was 0.06 $\log_{10}$ units), indicating symmetrical distributions with minimal skew

[3] The authors reported that all distributions were log-normal except for two virus datasets (HAdV and norovirus genogroup IA), which were both measured via molecular methods and were highly censored.

[4] In raw wastewater and tertiary-treated wastewater, coliphage distributions were found to have significant deviations from the log-normal distribution. In secondary-treated wastewater, distributions of all coliphages were found to be log-normally distributed. In primary-treated wastewater, distributions of somatic coliphages were log-normally distributed, but distributions of F-specific coliphages deviated from the lognormal distribution.

Relatively little attention has been given in the literature to the characterization of pathogen LRV distributions. Teunis et al. (2009) used a Bayesian model to show posterior distributions of pathogen concentrations and LRVs in several treatment processes used in drinking water treatment systems, including coagulation/sedimentation, coagulation/filtration, slow sand filtration, membrane filtration, and ozone disinfection. The authors reported distributions of LRVs that were bell-shaped but were slightly left skewed (the authors defined the LRV to be negative), displaying a tail toward higher removal. However, the data used by Teunis et al. (2009) were highly censored in most of the treated samples—for some treatment processes, 100% of treated samples were non-detects. For treatment processes such as coagulation-sedimentation, where all treated samples had detectable levels of viruses, LRV distributions were much more symmetrical. Pouillot et al. (2015) performed a meta-analysis of norovirus and male-specific coliphage concentrations and LRVs in biological wastewater treatment plants, also using a hierarchical Bayesian model. While the authors did not publish the posterior distributions for microbial concentrations or LRVs, the distributions were likely symmetrical since the medians were similar to the means and the widths between the 2.5th percentiles and the corresponding medians were similar to the widths between the medians and the corresponding 97.5th percentiles (Pouillot et al. 2015).

3.3.2 Methods for Handling Censored Data (Research Question 3b)

It is technically impossible to measure a pathogen concentration of zero—by definition, a concentration is a quantity per unit volume—and there are practical limitations to the volume of water that can be processed in a laboratory for pathogen analysis. Since pathogens are discrete, randomly distributed particles in water samples, when dealing with low concentrations and small sample volumes, there may be detection limits that are governed by the Poisson distribution (i.e., either the pathogen is there, or it is absent from the sample). If larger volume samples could be processed, it would be possible to detect lower concentrations of pathogens. However, there are also limitations associated with laboratory methods for concentrating and quantifying microorganisms from large volume samples. Thus, censored data are likely to occur from time to time in pathogen LRV validation studies.

Censored observations are defined as low concentrations with values that lie somewhere between zero and the method limit of detection (Helsel 2012). For some methods (such as qPCR-based methods), the limit of detection is not a strict limit, but rather a range of probabilities of detection, which can be estimated (Verbyla et al. 2016). The best way to handle non-detect sample results (i.e., censored data sets) is to avoid getting non-detects in the first place. This can be done by concentrating larger sample volumes while monitoring and maximizing percent recovery, as described previously. However, if data sets include non-detect values, appropriate data reporting and calculation methods need to be used to estimate key statistics such as mean, standard deviation, and percentiles, for the purposes of LRV crediting.

Based on the literature, methods of handling censored data can be broadly divided as statistical and substitution-based methods. With substitution-based methods, all non-detects are substituted with a value of either 0, the limit of detection (LOD), LOD/2, or LOD/√2. The

substitution method is not the best way to calculate mean, standard deviation, and percentiles when you have censored data, but it is acceptable under certain conditions. The advantage of the substitution method is its simplicity. One limitation of the substitution method is the lack of an ability to calculate a confidence interval around the estimate of the mean, the standard deviation, or the percentile estimate. Another limitation of the substitution method is that it introduces a bias in the estimate of the mean, standard deviation, or percentile. For instance, if the limit of detection is substituted for all non-detect samples, then the mean and especially the standard deviation will be overestimated.

The use of more advanced statistical methods is fundamentally a better approach that allows the calculation of more accurate estimates of key statistics (mean, standard deviation, percentiles, etc.) than the substitution methods, based on lower root mean square error (RMSE) (Huynh et al., 2014; Shoari et al., 2015). These methods work by using the uncensored data to characterize the features of the distribution based on the normal data quantiles. Gaps in the quantiles are left to account for the samples with concentrations that were below the limit of detection. Figure 3-3 shows a graphical representation of a censored data set with a total of 12 observations of pathogen gene copies (gc) detected using qPCR, four (33%) of which were below the limit of detection (0.10 $\log_{10}$(gc)/mL), where the MLE method was used to estimate a mean of 0.13 and a standard deviation of 0.056. Based on these values, the 5^{th} percentile of the data set is 0.059, which is well below the limit of detection of 0.1. The most appropriate method to use for censored data sets depends on several factors such as sample size, distribution, skewness, and censoring percentage (Helsel, 2012) (see Figure 3-4). If the distribution is unknown, it has been suggested to use a nonparametric method such as the Kaplan and Meier (KM) method since it does not assume a distribution (Nappier et al. 2019). Also, for low censoring (10%), imputation from a uniform distribution has been used (Canales et al., 2018).

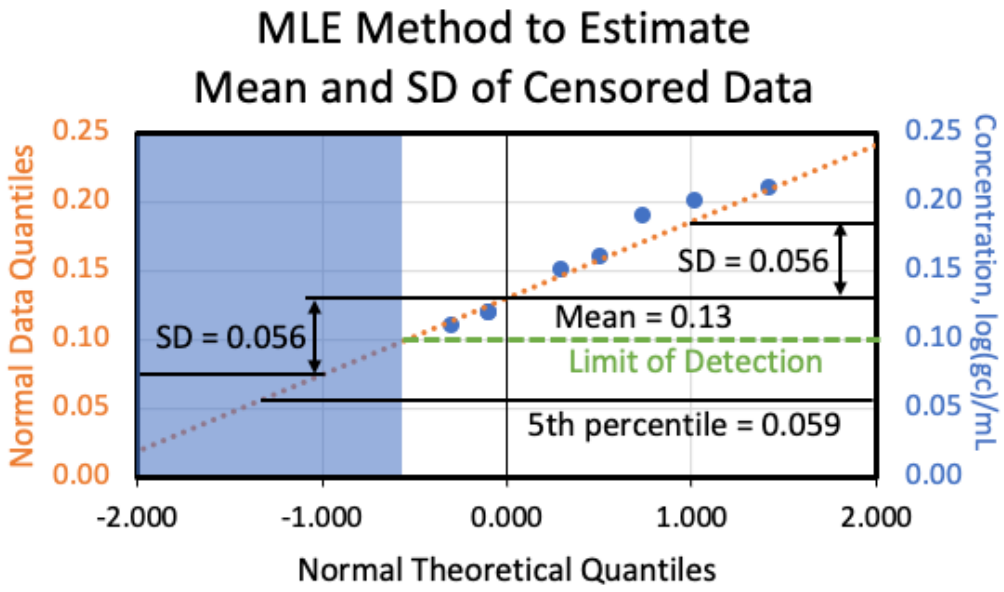

Figure 3-3. Example of the Use of the Maximum Likelihood Estimation (MLE) Method to Estimate the Mean, Standard Deviation, and 5th Percentile of a Censored Data Set of Log-Transformed Pathogen Concentrations.

This figure is an adaptation of an untitled image from page 122 of Assessment of Treatment Plant Performance and Water Quality Data by von Sperling et al. (2020) and is used under a Creative Commons Attribution License.

If the distribution is known (which, based on the results from Table 3-6, appears to be the case), then the maximum likelihood estimation (MLE) method (Cohen 1959; 1961) has been found to have a better result for lower skewed data, especially if the data follows a log-normal distribution (Canales et al., 2018; Shoari et al., 2015). Additionally, Canales et al. (2018) reported that for moderate to severe (35%-90%) censoring, the imputation method using MLE can be used to estimate distribution parameters, and for low (10%) censoring, the imputation method from a uniform distribution can be used. Moreover, regression on order statistics (ROS) and gamma regression on order statistics (GROS) have both been used when the data were highly skewed, regardless of the percentage of censoring (Shoari et al. 2015). Although these are not the only methods for handling censored data, they have been widely used in different studies of waterborne pathogens (Hogan et al. 2012; Rodriguez Alvarez et al. 2015; Masaka et al. 2021; Corrigan et al. 2021; Canales et al. 2018) and other studies with environmental data (Huynh et al. 2014).

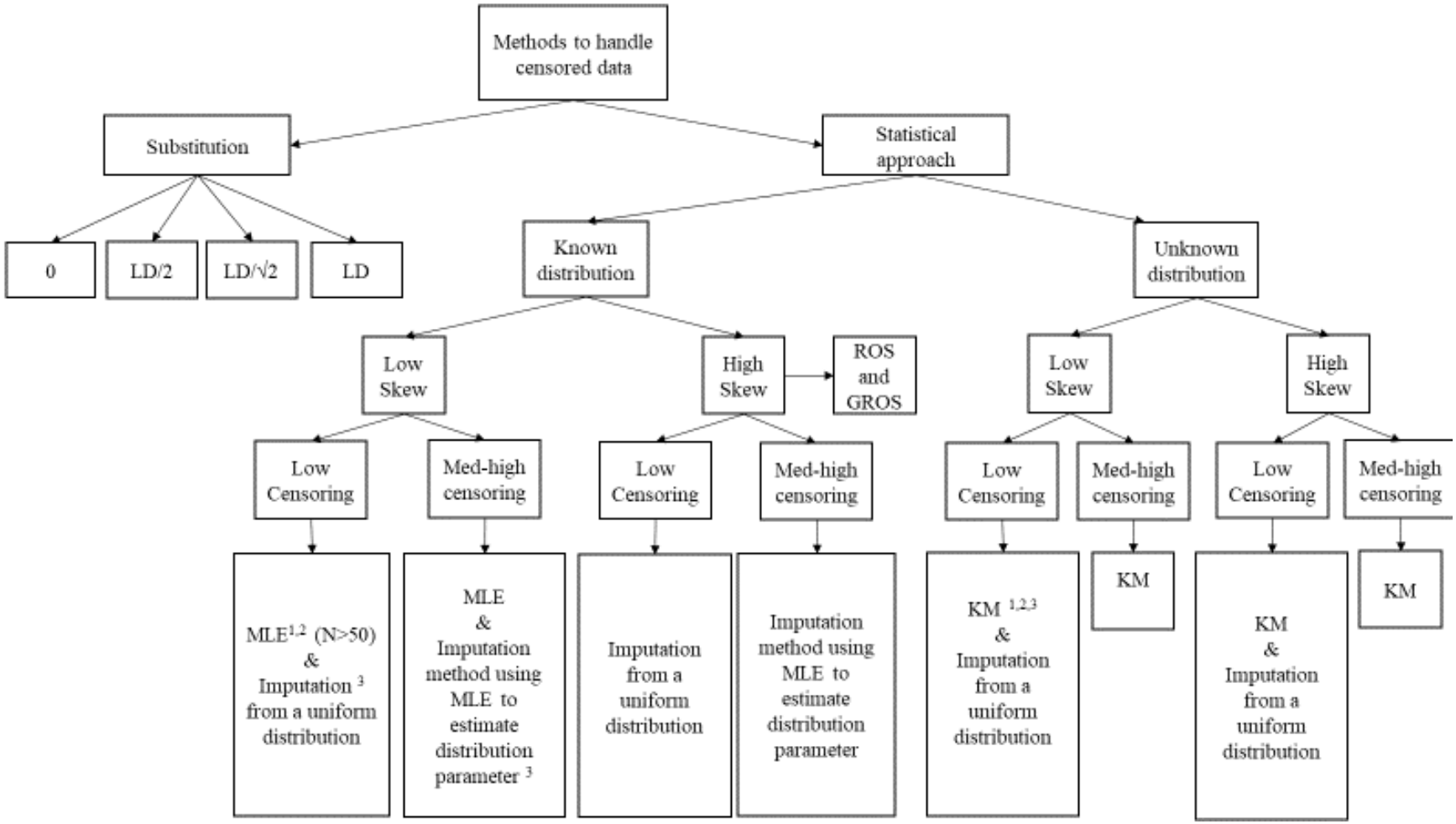

Figure 3-4. Decision Tree for the Use of Statistical Methods for Handling Censored Data Sets.

[1] Huynh et al. (2014); [2] Shoari et al. (2015); [3] Canales et al. (2018)

For example, Corrigan et al. (2021) and Hogan et al. (2012) both used the ROS method for handling censored data, although the percentage of their censored data varied from 31%-95%. Rodriguez-Alvarez et al. (2015) substituted non-detect data with half the LOD and used the KM method for data above the LOD. Nappier et al. (2019) used the MLE method since their data followed a lognormal distribution. Some authors have used different methods, such as Dila et al. (2018), who used a Tobit regression method, assuming a Weibull distribution to handle a censored data set of microbial source tracking markers. Based on the literature, the concentration of pathogens in wastewater generally follow a lognormal distribution. Therefore, it is suitable to use either the MLE method for data with low skewness or the ROS/GROS method for highly skewed data.

3.3.3 Calculation of Pathogen LRVs (Research Question 3c)

3.3.3.1 Definition of LRV

The pathogen LRV is defined as the $\log_{10}$ difference between pathogen concentrations at two points in a treatment system. However, there is a lack of consensus in the literature about how LRV statistics should be calculated from experimental data. In some studies, LRV statistics are calculated by treating influent and effluent samples as completely independent, assuming a distribution (e.g., lognormal) for influent and effluent pathogen concentrations, and characterizing the difference between those distributions. In other studies, influent and effluent samples are treated as paired, LRVs are calculated from each of these paired samples, and statistics are computed directly from this sample of calculated LRVs. Because many wastewater treatment processes have retention times of several hours or more, there has been critique that the effluent sample does not correspond directly to the influent sample. Some authors have accounted for mean HRTs when collecting grab samples at influent and effluent locations, others have used composite sampling. Even when composite samples are used, authors do not often account for HRT offsets, even though arguably this could influence the results. Unless the reactor hydraulics approach plug flow, offsetting sample collection times to account for HRT is not a perfect solution, as dispersion interferes with the ability to sample the same "plug" of wastewater at the influent and effluent locations. One study quantified human adenovirus, polyomavirus, *Microviridae* (somatic coliphages), and *E. coli* in an activated sludge system, finding no significant differences between LRVs calculated from grab samples collected with an HRT offset compared to grab samples collected simultaneously from the influent and effluent location, using paired sample statistical tests (Sidhu et al., 2017).

The $\log_{10}$ difference between concentrations at two points is mathematically equivalent to the difference between the $\log_{10}$-transformed concentrations (von Sperling et al., 2018). However, when concentration data are collected from multiple samples over a period of time (e.g., with N_o concentration measurements at the influent and N_e concentration measurements at the effluent), there are several different ways to calculate the central tendency and other statistics for the LRV. Depending on the approach used, the calculated SD and quantiles can be very different. If the dataset is not balanced (i.e., if the number of samples collected at the influent point is different from the number of samples collected at the effluent point), then even the

values for measures of central tendency (e.g., mean, median) can be different, depending on which approach is used.

3.3.3.2 Three Approaches for Calculating LRV Statistics

One common way that the LRV has been calculated in the peer-reviewed scientific literature and in LRV validation studies is by characterizing the influent and effluent concentration distributions, then treating the LRV as the difference between those two distributions. Using this approach, the influent and effluent samples are treated as completely independent from each other. The mean LRV is calculated by taking the mean of all $\log_{10}$-transformed influent and effluent concentrations, then calculating the difference between those means (Equation 3-3). The SD is calculated using Equation 3-6, then quantiles can be estimated using Equation 3-8. This will be referred to as the **complete independence approach**.

Another approach that has been commonly used in the scientific literature is to take the $\log_{10}$ difference of each pair of influent and effluent concentrations collected on the same day, then to calculate the statistics on those $\log_{10}$ differences (e.g., mean, SD, quantiles, etc.). Using this approach, the influent and effluent samples are treated as paired (Equation 3-4) (von Sperling et al., 2020). This will be referred to as the **paired samples approach**.

More recently, a new approach has been proposed for calculating LRV statistics (Tchobanoglous et al. 2022). This method, which is based on the proof that the difference of two correlated normal random variables is also normally distributed (Rabbani, 2017), accounts for the fact that the influent and effluent concentrations will likely correlate with each other. Using this approach, the mean is calculated using the same equation as with the complete independence method (Equation 3-3). However, the covariance of the data is calculated (Equation 3-5) and used to find the SD of the LRV (Equation 3-7). Quantiles can then be estimated using Equation 3-8. This will be referred to as the **covariance approach.**

$$\mathrm{LRV}_{\bar{C}} = \frac{\sum_{i=1}^{N_o} C_{o,i}}{N_o} - \frac{\sum_{i=1}^{N_e} C_{e,i}}{N_e} \qquad \text{(Equation 3-3)}$$

$$\overline{\mathrm{LRV}}_C = \frac{1}{N}\sum_{i=1}^{N} C_{o,i} - C_{e,i} \qquad \text{(Equation 3-4)}$$

$$CoV = \frac{1}{N-1}\sum_{i=1}^{N}\left(C_{o,i} - \frac{\sum_{i=1}^{N_o} C_{o,i}}{N_o}\right)\left(C_{e,i} - \frac{\sum_{i=1}^{N_e} C_{e,i}}{N_e}\right) \qquad \text{(Equation 3-5)}$$

$$SD_{LRV,IND} = \sqrt{{SD_{C_o}}^2 + {SD_{C_e}}^2} \qquad \text{(Equation 3-6)}$$

$$SD_{LRV,CoV} = \sqrt{{SD_{C_o}}^2 + {SD_{C_e}}^2 - 2CoV} \qquad \text{(Equation 3-7)}$$

$$Q_p = \mathrm{LRV}_{\bar{C}} + Z \cdot SD_{LRV} \qquad \text{(Equation 3-8)}$$

where $C_{o,i}$ is the $\log_{10}$-transformed microbial concentration at the influent for the i^{th} sample
$C_{e,i}$ is the $\log_{10}$-transformed microbial concentration at the effluent for the i^{th} sample
SD_{Co} is the standard deviation of the $\log_{10}$-transformed microbial concentrations at the influent
SD_{Ce} is the standard deviation of the $\log_{10}$-transformed microbial concentrations at the effluent
$SD_{LRV,IND}$ is the standard deviation of the LRV (using the complete independence approach)

$SD_{LRV,CoV}$ is the standard deviation of the LRV (using the covariance approach)
N_o is the number of samples analyzed at the influent
N_e is the number of samples analyzed at the effluent
N is equal to N_o and N_e (when $N_o = N_e$)
Q_p is the p^{th} quantile, where Z is the Z-score for the normal distribution

It should be noted that if the sample size N_o at the influent is equal to the sample size N_e at the effluent, then Equation 3-3 and Equation 3-4 will produce equivalent estimates of the mean LRV. However, the assumption of complete independence vs. paired samples affects the estimate of SD of the LRV, and consequently the estimate of LRV quantiles, such as the 5th percentile, will be different for the three methods described above.

3.3.3.3 The Complete Independence Approach (and the "Monte Carlo" Method)

Authors of some past LRV validation studies for water reuse systems have used an approach called the "Monte Carlo method" (*e.g.*, City of San Diego, 2018; Woodard & Curran, 2020). In this approach, a statistical method such as MLE is used to identify the mean and SD of $\log_{10}$-transformed pathogen concentrations based on samples collected at the influent and the effluent of the treatment process(es) being studied. Then, assuming the influent and effluent concentrations are log-normally distributed, a Monte Carlo simulation is used to predict the probability distribution of LRVs by selecting one influent and one effluent concentration from the distribution. This is typically repeated a total of 10,000 times, to construct a distribution of LRVs. Quantiles, such as the 5th percentile can be determined from that simulated distribution of the LRV. The use of a Monte Carlo simulation model is not actually needed in this case since the difference between two log-normal distributions can be determined analytically. For example, suppose X is normally distributed with mean μ_x and variance σ_x^2, and Y is normally distributed with mean μ_y and variance σ_y^2. Assuming X and Y are completely independent, X – Y will also be normally distributed with mean $\mu_x - \mu_y$ and variance $\sigma_x^2 + \sigma_y^2$ (Weisstein, 2022). Quantiles from the distribution of X – Y (e.g., the 5th percentile) can be calculated directly, using the equations described above.

This approach produces the lowest estimates of a low quantile (such as the 5th percentile) compared to the other approaches. However, there are several disadvantages to using this approach. First, the assumption that the LRV follows a normal distribution is not technically correct since the bounds of the normal distribution are negative infinity to infinity. For pathogens that cannot regrow outside of a human host, it is impossible for LRV to be less than zero. Second, the influent and effluent pathogen concentrations of a wastewater treatment process are not likely to be independent from each other. Pathogen concentrations in the influent often fluctuate seasonally, and when concentrations are higher in the influent, they may also be higher in the effluent. If the concentration of pathogens at the influent of the treatment process(es) is higher, it is possible (even probable) that the concentration of pathogens at the effluent of the treatment process(es) may also be higher. In fact, results from a meta-analysis of norovirus showed that concentrations in untreated wastewater were significantly higher between March and April (genogroup I) or between January and April (genogroup II) than they were during other months, but there was no significant seasonal fluctuations on the LRVs (Pouillot et al. 2015). In the same study, the authors found that male-specific coliphage concentrations in untreated wastewater were significantly higher from

February to June and significantly lower from August to December, but LRVs were significantly lower from February to June and significantly higher from August to December (Pouillot et al. 2015). The authors did not test correlation between influent and effluent concentrations, but the trends reported for seasonal fluctuations indicate that correlations likely exist.

3.3.3.4 The Paired Samples Approach

The paired samples approach is at the opposite end of the spectrum from the complete independence approach. This approach will typically produce the highest estimates of a low quantile (such as the 5th percentile) compared to the other approaches. One advantage of this approach is that the quantiles are calculated directly from the data, without making a distributional assumption about the LRV. This avoids the problems with the complete independence approach, which assumes an unbounded normal distribution that includes unrealistic values for LRV. There are, however, disadvantages to using the paired samples approach, especially for flow-through reactors with very long retention times. The influent water sample is not temporally aligned with the effluent water sample, especially when grab samples are used. As such, this approach is also likely incorrect for most wastewater treatment processes, due to hydraulic mixing that takes place within wastewater treatment reactors. No evidence was found for the use of this approach in practice for LRV validation studies, although it has been used in scientific publications (e.g., Sidhu et al., 2017).

The one situation when it may be more appropriate to use the paired samples approach over the other approaches is for a batch reactor. In this case, the influent sample (before treatment) can be matched with the effluent sample (after treatment).

3.3.3.5 Covariance Approach

The covariance approach generally produces an estimate of the 5th percentile that is somewhere between the values that would be calculated using the paired sample approach and the complete independence approach. Given the evidence of correlation between influent and effluent pathogen concentrations in wastewater treatment systems (e.g., Pouillot et al. 2015), the covariance approach appears to be the most appropriate approach to use for flow-through treatment systems. Tchobanoglous et al. (2022) recommended rank pairing the influent and effluent data when using the covariance approach, however there is not a lot of evidence to support the use of rank-pairing (as opposed to pairing samples based on the date of sample collection). The way samples are paired will affect estimates of percentiles of the LRV using this method. One disadvantage to this approach is that, like the complete independence approach, the covariance approach also assumes that the LRV follows the normal distribution, which is unbounded and includes values that may be unrealistic (such as negative removal) at the tails of the distribution.

3.3.3.6 Other Approaches: Bayesian Models

One limitation of the approaches described above are that they either rely on assumptions about the independence of samples or they rely on assumptions about the underlying distributions of pathogen concentration data. Bayesian statistics is an alternative approach for analyzing LRVs that is not limited by these assumptions. The three approaches described above (independence, paired samples, and covariance) are all part of the frequentist philosophy of

statistics, which assumes that parameters are fixed and that data vary. Bayesian statistics follow a different philosophy, which assumes that data is fixed and parameters vary.

Bayesian models allow for the characterization of LRV distributions without assuming a particular distribution such as the normal distribution. Bayesian models have been used to model distributions of pathogen concentrations and LRVs for treatment processes (Seis et al. 2020; Pouillot et al. 2015; Teunis et al. 2009). One advantage of Bayesian models is that they can be constructed using a hierarchical approach to separate out different sources of variability. Another advantage is that existing information about pathogen LRV distributions can be used as what is known as a prior distribution, to reduce the uncertainty associated with LRV estimates based on new data. Prior distributions must be assumed for the parameters being characterized in the Bayesian model. Data are then used to provide information to update the prior distributions and develop what are known as posterior distributions, which characterize uncertainty in the parameters of interest. However, the use of prior distributions can also be a disadvantage for inexperienced users. If there is no prior knowledge about the distribution of an LRV, then it is possible to choose what are known as flat or uninformative prior distributions, which are only supposed to have a negligible influence on the posterior distributions. However, choosing appropriate uninformative prior distributions requires experience. Another disadvantage of the use of Bayesian models is that their implementation is more complex and requires the use of Markov Chain Monte Carlo (MCMC), which sometimes also necessitates considerable computing power. As such, people using these methods must be well-trained in Bayesian statistical theory.

3.3.3.7 Unique Situations with Parallel Treatment Processes

There are some situations where the use of Equations 3-3 and 3-4 and the approaches described above is not so straightforward. For instance, consider a wastewater treatment system that has a common influent wastewater source that is split into parallel treatment trains with different unit treatment processes and different daily flow rates, where the effluent from all parallel trains is analyzed independently for pathogens, then blended at the end (e.g., the OCWD groundwater replenishment system [Polanco et al. 2022]). In this case, pathogen loadings can be calculated based on the flow rates and the pathogen concentrations, and the LRV can be calculated from the loadings (Equations 3-9 and 3-10). Alternatively, sampling plans can be designed to collect individual samples from the influent and effluent of each parallel treatment train, as well as the overall blended influent and the overall blended effluent, although this would increase the number of samples and the cost of the study.

$$\mathrm{LRV}_{\bar{L}} = \frac{\sum_{i=1}^{N_o} \log_{10}(C_{o,i}Q_{o,i})}{N_o} - \frac{\sum_{i=1}^{N_e} \log_{10}(C_{e,i}Q_{e,i})}{N_e} \qquad \text{(Equation 3-9)}$$

$$\overline{\mathrm{LRV}}_L = \frac{1}{N}\sum_{i=1}^{N}\left(\log_{10}\left(\frac{C_{o,i}Q_{o,i}}{C_{e,i}Q_{e,i}}\right)\right) \qquad \text{(Equation 3-10)}$$

where C_o is the (non-log-transformed) microbial concentration in the influent
C_e is the (non-log-transformed) microbial concentration in the effluent
Q_o is the influent flow rate
Q_e is the effluent flow rate
N_o is the number of samples analyzed at the influent point
N_e is the number of samples analyzed at the effluent point

N is equal to N_o and N_e (when $N_o = N_e$)

To assess these different approaches to calculate the LRV in systems with parallel treatment trains using different treatment processes, fictitious pathogen concentration and LRV distributions were modeled using Monte Carlo simulations in Excel with 10,000 iterations. Pathogen concentrations in untreated wastewater were randomly selected from a lognormal distribution and LRVs were randomly selected from a normal distribution. Flow rates were also simulated using a normal distribution. To make the simulations more realistic, the means and SDs of the LRVs and flow rates were changed between summer and winter seasons. One scenario was created with a single treatment process, and a second scenario was created with several treatment processes in parallel, each with different flow rates and different mean LRVs. The simulated influent and effluent concentrations were used to calculate LRV statistics, using Equations 3-3, 3-4, 3-9, and 3-10. For the scenario with multiple treatment processes in parallel, Equation 3-4 was used to calculate the LRV for each individual treatment process, then the overall LRV was estimated as the flow-weighted average of the individual LRVs.

When there was only one treatment process, the mean LRVs calculated using Equations 3-3, 3-4, 3-9, and 3-10 were identical. However, Equations 3-3 and 3-9 (the complete independence approach with either the concentrations or the loadings) produce different estimates of the mean LRV. This happens because the flow rate can vary independently from the pathogen concentrations, and the loading is a product of the concentration (which follows a skewed distribution) and the flow rate (which, in this simulation, was chosen from a normal distribution). The product of these two distributions is not normally distributed, which is why the estimated mean is different than the mean calculated using the concentrations. As such, the use of loadings to calculate LRVs should also be avoided, unless the paired samples approach is used (see Section 3.3.3.4). When it comes to controlling microbial risks from drinking water, pathogen concentrations are more important than pathogen loadings, since the ingested dose for a person drinking water is proportional to the pathogen concentration in the water.

In the simulations of multiple treatment trains in parallel, the flow-weighted average of LRVs for each parallel treatment train regularly overestimated the LRV relative to the overall LRV calculated based on the loadings in and out of the entire system. This is consistent with findings reported by Schmidt et al. (2020). Thus, the approach of calculating flow-weighted averages of LRVs measured from multiple parallel processes is incorrect and should be avoided.

3.4 Conclusions

3.4.1 Conclusions about Experimental Study Plan Design

Based on the findings from this study, sample sizes of 12 to 24 are recommended for validating LRV credits, potentially with a precision of 0.1 $\log_{10}$ units. However, the precision of the LRV credit being sought should be proportional to the width of the 95% confidence interval on the statistic used to claim LRV credits (e.g., the 5th percentile). The recommended sampling frequency is once or twice monthly, to include all seasons of the year and to capture a wide range of operating conditions. It is important to capture all characteristics of treatment plant

operation, including but not limited to variability in source water quality and operating conditions for all processes being studied. It is important to measure design, operational, and environmental factors known to directly influence pathogen reduction, as well as OMPs that correlate with LRVs. Due to hourly variations in pathogen concentrations in untreated wastewater, which can span orders of magnitude, the use of composite sampling may be helpful, especially in small systems that lack flow equalization, and for pathogens with low prevalence and high diurnal variability. The standardization of data collection protocols is recommended to compile a large data set for future meta-analysis and LRV modeling, which could potentially augment validation studies in the future.

3.4.2 Conclusions about Laboratory Methods and Costs

Pathogen LRV validation studies with 20 – 24 samples at two or three locations seeking credits for viruses and protozoan pathogens will typically cost around $300,000 but could cost up to $500,000 if all QA/QC protocols are followed. Typical non-laboratory (soft) costs ranged from approximately $100,000 to $300,000 per study, with approximately 10% corresponding to study planning, 25% to sample collection and shipping, 25% to data analysis, 15% to report writing, and 25% to time spent working with regulators and external advisory panels. The costs of studies could be reduced by ~$50,000 if study planning, data management, and data analysis approaches are standardized. Laboratory methods are mostly standardized, apart from the methods used to concentrate waterborne viruses. There are dozens of different virus concentration methods described in the literature, but the adsorption-extraction, ultrafiltration, ultracentrifugation, PEG precipitation, and skimmed milk flocculation methods have similar recoveries, and are the most suitable for concentrating viruses from wastewater. Concentrations should be corrected for recovery if there are large differences in efficiencies, and if the difference between the corrected LRV and the uncorrected LRV is greater than the level of precision used to issue the LRV credit (e.g., 0.1 $\log_{10}$ units).

3.4.3 Conclusions about Statistical Methods for Data Analysis

Pathogen concentration distributions are skewed and LRVs have symmetrical bell-shaped distributions. The few studies that used statistical analyses to assess pathogen concentration distributions have mostly concluded that the distributions are lognormal. Pathogen LRV validation studies should be carefully designed to avoid non-detect results, but when analyzing censored datasets, the MLE or ROS methods should be used when the data are lognormal, and the KM method should be used otherwise. The calculation of LRV percentiles for studies of most systems should consider correlation between influent and effluent concentrations by using the covariance approach. The paired samples approach is suitable for batch reactors and systems approaching plug flow hydraulics. Bayesian models can also be used if an experienced statistician is present on the data analysis team.

Industry experts concurred that web-based guidance materials can be helpful for practitioners and state regulators involved in conducting and reviewing pathogen LRV validation studies. Specifically, there is a need to standardize the processes of data collection and data analysis. An LRV validation study cost estimator tool has been developed to assist with study planning, and it is freely available at the following website: https://credit.waterpathogens.org/form/calculator.

APPENDIX A

Full List of References from Literature Review

The following is a full list of the references that were reviewed as part of the systematic literature review process, broken down by research question:

Research Question 1b. What microbial groups or process indicators should be measured?

References Cited in Section 3.1.2
52 papers were identified in the search. After initial screening, the full texts of 13 papers were reviewed. Additional articles were identified by reviewing works cited from other papers. Other papers were identified based on the authors' familiarity with other literature on the topic.

Identified in original search (indicates articles that were cited in this section of the report)*

Barrios-Hernández, M.L., Pronk, M., Garcia, H., Boersma, A., Brdjanovic, D., van Loosdrecht, M.C.M., Hooijmans, C.M., 2020. Removal of bacterial and viral indicator organisms in full-scale aerobic granular sludge and conventional activated sludge systems. Water Research X 6, 100040. https://doi.org/10.1016/j.wroa.2019.100040

Bauer, R., Dizer, H., Graeber, I., Rosenwinkel, K.-H., López-Pila, J.M., 2011. Removal of bacterial fecal indicators, coliphages and enteric adenoviruses from waters with high fecal pollution by slow sand filtration. Water Research 45, 439–452. https://doi.org/10.1016/j.watres.2010.08.047

Frederick, G.L., Lloyd, B.J., 1995. Evaluation of Serratia marcescens bacteriophage as a tracer and a model for virus removal in waste stabilisation ponds. Water Science and Technology, Waste Stabilisation Ponds and the Reuse of Pond Effluents 31, 291–302. https://doi.org/10.1016/0273-1223(95)00517-Q

Kauppinen, A., Martikainen, K., Matikka, V., Veijalainen, A.-M., Pitkänen, T., Heinonen-Tanski, H., Miettinen, I.T., 2014. Sand filters for removal of microbes and nutrients from wastewater during a one-year pilot study in a cold temperate climate. Journal of Environmental Management 133, 206–213. https://doi.org/10.1016/j.jenvman.2013.12.008

Lewis, G.D., Austin, F.J., Loutit, M.W., Sharples, K., 1986. Enterovirus removal from sewage. Water Research 20, 1291–1297. https://doi.org/10.1016/0043-1354(86)90160-0

Marti, E., Monclús, H., Jofre, J., Rodriguez-Roda, I., Comas, J., Balcázar, J.L., 2011. Removal of microbial indicators from municipal wastewater by a membrane bioreactor (MBR). Bioresource Technology 102, 5004–5009. https://doi.org/10.1016/j.biortech.2011.01.068

Papadimitriou, C.A., Papatheodoulou, A., Takavakoglou, V., Zdragas, A., Samaras, P., Sakellaropoulos, G.P., Lazaridou, M., Zalidis, G., 2010. Investigation of protozoa as indicators of wastewater treatment efficiency in constructed wetlands. Desalination 250, 378–382.

https://doi.org/10.1016/j.desal.2009.09.060

Reinoso, R., Torres, L.A., Bécares, E., 2008. Efficiency of natural systems for removal of bacteria and pathogenic parasites from wastewater. Science of The Total Environment 395, 80–86. https://doi.org/10.1016/j.scitotenv.2008.02.039

Sellaoui, L., Badawi, M., Monari, A., Tatarchuk, T., Jemli, S., Luiz Dotto, G., Bonilla-Petriciolet, A., Chen, Z., 2021. Make it clean, make it safe: A review on virus elimination via adsorption. Chemical Engineering Journal 412, 128682. https://doi.org/10.1016/j.cej.2021.128682

*Sidhu, J.P.S., Sena, K., Hodgers, L., Palmer, A., Toze, S., 2018. Comparative enteric viruses and coliphage removal during wastewater treatment processes in a sub-tropical environment. Sci. Total Environ. 616–617, 669–677. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.10.265

Stefanakis, A.I., Bardiau, M., Trajano, D., Couceiro, F., Williams, J.B., Taylor, H., 2019. Presence of bacteria and bacteriophages in full-scale trickling filters and an aerated constructed wetland. Science of The Total Environment 659, 1135–1145. https://doi.org/10.1016/j.scitotenv.2018.12.415

*Verbyla, M.E., Mihelcic, J.R., 2015. A review of virus removal in wastewater treatment pond systems. Water Res. 71, 107-124. https://doi.org/10.1016/j.watres.2014.12.031

*Wu, Z., Greaves, J., Arp, L., Stone, D., Bibby, K., 2020. Comparative fate of CrAssphage with culturable and molecular fecal pollution indicators during activated sludge wastewater treatment. Environ. Int. 136, 105452. https://doi.org/https://doi.org/10.1016/j.envint.2019.105452

Identified by reviewing works cited from other papers

Agulló-Barceló, M., Oliva, F., Lucena, F., 2013. Alternative indicators for monitoring Cryptosporidium oocysts in reclaimed water. Environ. Sci. Pollut. Res. 20, 4448–4454. https://doi.org/10.1007/s11356-012-1400-4

Amarasiri, M., Kitajima, M., Nguyen, T.H., Okabe, S., Sano, D., 2017. Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater reclamation: Review. Water Res. 121, 258–269. https://doi.org/10.1016/j.watres.2017.05.035

Dias, E., Ebdon, J., Taylor, H., 2018. The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. Water Res. 129, 172–179. https://doi.org/10.1016/j.watres.2017.11.022

McMinn, B.R., Ashbolt, N.J., Korajkic, A., 2017. Bacteriophages as indicators of faecal pollution and enteric virus removal. Lett. Appl. Microbiol. 65, 11–26. https://doi.org/10.1111/lam.12736

Stelma, G.N., 2018. Use of bacterial spores in monitoring water quality and treatment. J. Water Health 16, 491–500. https://doi.org/10.2166/wh.2018.013

Truchado, P., Garre, A., Gil, M.I., Simón-Andreu, P.J., Sánchez, G., Allende, A., 2021. Monitoring of human enteric virus and coliphages throughout water reuse system of wastewater treatment plants to irrigation endpoint of leafy greens. Sci. Total Environ. 782, 146837.

https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.146837

Wen, Q., Tutuka, C., Keegan, A., Jin, B., 2009. Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants. J. Environ. Manage. 90, 1442-1447. https://doi.org/10.1016/j.jenvman.2008.09.002

Identified due to authors' familiarity with other literature on the topic

Gerba, C.P., Betancourt, W.Q., Kitajima, M., 2017. How much reduction of virus is needed for recycled water: A continuous changing need for assessment? Water Res. 108, 25–31.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., Rose, J.B., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl Env. Microbiol 71, 3163-3170.

Momba, M., Edbon, J., Kamika, I., Verbyla, M., 2019. Using indicators to assess microbial treatment and disinfection efficacy, in: J.B. Rose and B. Jiménez-Cisneros, (eds) Global Water Pathogen Project (Ed.), A.Farnleitner, and A. Blanch (Eds) Part 2 Indicators and Microbial Source Tracking Markers. UNESCO, Michigan State University, E. Lansing, MI.

Naughton, C.C., Rousselot, O., 2017. Activated Sludge, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Pecson, B.M., Martin, L.V., Kohn, T., 2009. Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: Advantages and limitations of an enzymatic treatment to reduce false-positive results. Appl. Environ. Microbiol. 75, 5544–5554. https://doi.org/10.1128/AEM.00425-09

Rose, J.B., Jiménez-Cisneros, B.E. (Eds.), 2019. Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. Global Water Pathogen Project (GWPP); Michigan State University, UNESCO, E. Lansing, MI, USA.

Verbyla, M.E., Rousselot, O., 2018. Membrane Bioreactors, in: Rose, J.B., Jiménez-Cisneros, B. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Wigginton, K.R., Pecson, B.M., Sigstam, T., Bosshard, F., Kohn, T., 2012. Virus inactivation mechanisms: Impact of disinfectants on virus function and structural integrity. Environ. Sci. Technol. 46, 12069–12078. https://doi.org/10.1021/es3029473

Research Question 1c. Where, how, when, and how frequently should samples be collected for site specific studies, based on temporal trends in pathogen concentrations and LRVs?

References Cited in Section 3.1.3

107 papers were identified in the search. After initial screening, the full texts of 13 papers were reviewed. Additional articles were identified by reviewing works cited from other papers. Other papers were identified based on the authors' familiarity with other literature on the topic.

Identified in original search (indicates articles that were cited in this section of the report)*

Arora, S., Kazmi, A.A., 2015. The effect of seasonal temperature on pathogen removal efficacy of vermifilter for wastewater treatment. Water Research 74, 88–99. https://doi.org/10.1016/j.watres.2015.02.001

Ballesté, E., Pascual-Benito, M., Martín-Díaz, J., Blanch, A.R., Lucena, F., Muniesa, M., Jofre, J., García-Aljaro, C., 2019. Dynamics of crAssphage as a human source tracking marker in potentially faecally polluted environments. Water Research 155, 233–244. https://doi.org/10.1016/j.watres.2019.02.042

*Barril, P.A., Fumian, T.M., Prez, V.E., Gil, P.I., Martínez, L.C., Giordano, M.O., Masachessi, G., Isa, M.B., Ferreyra, L.J., Ré, V.E., Miagostovich, M., Pavan, J.V., Nates, S.V., 2015. Rotavirus seasonality in urban sewage from Argentina: Effect of meteorological variables on the viral load and the genetic diversity. Environmental Research 138, 409–415. https://doi.org/10.1016/j.envres.2015.03.004

Campos, C.J.A., Avant, J., Lowther, J., Till, D., Lees, D.N., 2016. Human norovirus in untreated sewage and effluents from primary, secondary and tertiary treatment processes. Water Research 103, 224–232. https://doi.org/10.1016/j.watres.2016.07.045

El Hamouri, B., Khallayoune, K., Bouzoubaa, K., Rhallabi, N., Chalabi, M., 1994. High-rate algal pond performances in faecal coliforms and helminth egg removals. Water Research 28, 171–174. https://doi.org/10.1016/0043-1354(94)90131-7

Francy, D.S., Stelzer, E.A., Bushon, R.N., Brady, A.M.G., Williston, A.G., Riddell, K.R., Borchardt, M.A., Spencer, S.K., Gellner, T.M., 2012. Comparative effectiveness of membrane bioreactors, conventional secondary treatment, and chlorine and UV disinfection to remove microorganisms from municipal wastewaters. Water Research 46, 4164–4178. https://doi.org/10.1016/j.watres.2012.04.044

Greenwald, H.D., Kennedy, L.C., Hinkle, A., Whitney, O.N., Fan, V.B., Crits-Christoph, A., Harris-Lovett, S., Flamholz, A.I., Al-Shayeb, B., Liao, L.D., Beyers, M., Brown, D., Chakrabarti, A.R., Dow, J., Frost, D., Koekemoer, M., Lynch, C., Sarkar, P., White, E., Kantor, R., Nelson, K.L., 2021. Tools for interpretation of wastewater SARS-CoV-2 temporal and spatial trends demonstrated with data collected in the San Francisco Bay Area. Water Research X 12, 100111. https://doi.org/10.1016/j.wroa.2021.100111

Li, B., Di, D.Y.W., Saingam, P., Jeon, M.K., Yan, T., 2021. Fine-Scale Temporal Dynamics of SARS-CoV-2 RNA Abundance in Wastewater during A COVID-19 Lockdown. Water Research 197, 117093. https://doi.org/10.1016/j.watres.2021.117093

*Madoux-Humery, A.-S., Dorner, S., Sauvé, S., Aboulfadl, K., Galarneau, M., Servais, P., Prévost, M., 2013. Temporal variability of combined sewer overflow contaminants: Evaluation of wastewater micropollutants as tracers of fecal contamination. Water Research 47, 4370–4382.

https://doi.org/10.1016/j.watres.2013.04.030

Pascual-Benito, M., Nadal-Sala, D., Tobella, M., Ballesté, E., García-Aljaro, C., Sabaté, S., Sabater, F., Martí, E., Gracia, C.A., Blanch, A.R., Lucena, F., 2020. Modelling the seasonal impacts of a wastewater treatment plant on water quality in a Mediterranean stream using microbial indicators. Journal of Environmental Management 261, 110220. https://doi.org/10.1016/j.jenvman.2020.110220

Rames, E., Roiko, A., Stratton, H., Macdonald, J., 2016. Technical aspects of using human adenovirus as a viral water quality indicator. Water Research 96, 308–326. https://doi.org/10.1016/j.watres.2016.03.042

Singh, V., Suthar, S., 2021. Occurrence, seasonal variations, and ecological risk of pharmaceuticals and personal care products in River Ganges at two holy cities of India. Chemosphere 268, 129331. https://doi.org/10.1016/j.chemosphere.2020.129331

*Tolouei, S., Burnet, J.-B., Autixier, L., Taghipour, M., Bonsteel, J., Duy, S.V., Sauvé, S., Prévost, M., Dorner, S., 2019. Temporal variability of parasites, bacterial indicators, and wastewater micropollutants in a water resource recovery facility under various weather conditions. Water Research 148, 446–458. https://doi.org/10.1016/j.watres.2018.10.068

Identified by reviewing works cited from other papers

Casanovas-Massana, A., Costa, F., Riediger, I.N., Cunha, M., de Oliveira, D., Mota, D.C., Sousa, E., Querino, V.A., Nery, N., Reis, M.G., Wunder, E.A., Diggle, P.J., Ko, A.I., 2018. Spatial and temporal dynamics of pathogenic Leptospira in surface waters from the urban slum environment. Water Res. 130, 176–184. https://doi.org/https://doi.org/10.1016/j.watres.2017.11.068

Guo, B., Liu, C., Gibson, C., Frigon, D., 2019. Wastewater microbial community structure and functional traits change over short timescales. Sci. Total Environ. 662, 779–785. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.01.207

Morton, V.K., Thomas, M.K., McEwen, S.A., 2015. Estimated hospitalizations attributed to norovirus and rotavirus infection in Canada, 2006-2010. Epidemiol. Infect. 143, 3528–3537. https://doi.org/10.1017/S0950268815000734

Sidhu, J.P.S., Ahmed, W., Palmer, A., Smith, K., Hodgers, L., Toze, S., 2017. Optimization of sampling strategy to determine pathogen removal efficacy of activated sludge treatment plant. Environ. Sci. Pollut. Res. 24, 19001–19010. https://doi.org/10.1007/s11356-017-9557-5

Identified due to authors' familiarity with other literature on the topic

Ahmed, W., Bivins, A., Bertsch, P.M., Bibby, K., Gyawali, P., Sherchan, S.P., Simpson, S.L., Thomas, K. V., Verhagen, R., Kitajima, M., Mueller, J.F., Korajkic, A., 2021. Intraday variability of indicator and pathogenic viruses in 1-h and 24-h composite wastewater samples: Implications for wastewater-based epidemiology. Environ. Res. 193, 110531. https://doi.org/10.1016/j.envres.2020.110531

Chalapati Rao, V., Metcalf, T.G., Melnick, J.L., 1987. Removal of indigenous rotaviruses during primary settling and activated-sludge treatment of raw sewage. Water Res. 21, 171–177. https://doi.org/10.1016/0043-1354(87)90046-7

Curtis, K., Keeling, D., Yetka, K., Larson, A., Gonzalez, R., 2020. Wastewater SARS-CoV-2 Concentration and Loading Variability from Grab and 24-Hour Composite Samples. medRxiv 2020.07.10.20150607.

Research Question 1d. What type of ongoing performance monitoring should be required?

References Cited in Section 3.1.4

32 papers were identified in the search. After initial screening, the full texts of 6 papers were reviewed. No qualifying articles were identified after that review. As such, articles were identified based on the authors' familiarity with literature on the topic (e.g., through the Global Water Pathogen Project literature review).

Identified in original search (but not cited)

Carvajal, G., Roser, D.J., Sisson, S.A., Keegan, A., Khan, S.J., 2017. Bayesian belief network modelling of chlorine disinfection for human pathogenic viruses in municipal wastewater. Water Research 109, 144–154. https://doi.org/10.1016/j.watres.2016.11.008

Cruz, M.C., Romero, L.C., Vicente, M.S., Rajal, V.B., 2017. Statistical approaches to understanding the impact of matrix composition on the disinfection of water by ultrafiltration. Chemical Engineering Journal 316, 305–314. https://doi.org/10.1016/j.cej.2017.01.081

Munasinghe-Arachchige, S.P., Delanka-Pedige, H.M.K., Henkanatte-Gedera, S.M., Tchinda, D., Zhang, Y., Nirmalakhandan, N., 2019. Factors contributing to bacteria inactivation in the Galdieria sulphuraria-based wastewater treatment system. Algal Research 38, 101392. https://doi.org/10.1016/j.algal.2018.101392

Nordgren, J., Matussek, A., Mattsson, A., Svensson, L., Lindgren, P.-E., 2009. Prevalence of norovirus and factors influencing virus concentrations during one year in a full-scale wastewater treatment plant. Water Research 43, 1117–1125. https://doi.org/10.1016/j.watres.2008.11.053

Almasi, A., Pescod, M.B., 1996. Pathogen removal mechanisms in anoxic wastewater stabilization ponds, Water Science and Technology 33. https://doi.org/10.1016/0273-1223(96)00348-4

Smith, S.R., Lang, N.L., Cheung, K.H.M., Spanoudaki, K., 2005. Factors controlling pathogen destruction during anaerobic digestion of biowastes. Waste Management 25, 417–425. https://doi.org/10.1016/j.wasman.2005.02.010

Articles that were cited in this section of the report

Bali, M., Gueddari, M., Boukchina, R., 2011. Removal of contaminants and pathogens from secondary effluents using intermittent sand filters. Water Sci. Technol. 64, 2038–2043.

https://doi.org/10.2166/wst.2011.448

Hai, F.I., Riley, T., Shawkat, S., Magram, S.F., Yamamoto, K., 2014. Removal of pathogens by membrane bioreactors: a review of the mechanisms, influencing factors and reduction in chemical disinfectant dosing. Water 6, 3603-3630.

Koivunen, J., Siitonen, A., Heinonen-Tanski, H., 2003. Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units. Water Res. 37, 690-698. https://doi.org/10.1016/S0043-1354(02)00305-6

Oakley, S.M., 2018. Preliminary Treatment and Primary Settling, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Oakley, S.M., von Sperling, M., 2017. Media Filters, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Naughton, C.C., Rousselot, O., 2017. Activated Sludge, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Tonon, D., Tonetti, A.L., Coraucci Filho, B., Bueno, D.A.C., 2015. Wastewater treatment by anaerobic filter and sand filter: Hydraulic loading rates for removing organic matter, phosphorus, pathogens and nitrogen in tropical countries. Ecol. Eng. 82, 583–589. https://doi.org/10.1016/j.ecoleng.2015.05.018

Verbyla, M.E., Rousselot, O., 2018. Membrane Bioreactors, in: Rose, J.B., Jiménez-Cisneros, B. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Other articles cited in this section

Weinroth, M.D., Belk, A.D., Belk, K.E., 2018. History, development, and current status of food safety systems worldwide. Animal Frontiers 8, 9–15. https://doi.org/10.1093/af/vfy016

Polanco, J., Safarik, J., Plumlee, M.H., 2022. *Demonstrating Virus Log Removal Credit for Wastewater Treatment and Reverse Osmosis for Potable Reuse at OCWD*. Water Research Foundation, Alexandria, VA, USA.

City of San Diego, 2018. Draft Title 22 Engineering Report North City Pure Water Project. San Diego, CA, USA.

Research Question 2a. What are the current practices for sample concentration, extraction, and quantification of pathogens and other microbial process indicators (viruses in particular)?

References Cited in Section 3.2.1

64 papers were identified in the search. After initial screening, the full texts of 21 papers were reviewed. Additional articles were identified by reviewing works cited from other papers.

Identified in original search (indicates articles that were cited in this section of the report)*

*Ahmed, W., Bivins, A., Simpson, S.L., Smith, W.J.M., Metcalfe, S., McMinn, B., Symonds, E.M., Korajkic, A., 2021. Comparative analysis of rapid concentration methods for the recovery of SARS-CoV-2 and quantification of human enteric viruses and a sewage-associated marker gene in untreated wastewater. Science of The Total Environment 799, 149386. https://doi.org/10.1016/j.scitotenv.2021.149386

Calgua, B., Fumian, T., Rusiñol, M., Rodriguez-Manzano, J., Mbayed, V.A., Bofill-Mas, S., Miagostovich, M., Girones, R., 2013a. Detection and quantification of classic and emerging viruses by skimmed-milk flocculation and PCR in river water from two geographical areas. Water Research 47, 2797–2810. https://doi.org/10.1016/j.watres.2013.02.043

*Calgua, B., Rodriguez-Manzano, J., Hundesa, A., Suñen, E., Calvo, M., Bofill-Mas, S., Girones, R., 2013b. New methods for the concentration of viruses from urban sewage using quantitative PCR. Journal of Virological Methods 187, 215–221. https://doi.org/10.1016/j.jviromet.2012.10.012

*Crank, K., Li, X., North, D., Ferraro, G.B., Iaconelli, M., Mancini, P., La Rosa, G., Bibby, K., 2020. CrAssphage abundance and correlation with molecular viral markers in Italian wastewater. Water Research 184, 116161. https://doi.org/10.1016/j.watres.2020.116161

Dubois, E., Hennechart, C., Deboosère, N., Merle, G., Legeay, O., Burger, C., Le Calvé, M., Lombard, B., Ferré, V., Traoré, O., 2006. Intra-laboratory validation of a concentration method adapted for the enumeration of infectious F-specific RNA coliphage, enterovirus, and hepatitis A virus from inoculated leaves of salad vegetables. International Journal of Food Microbiology 108, 164–171. https://doi.org/10.1016/j.ijfoodmicro.2005.11.007

Fonseca, M.S., Machado, B.A.S., Rolo, C. de A., Hodel, K.V.S., Almeida, E. dos S., de Andrade, J.B., 2022. Evaluation of SARS-CoV-2 concentrations in wastewater and river water samples. Case Studies in Chemical and Environmental Engineering 6, 100214. https://doi.org/10.1016/j.cscee.2022.100214

Girón-Guzmán, I., Díaz-Reolid, A., Cuevas-Ferrando, E., Falcó, I., Cano-Jiménez, P., Comas, I., Pérez-Cataluña, A., Sánchez, G., 2023. Evaluation of two different concentration methods for surveillance of human viruses in sewage and their effects on SARS-CoV-2 sequencing. Science of The Total Environment 862, 160914. https://doi.org/10.1016/j.scitotenv.2022.160914

Hsu, B.-M., Chen, C.-H., Kung, C.-M., Wan, M.-T., Shen, S.-M., 2007. Evaluation of enterovirus recovery in surface water by different adsorption and elution procedures. Chemosphere 66, 964–969. https://doi.org/10.1016/j.chemosphere.2006.06.054

*Jafferali, M.H., Khatami, K., Atasoy, M., Birgersson, M., Williams, C., Cetecioglu, Z., 2021. Benchmarking virus concentration methods for quantification of SARS-CoV-2 in raw wastewater. Science of The Total Environment 755, 142939.

https://doi.org/10.1016/j.scitotenv.2020.142939

Kabdaşlı, I., Tünay, O., 2021. Concentration techniques tailored for the detection of SARS-CoV-2 genetic material in domestic wastewater and treatment plant sludge: A review. Journal of Environmental Chemical Engineering 9, 106296. https://doi.org/10.1016/j.jece.2021.106296

*Kaya, D., Niemeier, D., Ahmed, W., Kjellerup, B.V., 2022. Evaluation of multiple analytical methods for SARS-CoV-2 surveillance in wastewater samples. Science of The Total Environment 808, 152033. https://doi.org/10.1016/j.scitotenv.2021.152033

*Kevill, J.L., Pellett, C., Farkas, K., Brown, M.R., Bassano, I., Denise, H., McDonald, J.E., Malham, S.K., Porter, J., Warren, J., Evens, N.P., Paterson, S., Singer, A.C., Jones, D.L., 2022. A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater. Science of The Total Environment 808, 151916. https://doi.org/10.1016/j.scitotenv.2021.151916

LaTurner, Z.W., Zong, D.M., Kalvapalle, P., Gamas, K.R., Terwilliger, A., Crosby, T., Ali, P., Avadhanula, V., Santos, H.H., Weesner, K., Hopkins, L., Piedra, P.A., Maresso, A.W., Stadler, L.B., 2021. Evaluating recovery, cost, and throughput of different concentration methods for SARS-CoV-2 wastewater-based epidemiology. Water Research 197, 117043. https://doi.org/10.1016/j.watres.2021.117043

*Lu, D., Huang, Z., Luo, J., Zhang, X., Sha, S., 2020. Primary concentration – The critical step in implementing the wastewater based epidemiology for the COVID-19 pandemic: A mini-review. Science of The Total Environment 747, 141245. https://doi.org/10.1016/j.scitotenv.2020.141245

*McMinn, B.R., Korajkic, A., Kelleher, J., Herrmann, M.P., Pemberton, A.C., Ahmed, W., Villegas, E.N., Oshima, K., 2021. Development of a large volume concentration method for recovery of coronavirus from wastewater. Science of The Total Environment 774, 145727. https://doi.org/10.1016/j.scitotenv.2021.145727

*Oh, C., Kim, K., Araud, E., Wang, L., Shisler, J.L., Nguyen, T.H., 2022. A novel approach to concentrate human and animal viruses from wastewater using receptors-conjugated magnetic beads. Water Research 212, 118112. https://doi.org/10.1016/j.watres.2022.118112

Pérez-Méndez, A., Chandler, J.C., Bisha, B., Goodridge, L.D., 2014. Evaluation of an anion exchange resin-based method for concentration of F-RNA coliphages (enteric virus indicators) from water samples. Journal of Virological Methods 204, 109–115. https://doi.org/10.1016/j.jviromet.2014.03.024

Rhodes, E.R., Huff, E.M., Hamilton, D.W., Jones, J.L., 2016. The evaluation of hollow-fiber ultrafiltration and celite concentration of enteroviruses, adenoviruses and bacteriophage from different water matrices. Journal of Virological Methods 228, 31–38. https://doi.org/10.1016/j.jviromet.2015.11.003

*Rusiñol, M., Martínez-Puchol, S., Forés, E., Itarte, M., Girones, R., Bofill-Mas, S., 2020. Concentration methods for the quantification of coronavirus and other potentially pandemic enveloped virus from wastewater. Current Opinion in Environmental Science & Health 17, 21–28. https://doi.org/10.1016/j.coesh.2020.08.002

Villar, L.M., de Paula, V.S., Diniz-Mendes, L., Lampe, E., Gaspar, A.M.C., 2006. Evaluation of methods used to concentrate and detect hepatitis A virus in water samples. Journal of Virological Methods 137, 169–176. https://doi.org/10.1016/j.jviromet.2006.06.008

Zheng, X., Wang, M., Deng, Y., Xu, X., Lin, D., Zhang, Y., Li, S., Ding, J., Shi, X., Yau, C.I., Poon, L.L.M., Zhang, T., 2022. A rapid, high-throughput, and sensitive PEG-precipitation method for SARS-CoV-2 wastewater surveillance. Water Research 119560. https://doi.org/10.1016/j.watres.2022.119560

Identified by reviewing works cited from other papers

Ahmed, W., Bertsch, P., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J., Simpson, S., Smith, W.J.M., Symonds, E.M., Thomas, K. V., Verhagen, R., Kitajima, M., 2020. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. Sci. Total Environ. 739, 139960. https://doi.org/10.1016/j.scitotenv.2020.139960

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405. https://doi.org/10.1016/j.scitotenv.2020.140405

Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S., Honda, R., 2021. Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. Sci. Total Environ. 758, 143578. https://doi.org/10.1016/j.scitotenv.2020.143578

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in the Netherlands. Environ. Sci. Technol. Lett. 7, 511–516. https://doi.org/10.1021/acs.estlett.0c00357

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 181. https://doi.org/10.1016/j.watres.2020.115942

Ye, Y., Ellenberg, R.M., Graham, K.E., Wigginton, K.R., 2016. Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. Environ. Sci. Technol. 50, 5077–5085. https://doi.org/10.1021/acs.est.6b00876

Research Question 3a. How are concentration and LRV data distributed?

References Cited in Section 3.3.1

4 papers were identified in the search. After initial screening, the full texts of all 4 papers were reviewed. Additional articles were identified by reviewing works cited from other papers. Other papers were identified based on the authors' familiarity with other literature on the topic.

Identified in original search (indicates articles that were cited in this section of the report)*

*Eftim, S.E., Hong, T., Soller, J., Boehm, A., Warren, I., Ichida, A., Nappier, S.P., 2017.

Occurrence of norovirus in raw sewage – A systematic literature review and meta-analysis. Water Research 111, 366–374. https://doi.org/10.1016/j.watres.2017.01.017

*Jones, K.R., Eftim, S., Lindahl, A.J., Black, S., Nappier, S.P., 2022. Occurrence of coliphage in effluent: A systematic literature review and meta-analysis. Hygiene and Environmental Health Advances 3, 100014. https://doi.org/10.1016/j.heha.2022.100014

Seis, W., Rouault, P., Medema, G., 2020. Addressing and reducing parameter uncertainty in quantitative microbial risk assessment by incorporating external information via Bayesian hierarchical modeling. Water Research 185, 116202. https://doi.org/10.1016/j.watres.2020.116202

*Nappier, S.P., Hong, T., Ichida, A., Goldstone, A., Eftim, S.E., 2019. Occurrence of coliphage in raw wastewater and in ambient water: A meta-analysis. Water Research 153, 263–273. https://doi.org/10.1016/j.watres.2018.12.058

Identified by reviewing works cited from other papers

Pouillot, R., Van Doren, J.M., Woods, J., Plante, D., Smith, M., Goblick, G., Roberts, C., Locas, A., Hajen, W., Stobo, J., White, J., Holtzman, J., Buenaventura, E., Burkhardt, W., Catford, A., Edwards, R., DePaola, A., Calci, K.R., 2015. Meta-Analysis of the Reduction of Norovirus and Male-Specific Coliphage Concentrations in Wastewater Treatment Plants. Appl Environ Microbiol 81, 4669–4681. https://doi.org/10.1128/AEM.00509-15

Teunis, P.F., Rutjes, S.A., Westrell, T., de Roda Husman, A.M., 2009. Characterization of drinking water treatment for virus risk assessment. Water Res 43, 395–404. https://doi.org/10.1016/j.watres.2008.10.049

Identified due to authors' familiarity with other literature on the topic

Benke, K.K., Hamilton, A.J., 2008. Quantitative microbial risk assessment: Uncertainty and measures of central tendency for skewed distributions. Stoch. Environ. Res. Risk Assess. 22, 533–539. https://doi.org/10.1007/s00477-007-0171-9

Haas, C.N., 1996. How to average microbial densities to characterize risk. Water Res. 30, 1036–1038. https://doi.org/10.1016/0043-1354(95)00228-6

Karavarsamis, N., Hamilton, A.J., 2010. Estimators of annual probability of infection for quantitative microbial risk assessment. J. Water Health 08, 365. https://doi.org/10.2166/wh.2010.045

McBride, G.B., Stott, R., Miller, W., Bambic, D., Wuertz, S., 2013. Discharge-based QMRA for estimation of public health risks from exposure to stormwater-borne pathogens in recreational waters in the United States. Water Res. 47, 5282–5297. https://doi.org/10.1016/j.watres.2013.06.001

Schoen, M.E., Ashbolt, N.J., Jahne, M.A., Garland, J., 2017. Risk-based enteric pathogen reduction targets for non-potable and direct potable use of roof runoff, stormwater, and greywater. Microb. Risk Anal. 5, 32–43. https://doi.org/10.1016/j.mran.2017.01.002

von Sperling, M., Verbyla, M.E., Mihelcic, J.R., 2018. Understanding Pathogen Reduction in Sanitation Systems: Units of Measurement , Expressing Changes in Concentrations, and Kinetics, in: Rose, J.B., JImenez-Cisneros, B.E. (Eds.), Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. Michigan State University, UNESCO, E. Lansing, MI, USA.

Research Question 3b. What statistical methods should be used to handle left-censored data?

References Cited in Section 3.3.2

80 papers were identified in the search. After initial screening, the full text of 11 papers were reviewed. Additional articles were identified by reviewing works cited from other papers. Other papers were identified based on the authors' familiarity with other literature on the topic.

Identified in original search (indicates articles that were cited in this section of the report)*

*Canales, R.A., Wilson, A.M., Pearce-Walker, J.I., Verhougstraete, M.P., Reynolds, K.A., 2018. Methods for Handling Left-Censored Data in Quantitative Microbial Risk Assessment. Appl Environ Microbiol 84, e01203-18. https://doi.org/10.1128/AEM.01203-18

*Dila, D.K., Corsi, S.R., Lenaker, P.L., Baldwin, A.K., Bootsma, M.J., McLellan, S.L., 2018. Patterns of Host-Associated Fecal Indicators Driven by Hydrology, Precipitation, and Land Use Attributes in Great Lakes Watersheds. Environ. Sci. Technol. acs.est.8b01945. https://doi.org/10.1021/acs.est.8b01945

*Jahne, M.A., Brinkman, N.E., Keely, S.P., Zimmerman, B.D., Wheaton, E.A., Garland, J.L., 2020. Droplet digital PCR quantification of norovirus and adenovirus in decentralized wastewater and graywater collections: Implications for onsite reuse. Water Research 169, 115213. https://doi.org/10.1016/j.watres.2019.115213

*Nappier, S.P., Hong, T., Ichida, A., Goldstone, A., Eftim, S.E., 2019. Occurrence of coliphage in raw wastewater and in ambient water: A meta-analysis. Water Research 153, 263–273. https://doi.org/10.1016/j.watres.2018.12.058

*Pouillot, R., Van Doren, J.M., Woods, J., Plante, D., Smith, M., Goblick, G., Roberts, C., Locas, A., Hajen, W., Stobo, J., White, J., Holtzman, J., Buenaventura, E., Burkhardt, W., Catford, A., Edwards, R., DePaola, A., Calci, K.R., 2015. Meta-Analysis of the Reduction of Norovirus and Male-Specific Coliphage Concentrations in Wastewater Treatment Plants. Appl Environ Microbiol 81, 4669–4681. https://doi.org/10.1128/AEM.00509-15

*Staley, C., Gordon, K.V., Schoen, M.E., Harwood, V.J., 2012. Performance of Two Quantitative PCR Methods for Microbial Source Tracking of Human Sewage and Implications for Microbial Risk Assessment in Recreational Waters. Appl Environ Microbiol 78, 7317–7326. https://doi.org/10.1128/AEM.01430-12

*Hogan, J.N., Daniels, M.E., Watson, F.G., Conrad, P.A., Oates, S.C., Miller, M.A., Hardin, D., Byrne, B.A., Dominik, C., Melli, A., Jessup, D.A., Miller, W.A., 2012. Longitudinal Poisson Regression To Evaluate the Epidemiology of Cryptosporidium, Giardia, and Fecal Indicator Bacteria in Coastal California Wetlands. Appl Environ Microbiol 78, 3606–3613.

https://doi.org/10.1128/AEM.00578-12

*Nappier, S.P., Hong, T., Ichida, A., Goldstone, A., Eftim, S.E., 2019. Occurrence of coliphage in raw wastewater and in ambient water: A meta-analysis. Water Research 153, 263–273. https://doi.org/10.1016/j.watres.2018.12.058

*Rodriguez-Alvarez, M.S., Weir, M.H., Pope, J.M., Seghezzo, L., Rajal, V.B., Salusso, M.M., Moraña, L.B., 2015. Development of a relative risk model for drinking water regulation and design recommendations for a peri urban region of Argentina. International Journal of Hygiene and Environmental Health 218, 627–638. https://doi.org/10.1016/j.ijheh.2015.06.007

*Masaka, E., Reed, S., Davidson, M., Oosthuizen, J., 2021. Opportunistic Premise Plumbing Pathogens. A Potential Health Risk in Water Mist Systems Used as a Cooling Intervention. Pathogens 10, 462. https://doi.org/10.3390/pathogens10040462

*Corrigan, J.A., Butkus, S.R., Ferris, M.E., Roberts, J.C., 2021. Microbial Source Tracking Approach to Investigate Fecal Waste at the Strawberry Creek Watershed and Clam Beach, California, USA. IJERPH 18, 6901. https://doi.org/10.3390/ijerph18136901

Identified by reviewing works cited from other papers

Huynh, T., Ramachandran, G., Banerjee, S., Monteiro, J., Stenzel, M., Sandler, D.P., Engel, L.S., Kwok, R.K., Blair, A., Stewart, P.A., 2014. Comparison of methods for analyzing left-censored occupational exposure data. Ann. Occup. Hyg. 58, 1126–1142. https://doi.org/10.1093/annhyg/meu067

Shoari, N., Dubé, J.S., Chenouri, S., 2015. Estimating the mean and standard deviation of environmental data with below detection limit observations: Considering highly skewed data and model misspecification. Chemosphere 138, 599–608. https://doi.org/10.1016/j.chemosphere.2015.07.009

Identified due to authors' familiarity with other literature on the topic

Helsel, D.R., 2012. *Statistics for censored environmental data using Minitab and R*. 2nd Ed., John Wiley & Sons, Hoboken, NJ, USA. ISBN 978-0-470-47988-9

Cohen, A.C., 1959. Simplified estimators for the normal distribution when samples are singly censored or truncated. Technometrics 1, 217–237.

Cohen, A.C., 1961. Tables for maximum likelihood estimates singly truncated and singly censored samples. Technometrics 3, 535–541.

APPENDIX B

Estimation of 95% Confidence Intervals on a Percentile (Parametric Method)

The confidence interval around the estimate of a quantile can be estimated parametrically as long as the underlying distribution of the variable is Gaussian (Ialongo, 2019). This is the case for a $\log_{10}$ reduction value (LRV) of two pathogen concentrations; as indicated in this report, the LRV follows a normal distribution when the pathogen concentrations are lognormally distributed. To calculate the confidence interval around a percentile, Ialongo (2019) defined the V-statistic as the difference between the estimate of the q^{th} percentile (based on a statistical sample) and the true underlying value of the q^{th} percentile, divided by the sample standard deviation:

$$V = \frac{\hat{x}_r - X_r}{s} \qquad \text{(Equation B-1)}$$

where: r = an element within an ordered data set of size N whose value is larger than or equal to that of $q = r/N$ elements (i.e., $x_1 \leq x_2 \leq \ldots \leq x_r \leq \ldots \leq x_{n-1} \leq x_n$)

$\hat{x}_r$ = the sample estimate of the q^{th} percentile

X_r = the true underlying q^{th} percentile

s = sample standard deviation

It has been shown that V follows a Student's t-distribution with $n - 1$ degrees of freedom and a non-centrality parameter equal to $\lambda = -zN^{0.5}$ (Ialongo, 2019), where z is the normal score for the q^{th} percentile and N is the sample size. Correspondingly, the probability that V is between $\alpha/2$ and $1 - \alpha/2$ is approximately equal to $1 - \alpha$:

$$P\left(\frac{\alpha}{2} < V < 1 - \frac{\alpha}{2}\right) \cong (1 - \alpha) \qquad \text{(Equation B-2)}$$

Given Equations A-1 and A-2, the upper and lower confidence limits on q can be defined as follows:

$$\text{Confidence Interval } (q) = \left[m - t_{1-\frac{\alpha}{2},\lambda} \cdot s \cdot n^{-0.5}, m - t_{\frac{\alpha}{2},\lambda} \cdot s \cdot n^{-0.5}\right] \qquad \text{(Equation B-3)}$$

As an example, suppose the 5th percentile of the LRV is estimated from 20 influent and effluent samples, where the mean LRV is 3-$\log_{10}$ with a standard deviation of 0.9 (CoV = 0.9 / 3.0 = 0.3). The following simulation with 10,000 iterations indicates that the width of the 95% confidence interval around the 5th percentile estimate is 1.31. The true value of the 5th percentile for this scenario is 1.5, and the average lower and upper 95% confidence limits, respectively, were 0.7 and 2.0. The fraction of times the calculated 95% confidence intervals included the true 5th percentile was 0.9464, very close to 95%. Statistical theory tells us that the confidence interval

of an estimated 5th percentile will contain the true underlying value of the 5th percentile with a probability of 95% (with the other 5% representing the alpha error).

```
P=0.05         #quantile of the LRV to be estimated (5th percentile)
alpha=0.05     #alpha error
cl=1-alpha     #confidence level
sims<-10000    #number of iterations in the simulation
n<-20          #sample size
lrv<-3         #true mean LRV
cv<-0.3        #true coefficient of variation (CoV) of the LRV
lrvsd<-cv*lrv #standard deviation (calculated as LRV multiplied by CoV)

#PARAMETRIC METHOD (see Ialongo, 2019)
diff.par<-NA;prob<-NA;upper<-NA;lower<-NA;alphaError=NA
true.5th<-lrv+qnorm(p)*lrvsd
for(i in 1:sims){
  x<-rnorm(n,lrv,lrvsd)
  m<-mean(x)
  s<-sd(x)
  lamb <- -qnorm(p)*n^0.5
  lower[i] <- m-qt(p=1-alpha/2,df=n-1,ncp=lamb)*s*n^-0.5
  upper[i] <- m-qt(p=alpha/2,df=n-1,ncp=lamb)*s*n^-0.5
  diff.par[i] <- upper[i]-lower[i]
  alphaError[i] <- !(true.5th<upper[i]&true.5th>lower[i])
}

lrv+qnorm(p)*lrvsd    #this is the true underlying 5th percentile of the LRV

## [1] 1.519632

1-sum(alphaError)/sims #percentage of times the CI included the true quantile

## [1] 0.9464

mean(lower)           #the mean of the lower confidence limits calculated

## [1] 0.7156367

mean(upper)           #the mean of the upper confidence limits calculated

## [1] 2.029351

mean(diff.par)        #the mean of the confidence interval width

## [1] 1.313714
```

APPENDIX C

Estimation of 95% Confidence Intervals on a Percentile (Nonparametric Method)

The nonparametric "exact" method to estimate 95% confidence intervals on a quantile, described by Beran and Hall (1993), was implemented using the *eqnpar* command from the *EnvStats* package in R (Millard and Kowarick 2022). This method cannot be used for low quantiles when the sample size is very low. For example, it does not work for the 5th percentile when the sample size is 48 or below. Therefore, Table C-1 shows results of a simulation of the 95% confidence interval on the estimate of the 25th percentile, instead of the 5th percentile.

Table C-1. Sensitivity Analysis for the Confidence Interval Using T=the Nonparametric Exact Method

Simulation No.	Sample size	Mean LRV	Standard Deviation	Coefficient of Variation (CoV)	Width of the 95% confidence interval on the 25th percentile of the LRV
1	12	1.0	0.1	10%	0.17
2	12	1.0	0.3	30%	0.52
3	12	1.0	0.5	50%	0.86
4	12	3.0	0.3	10%	0.52
5	12	3.0	0.9	30%	1.55
6	12	3.0	1.5	50%	2.60
7	24	1.0	0.1	10%	0.12
8	24	1.0	0.3	30%	0.36
9	24	1.0	0.5	50%	0.59
10	24	3.0	0.3	10%	0.36
11	24	3.0	0.9	30%	1.06
12	24	3.0	1.5	50%	1.78
13	48	1.0	0.1	10%	0.09
14	48	1.0	0.3	30%	0.26
15	48	1.0	0.5	50%	0.43
16	48	3.0	0.3	10%	0.25
17	48	3.0	0.9	30%	0.77
18	48	3.0	1.5	50%	1.27

APPENDIX D

User Manual for the LRV Credit Validation Study Cost Estimator

The web-based Log_{10} Reduction Value (LRV) Credit Validation Study Cost Estimating Tool is available for free at the following website: https://credit.waterpathogens.org/form/calculator. The following is a user manual for this tool.

Introduction

The Cost Estimating Tool is a web-based tool developed for use by people who are planning a validation study to seek LRV credits for a wastewater treatment system that is part of a reuse project. The tool is designed to provide a cost estimate for completing LRV validation studies based on the user's specified pathogen or microbial indicator groups, the number of sample locations, and the number of sample sets. The tool allows the user to input assumptions about the soft costs, generally based on whether the cost of labor is considered to be high, medium, or low.

Getting Started

To use the tool, complete the following actions:

1. **Select Microbial Groups.** Click on the checkboxes next to the microbial groups you propose to study as part of your LRV validation plan.
2. **Input Number of Sample Locations.** Input the number of different locations you plan to collect wastewater samples from as part of the validation plan. Note, you should have at least two locations (upstream and downstream of the treatment processes being studied/validated), but you may have more than two (e.g., intermediate points or more than one influent/effluent. You may either type the number or use the up/down buttons next to the text entry window.
3. **Input Number of Sample Sets.** Type the number of sample sets you plan to collect for the validation study. A sample set is defined as a date of sampling, where you collect samples at each of the different sampling locations specified in Step 2. Note that a typical sample size ranges from 12 sample sets to 24 sample sets. You may either type the number or use the up/down buttons next to the text entry window.
4. **Select Assumption for Soft Costs.** Click the drop-down window to select your assumption for the soft costs associated with LRV validation studies (e.g., costs associated with people-hours to plan the study, collect the samples, analyze data, write the report, and interact with regulators or advisory panels). These costs are generally driven by salaries and the cost of labor in your region or at your organization.
5. **Click Submit to See Your Estimate!** Once you have entered the above information, click on the "Submit" button to see your cost estimate, broken down by the following cost categories:

- *Study planning*. These are the costs associated with developing the validation plan. They mostly consist of person-hours of time to write the plan and get it approved. It includes a base rate for employee time plus overhead. Note that this cost item might be greatly reduced if data reporting and calculation protocols are standardized using the recommendations presented in this report.
- *Sample collection*. These are the costs associated with collecting the wastewater samples from the treatment facility and preparing them to ship to the laboratory. It includes person-hours of time plus field equipment such as tubing, pumps, etc.
- Shipping samples. These are the fees associated with shipping wastewater samples overnight on dry ice to a laboratory.
- *Laboratory fees*. These are the fees charged by the commercial laboratories to analyze wastewater samples for the parameters requested. The costs are based on 2021 rates gathered from 15 different laboratories based in different locations throughout the United States, for the following analyses: EPA Methods 1602/1642 (coliphage), 1615 (enteric viruses by cultivation and RT-qPCR), 1623/1623.1/1693 (*Cryptosporidium*/*Giardia*), Standard Methods protocols SM 9223B (for E. coli), SM 9510 (for enteric viruses), and SM 9711B (for *Giardia* and *Cryptosporidium*). The availability of testing varies greatly by pathogen. Cost estimates were available from eight laboratories for *E. coli* analysis, five laboratories for *Cryptosporidium*/*Giardia* analysis, four laboratories for (culture-based) enteric virus analysis, and two laboratories for coliphage analysis. Costs were also gathered for some non-standardized PCR-based methods for quantifying enteric viruses in wastewater samples from commercial and academic laboratories that have offered this service in the past.
- *Data analysis*. These are the costs associated with the person-hours of time required to analyze LRV validation study data. They include a base rate for employee time plus overhead. Note that this cost item might be greatly reduced if data reporting and calculation protocols are standardized using the recommendations presented in this report.
- *Report writing*. These are the costs associated with the person-hours of time required to analyze LRV validation study data. They include a base rate for employee time plus overhead.
- *Advisory panel*. These are the costs associated with the person-hours of time required to interact with independent external advisory panels and with the state regulatory agency. Note that this cost item might be greatly reduced if data reporting and calculation protocols are standardized using the recommendations presented in this report.

More Advanced Use

There is some flexibility related to the assumptions used in the cost estimate for this tool. The cost data can be edited and updated. If you are interested in doing a more detailed cost estimate, please contact Matthew E. Verbyla (mverbyla@sdsu.edu) to schedule a meeting to discuss a specific case study.

References

Agulló-Barceló, M., Oliva, F., Lucena, F., 2013. Alternative indicators for monitoring Cryptosporidium oocysts in reclaimed water. Environ. Sci. Pollut. Res. 20, 4448–4454. https://doi.org/10.1007/s11356-012-1400-4

Ahmed, W., Bertsch, P., Bivins, A., Bibby, K., Farkas, K., Gathercole, A., Haramoto, E., Gyawali, P., Korajkic, A., McMinn, B.R., Mueller, J., Simpson, S., Smith, W.J.M., Symonds, E.M., Thomas, K. V., Verhagen, R., Kitajima, M., 2020. Comparison of virus concentration methods for the RT-qPCR-based recovery of murine hepatitis virus, a surrogate for SARS-CoV-2 from untreated wastewater. Sci. Total Environ. 739, 139960. https://doi.org/10.1016/j.scitotenv.2020.139960

Ahmed, W., Bivins, A., Bertsch, P.M., Bibby, K., Gyawali, P., Sherchan, S.P., Simpson, S.L., Thomas, K. V., Verhagen, R., Kitajima, M., Mueller, J.F., Korajkic, A., 2021a. Intraday variability of indicator and pathogenic viruses in 1-h and 24-h composite wastewater samples: Implications for wastewater-based epidemiology. Environ. Res. 193, 110531. https://doi.org/10.1016/j.envres.2020.110531

Ahmed, W., Bivins, A., Simpson, S.L., Smith, W.J.M., Metcalfe, S., McMinn, B., Symonds, E.M., Korajkic, A., 2021b. Comparative analysis of rapid concentration methods for the recovery of SARS-CoV-2 and quantification of human enteric viruses and a sewage-associated marker gene in untreated wastewater. Sci. Total Environ. 799, 149386. https://doi.org/10.1016/j.scitotenv.2021.149386

Amarasiri, M., Kitajima, M., Nguyen, T.H., Okabe, S., Sano, D., 2017. Bacteriophage removal efficiency as a validation and operational monitoring tool for virus reduction in wastewater reclamation: Review. Water Res. 121, 258–269. https://doi.org/10.1016/j.watres.2017.05.035

Australian Water Recycling Centre of Excellence, 2016. *Protocol Template*. WaterVal Streamlining Technology Validation, Australian Water Recycling Centre of Excellence, Brisbane, Australia. https://members.waterra.au/Members/Members/Publications/WaterVal.aspx

Bali, M., Gueddari, M., Boukchina, R., 2011. Removal of contaminants and pathogens from secondary effluents using intermittent sand filters. Water Sci. Technol. 64, 2038–2043. https://doi.org/10.2166/wst.2011.448

Barril, P.A., Fumian, T.M., Prez, V.E., Gil, P.I., Mart\'\inez, L.C., Giordano, M.O., Masachessi, G., Isa, M.B., Ferreyra, L.J., Ré, V.E., Miagostovich, M., Pavan, J. V, Nates, S.V., 2015. Rotavirus seasonality in urban sewage from Argentina: effect of meteorological variables on the viral load and the genetic diversity. Environ. Res. 138, 409–415. https://doi.org/10.1016/j.envres.2015.03.004

Benke, K.K., Hamilton, A.J., 2008. Quantitative microbial risk assessment: Uncertainty and measures of central tendency for skewed distributions. Stoch. Environ. Res. Risk Assess. 22, 533–539. https://doi.org/10.1007/s00477-007-0171-9

Beran, R., Hall, P., 1993. Interpolated Nonparametric Prediction Intervals and Confidence Intervals. Journal of the Royal Statistical Society: Series B (Methodological) 55, 643–652. https://doi.org/10.1111/j.2517-6161.1993.tb01929.

Bivins, A., Greaves, J., Fischer, R., Yinda, K.C., Ahmed, W., Kitajima, M., Munster, V.J., Bibby, K., 2020. Persistence of SARS-CoV-2 in Water and Wastewater. Environ. Sci. Technol. Lett. 7, 937–942. https://doi.org/10.1021/acs.estlett.0c00730

Branch, A., Trinh, T., Ta, T.M., Leslie, G., Le-Clech, P., 2021. Log removal values in membrane bioreactors: Correlation of surrogate monitoring and operational parameters. J. Water Process Eng. 41, 102032. https://doi.org/10.1016/j.jwpe.2021.102032

Calgua, B., Rodriguez-Manzano, J., Hundesa, A., Sunen, E., Calvo, M., Bofill-Mas, S., Girones, R., 2013. New methods for the concentration of viruses from urban sewage using quantitative PCR, in: Journal of Virological Methods. 2012 Elsevier B.V, Netherlands, pp. 215–21.

California Code of Regulations. (CCR). 2023. Water Recycling Criteria. Title 22, Division 4, Chapter 3 (22 CCR §60301 - 60355). https://govt.westlaw.com/calregs/Browse/Home/California/CaliforniaCodeofRegulations.

California Code of Regulations (CCR). 2014a. Pathogenic Microorganism Control, Title 22, Division 4, Chapter 3, Article 5.1. Indirect Potable Reuse: Groundwater Replenishment - Surface Application (22 CCR §60320.108). https://govt.westlaw.com/calregs/Browse/Home/California/CaliforniaCodeofRegulations.

California Code of Regulations. (CCR). 2014b. Pathogenic Microorganism Control, Title 22, Division 4, Chapter 3, Article 5.2. Indirect Potable Reuse: Groundwater Replenishment - Subsurface Application (22 CCR §60320.208). https://govt.westlaw.com/calregs/Browse/Home/California/CaliforniaCodeofRegulations.

California Code of Regulations. (CCR). 2018. Pathogenic Microorganism Control, Title 22, Division 4, Chapter 3, Article 5.3. Indirect Potable Reuse: Surface Water Augmentation (22 CCR §60320.308). https://govt.westlaw.com/calregs/Browse/Home/California/CaliforniaCodeofRegulations.

California State Water Resources Control Board (SWRCB), 2018. *A proposed framework for regulating direct potable reuse in California*. California Environmental Protection Agency, Division of Drinking Water, Sacramento, CA.

California State Water Resources Control Board (SWRCB), 2019. *A proposed framework for regulating direct potable reuse in California*. 2nd Edition. California Environmental Protection Agency, Division of Drinking Water, Sacramento, CA.

California State Water Resources Control Board (SWRCB), 2021. *A Proposed Framework of Regulating Direct Potable Reuse in California 2nd Edition Addendum – Early Draft of Anticipated Criteria for Direct Potable Reuse*. Division of Drinking Water, Sacramento, CA, USA.

Canales, R.A., Wilson, A.M., Pearce-Walker, J.I., Verhougstraete, M.P., Reynolds, K.A., 2018. Methods for handling left-censored data in quantitative microbial risk assessment. Appl. Environ. Microbiol. 84, 1–10. https://doi.org/10.1128/AEM.01203-18

Canchola, J.A., Tang, S., Hemyari, P., Paxinos, E., Marins, E., 2017. Correct use of percent coefficient of variation (%CV) formula for log-transformed data. *MOJ Proteomics Bioinformatics*, 6(4), 316–317. https://doi.org/10.15406/mojpb.2017.06.00200.

Carollo, 2017. Advanced Water Purification Facility: Indirect Potable Reuse Engineering Report. Volume 1 of 2. Oxnard, CA, USA: Carollo.

Casanovas-Massana, A., Costa, F., Riediger, I.N., Cunha, M., de Oliveira, D., Mota, D.C., Sousa, E., Querino, V.A., Nery, N., Reis, M.G., Wunder, E.A., Diggle, P.J., Ko, A.I., 2018. Spatial and temporal dynamics of pathogenic Leptospira in surface waters from the urban slum environment. Water Res. 130, 176–184. https://doi.org/https://doi.org/10.1016/j.watres.2017.11.068.

Cel Analytical, 2021. Quality Assurance Project Plan: Analytical Microbiology Services, Version 5.0. San Francisco, CA, USA: Cel Analytical.

Chalapati Rao, V., Metcalf, T.G., Melnick, J.L., 1987. Removal of indigenous rotaviruses during primary settling and activated-sludge treatment of raw sewage. Water Res. 21, 171–177. https://doi.org/10.1016/0043-1354(87)90046-7.

City of San Diego, 2018. Draft Title 22 Engineering Report North City Pure Water Project. San Diego, CA, USA: City of San Diego.

Cohen, A.C., 1959. Simplified estimators for the normal distribution when samples are singly censored or truncated. Technometrics 1, 217–237.

Cohen, A.C., 1961. Tables for maximum likelihood estimates singly truncated and singly censored samples. Technometrics 3, 535–541.

Corrigan, J.A., Butkus, S.R., Ferris, M.E., Roberts, J.C., 2021. Microbial Source Tracking Approach to Investigate Fecal Waste at the Strawberry Creek Watershed and Clam Beach, California, USA. IJERPH 18, 6901. https://doi.org/10.3390/ijerph18136901.

Crank, K., Li, X., North, D., Ferraro, G.B., Iaconelli, M., Mancini, P., La Rosa, G., Bibby, K., 2020. CrAssphage abundance and correlation with molecular viral markers in Italian wastewater. Water Res. 184, 116161. https://doi.org/10.1016/j.watres.2020.116161.

Curtis, K., Keeling, D., Yetka, K., Larson, A., Gonzalez, R., 2020. Wastewater SARS-CoV-2 Concentration and Loading Variability from Grab and 24-Hour Composite Samples. medRxiv 2020.07.10.20150607.

de Oliveira, L.C., Torres-Franco, A.F., Lopes, B.C., Santos, B.S.Á. da S., Costa, E.A., Costa, M.S.,

Reis, M.T.P., Melo, M.C., Polizzi, R.B., Teixeira, M.M., Mota, C.R., 2021. Viability of SARS-CoV-2 in river water and wastewater at different temperatures and solids content. Water Res. 195, 117002. https://doi.org/10.1016/j.watres.2021.117002.

Dias, E., Ebdon, J., Taylor, H., 2018. The application of bacteriophages as novel indicators of viral pathogens in wastewater treatment systems. Water Res. 129, 172–179. https://doi.org/10.1016/j.watres.2017.11.022.

Dila, D.K., Corsi, S.R., Lenaker, P.L., Baldwin, A.K., Bootsma, M.J., McLellan, S.L., 2018. Patterns of Host-Associated Fecal Indicators Driven by Hydrology, Precipitation, and Land Use Attributes in Great Lakes Watersheds. Environ. Sci. Technol. 52(20), 11500–11509. https://doi.org/10.1021/acs.est.8b01945.

Eftim, S.E., Hong, T., Soller, J., Boehm, A., Warren, I., Ichida, A., Nappier, S.P., 2017. Occurrence of norovirus in raw sewage – A systematic literature review and meta-analysis. Water Research 111, 366–374. https://doi.org/10.1016/j.watres.2017.01.017.

Falk, C.C., Karanis, P., Schoenen, D., Seitz, H.M., 1998. Bench scale experiments for the evaluation of a membrane filtration method for the recovery efficiency of Giardia and Cryptosporidium from water. Water Res. 32, 565–568.

Gerba, C.P., Betancourt, W.Q., Kitajima, M., 2017. How much reduction of virus is needed for recycled water: A continuous changing need for assessment? Water Res. 108, 25–31.

Gerba, C.P., Betancourt, W.Q., Kitajima, M., Rock, C.M., 2018. Reducing uncertainty in estimating virus reduction by advanced water treatment processes. Water Res. 133, 282–288. https://doi.org/10.1016/j.watres.2018.01.044.

Guo, B., Liu, C., Gibson, C., Frigon, D., 2019. Wastewater microbial community structure and functional traits change over short timescales. Sci. Total Environ. 662, 779–785. https://doi.org/https://doi.org/10.1016/j.scitotenv.2019.01.207.

Haas, C.N., 1996. How to average microbial densities to characterize risk. Water Res. 30, 1036–1038. https://doi.org/10.1016/0043-1354(95)00228-6.

Hai, F.I., Riley, T., Shawkat, S., Magram, S.F., Yamamoto, K., 2014. Removal of pathogens by membrane bioreactors: a review of the mechanisms, influencing factors and reduction in chemical disinfectant dosing. Water 6, 3603-3630.

Haramoto, E., Malla, B., Thakali, O., Kitajima, M., 2020. First environmental surveillance for the presence of SARS-CoV-2 RNA in wastewater and river water in Japan. Sci. Total Environ. 737, 140405. https://doi.org/10.1016/j.scitotenv.2020.140405.

Harwood, V.J., Levine, A.D., Scott, T.M., Chivukula, V., Lukasik, J., Farrah, S.R., Rose, J.B., 2005. Validity of the indicator organism paradigm for pathogen reduction in reclaimed water and public health protection. Appl Env. Microbiol 71, 3163-3170.

Hata, A., Hara-Yamamura, H., Meuchi, Y., Imai, S., Honda, R., 2021. Detection of SARS-CoV-2 in wastewater in Japan during a COVID-19 outbreak. Sci. Total Environ. 758, 143578. https://doi.org/10.1016/j.scitotenv.2020.143578.

Helsel, D., 2012. *Statistics for censored environmental data using Minitab and R*. 2nd Ed., John Wiley & Sons, Inc. Hoboken, NJ.

Henderson, L.K., Craig, J.C., Willis, N.S., Tovey, D., Webster, A.C., 2010. How to write a Cochrane systematic review: Doing a Cochrane systematic review. Nephrology 15, 617–624. https://doi.org/10.1111/j.1440-1797.2010.01380.x.

Hogan, J.N., Daniels, M.E., Watson, F.G., Conrad, P.A., Oates, S.C., Miller, M.A., Hardin, D., Byrne, B.A., Dominik, C., Melli, A., Jessup, D.A., Miller, W.A., 2012. Longitudinal Poisson Regression To Evaluate the Epidemiology of Cryptosporidium, Giardia, and Fecal Indicator Bacteria in Coastal California Wetlands. Appl Environ Microbiol 78, 3606–3613. https://doi.org/10.1128/AEM.00578-12.

Huynh, T., Ramachandran, G., Banerjee, S., Monteiro, J., Stenzel, M., Sandler, D.P., Engel, L.S., Kwok, R.K., Blair, A., Stewart, P.A., 2014. Comparison of methods for analyzing left-censored occupational exposure data. Ann. Occup. Hyg. 58, 1126–1142. https://doi.org/10.1093/annhyg/meu067.

Ialongo, C., 2019. Confidence interval for quantiles and percentiles. Biochem. Medica 29, 1–13. https://doi.org/10.11613/BM.2019.010101.

Jafferali, M.H., Khatami, K., Atasoy, M., Birgersson, M., Williams, C., Cetecioglu, Z., 2021. Benchmarking virus concentration methods for quantification of SARS-CoV-2 in raw wastewater. Sci. Total Environ. 755, 142939. https://doi.org/10.1016/j.scitotenv.2020.142939.

Jones, K.R., Eftim, S., Lindahl, A.J., Black, S., Nappier, S.P., 2022. Occurrence of coliphage in effluent: A systematic literature review and meta-analysis. Hygiene and Environmental Health Advances 3, 100014. https://doi.org/10.1016/j.heha.2022.100014.

Kantor, R.S., Nelson, K.L., Greenwald, H.D., Kennedy, L.C., 2021. Challenges in Measuring the Recovery of SARS-CoV-2 from Wastewater. Environ. Sci. Technol. 55, 3514–3519. https://doi.org/10.1021/acs.est.0c08210.

Karavarsamis, N., Hamilton, A.J., 2010. Estimators of annual probability of infection for quantitative microbial risk assessment. J. Water Health 08, 365. https://doi.org/10.2166/wh.2010.045.

Kaya, D., Niemeier, D., Ahmed, W., Kjellerup, B. V., 2022. Evaluation of multiple analytical methods for SARS-CoV-2 surveillance in wastewater samples. Sci. Total Environ. 808, 152033. https://doi.org/10.1016/j.scitotenv.2021.152033.

Kevill, J.L., Pellett, C., Farkas, K., Brown, M.R., Bassano, I., Denise, H., McDonald, J.E., Malham,

S.K., Porter, J., Warren, J., Evens, N.P., Paterson, S., Singer, A.C., Jones, D.L., 2022. A comparison of precipitation and filtration-based SARS-CoV-2 recovery methods and the influence of temperature, turbidity, and surfactant load in urban wastewater. Sci. Total Environ. 808, 151916. https://doi.org/10.1016/j.scitotenv.2021.151916.

Khan, K.S., Kunz, R., Kleijnen, J., Antes, G., 2003. Five Steps to Conducting a Systematic Review. J R Soc Med 96, 118–121. https://doi.org/10.1177/014107680309600304.

Koivunen, J., Siitonen, A., Heinonen-Tanski, H., 2003. Elimination of enteric bacteria in biological-chemical wastewater treatment and tertiary filtration units. Water Res. 37, 690-698. https://doi.org/10.1016/S0043-1354(02)00305-6.

Leverenz, H.L., Tchobanoglous, G., Asano, T., 2011. Direct potable reuse: A future imperative. J. Water Reuse Desalin. 1, 2–10. https://doi.org/10.2166/wrd.2011.000.

Lewis, G. D., Metcalf, T. G. (1988). Polyethylene glycol precipitation for recovery of pathogenic viruses, including hepatitis A virus and human rotavirus, from oyster, water, and sediment samples. *Applied and Environmental Microbiology*, 54(8), 1983-1988.

Li, B., Di, D.Y.W., Saingam, P., Jeon, M.K., Yan, T., 2021. Fine-Scale Temporal Dynamics of SARS-CoV-2 RNA Abundance in Wastewater during A COVID-19 Lockdown. Water Res. 197, 117093. https://doi.org/https://doi.org/10.1016/j.watres.2021.117093.

Lu, D., Huang, Z., Luo, J., Zhang, X., Sha, S., 2020. Primary concentration – The critical step in implementing the wastewater based epidemiology for the COVID-19 pandemic: A mini-review. Sci. Total Environ. 747, 141245. https://doi.org/https://doi.org/10.1016/j.scitotenv.2020.141245.

Madoux-Humery, A.-S., Dorner, S., Sauvé, S., Aboulfadl, K., Galarneau, M., Servais, P., Prévost, M., 2013. Temporal variability of combined sewer overflow contaminants: Evaluation of wastewater micropollutants as tracers of faecal contamination. Water Res. 47, 4370–4382.

Masaka, E., Reed, S., Davidson, M., Oosthuizen, J., 2021. Opportunistic Premise Plumbing Pathogens. A Potential Health Risk in Water Mist Systems Used as a Cooling Intervention. Pathogens 10, 462. https://doi.org/10.3390/pathogens10040462.

McBride, G.B., Stott, R., Miller, W., Bambic, D., Wuertz, S., 2013. Discharge-based QMRA for estimation of public health risks from exposure to stormwater-borne pathogens in recreational waters in the United States. Water Res. 47, 5282–5297. https://doi.org/10.1016/j.watres.2013.06.001.

McMinn, B.R., Ashbolt, N.J., Korajkic, A., 2017. Bacteriophages as indicators of faecal pollution and enteric virus removal. Lett. Appl. Microbiol. 65, 11–26. https://doi.org/10.1111/lam.12736.

McMinn, B.R., Korajkic, A., Kelleher, J., Herrmann, M.P., Pemberton, A.C., Ahmed, W., Villegas, E.N., Oshima, K., 2021. Development of a large volume concentration method for recovery of

coronavirus from wastewater. Sci. Total Environ. 774, 145727. https://doi.org/10.1016/j.scitotenv.2021.145727.

Medema, G., Heijnen, L., Elsinga, G., Italiaander, R., Brouwer, A., 2020. Presence of SARS-Coronavirus-2 RNA in Sewage and Correlation with Reported COVID-19 Prevalence in the Early Stage of the Epidemic in the Netherlands. Environ. Sci. Technol. Lett. 7, 511–516. https://doi.org/10.1021/acs.estlett.0c00357.

Millard, S.P., Kowarik, A., 2022. *Package 'EnvStats': Package for Environmental Statistics, Including US EPA Guidance*. Version 2.7.0. https://github.com/alexkowa/EnvStats

Moher, D., Shamseer, L., Clarke, M., Ghersi, D., Liberati, A., Petticrew, M., Shekelle, P., Stewart, L.A., PRISMA-P Group, 2015. Preferred reporting items for systematic review and meta-analysis protocols (PRISMA-P) 2015 statement. Systematic Reviews 4, 1. https://doi.org/10.1186/2046-4053-4-1.

Momba, M., Edbon, J., Kamika, I., Verbyla, M., 2019. Using indicators to assess microbial treatment and disinfection efficacy, in: J.B. Rose and B. Jiménez-Cisneros, (eds) Global Water Pathogen Project (Ed.), A.Farnleitner, and A. Blanch (Eds) Part 2 Indicators and Microbial Source Tracking Markers. UNESCO, Michigan State University, E. Lansing, MI.

Morton, V.K., Thomas, M.K., McEwen, S.A., 2015. Estimated hospitalizations attributed to norovirus and rotavirus infection in Canada, 2006-2010. Epidemiol. Infect. 143, 3528–3537. https://doi.org/10.1017/S0950268815000734.

Nappier, S.P., Hong, T., Ichida, A., Goldstone, A., Eftim, S.E., 2019. Occurrence of coliphage in raw wastewater and in ambient water: A meta-analysis. Water Research 153, 263–273. https://doi.org/10.1016/j.watres.2018.12.058.

Naughton, C.C., Rousselot, O., 2017. Activated Sludge, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Nellor Environment Associates, Trussell Technologies, TODD Groundwater, 2019. Final Engineering Report: Monterey One Water Pure Water Monterey Groundwater Replenishment Project. Monterey, CA, USA: Nellor Environmental; Trussell Technologies; TODD Groundwater.

NWRI, 2012. *Examining the Criteria for Direct Potable Reuse: Recommendations of an NWRI Independent Advisory Panel*. National Water Research Institute

NWRI, WateReuse Foundation, AWWA, WEF, 2015. *Framework for Direct Potable Reuse*. WateReuse Association

NWRI, 2022. *Memorandum of Findings: Expert Panel Preliminary Findings and Recommendations on Draft DPR Criteria*. Report published by the National Water Research

Institute, Fountain Valley, CA, USA.

Oakley, S.M., 2018. Preliminary Treatment and Primary Settling, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Oakley, S.M., von Sperling, M., 2017. Media Filters, in: Mihelcic, J.R., Verbyla, M.E. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Oh, C., Kim, K., Araud, E., Wang, L., Shisler, J.L., Nguyen, T.H., 2022. A novel approach to concentrate human and animal viruses from wastewater using receptors-conjugated magnetic beads. Water Res. 212, 118112. https://doi.org/10.1016/j.watres.2022.118112.

Pecson, B.M., Martin, L.V., Kohn, T., 2009. Quantitative PCR for determining the infectivity of bacteriophage MS2 upon inactivation by heat, UV-B radiation, and singlet oxygen: Advantages and limitations of an enzymatic treatment to reduce false-positive results. Appl. Environ. Microbiol. 75, 5544–5554. https://doi.org/10.1128/AEM.00425-09.

Pecson, B.M., Darby, E., Danielson, R., Dearborn, Y., Di Giovanni, G., Jakubowski, W., Leddy, M., Lukasik, G., Mull, B., Nelson, K.L., Oliveiri, A., Rock, C., Slifko, T., 2022. Distributions of waterborne pathogens in raw wastewater based on a 14-month, multi-site monitoring campaign. *Water Research*, 213, 118170. https://doi.org/10.1016/j.watres.2022.118170.

Pecson, B.M., Ashbolt, N., Haas, C., Slifko, T., Kaufmann, A., Gerrity, D., Seto, E., Olivieri, A., 2021a. *Tools to evaluate quantitative microbial risk and plant performance/reliability (DPR-1)*. WRF Project No. 4951, The Water Research Foundation, Alexandria, VA.

Pecson, B.M., Darby, E., Di Giovanni, G., Leddy, M., Nelson, K.L., Rock, C., Slifko, T., Jakubowski, W., Olivieri, A., 2021b. *Pathogen monitoring in untreated wastewater (DPR-2)*. WRF Project Number 4989, The Water Research Foundation, Alexandria, VA.

Polanco, J., Safarik, J., Plumlee, M.H., 2022. *Demonstrating Virus Log Removal Credit for Wastewater Treatment and Reverse Osmosis for Potable Reuse at OCWD*. Water Research Foundation, Alexandria, VA, USA.

Pouillot, R., Van Doren, J.M., Woods, J., Plante, D., Smith, M., Goblick, G., Roberts, C., Locas, A., Hajen, W., Stobo, J., White, J., Holtzman, J., Buenaventura, E., Burkhardt, W., Catford, A., Edwards, R., DePaola, A., Calci, K.R., 2015. Meta-Analysis of the Reduction of Norovirus and Male-Specific Coliphage Concentrations in Wastewater Treatment Plants. Appl Environ Microbiol 81, 4669–4681. https://doi.org/10.1128/AEM.00509-15.

Pullin, A.S., Stewart, G.B., 2006. Guidelines for Systematic Review in Conservation and Environmental Management. Conservation Biology 20, 1647–1656.

https://doi.org/10.1111/j.1523-1739.2006.00485.x.

Rabbani, S., 2017. *Proof that the Difference of Two Correlated Normal Random Variables is Normal*. https://srabbani.com/.

Randazzo, W., Truchado, P., Cuevas-Ferrando, E., Simón, P., Allende, A., Sánchez, G., 2020. SARS-CoV-2 RNA in wastewater anticipated COVID-19 occurrence in a low prevalence area. Water Res. 181. https://doi.org/10.1016/j.watres.2020.115942.

Rodriguez-Alvarez, M.S., Weir, M.H., Pope, J.M., Seghezzo, L., Rajal, V.B., Salusso, M.M., Moraña, L.B., 2015. Development of a relative risk model for drinking water regulation and design recommendations for a peri urban region of Argentina. International Journal of Hygiene and Environmental Health 218, 627–638. https://doi.org/10.1016/j.ijheh.2015.06.007.

Rose, J.B., Farrah, S.R., Harwood, V.J., Levine, A., Lukasik, J., Menendez, P., Scott, T.M., 2004. *Reduction of pathogens, indicator bacteria, and alternative indicators by wastewater treatment and reclamation processes*. WERF Report: Water for Reuse (00-PUM-2T). IWA Publishing, London, UK. https://doi.org/10.2166/9781780404370.

Rose, J.B., Jiménez-Cisneros, B.E. (Eds.), 2019. Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. Global Water Pathogen Project (GWPP); Michigan State University, UNESCO, E. Lansing, MI, USA.

Rose, J.B., Hofstra, N., Murphy, H.M., Verbyla, M.E., 2019. What is safe sanitation? ASCE Journal of Environmental Engineering, 145(12), 02519002. https://doi.org/10.1061/(ASCE)EE.1943-7870.0001599.

Rusiñol, M., Martínez-Puchol, S., Forés, E., Itarte, M., Girones, R., Bofill-Mas, S., 2020. Concentration methods for the quantification of coronavirus and other potentially pandemic enveloped virus from wastewater. Curr. Opin. Environ. Sci. Heal. 17, 21–28. https://doi.org/10.1016/j.coesh.2020.08.002.

Schmidt, P.J., Anderson, W.B., Emelko, M.B., 2020. Describing water treatment process performance: Why average log-reduction can be a misleading statistic. Water Res. 176, 115702. https://doi.org/10.1016/j.watres.2020.115702.

Schoen, M.E., Ashbolt, N.J., Jahne, M.A., Garland, J., 2017. Risk-based enteric pathogen reduction targets for non-potable and direct potable use of roof runoff, stormwater, and greywater. Microb. Risk Anal. 5, 32–43. https://doi.org/10.1016/j.mran.2017.01.002.

Seis, W., Rouault, P., Medema, G., 2020. Addressing and reducing parameter uncertainty in quantitative microbial risk assessment by incorporating external information via Bayesian hierarchical modeling. Water Research 185, 116202. https://doi.org/10.1016/j.watres.2020.116202.

Shepherd, K.M., Wyn-Jones, A.P., 1996. An evaluation of methods for the simultaneous

detection of Cryptosporidium oocysts and Giardia cysts from water. Appl. Environ. Microbiol. 62, 1317–1322. https://doi.org/10.1128/aem.62.4.1317-1322.1996.

Shoari, N., Dubé, J.S., Chenouri, S., 2015. Estimating the mean and standard deviation of environmental data with below detection limit observations: Considering highly skewed data and model misspecification. Chemosphere 138, 599–608. https://doi.org/10.1016/j.chemosphere.2015.07.009.

Sidhu, J.P.S., Ahmed, W., Palmer, A., Smith, K., Hodgers, L., Toze, S., 2017. Optimization of sampling strategy to determine pathogen removal efficacy of activated sludge treatment plant. Environ. Sci. Pollut. Res. 24, 19001–19010. https://doi.org/10.1007/s11356-017-9557-5.

Sidhu, J.P.S., Sena, K., Hodgers, L., Palmer, A., Toze, S., 2018. Comparative enteric viruses and coliphage removal during wastewater treatment processes in a sub-tropical environment. Sci. Total Environ. 616–617, 669–677. https://doi.org/https://doi.org/10.1016/j.scitotenv.2017.10.265.

Soller, J.A., Eftim, S.E., Warren, I., Nappier, S.P., 2017. Evaluation of microbiological risks associated with direct potable reuse. Microb. Risk Anal. 5, 3–14. https://doi.org/10.1016/j.mran.2016.08.003.

Stelma, G.N., 2018. Use of bacterial spores in monitoring water quality and treatment. J. Water Health 16, 491–500. https://doi.org/10.2166/wh.2018.013.

Tchobanoglous, G., Kenny, J., Leverenz, H., Oliveri, A., 2022. Establishing log reduction values for wastewater treatment processes from ambient influent and effluent pathogen monitoring data. *Front. Environ. Sci.* 10, 940014. https://doi.org/10.3389/fenvs.2022.940014.

Teunis, P.F., Rutjes, S.A., Westrell, T., de Roda Husman, A.M., 2009. Characterization of drinking water treatment for virus risk assessment. Water Res 43, 395–404. https://doi.org/10.1016/j.watres.2008.10.049.

Tolouei, S., Burnet, J.-B., Autixier, L., Taghipour, M., Bonsteel, J., Duy, S.V., Sauvé, S., Prévost, M., Dorner, S., 2019. Temporal variability of parasites, bacterial indicators, and wastewater micropollutants in a water resource recovery facility under various weather conditions. Water Res. 148, 446–458. https://doi.org/https://doi.org/10.1016/j.watres.2018.10.068.

Tonon, D., Tonetti, A.L., Coraucci Filho, B., Bueno, D.A.C., 2015. Wastewater treatment by anaerobic filter and sand filter: Hydraulic loading rates for removing organic matter, phosphorus, pathogens and nitrogen in tropical countries. Ecol. Eng. 82, 583–589. https://doi.org/10.1016/j.ecoleng.2015.05.018.

Truchado, P., Garre, A., Gil, M.I., Simón-Andreu, P.J., Sánchez, G., Allende, A., 2021. Monitoring of human enteric virus and coliphages throughout water reuse system of wastewater treatment plants to irrigation endpoint of leafy greens. Sci. Total Environ. 782, 146837. https://doi.org/https://doi.org/10.1016/j.scitotenv.2021.146837.

US EPA, 1991. *Guidance Manual for Compliance with the Filtration and Disinfection Requirements for Public Water Systems Using Surface Water Sources*. Washington, D.C.: US EPA.

US EPA. 1992. *Guidelines for Water Reuse*. EPA/625/R-92/004. US EPA.

US EPA, 1999a. *Disinfection Profiling and Benchmarking Guidance Manual*. Washington, D.C.: US EPA.

US EPA. 1999b. *Alternative Disinfectants and Oxidants Guidance Manual*. EPA 815-R-99-014. Washington, D.C.: US EPA.

US EPA, 2003. *LT1ESWTR Disinfection Profiling and Benchmarking: Technical Guidance Manual*. Washington, D.C.: US EPA.

US EPA. 2004. *Guidelines for Water Reuse*. EPA 625/R-04/108. Washington, D.C.: US EPA.

US EPA, 2005. Method 1623: Cryptosporidium and Giardia in Water by Filtration/IMS/FA. EPA 815-R-05-002. US EPA.

US EPA. 2006a. *National Primary Drinking Water Regulations: Long Term 2 Enhanced Surface Water Treatment Rule*; Final Rule. 71 FR 654. January 5, 2006. US EPA.

US EPA. 2006b. *National Primary Drinking Water Regulation: Ground Water Rule; Final Rule*. 71 FR 65573. November 8, 2006. US EPA.

US EPA, 2006c. *Ultraviolet Disinfection Guidance Manual*. Washington, D.C.: US EPA.

US EPA, 2010. *Long Term 2 Enhanced Surface Water Treatment Rule Toolbox Guidance Manual.* Washington, D.C.: US EPA.

US EPA, 2012a. *Guidelines for Water Reuse*. EPA/600/R-12/618. Washington, D.C.: US EPA.

US EPA, 2012b. Method 1623.1 Cryptosporidium and Giardia in Water by FIltration/IMS/FA. Washington, D.C.: US EPA.

US EPA, 2014. Method 1693: Cryptosporidium and Giardia in Disinfected Wastewater by Concentration / IMS / IFA, EPA 821-R-14-013. Washington, D.C.: US EPA.

Fout, Shay, N. Brinkman, J. Cashdollar, S. Griffin, B. McMinn, E. Rhodes, E. Varughese, M. Karim, A. Grimm, S. Spencer, AND M. Borchardt. 2014. Method 1615 Measurement of Enterovirus and Norovirus Occurrence in Water by Culture and RT-qPCR. Washington, D.C.: U.S. Environmental Protection Agency.

US EPA, CDM Smith, 2018. *Potable Reuse Compendium*. Washington, D.C.: US EPA; Boston, MA: CDM Smith.

Venthic. 2023. Venthic. Venthic Technologies. https://www.venthic.com/.

Verbyla, M.E., Mihelcic, J.R., 2015. A review of virus removal in wastewater treatment pond systems. Water Res. 71, 107-124. https://doi.org/10.1016/j.watres.2014.12.031.

Verbyla, M.E., Rousselot, O., 2018. Membrane Bioreactors, in: Rose, J.B., Jiménez-Cisneros, B. (Eds.), Water and Sanitation for the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. J.B. Rose and B. Jiménez-Cisneros (eds), Global Water Pathogen Project (GWPP), UNESCO, Michigan State University, East Lansing, MI, USA.

Verbyla, M.E., Symonds, E.M., Kafle, R.C., Cairns, M.R., Iriarte, M., Mercado Guzmán, A., Coronado, O., Breitbart, M., Ledo, C., Mihelcic, J.R., 2016. Managing Microbial Risks from Indirect Wastewater Reuse for Irrigation in Urbanizing Watersheds. Environ. Sci. Technol. 50. https://doi.org/10.1021/acs.est.5b05398.

von Sperling, M., Verbyla, M.E., Mihelcic, J.R., 2018. Understanding Pathogen Reduction in Sanitation Systems: Units of Measurement , Expressing Changes in Concentrations, and Kinetics, in: Rose, J.B., JImenez-Cisneros, B.E. (Eds.), Sanitation and Disease in the 21st Century: Health and Microbiological Aspects of Excreta and Wastewater Management. Michigan State University, UNESCO, E. Lansing, MI, USA.

von Sperling, M., Verbyla, M.E., Oliveira, S.M.A.C., 2020. *Assessment of Treatment Plant Performance and Water Quality Data: A Guide for Students, Researchers and Practitioners, Assessment of Treatment Plant Performance and Water Quality Data: A Guide for Students, Researchers and Practitioners*. IWA Publishing, London, UK. https://doi.org/10.2166/9781780409320.

Wen, Q., Tutuka, C., Keegan, A., Jin, B., 2009. Fate of pathogenic microorganisms and indicators in secondary activated sludge wastewater treatment plants. J. Environ. Manage. 90, 1442-1447. https://doi.org/10.1016/j.jenvman.2008.09.002.

Weinroth, M.D., Belk, A.D., Belk, K.E., 2018. History, development, and current status of food safety systems worldwide. Animal Frontiers 8, 9–15. https://doi.org/10.1093/af/vfy016.

Weisstein, E.W., 2022. *Normal Difference Distribution*. From MathWorld--A Wolfram Web Resource. Available at: https://mathworld.wolfram.com/NormalDifferenceDistribution.html. (accessed on December 21, 2022).

Wigginton, K.R., Pecson, B.M., Sigstam, T., Bosshard, F., Kohn, T., 2012. Virus inactivation mechanisms: Impact of disinfectants on virus function and structural integrity. Environ. Sci. Technol. 46, 12069–12078. https://doi.org/10.1021/es3029473.

Woodard & Curran, 2020. Pure Water Oceanside Title 22 Engineering Report. Technical Report. Oceanside, CA, USA.

Wu, Z., Greaves, J., Arp, L., Stone, D., Bibby, K., 2020. Comparative fate of CrAssphage with culturable and molecular fecal pollution indicators during activated sludge wastewater treatment. Environ. Int. 136, 105452.

https://doi.org/https://doi.org/10.1016/j.envint.2019.105452.

Wurtzer, S., Waldman, P., Ferrier-Rembert, A., Frenois-Veyrat, G., Mouchel, J.M., Boni, M., Maday, Y., Marechal, V., Moulin, L., 2021. Several forms of SARS-CoV-2 RNA can be detected in wastewaters: Implication for wastewater-based epidemiology and risk assessment. Water Res. 198, 117183. https://doi.org/10.1016/j.watres.2021.117183.

Ye, Y., Ellenberg, R.M., Graham, K.E., Wigginton, K.R., 2016. Survivability, Partitioning, and Recovery of Enveloped Viruses in Untreated Municipal Wastewater. Environ. Sci. Technol. 50, 5077–5085. https://doi.org/10.1021/acs.est.6b00876.

Weinroth, M. D., Belk, A. D., & Belk, K. E. (2018). History, development, and current status of food safety systems worldwide. *Animal Frontiers*, 8(4), 9-15.

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