

Normalizing Wastewater Data

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Key Points

- **The choice of normalization approach is highly-dependent on the presumed environmental effects and practical constraints of the specific wastewater-based epidemiology (WBE) application, and no single answer is best. Presenting data without normalization can also be a valid approach.**
- Normalization refers to the process of adjusting wastewater data so that changes in reported concentrations of a target analyte or biomarker better reflect meaningful changes in the community. Normalization can be used to account for a variety of environmental effects, including in-lab, in-sewer, and in-human effects.
- In this white paper, we propose a framework for selecting and evaluating normalization approaches for any WBE application. At minimum, a normalization approach must be theoretically justified and logistically practical. Additionally, normalization approaches may provide direct estimates of the effect being accounted for, improve representation of external trends, and/or improve the interpretability of the data.
- Normalization works by scaling laboratory measurements using either (i) marker normalization, in which markers measured in the same sample as the analyte of interest are used to account for the environmental effects, or (ii) metadata normalization, in which metadata derived from the community or other external sources is used to account for effects.
- Marker normalization can be applied to any wastewater sample regardless of where it was collected and without the need for additional metadata like flow or population, making it an extremely versatile normalization approach. It can address all types of environmental effects, unlike metadata normalization which is limited to in-sewer and in-human effects. However, marker normalization requires additional lab measurements and may therefore be less practical than metadata normalization for some applications.

Normalization in wastewater-based epidemiology

Wastewater-based epidemiology (WBE) has demonstrated its potential as a powerful public health surveillance tool during the Covid-19 pandemic. This successful application of WBE has invigorated efforts to expand wastewater surveillance to public health applications beyond Covid-19. However, one of the major challenges in interpreting wastewater-based surveillance data is ensuring comparability of the data produced between samples (Kumblathan 2021). For example, variation in daily inflow or differences between population counts affect laboratory measurements and complicate comparison across samples.

Normalization refers to the process of adjusting wastewater data so that changes in lab measurements better reflect meaningful changes in the community where the target biomarker is being measured. Ideally, normalization enables or improves the comparability of concentrations across different time points and locations, and in some cases can even be used to compare results generated by different laboratory protocols (Biobot 2022).

Why normalize wastewater data?

By the time target biomarkers are measured in a laboratory, they have already been subjected to a number of environmental effects that obscure the interpretation of meaningful community trends. For example, variation in dilution or degradation would appear as if the biomarker's prevalence is changing within a community when it may actually be static. Normalization addresses key interpretation challenges of wastewater data by standardizing these effects across samples to facilitate comparisons (Hoar 2022, O'Brien 2017). We categorize the primary environmental effects that impact interpretation of wastewater data into three groups:

- ① **In-lab effects**, which include any gains or losses related to sample processing within the laboratory. For example: process or extraction inefficiencies that lead to measured concentrations that no longer reflect the true concentration of the sample; instrument biases leading to systematically increased measured concentrations; day-to-day fluctuations in lab reagent efficiencies; and systematic differences between different protocols across labs.
- ② **In-sewer effects**, which include any physical or chemical transformations to the target that occur in transit from source to sampling location and lab. For example: degradation of viruses or analytes as they travel in the sewer network to the sampling point; chemical transformation of a metabolite back into its parent compound or another metabolite; partitioning of the analyte between liquid and solid fractions in the wastewater; dilution due to precipitation events, inflow and infiltration, or industrial discharges; and degradation of analytes during transit to the lab.
- ③ **In-human effects**, which include anything that happens before the sample is introduced into the wastewater or independently of it. For example: differences in drug administration and the respective impact on excretion rates; fluctuating populations in the catchment being sampled related to commuting patterns; transient populations within a community, for example related to a one-off entertainment event or seasonal tourism; and different viral shedding rates between infected individuals leading to variable per-capita contributions of virus into the wastewater system.

Different WBE practitioners and consumers of WBE data may place different levels of importance on each of these effects. A wastewater testing laboratory would be very motivated to normalize for lab effects, but less concerned with normalization approaches that address upstream in-human effects because those are out of their scope of work and responsibility. In contrast, an epidemiologist in a public health department likely cares much more about understanding how upstream in-human effects like fluctuating population are accounted for in the data than specifics about lab process efficiencies.

A normalization approach must be theoretically justified & logistically practical

The ideal normalization method would allow us to accurately quantify real world phenomena from wastewater data. Unfortunately, this idealized approach is likely impossible to achieve due to the number of environmental effects that alter the target analyte or pathogen as it travels from person to laboratory result (Castiglioni 2013, Feng 2021). Normalization can be used to account for these effects; however, we find that it is often a multi-faceted process, where no single approach is best and the choice of normalization approach is highly-dependent on the environmental effects trying to be addressed and practical constraints of the specific WBE application. Importantly, not applying any normalization is also a valid choice for many applications. Here, we propose a framework for selecting and evaluating normalization approaches for any WBE application.

The first consideration is that at minimum, a normalization approach must be theoretically justified and logistically practical. For example, a good marker for population-focused normalization should be excreted by most people or by a consistent fraction of people, have limited non-human contributions, and show little seasonal variation. Ideally, a normalization marker should exhibit few systematic biases related to demographics or behavioral patterns that may be of interest for wastewater monitoring. From a practical perspective, normalization markers should be compatible with existing analytical methods and not add substantial logistical or financial burdens on existing operational processes.

How do we evaluate different normalization approaches?

In addition to theoretical and practical justification, normalization approaches can be evaluated and compared along multiple axes. These axes, which are presented below, can serve as a framework for determining which specific normalization approach and markers to use. Importantly, a normalization approach does not need to hit all of these criteria. An important consideration for all three data-driven evaluation approaches mentioned below is that a variety of sampling locations need to be included in any analysis, since the effects of normalization may vary drastically between different sampling locations.

Provides direct estimate of the effect

The most easily interpretable approach towards normalization is to directly estimate and correct for a known effect. However, this approach is also the most challenging to develop and validate, in part due to the lack of gold standards or baselines that can be used to benchmark the correction. Despite this challenge, a substantial amount of WBE literature focuses on this axis of evaluation. For example, studies use wastewater parameters to estimate a sample's contributing population (Hoar 2022) or combine multiple analyte measurements into a prediction of the transient population (O'Brien 2013). These models can be used as a direct substitute for the contributing population in a given wastewater sample. Other work attempts to directly model in-sewer effects by calculating degradation of individual analytes in a variety of sewer conditions (McCall 2016, van Nuijs 2011). These models can then theoretically be applied to correct for degradation or biotransformation experienced by analytes as they travel to the sampling location.

Improves representation of external trends

An alternative way to evaluate a given normalization approach is to show that normalized data better reflects expected external trends. For example, normalization can improve correlations with an independent proxy marker for an epidemiological phenomenon of interest. Studies evaluating normalization in Covid-19 monitoring often compare the correlation of normalized and unnormalized wastewater measurements with reported clinical cases (Duvall et

2022, Feng 2021, Wolfe 2021). Evaluating normalization along this axis is beneficial, because it directly addresses the question at the heart of normalization: is normalization improving our ability to interpret the epidemiological trends within and across locations?

However, this type of evaluation is not applicable for many applications of WBE. First, proxy markers or gold standards are not available for many WBE use cases. For example, in the case of opioid monitoring, few gold-standard datasets exist that reflect the true underlying amount of opioid use disorder in a community (Reuter 2021). Additionally, epidemiological dynamics may be too slow for correlations to capture meaningful changes in a reasonable time period. Trends in opioid use change over the course of years rather than weeks, meaning that evaluating normalization markers via correlation improvements would require a multi-year study to yield meaningful correlations, which is practically and logistically not feasible.

Improves interpretability of the data

A third way to evaluate normalization is to determine whether it improves the overall interpretability of the wastewater data. For example, normalization might reduce unwanted noise or variability within a location or remove systematic location-specific biases in average concentration levels. Normalization may also recover a constant trend for a marker which is known to be stable across time. This axis is purposefully left open-ended, as the field discovers new and better ways to evaluate the impact of normalization without external gold-standards against which to compare.

How do we apply normalization to wastewater data?

Normalization works by scaling laboratory measurements using either (i) marker normalization, in which markers measured concurrently with the analyte of interest are used to account for the environmental effects, or (ii) metadata normalization, in which metadata derived from the community or other external sources is used to directly estimate or otherwise account for effects.

Both of these approaches attempt to standardize wastewater data to facilitate community comparisons and are often used in combination to overcome different environmental effects. For example, models to predict population based on population markers may rely on external metadata during development and validation phases, but then simply use biomarkers measured alongside the target analyte of interest in their routine application (Hoar 2022). Similarly, biomarker normalization can use an externally-derived reference value of the normalization marker to convert unitless ratios back into human-interpretable units (Duvall 2022). Our goal in proposing these two broad categories of normalization approaches is not to draw strict boundaries between them, but rather provide a way to reason about different approaches and evaluate their tradeoffs.

Marker normalization

Marker normalization attempts to address environmental effects through the use of a normalization marker derived from the sample itself. In theory, if the normalization marker experiences similar environmental effects as the target biomarker of interest, then it can be used as a reference to account for those effects. For example, if a precipitation event led to a more diluted sample on a given day, both the biomarker of interest and the normalization marker would have lower measured concentrations. Dividing the analyte by the normalization marker would, in effect, cancel out the dilution and provide a constant ratio between the two measurements. Normalization markers may be commonly excreted biological markers (e.g. crASSphage, RNaseP, pepper mild mottle virus) or chemical analytes (e.g. creatinine, atenolol, nicotine) (Daughton 2020, O'Brien 2017).

Marker normalization can address all types of environmental effects:

- ① **In-lab effects**, with normalization markers that experience similar procedural losses as the target of interest to provide a reference to correct for those losses. These markers can be native to the sample, but are often added prior to sample preparation. Examples of these markers include heavy-labeled chemicals for LC-MS analysis or spike-in recovery controls for qPCR.
- ② **In-sewer effects**, with markers that undergo similar transformations, partitioning, or dilution and can therefore be used as a surrogate to normalize those occurrences across samples.
- ③ **In-human effects**, with markers that are consistently excreted across communities and can be used as surrogates to address fluctuating population. These markers include commonly-used pharmaceuticals and their metabolites (e.g. atenolol), endogenous metabolites (e.g. creatinine), ubiquitous human-associated viruses or bacteria (e.g. pepper mild mottle virus, crASSphage) or other human-associated biological markers (e.g. RNaseP).

Metadata normalization

Metadata normalization attempts to quantify environmental effects using external data sources separate from measurements derived from the sample itself. A common application of this approach is in standard mass balance normalization: using catchment populations and measured inflow data to convert sample concentrations (mass/volume) to community mass loads per capita (mass/person/day) (Zuccato 2008). Flow and population values can be directly measured or theoretically modeled from a combination of wastewater parameters, for example population markers, ammonia, total suspended solids, or biological oxygen (O'Brien 2013, Hoar 2022). Theoretical models may use a combination of marker and metadata normalization.

Metadata normalization focuses on accounting for events that occur prior to laboratory analysis, and is therefore limited to addressing in-sewer and in-human effects:

- ① **In-sewer effects**, for example by using flow to account for dilution by calculating the total massload entering a wastewater treatment plant. In addition, the degradation of target analytes can be modeled to predict the extent of degradation during transit given a certain wastewater residence time (McCall 2016, van Nuijs 2011). By combining external metadata like residence time with these models, the amount of analyte lost during transit can be calculated and used to infer the amount of total analyte entering the sewer system.
- ② **In-human effects**, for example by using the estimated catchment population provided by wastewater treatment plant operators to account for different populations across sampling sites. These metrics are used to calculate massload per capita, which facilitates community comparison.

What are the tradeoffs between marker and metadata normalization?

Marker and metadata normalization are widely used in WBE. Determining which is appropriate for a given application requires weighing tradeoffs, and there is no one best approach for all applications. Here, we provide an overview of the benefits and tradeoffs of each approach and present case studies applying this framework to two common normalization approaches.

Marker normalization

Benefits	Tradeoffs
Does not require external sample metadata, expanding applicability to any type of wastewater sample	Results in unitless ratios unless additional unit conversion is applied
Can account for in-lab effects	Needs additional lab measurements
Easier to account for all three categories of environmental effects at once (in-lab, in-sewer, and in-human)	Variability of normalization marker measurement carries over into the normalization
	Requires assumption that normalization marker and target analyte undergo similar environmental transformations

Marker normalization can be applied to any wastewater sample regardless of where it was collected and without the need for additional metadata like flow or population. Because normalization markers are measured from the same wastewater sample as the target analyte or biomarker, the only thing needed to perform biomarker normalization is the sample itself. Additionally, marker normalization can account for all types of effects (in-lab, in-sewer, and in-human), so long as the effects on the marker used for normalization are similar to the effects on the target biomarker. However, results are reported as a unitless ratio, which can be hard to interpret or communicate. Additional transformations like converting back to interpretable units using a reference value can overcome this challenge (Duvall 2022). Normalization markers should also have similar precision to the target analyte, otherwise the variability introduced by the normalization marker may overshadow benefits of normalization. Finally, biomarker normalization requires additional lab measurements, which may require the development of new lab protocols.

Metadata normalization

Benefits	Tradeoffs
Yields intuitive units	Requires external metadata to represent an environmental effect
Does not need additional lab measurements	Cannot account for in-lab or in-sewer effects; few models have been developed to address these effects
	Requires assumption that metadata adequately reflects environmental effects and is provided consistently and accurately

The primary benefit of metadata normalization is that it often yields relatively interpretable units like total copies of virus or mass of a drug per capita. In addition, metadata normalization does not require additional lab measurements beyond the target biomarker of interest, which may make it more broadly applicable across a variety of lab protocols. However, metadata normalization requires metadata that accurately represents the environmental effects that are attempting to be modeled. These metrics, such as flow, are not always available for sampling sites (e.g. manholes

without flow meters, building cleanouts with flow too low for standard flow meters), and can be difficult to collect consistently from sampling locations. Models such as those measuring degradation are usually developed in controlled environments and can lack validation across multiple sewers systems to validate their efficacy on all potential wastewater samples. Finally, in most cases metadata normalization does not account for laboratory effects.

Normalization case studies

Marker normalization: pepper mild mottle virus normalization

As an example, we can apply the framework proposed above to evaluate normalization by pepper mild mottle virus (PMMoV), which is currently one of the most commonly-used normalization approaches in Covid-19 wastewater monitoring.

What makes PMMoV normalization a suitable normalization approach?

PMMoV is one of the most commonly excreted human viruses and can be measured using the same assay as SARS-CoV-2, making it well-justified and extremely practical (Duvall et al. 2022, Wu 2020). However, organizations who are aggregating data from a variety of different labs may view PMMoV normalization as impractical because it requires all contributing labs to measure PMMoV in addition to SARS-CoV-2.

How well does PMMoV work as a normalization marker?

- Direct estimation: PMMoV does not directly estimate in-human, in-sewer, or in-lab effects.
- Representation of external trends: PMMoV normalization improves correlations with clinical Covid-19 cases when comparing across locations. However, it does not necessarily improve correlations within individual locations' time series: PMMoV has been shown to both improve and worsen correlations with clinical cases within individual locations (Duvall et al. 2022, Feng 2021, Wolfe 2021).
- Improving data interpretability: We have found that in certain laboratory protocols with low measurement variability, PMMoV normalization reduces the sample-to-sample variability of the normalized SARS-CoV-2 concentrations.

What effects can PMMoV normalization address?

- In-lab effects: PMMoV corrects for lab effects, as PMMoV and SARS-CoV-2 are measured with the same protocol and therefore expected to experience similar losses during laboratory processing.
- In-sewer effects: PMMoV and SARS-CoV-2 may experience similar degradation in the wastewater network.
- In-human effects: PMMoV is a commonly excreted dietary virus and serves as a reasonable proxy for fluctuating populations.

Overall, PMMoV's primary strengths as a normalization marker are that it is theoretically justified, practically superior for organizations monitoring a large variety of sampling location types (e.g. manholes in addition to wastewater treatment plants), and that it improves our ability to compare wastewater measurements across locations (as shown by the improved correlations across locations).

Metadata normalization: normalization with population and flow metadata

This framework can also be used to evaluate normalization using sample metadata typically tracked by wastewater treatment plants: total volumetric inflow and estimated catchment population. This approach is commonly used to report the total massload of substances entering a wastewater system, and is another popular normalization method in WBE applications (Zuccato 2008).

What makes population/flow normalization a suitable normalization approach?

Using population and flow data is theoretically justified to determine the total massload for a community based on simple mass balance. From a practical perspective, these metrics are often not available for samples collected at manholes or buildings, which limits practicality to wastewater treatment plant samples only.

How well does population/flow normalization work?

- Direct estimation: Flow measurements provided by wastewater treatment plants are a direct measurement of the total volume, which can be used to account for differences in sewer inflow across different days and locations. Additionally, estimates of the population provide a means of directly accounting for population differences across locations, but usually cannot account for fluctuating or transient populations within a catchment.
- Representation of external trends: The impact of flow/population normalization on correlations with Covid-19 incidence and proxy markers for substance use (pharmaceutical sales, drug seizures, etc) has been evaluated. One study found improved correlations with Covid-19 cases after flow/population normalization, while the impact of normalization on correlations with substance use proxy markers yielded mixed results (Feng 2021, Baz-Lomba 2016).
- Improving data interpretability: population/flow normalization converts measured concentration values to total mass entering a wastewater treatment plant per person in a catchment area, which can be a more interpretable unit than concentration.

What effects can population/flow normalization address?

- In-lab effects: population/flow normalization does not address in-lab effects.
- In-sewer effects: population/flow normalization does not address in-sewer effects like degradation, but it can address variability in flow if the provided flow rates are accurate.
- In-human effects: population/flow normalization directly addresses upstream variability due to population differences between locations. Using static estimates of population (e.g. catchment population) does not address fluctuating populations within a given wastewater catchment, but using dynamic estimates of populations (e.g. estimates based on mobility or cell phone data) could.

Overall, normalization with population and flow metadata provides a direct estimate for flow variations across time and locations and population differences between communities. This approach improves the interpretability of wastewater data by reporting comprehensible units and is theoretically justified, but has limited applicability beyond samples that have flow and population data readily available. It also does not account for any in-lab effects.

Conclusion

Normalizing wastewater data can improve the interpretability of wastewater data, ensuring that changes in wastewater measurements reflect meaningful changes in the community. Normalization can address multiple types of environmental effects including in-lab, in-sewer, and in-human effects, and can be applied to many types of WBE applications. Therefore, there is no one best approach for the normalization of wastewater data. We present a theoretical framework for evaluating different normalization approaches: at minimum, a normalization approach must be theoretically justified and logistically practical, and beyond this minimal requirement, there are multiple ways to evaluate and compare different normalization approaches. We also provide a broad categorization of normalization approaches into marker and metadata normalization. Neither approach is superior for all applications, and the choice of normalization approach is highly-dependent on the presumed environmental effects and practical constraints of the specific wastewater-based epidemiology (WBE) application.

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