Variability in Normalization Methods of COVID-19 Wastewater Surveillance

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Abstract

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Wastewater surveillance for SARS-CoV-2 provides an approach for assessing the infection burden across a sewer service area. For these data to be useful for public health, measurement variability in relation to normalization methods need to be established. While the relationships between wastewater SARS-CoV-2 concentrations and COVID-19 incidence are now being reported widely in the literature, most studies are analyzed using a variety of different data normalization techniques leading to inconsistency and limited applicability when comparing data to other systems or studies.This work examines the variability and correlation of SARS-CoV-2 wastewater concentration normalization methods to improve confidence in these data for public health surveillance. In this study, we focused on better defining variability in the wastewater measurements, Total Suspended Solids (TSS), Chemical Oxygen Demand (COD), and the Pepper Mild Mottle Virus (PMMoV) to improve confidence in the interpretation of data and to provide evidence to support different study analysis options. This study found that the COD normalization method has the strongest correlation to our comparative normalization method of flow and population. TSS as a method also looked promising as a normalization method for wastewater surveillance with a moderate correlation. PMMoV had the lowest correlation. These results can help facilitate future wastewater surveillance method standardization by informing data analysis techniques utilized as well as illustrate variability between methods in general.

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Chapter 1: Introduction

1.1 Background

Pathogenic viruses represent one of the greatest threats to human health on a global scale. Halting or limiting the spread of these highly contagious diseases, as seen throughout the COVID-19 global pandemic, is notoriously difficult (1). Effective surveillance (clinical and environmental) and monitoring are therefore among the most successful strategies when attempting to control these viruses. Monitoring wastewater from urban areas has been used to detect the arrival and decline of pathogens for decades, and has subsequently been productive in discovering the presence of SARS-CoV-2 (1). This sort of wastewater based epidemiology is useful in identifying emerging and re-emerging pathogens in a community setting and can potentially serve as an early warning system when compared to clinical COVID-19 positive test results (1). More research on the surveillance of viruses in wastewater using accurate and validated methods, as well as subsequent risk analysis and modeling is of the utmost importance in understanding the dynamics of viral outbreaks.

Municipal wastewater harbors a large variety of pathogenic viruses. Extensive research has been undertaken on the persistence of human enteric viruses, transmitted via the fecal-oral route, in wastewater and in the aquatic environment (1). Enveloped viruses, such as coronavirus, which rapidly inactivate without a host, have also been found in wastewater. Temporal changes in viral concentrations in wastewater can therefore indicate the presence or absence of a virus, related outbreaks in the population, and their effect on public health (1). This is why domestic wastewater monitoring is an important tool to assess and mitigate viral outbreaks in a community.

A recent study conducted by the University of Arizona utilized wastewater based epidemiology paired with clinical testing as a surveillance strategy to monitor COVID-19 prevalence in a dormitory community (3). Positive SARS-CoV-2 RNA detection in wastewater led to prompt testing of all residents and the identification and isolation of three infected individuals which averted potential disease transmission (3). Another recent study in Pakistan indicated that SARS-CoV-2 detection through wastewater surveillance has an epidemiologic potential that can be used as an early warning system to monitor viral tracking and circulation in cities with lower COVID-19 disease burden or heavily populated areas where door-to-door tracing may not be possible (3).

Generating reliable data that can be interpreted by public health practitioners is critical for implementing SARS-CoV-2 wastewater surveillance. Currently, there is no standard method for SARS-CoV-2 measurements in wastewater; for collecting, process, or analyzing data. However, research is underway to evaluate concentration methods for these viruses. In addition, normalizing SARS-CoV-2 RNA concentrations to wastewater treatment plant flow and population served or to the estimated amount of human fecal material in the samples (fecal strength) is needed to compare across temporal samples and different treatment plants and systems (4). Other methods of normalization are also needed when the system's flow is not sufficient, this is often the case in low-middle income countries and rural areas where flow may be too variable and large municipal systems dont exist. The normalization of cumulative curves for Total Suspended Solids (TSS) and Chemical oxygen demand (COD) has also been utilized for adjustments. Another candidate for normalizing SARS-CoV-2 concentration data is the pepper mild mottle virus (PMMoV), found in high concentrations in untreated sewage. This fecal marker is introduced into humans through the diet and it has been thought attractive because, as a single-stranded RNA virus, it may also serve as an RNA recovery control. The need for standardizing these normalization techniques is critical for both an accurate and applicable future in wastewater surveillance and is necessary to the production of data that looks, reads, and can be utilized the same way across all systems and disciplines (4).

The burden of viral disease is a global concern. Due to their unique properties, viruses have a particular relevance when analyzing the interaction among humans, animals, and the environment. Viruses are small compared to other pathogens, facilitating transport in the environment (5). Moreover, their resistance to disinfection and ability to survive for prolonged periods in water and solids make their transmission from the environment to suitable hosts likely (5). This is compounded by their low infectious dose, inability to be treated by antibiotics, and their proclivity for adaptive mutation. Additionally, viruses do not replicate outside their host cells, therefore detection in environmental samples can be directly related to the human or animal population that excreted these viruses (5).

1.2 Popular SARS-CoV-2 Wastewater Indicators used to Normalize data

If the number of people contributing to the sewershed is expected to change over the surveillance period (due to tourism, weekday commuters, temporary workers, etc.), human fecal normalization may be important for interpreting SARS-CoV-2 concentrations and comparing concentrations between sewage samples over time (4). Human fecal normalization targets are organisms or compounds specific to human feces that can be measured in wastewater to estimate its human fecal content. While there is no one agreed upon method, you can normalize by human fecal content by dividing non-normalized wastewater concentrations by the human marker concentrations, resulting in a unitless ratio (4)(29). This ratio may also account for viral losses in the sewage system and viral recovery through laboratory processes.

These various parameters range from nutrient concentrations to flow volume. However, the impact of the method chosen on the strength of relationship with clinical case numbers has yet to be thoroughly explored. Herein, data on the concentration of SARS-CoV-2 in wastewater were normalized by the indicators of wastewater treatment plant (WWTP) flow and population size, PMMoV, TSS, and COD.

Flow and Population Size: To compare viral wastewater concentrations over time, one may normalize estimated viral concentrations by daily wastewater flow to account for changes in wastewater contributions (3). This normalization provides data in units of viral gene copies per liter per day (6). To compare viral levels across sampling locations in this case it is important to also normalize viral concentrations by the number of people served by the sewer system, resulting in units of viral gene copies per liter per person contributing to the sewershed per day (6).

PMMoV: A popular model used as a human fecal normalization target is the Pepper mild mottle virus (PMMoV). This method was recently found to be the most abundant RNA virus in human feces, and is a plant virus belonging to the genus *Tobamovirus* in the family *Virgaviridae* (7). These large quantities can get up to 10^9 gene copies/g feces and is often utilized as a marker of wastewater pollution in environmental waters. When in human feces, it is of dietary origin from peppers and their processed products, and is excreted by a large proportion of healthy human populations, but rarely found in animal feces (7). Over the past decade, this virus has been increasingly attracting research attention as a potential viral indicator for human fecal pollution in aquatic environments and water treatment systems. Results presented in the literature reveal that PMMoV is globally distributed and present in various water sources in greater abundance than human pathogenic viruses, without substantial seasonal fluctuations (7). Several studies report that increased concentrations of PMMoV tend to be correlated with increased fecal contamination in general, along with more frequent detection of pathogenic enteric viruses (7). PMMoV also exhibits stability in water under various environmental conditions but also comes with inherent variability in its concentration in feces and wastewater indicating that it should be used more so as an indicator instead of an actual mode of normalization in wastewater data. Other causes for concern with this method include the effects of freezing and thawing may have varying degrees of impact on the viruses, and further research to support the development of a more complete understanding of this impact is necessary (29). Several studies have reported that PMMoV shows minimal geographic and seasonal variation (7)(29). However, PMMoV concentrations originate from the consumption of PMMoV infected plants which may vary between regions due to social and cultural variations influencing food habits (7).

TSS: Wastewater contains a variety of solid materials ranging from rags, which are any fibrous materials that do not break down after being flushed down your plumbing, to colloidal material. In characterization of wastewater, coarse materials are usually removed before the sample is analyzed for solids (8). TSS means "Total Suspended Solids" and is the measure of the solid waste coming into the wastewater plant as opposed to the liquid and dissolved matter (8)(36). This is typically measured as the portion of total solids retained on a glass fiber filter with a specific pore size (0.45 um), that is measured after being dried at a specific temperature (105 degrees C) (8). The amount of TSS within the influent composition of wastewater plants can help to compare to viral gene copies within samples to normalize data.

COD: Chemical Oxygen Demand (COD) is a measure of the ability of a waste to pollute. That is, if a waste source were to be discharged into a body of water, it would consume a measurable amount of oxygen depriving aquatic life of that oxygen (8)(36). The COD test is used to measure the oxygen equivalent of the organic material in wastewater that can be oxidized chemically using dichromate in an acid solution (8)(37). COD will typically decrease as untreated wastewater settles and before final effluent flow (8). This sort of measure can also be utilized in data normalization methods where a comparison can be made based on gene copies of SARS-COV-2 within the wastewater.

1.3 One Health

One Health is a relatively new integrative approach for addressing public and global health challenges. A key component to this approach is the notion that human health, animal health, and environmental health are all innately interrelated (28)(31). One Health interventions have proven to be effective in controlling outbreak in regards to zoonotic disease, antibiotic resistance, and foodborne illness (11)(5). Environmental engineers and environmental scientists hold a critical role in the further development of One Health approaches that include water-related transport and transmission of human, animal, and zoonotic viruses. This sort of work is applicable to a wide range of viruses that are found in human and animal excrement since contaminated water based surveillance systems may be used for early detection of viral disease (13). One Health can be applied by water based surveillance systems for early detection at a population level and implementation of intervention approaches to block the critical pathways of exposure.

1.4 Specific Aims

With the existing wastewater data from the Department of Health, and the King County data from the City of Seattle, the aim in this study is to conduct a comparative analysis in order to test the residual significance of different normalization techniques in regards to wastewater surveillance data and COVID-19. The hypothesis here is that there will be a statistically significant comparison between all of these techniques in varying degrees showing their modality in wastewater surveillance data normalization.

Aim 1: Conduct a comparative analysis of the different normalization techniques using the DOH data and Seattle King County data. The goal here is to use the data collected to compare several normalization techniques in relation to wastewater levels of SARS-CoV-2 normalized to wastewater flow.

Aim 2: One Health Application. The nature of wastewater surveillance brings together human, animal, and environmental health aspects in a One Health manner. For this reason, I will do a brief literature review search in order to apply wastewater based methods to environmental and animal health in relation to SARS-CoV-2. This review will include the significance of One Health and SARS-CoV-2 as well as its application on wastewater surveillance in general. Foci will include farmed animals, companion animals, zoo animals, wildlife, and variant potential.

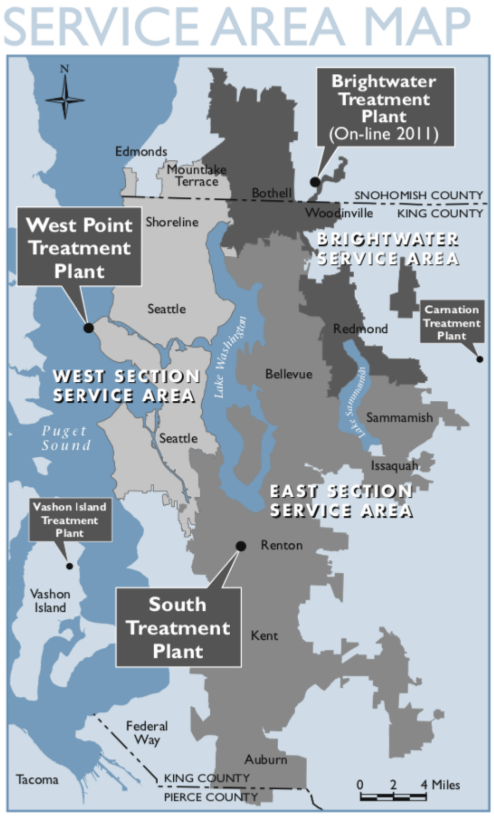
Chapter 2: Normalization Methods and Approach

2.1 Data Utilized

The data utilized for this project included work from the Department of Health (DOH) as well as that from Seattle King County. The data originating from DOH was part of the Washington State Wastewater Surveillance Program and was an aggregate of 3 counties and 6 sites. This data was obtained through an extensive Data Sharing Agreement (DSA). Benton county included Kennewick Wastewater Treatment Plant (WWTP) with population (p) of 87692.131. City of West Richland WWTP (p= 16785.21401), and City of Richland WTTP (p= 60466.05101). Franklin county included the City of Pasco WWTP (p= 79061.73203). Spokane included the Riverside Park Water Reclamation Facility (p= 270522.765) and Spokane County Regional Water Reclamation Facility (p= 118983.073).The data utilized were collected from October 2021 to April 30, 2022 and included COVID-19 case counts, population estimates by wastewater plant service area, wastewater variables by service area, and spatial data by service area. Variables utilized from this data included; Date (specimen collection date), Service Area, Case counts (per service area, suppression for case counts under 10), Population Estimate (per service area, COVID-19 cases and wastewater values - suppression of data for population under 3,000), Concentration N1, Concentration N2, Flow Rate, TSS, and COD. The case, wastewater and spatial data can be linked by facility ID and facility name (or facility short name). General influent flow in millions of liters per day (MLD) for the DOH data averaged for Benton is 19.99 MLD, Franklin 18.17 MLD, Spokane 68.70 MLD. All data provided was de-identified and aggregated with all days/geographies with less than 10 people reported as less than 10 rather than a real number to ensure the identity of individuals cannot be discovered.

The Seattle King County data arepart of a project for Biobot Analytics, an organization working to advance Wastewater as a Public Health Platform. The data received from this organization came from their recent work on COVID-19 Wastewater Monitoring in the United States. We were particularly interested in these data due to the nature of the normalization technique deployed, PMMoV. This work included data from the three major wastewater treatment plants in King County; Brightwater (p= 189000), Westpoint (p= 1400000), and South Plant (p= 1400000). The data utilized is from a three month period starting on June 14th, 2021 and ending on August 16th, 2021. The variables used from this data set included sample collection date, treatment plant name, service area population estimate, SARS-CoV-2 genome copies/L sewage (Virus Concentration), PMMOV Normalized SARS-CoV-2 genome copies/L sewage (Virus Concentration), flow, and TSS. Influent flow averages for this data included Brightwater at 61.34 MLD, South Plant at 201.22 MLD, and West point at 65.49 MLD. Seattle King County Data have smaller concentrations of non-normalized SARS-CoV-2 viral concentrations in their wastewater with an average of 10^5 gene copies per liter while DOH has higher with average of 10^7 gene copies per liter. This could be due to 1) water dilution factor difference, where Seattle King County data was collected in Seattle's driest months, 2) general higher case counts in the Eastern Washington DOH study site locations, and/or 3) sample timeframe being so much longer for the DOH data.

Map 1: Service area of the three most prominent wastewater treatment plants in King County



*This map covers the service area of the three most prominent wastewater treatment plants in King County; Brightwater (population served 189,000 people), West Point (population served 1,400,000 people), and South Plant (population served 1,400,000 people). (*Water Technology, 2022*)

2.2 Methods of Data Normalization

Flow and Population: To compare viral wastewater concentrations over time it is important to first normalize estimated viral concentrations to daily wastewater flow to account for changes in wastewater contributions. This normalization provides data in units of viral gene copies per day (Equation 1):

Equation 1: *Flow MLD x SARS-CoV-2 genome copies/L sewage "Virus Concentration" = Flow Normalized (gene copies per day)*

To compare viral levels across sampling locations it is also important to normalize viral concentrations by the number of people served by the sewer system, resulting in units of viral gene copies per person contributing to the sewershed per day. This normalization will be used as the base for our other indicators to be compared to. This normalization provides data in gene copies per person contributing to the sewershed per day (Equation 2):

Equation 2: *(Flow MLD * SARS-CoV-2 genome copies/L sewage "Virus Concentration")/ Population served estimate x 100000 (to account for flow) = Normalized by Flow and Population (gene copies per person contributing to the sewershed per day)*

TSS and COD Method: Other possible indicators utilized for the normalization of wastewater viral concentrations include total suspended solids (TSS) and chemical oxygen demand (COD). These normalization methods provide gene copies SARS-CoV-2 per milligram of TSS or COD (Equation 3,4):

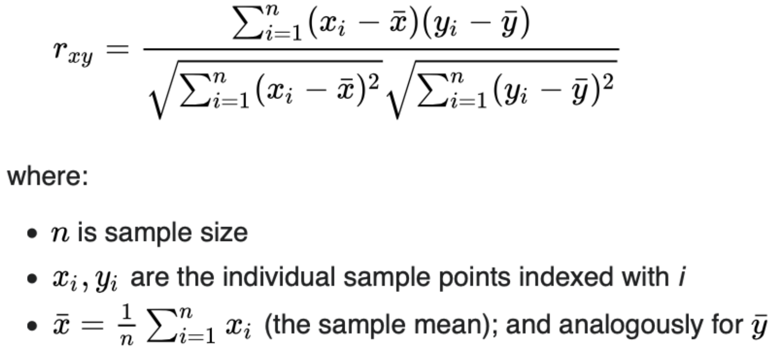
Equation 3: *SARS-CoV-2 genome copies/L sewage "Virus Concentration" / TSS Mg/L= TSS TSS Normalized (gc SARS-CoV-2 per mg TSS)*

Equation 4: *SARS-CoV-2 genome copies/L sewage "Virus Concentration" / COD Mg/L= COD Normalized (gc SARS-CoV-2 per mg COD)*

PMMoV Method: A fecal marker was utilized as a normalization indicator is the Pepper Mild Mottle Virus (PMMoV). We looked at the SARS-CoV-2 gene copies per liter normalized to PMMoV. This calculation was performed by Seattle King County and then used for our comparisons.

Comparison Between Normalization Methods: After data normalization a Pearson correlation test was used to assess the type of relationship between the different normalization approaches of SARS-CoV-2 in wastewater and indicator data. Using the *cor()* function in the program R studio the Pearson's correlation coefficient formula can be obtained by substituting estimates of the covariances and variances based on the sample into the formula below (Equation 5)(Appendix A).

Equation 5:



$$r_{xy} = \frac{\sum_{i=1}^{n}(x_i - \bar{x})(y_i - \bar{y})}{\sqrt{\sum_{i=1}^{n}(x_i - \bar{x})^2}\sqrt{\sum_{i=1}^{n}(y_i - \bar{y})^2}}$$

where:

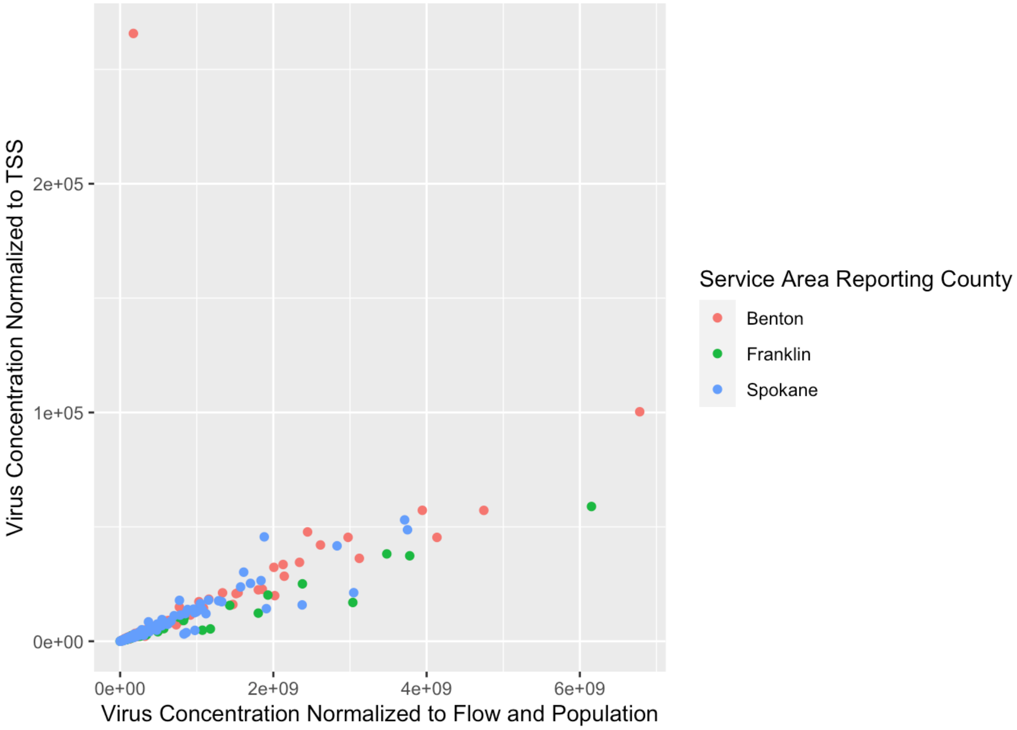
- n is sample size
- x_i, y_i are the individual sample points indexed with *i*
- $\bar{x} = \frac{1}{n}\sum_{i=1}^{n} x_i$ (the sample mean); and analogously for $\bar{y}$

Chapter 3: Results and Discussion

3.1 DOH Results

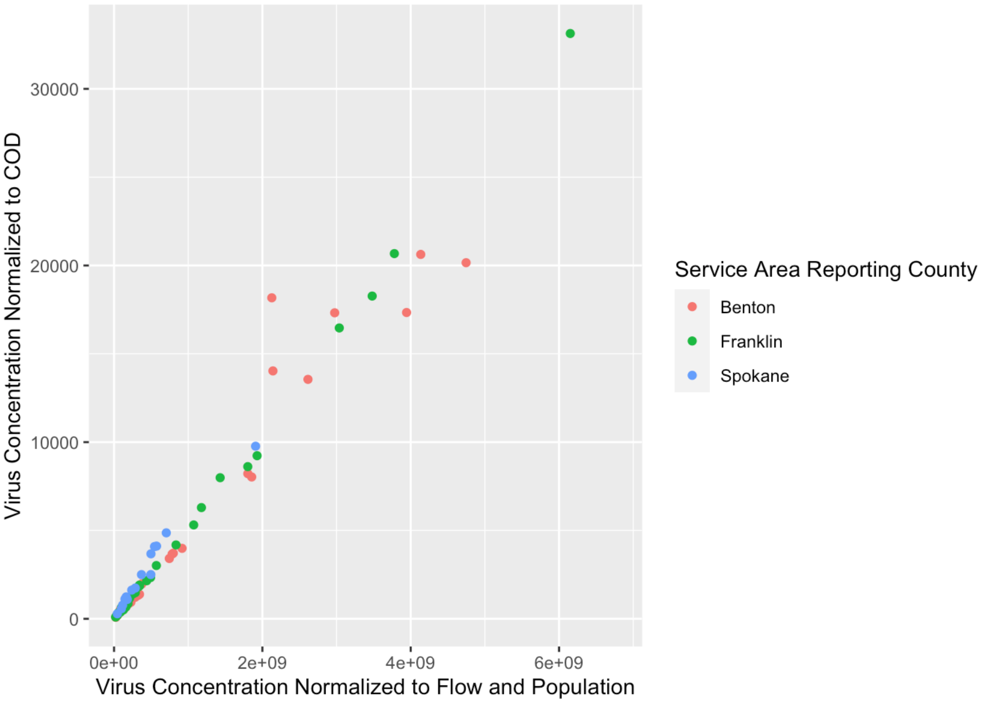
Results from the DOH are represented by the cumulative site average of the three service area reporting counties within the data set Benton, Franklin, and Spokane. The goal of these comparisons is to test TSS and COD as a surrogate indicator for normalization against the flow and population normalization to assess utility. In figures (1,2,3) "Virus Concentration" refers to the gene copies of SARS-CoV-2 per liter of wastewater.

Figure 1: SARS-CoV-2 viral concentrations (GC/L) normalized by flow and population compared against SARS-CoV2 GC/L normalized to TSS (DOH)



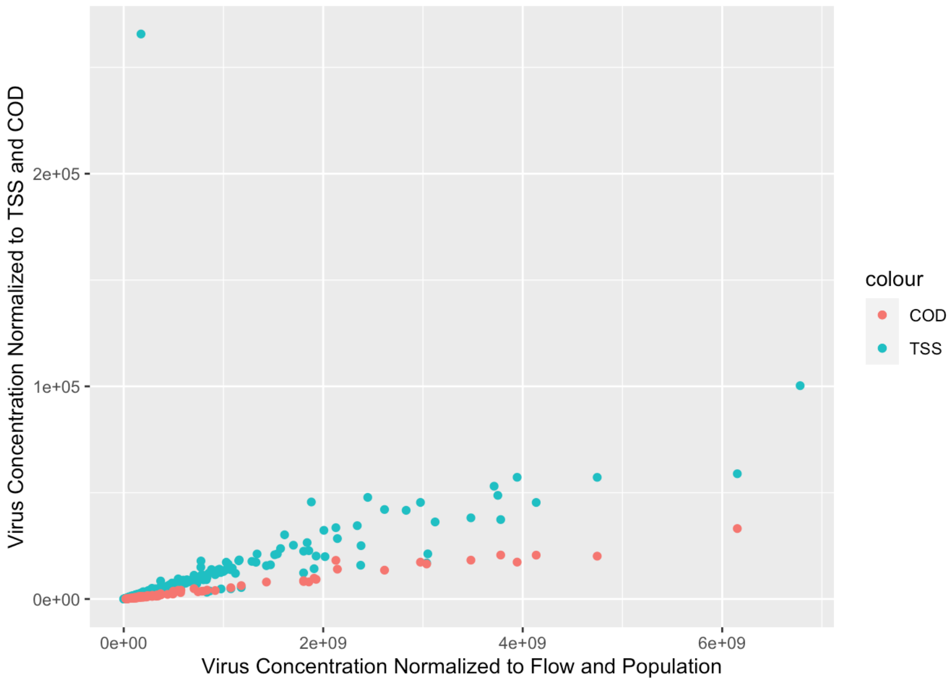
*DOH residual plot comparing the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastwater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day) compared against SARS-CoV2 GC/L normalized to TSS (gene copies of SARS-CoV-2 per mg TSS). Resulting in a correlation coefficient (r) of 0.5834789.

Figure 2: SARS-CoV-2 viral concentrations (GC/L) normalized by flow and population compared against SARS-CoV2 GC/L normalized to COD (DOH)



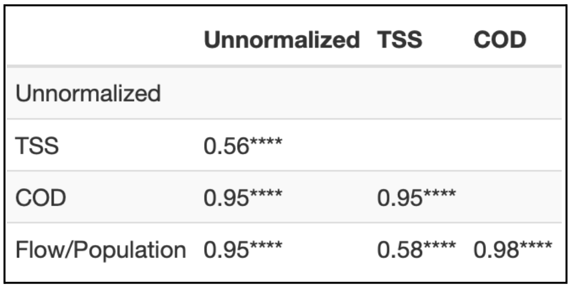
*DOH residual plot comparing the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastewater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day) compared against SARS-CoV2 GC/L normalized to COD (gene copies of SARS-CoV-2 per mg COD). Resulting in a correlation coefficient (r) of 0.9845687.

Figure 3: SARS-CoV-2 viral concentrations (GC/L) normalized by flow and population compared against SARS-CoV2 GC/L normalized to TSS and COD (DOH)



*DOH residual plot comparing the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastewater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day) compared against SARS-CoV2 GC/L normalized to TSS and COD (gene copies of SARS-CoV-2 per mg COD).

Table 1: Correlation Coefficient Chart (DOH)



	Unnormalized	TSS	COD
Unnormalized			
TSS	0.56****		
COD	0.95****	0.95****	
Flow/Population	0.95****	0.58****	0.98****

*This correlation chart indicates the association between correlation coefficients comparing each normalized indicator to the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastewater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day). Asterisk indicate; p<.0001=****,p<.001=***,p<.01=**,p<.05=*.

As seen in Figure 1 SARS-CoV-2 gene copies per mg of TSS were plotted against flow normalization of gene copies per person contributing to the sewershed per day. This comparison *(r =0.5834789),* averaging all three counties, indicates that a positive increase in the TSS normalized SARS-CoV-2 viral concentrations corresponds to the increase of flow normalized SARS-CoV-2 viral concentrations by population, thus implying a direct relationship between the variables. This relationship, with a magnitude above 0.5 and below 0.7 indicates a moderate correlation.

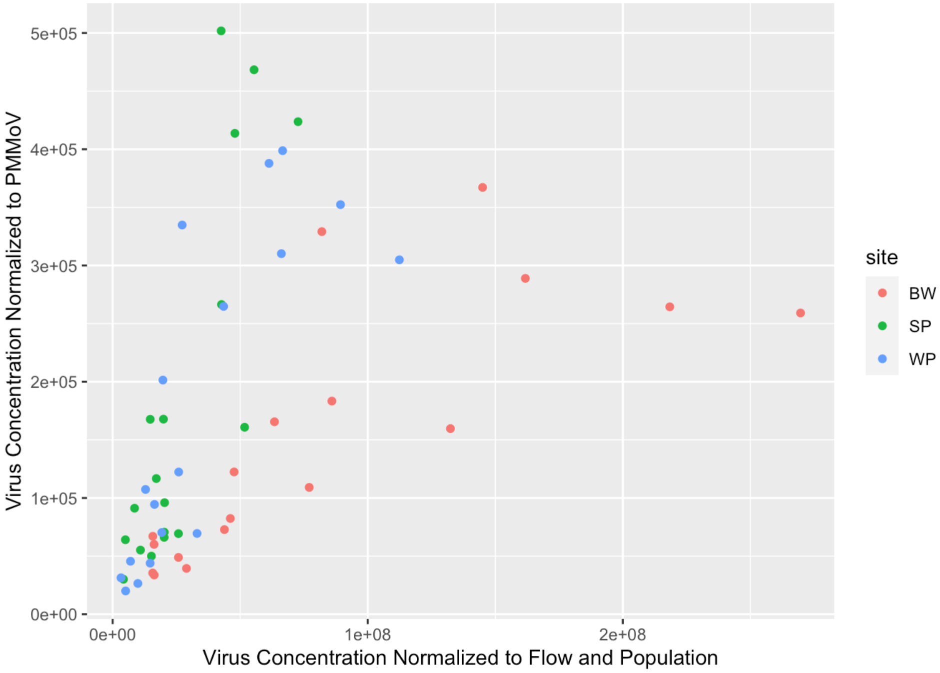
Also seen in the graph above (Figure 2) SARS-CoV-2 gene copies per mg of COD were plotted against flow normalization of gene copies per person contributing to the sewershed per day. This comparison *(r =0.9845687),* averaging all three counties, indicates that a positive increase in the COD normalized SARS-CoV-2 viral concentrations corresponds to the increase of flow normalized SARS-CoV-2 viral concentrations by population, thus implying a direct relationship between the variables. This relationship suggests a very strong, positive association between two variables.

By looking at the correlation coefficients (Table 1) it can be concluded from this DOH dataset that COD has the strongest correlation to our comparative normalization method of flow and population, where p=0.98 (p<.0001). TSS with a p=0.58 (p<.0001) indicated a moderate correlation when compared to the SARS-COV-2 viral concentrations normalized to flow and population .

3.2 Seattle King County Results

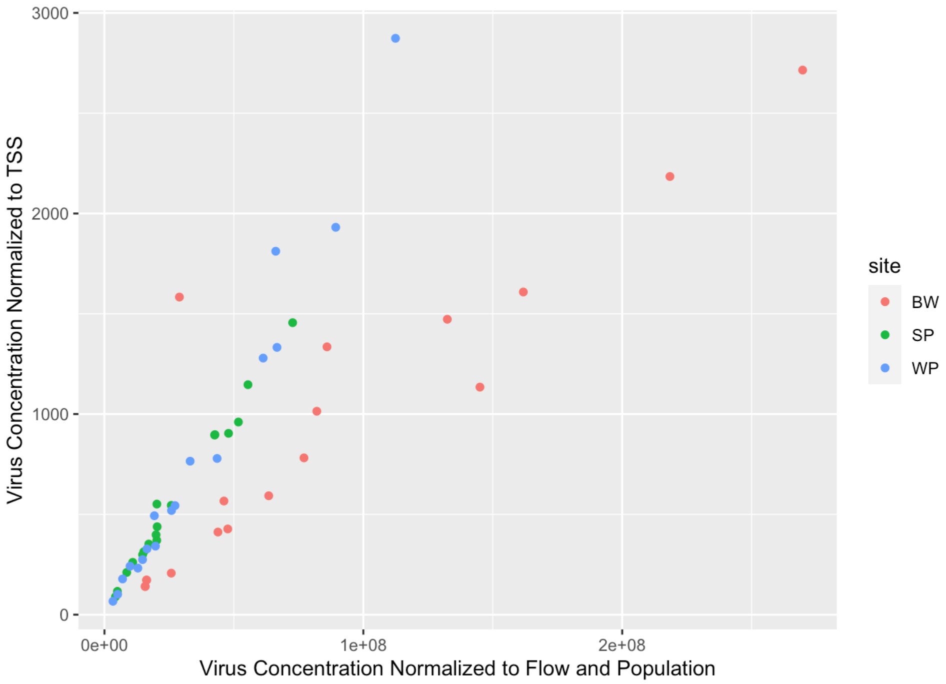
The following are results on the cumulative site average of the three reporting wastewater treatment plants within the data set Brightwater, West Point, and South Plant. The goal of these comparisons is to test PMMoV and TSS as a surrogate indicator for normalization against the flow and population normalization to assess utility. In figures (4,5) “Virus Concentration” refers to the gene copies of SARS-CoV-2 per liter of wastewater.

Figure 4: SARS-CoV-2 viral concentrations (GC/L) normalized by flow and population compared against SARS-CoV2 GC/L normalized to PMMoV (Seattle King County)



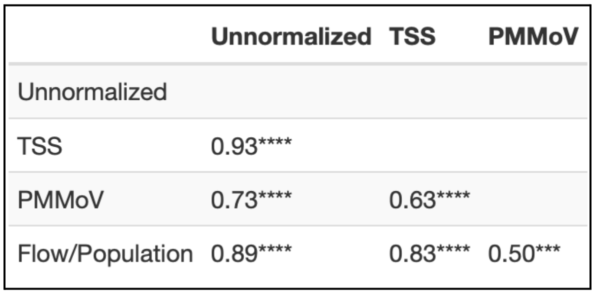
*Seattle King County residual plot comparing the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastewater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day) compared against SARS-CoV2 GC/L normalized to PMMoV concentrations (GC/L). Resulting in a correlation coefficient (r) of 0.4988427.

Figure 5: SARS-CoV-2 viral concentrations (GC/L) normalized by flow and population compared against SARS-CoV2 GC/L normalized to TSS (Seattle King County)



*Seattle King County residual plot comparing the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastewater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day) compared against SARS-CoV2 GC/L normalized to TSS (gene copies of SARS-CoV-2 per mg TSS). Resulting in a correlation coefficient (r) of 0.7263636.

Table 2: Correlation Coefficient Chart (Seattle King County)



	Unnormalized	TSS	PMMoV
Unnormalized			
TSS	0.93****		
PMMoV	0.73****	0.63****	
Flow/Population	0.89****	0.83****	0.50***

*This correlation chart indicates the association between correlation coefficients comparing each normalized indicator to the averaged N1 and N2 SARS-CoV-2 viral concentrations in gene copies per liter of wastwater (GC/L) normalized by flow and population (gene copies per person contributing to the sewershed per day). Asterisk indicate; p<.0001=****,p<.001=***,p<.01=**,p<.05=*.

As seen in the figure above SARS-CoV-2 gene copies per liter of sewage normalized to PMMoV concentration were plotted against flow normalization of gene copies per person contributing to the sewershed per day (Figure 4). This comparison *(r* =0.4988427*),* averaging all three wastewater treatment plants, indicates that a positive increase in the PMMOV normalized SARS-CoV-2 viral concentrations corresponds to the increase of flow normalized SARS-CoV-2 viral concentrations by population, thus implying a direct relationship between the variables. This being said this relationship, with a magnitude below 0.5 indicated a weak to moderate level of correlation.

SARS-CoV-2 gene copies per mg of TSS were also plotted against flow normalization of gene copies per person contributing to the sewershed per day (Figure 5). This comparison *(r =0.7263636),* averaging all three wastewater treatment plants, indicates that a positive increase in the TSS normalized SARS-CoV-2 viral concentrations corresponds to the increase of flow normalized SARS-CoV-2 viral concentrations by population, thus implying a direct relationship between the variables. This relationship, with a magnitude above 0.7 indicates a moderate to strong correlation.

Looking at the correlation coefficients it can be concluded from this Seattle King County dataset that TSS has the strongest correlation to our comparative normalization method of flow and population, where p=0.83 (p<.0001). PMMoV with a p=0.50 (p<.0001) indicated a moderate to low correlation when compared to the SARS-COV-2 viral concentrations normalized to flow and population (Table 2).

3.3 Discussion

As has been established, wastewater research is of the utmost of importance as a public health measure. This sort of surveillance is relatively novel and has seen new application through the onset of the COVID-19 pandemic. Due to the novel nature of this work and its application to SARS-CoV-2, a few issues can be seen in the normalization of this data. The biggest hurdle is there is no standardized method for the collection and analysis of wastewater, in this sense it is difficult to directly compare data and trends for consistency. Other issues include the over-interpretation and extrapolation of data, the limitations of health data in many areas, and the uncertainty in changing factors such as vaccinations and variants.

By identifying the variability in alternative normalization methods recommendations can be made for future studies. This sort of study will also be a first step in the standardization of wastewater surveillance data in general. In regards to population normalization of viral loads, these sorts of methods might increase in importance as populations grow and census data continues to deviate from the true population. Particularly in places with high diurnal variation where the population tends to fluctuate. Also, population dynamics may have a different impact on SARS-CoV-2 wastewater data as more people are vaccinated and boosted, resulting in decreased virus prevalence in communities. This is important because depending on the sensitivity of the techniques utilized wastewater surveillance is a great option to monitor these low levels of virus throughout a community.

This study had a few limitations, some of which can be corrected for in future studies, but others of which are simply inherent to environmental sampling work. One major limitation to this study was due to data availability. Due to the fact wastewater surveillance is a growing field of study the number of accessible, comparable and robust data sets is limited. It was for this reason why we utilized the PMMoV data from Seattle King County Data and the other indicator data from DOH. Though these two data sets are not comparable to each other by nature they are relevant individually and can help to assess the variability in normalization.

Another limitation can be attributed to missing data variables. The data from the DOH is from a new and ongoing project with the Washington State Wastewater Surveillance Program. Due to the novel nature of this project as well as the time consuming and laborious nature of wastewater surveillance there were many concentration variables that were yet to be processed, somewhat limiting our sample size for this analysis.

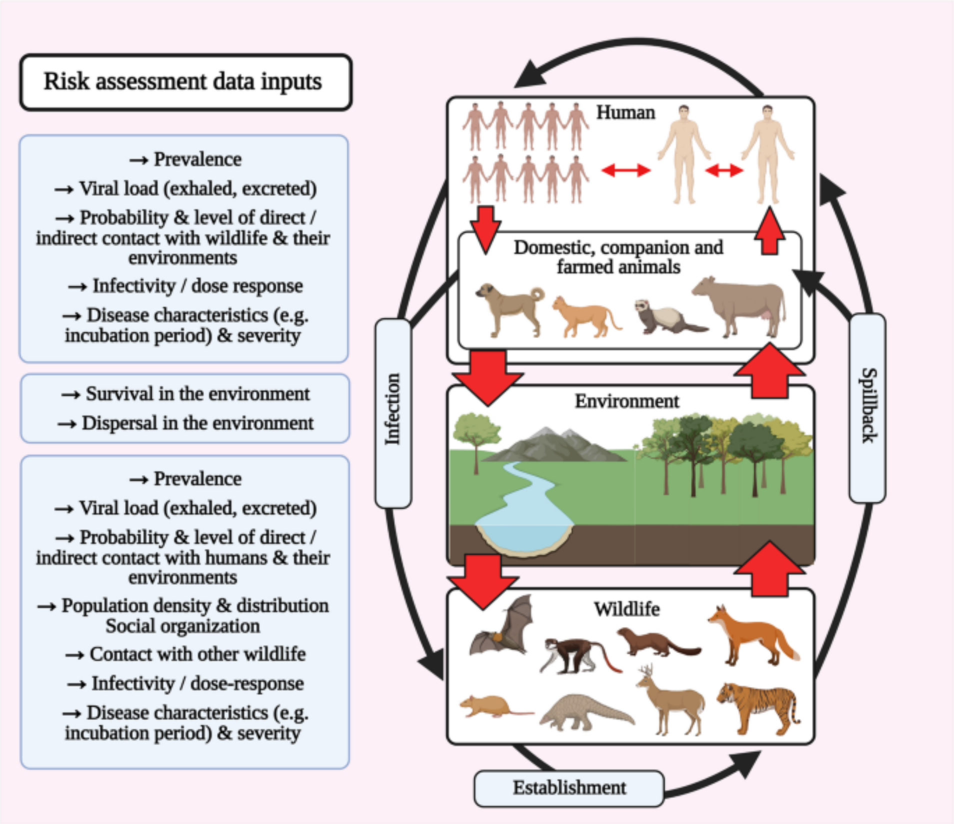
Based on the results of this study a few recommendations can be made regarding appropriate protocol in conducting wastewater data normalization. First of all, if a system has major flow data available then it is most appropriate to normalize wastewater data by flow and population in order to account for viral losses in systems and viral recovery through laboratory processes. It is important to also note that indicator measures should not be used mutually exclusively but deployed based on the sampling system. For example, if you are conducting wastewater sampling in a system with no or low flow measurement and the area is lacking in infrastructure, PMMoV, TSS and/or COD could be utilized. Generally speaking, though PMMoV, TSS, and COD have their utility, there just isn't enough wastewater surveillance data in respect to SARS-CoV-2 to use these different measures to normalize data yet. Instead these measures should be used as indicators of performance to flag methods or samples in comparison. This means instead of taking the variable of interest and dividing the non-normalized concentrations by it in order to create a unitless ratio for normalization, that variable or measure could be used as a comparison tool to test the performance of the original data.

Chapter 4: One Health

4.1 Background and Significance of One Health and Natural SARS-CoV-2 Infection

The One Health approach addresses shared health threats by recognizing the interconnection between people, animals, and the environment. This approach, though often left out, is more important than ever as we navigate the COVID-19 pandemic. The virus that causes COVID-19, SARS-CoV-2, is a zoonotic virus, which means we believe it was originally spread from animals to humans (9), and has proven possible for bidirectional transmission between humans and non-human animals (Figure 6). As more animals are reported infected with the COVID-19 virus, it becomes increasingly clear that a One Health approach is crucial to address new disease threats that affect both people and animals (28). More than 400 animals from 29 countries have been reported infected, including nearly 300 animals in the United States, as well as thousands of mink in mink farms in the U.S. and abroad (as of November 2021) (9). The virus has infected farm animals, wildlife, companion animals, and zoo inhabitants, including cats, dogs, tigers, lions, gorillas, white-tailed deer, mink, and others. Most of these animals became infected after contact with humans who had COVID-19 (9). Although animals do not appear to play a significant role in the spread of the virus among people currently, One Health investigations and animal surveillance are crucial in evaluating transmission of SARS-CoV-2 between people and animals. This will increase our understanding about the range of animals that can be infected and the risks of potential establishment of new hosts and reservoirs where the virus could hide, mutate, and potentially re-emerge as a new variant in the human population. One of the lessons learned from COVID-19 is that emerging zoonotic infectious diseases are here to stay and fighting new disease threats such as COVID-19, Ebola, and Zika requires One Health collaboration across human, animal, and environmental health organizations (9). In order to investigate the application of One Health and wastewater surveillance it is important to look back through the existing literature surrounding the issue in order to see the full picture and extract importance.

Figure 6: One Health risk assessment data output diagram



Potential pathways of SARS-CoV-2 transmission in a multi-host system and sources of data for the assessment of associated risks

(Assessing the Risks of SARS-COV-2 in Wildlife - One Health Outlook, 2020)

4.2 Methods of Literature Review

A brief literature review was conducted in order to further investigate the relationship between the One Health framework and wastewater surveillance. For this review I utilized the search services Google Scholar, PubMed, and the University of Washington libraries. Key search words and phrases I used as search statements included, *One Health, Wastewater Surveillance, COVID-19, SARS-CoV-2 in Wastewater, COVID-19 infection in animals, Farms, Wildlife, Zoos, Pets, Viral transmission pathways, Zoonoses, Reverse zoonosis, COVID-19 Variant potential,* as well as variations of those listed above. From this search I found 98 studies and journals on the topic and utilized and cited 15 from these articles.

4.3 Farmed Animals

Due to the nature of the living conditions of some farmed animals as well as their susceptibility to the virus they can be of particular concern when considering SARS-CoV-2 and potential risk. So far the largest outbreaks of COVID-19 through farm animals has been seen within Mink populations. The housing of mink at unnaturally high densities and the spatial structure of farms may facilitate rapid spread and persistence of the virus in these captive populations.

This species, *Neovison vison*, or more commonly known as the Mink, is a small animal belonging to the family Mustelidae (10). Natural transmission of SARS-CoV-2 has taken place amongst farmed mink following initial introduction from farm workers in Denmark as well as other European countries. Transmission amongst separately housed mink suggests spread by fomites, respiratory droplets or aerosols which is consistent with widespread detection of viral RNA during environmental sampling of infected premises (10). Mink farms provide the only current source of evidence for maintenance of naturally acquired infection in an animal population and spillback to humans. Minks have exhibited virus replication and shed viruses in nasal discharges, saliva, feces and urine for up to 8 days (11). Infection reported on these mink farms in Denmark resulted in culling of millions of minks on many farms.

In regards to control, according to Astrid Iversen, a virologist at the University of Oxford, UK, who said due to rapid and uncontrollable spread of COVID-19 virus in minks, which makes animals a massive viral source that can easily infect people, culling of animals is probably essential (12). However, there are a lot of animal welfare and animal rights concerns.

COVID-19 has yet to cause disastrous infection in commercial livestock and poultry. In fact, early experiments suggest that poultry are not susceptible to early strains of the virus and are thus unlikely to play a role in COVID-19 spread. However, in order to avoid outbreaks that can lead to such destruction as the Highly Pathogenic Avian Influenza (HPAI) or African Swine Fever (ASF) it is crucial we continue to study the transmission mechanism of COVID-19 among group animals and farms (13). As of now, we know that the latency period for SARS-CoV-2 is similar in humans and animals which ranges from 3–7 days to up to 14 days while the symptoms in animals are not certain, some developed dry cough with sneezing and lethargic signs. Currently, the common diagnostic tests used to test animals for SARS-CoV-2 include virus neutralizing antibody test and reverse transcriptase polymerase chain reaction (RT-PCR) (13). Before the right vaccine is successfully developed and marketed, conventional and emerging measures of on-farm biosecurity may help prevent transmission of COVID-19 among farm animals.

Farmed animal populations hold good potential for wastewater surrviellance opportunities. These animals are in controlled environments in close proximity to humans. This proximity helps in the initiation of frequent sampling at longer durations. Farms also often have their own independent wastewater systems or at the least have knowledge of which system their wastewater is directed to, creating a productive sampling environment.

4.4 Wildlife

Wildlife populations, by nature, are inherently difficult to sample, prohibiting us from the full picture in regards to outbreak potential amongst these vulnerable populations. Since human influence occurs across almost all human ecosystems, the increased interaction with wild animals is almost impossible to avoid. So if and when SARS-CoV-2 breaks the species barrier again, from humans to wildlife, it is imperative to carry out studies on the identification of the wild species at risk, and subsequently study the impact on their populations.

This sort of potential spillover of a human pandemic-type virus to wild animals via reverse zoonosis can have drastic consequences on their populations. A recent example occurred with the Ebola pandemic where over 5000 gorillas were lost over the course of 2 years after a human strain of the virus was transmitted to a gorilla (14). There is a need to prevent such spillover events from occurring to protect vulnerable wildlife species around the world and that begins with assessing the possibility of it occurring for species in proximity to humans. The transmission of the SARS-CoV-2 virus from humans to other vulnerable species in close contact to humans has already been observed. Studies thus far have described how ACE2 (the host receptor targeted by SARS-CoV-2) variability has contributed to why certain species are susceptible and others are not (15). In addition, a report utilizing a similar approach suggested that only mammals were predicted to be highly susceptible to the SARS-CoV-2 virus (16). In support of these analyses, chickens were recently found to not be susceptible to the virus (17). As we continue to gain knowledge from such research we can begin to narrow down species of concern in wild populations.

Recent studies have also considered the possibility that contamination of aquatic systems with feces from infected humans could provide a route for spillover into wild marine species including cetaceans and seals (18)(30). Though this type of research has not been extensive, it brings up an important question. No work has been done that suggests the transmission of SARS-CoV-2 infection directly from wastewater to host but future research should be conducted regarding marine mammals and infectious dose. Since marine mammals spend the majority if not all of their time in aquatic environments the potential viral load they are exposed to is drastic. This being said, in order for a viral outbreak in marine mammal populations to occur there would have to be a "perfect storm" of direct exposure to a large wastewater viral load source that has not been treated and has enough live virus intact. And even if this were the case the potential for these marine mammals to spread the virus to each other is another question that would need to be asked.

A lot of speculation has been derived from the prospective "leap" of the SARS-COV-2 virus to the human population from wildlife. Though most researchers agree the virus originated from a wild bat found in a wet market in Wuhan, China, the history of how this transmission was possible is up for debate. SARS-CoV-2 is a betacoronavirus (β-CoV), closely related to SARS-CoV and MERS-CoV which have also caused serious outbreaks of disease in human populations (19). All are thought to have originated in bats, with evidence of intermediate or bridge hosts being responsible for transmission to humans (19). Masked palm civets (*Paguma larvata*) were identified as the proximal source of SARS (Severe Acute Respiratory Syndrome) in humans, and dromedary camels (*Camelus dromedarius*) are a reservoir and source of MERS (Middle Eastern Respiratory Syndrome) in humans (19). Malayan pangolins (*Manis javanica*) have been the subject of some speculation on the basis of infection with a closely related coronavirus in animals seized in southern China (19). Sequence analysis of the spike glycoprotein (S) of SARS-CoV-2 and related coronaviruses suggest a series of recombination events between bat and pangolin coronaviruses may have eventually led to the emergence of this novel coronavirus (19). However, raccoon dogs (*Nyctereutes procyonoides*), which were identified as possible intermediate hosts for the SARS pandemic of 2002–2003 have also been suggested as candidate intermediate hosts for SARS-CoV-2 as experimental infection resulted in intense viral shedding (19). Both pangolins and raccoon dogs have been found in wildlife markets in Southern China, along with many other wild mammals.

The important role wet markets play in the transmission of zoonotic pathogens is undeniable. These markets are unique epicenters for transmission of potential viral pathogens where new genes may be acquired or existing genes modified through various mechanisms such as genetic reassortment, recombination, and mutation (44). The wildlife found in these places of gathering are diverse in nature and entirely unregulated when it comes to disease. At close proximity to humans, with high viral burden or strains of higher transmission efficiency, the facilitated transmission of viruses to humans is unavoidable (44).

As we discover more about how this virus came to be amongst the human population and how it is or will circulate amongst wildlife populations it is important to address host susceptibility. The behavior and demography of a wild population must align with pathogen characteristics to result in a successful host jumping event (20). Therefore, in order to determine the most likely species of wild mammal and circumstances whereby they might play a role in the epidemiology of SARS-CoV-2, we need to look beyond the evidence for susceptibility to infection alone. One study which recognized the importance of host susceptibility covered the recent COVID-19 infections in North American White Tailed Deer *(Odocoileus virginianus).* This study illustrated the importance of this species population dynamics and movement patterns, often in groups or herds. It's not yet clear whether the virus can spread in long chains of infection among deer, or whether deer-to-human transmission could spark outbreaks either from park visitors, rangers, or hunters (43). But researchers are growing increasingly concerned about the animals becoming a viral reservoir, serving as a recalcitrant source of outbreaks and potentially breeding new variants. Some researchers think that the highly infectious Omicron variant spent time in a wild animal reservoir such as the White Tail Deer before popping up in people (43).

Deploying wastewater surveillance methods for wildlife populations has many limitations. Due to the nature of wild population dynamics and difficulty in identifying wildlife wastewater sources it can inhibit the ability for sample collection. It is not without utility however. In a study on ecological surveillance of bat coronaviruses in Sarawak, Malaysian Borneo two subgenera, namely Sarbecoviruses and Merbecoviruses were found. These subgenera have a high zoonotic potential and have been associated with three separate spillover events in the past 2 decades, making surveillance of bat-CoVs crucial for the prevention of the next epidemic as well as variant development (41). The study was aimed to elucidate the presence of coronavirus in fresh bat guano and is a great example of how wastewater surveillance can be deployed in these wild systems (41).

4.5 Companion Pets

Thus far in the COVID-19 pandemic there has been no transmission of SARS-CoV-2 from pets to humans to date. It is also important to note that transmission from people to pets is rare but has been seen in companion pets such as cats and dogs (31). In a study carried out by the Pasteur Institute in France published in April 2020, 21 domestic animals were tested for the SARS-CoV-2 virus, including 9 cats and 12 dogs that lived in close contact with their guardians, a total of 20 veterinary students (21). Among the students, two tested positive for SARS-CoV-2 by RT-PCR, and 11 out of 18 showed clinical signs of COVID-19 (21), while the other 7 tested negative. Among the animals, three cats showed respiratory and gastrointestinal symptoms (21). Despite the proximity to infected guardians, no dog or cat tested positive for SARS-CoV-2 by RT-PCR nor showed antibodies to SARS-CoV-2 in an immunoprecipitation assay (21). Studies like this are important to understand the capacity in which these animals can be affected in order to better protect them from the virus.

The first report of SARS-CoV-2 infection in dogs occurred in Hong Kong, China, by the Hong Kong Agriculture, Fisheries, and Conservation Department (AFCD) (22). A 17 year old male Pomeranian with several comorbidities was asymptomatic and quarantined on February 26, 2020, after its owner was diagnosed with COVID-19 (22). On March 18, an asymptomatic 2.5 year old male German shepherd dog tested positive for SARS-CoV-2 by RT-qPCR in nasal and oral swabs; the two dogs had detectable antibodies against SARS-CoV-2 (22). In addition, viral sequences were identical to the virus identified in the respective owner cases, suggesting human to animal transmission (22). Also, in Hong Kong, nasal and oral swab and fecal samples from a clinically healthy cat tested positive for SARS-CoV-2 by RT-qPCR (22). The owner had been hospitalized with COVID-19. Until April 15, 2020, the Hong Kong Agriculture, Fisheries, and Conservation Department tested 17 cats from guardians positive for COVID-19, and only the cat mentioned above was positive.

Though there is yet to be any suggestion of SARS-COV-2 viral transmission between companion pets and humans, misinformation and fear have led to both unnecessary culling and policy change. An example of this was in December of 2022 the city of Hong Kong culled more than 2,000 hamsters and banned the import of small animals after a pet shop worker, a customer, and at least 11 hamsters tested positive for the Delta variant of the coronavirus (23). Also, across the United States a major influx of abandoned dogs and cats were observed after initial news broke of pets testing positive for the virus (24). For these reasons it is important we continue our research as well as utilize protection measures to inhibit the virus from impacting our pet populations.

Wastewater surveillance can be utilized through companion pets, providing valuable information on how these animals' health is impacted by their owners. Pets are the animals in our society that often hold the most value and spend the most physical time with humans (31). This can be advantageous for wastewater sampling purposes as well as contributes to the potential for robust studies with long durations. However, since pets are typically privately owned it is important that recruitment and deployment of surveillance is done with care.

4.6 Zoo Animals

For obvious reasons similar to wildlife, naturally acquired infections of SARS-CoV-2 have been seen in an array of zoo animals across the world. So far in tigers (*Panthera tigris*), lions (*Panthera leo*), a puma (*Puma concolor*), snow leopard (*Panthera uncia*), Canada lynx, spotted hyena, Hippos, and Western lowland gorillas (*Gorilla gorilla*) (11). The majority of these infections were linked back to an infected zookeeper who had close contact with the animals. So far in zoo animals there has been no outbreaks from animal to animal transmission within groups of captive felids and primates, but it seems plausible given evidence for virus shedding in feces and respiratory secretions (11).

Zoos hold a particularly significant opportunity in deploying wastewater technologies and protecting animals from COVID-19 due to often well funded operations as well as access to captive and healthy animals. Zoos across the world are vital in the collaboration and funding of conservation programs, wildlife rescue, as well as animal medicine and health work (32). An example of such work can be seen from veterinary researchers at Kansas State University who are working to protect zoo animals from the spread of SARS-CoV-2 through vaccination (25). This developmental work on a COVID-19 vaccine for animals is an important step to protect susceptible animal species against the virus both in zoos as well as other captive environments. Wastewater surveillance can also be adapted and expanded upon with collaborations between government agencies and private and public zoos across the world in order to protect their animal occupants as well as provide vital data.

4.7 Variant Potential

The World Health Organization (WHO) warned in March of 2022 that animal reservoirs could lead to "potential acceleration of virus evolution" and new variants (42). The agency noted the large numbers of infected animals, and it urged countries to increase their monitoring of mammalian species for SARS-CoV-2 and suspend the sale of live, wild mammals in food markets as an emergency measure (42). The CDC also endorsed efforts to track the virus in animals, even as it described the risk of transmission to humans as "low." This new urgency to track this virus as well as future variants is of the utmost importance in regards to surveillance.

4.8 Wastewater Surveillance Environmental Application

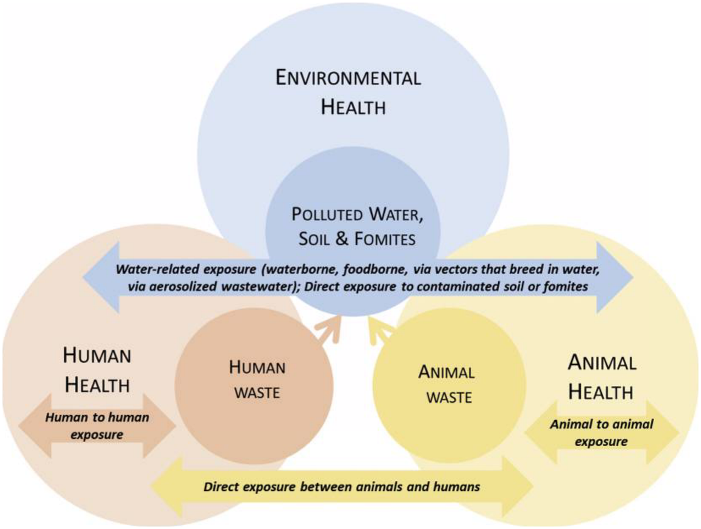
Much of the current work using the One Health approach is focused upon the exposure pathway between humans and animals, while the water related exposure pathway has not been thoroughly investigated from a One Health perspective (5). The investigation and surveillance of critical environmental reservoirs and pathways will be key in allowing for early detection of outbreaks in regards to zoonosis. Monitoring viral disease indicators like viral load in wastewater through environmental sampling, as well as collecting clinical samples from infected people, livestock, and humans will be pertinent to help to identify peaks in viral concentrations that in turn can be related to early signals of disease outbreaks (5)(13). Livestock, zoo animals, and companion pets have the most potential for a successful wastewater surveillance application due to the fact they are captive and controlled, in close proximity to humans, have independent wastewater systems in place, and can be sampled at a more frequent rate at longer durations.

The central premise of this kind of surveillance approach is that community fecal contamination represents a snapshot of the status of public health and animal health (5). Traditionally, human and animal disease detection and management systems are based on diagnostic analyses of clinical samples. However, these systems fail to detect early warnings of public health threats at a wide population level and fail to predict outbreaks in a timely manner. Wastewater analysis, manure analysis, or polluted water analysis is equivalent to obtaining and analyzing a community based urine and fecal sample of the representative watershed (5). Monitoring temporal changes in pathogen concentration and diversity excreted in a sewage watershed allows for a view into the critical moments before the onset of an outbreak. In addition, carefully designed spatial sampling will allow detection of locations where an outbreak may begin to develop and spread (5)(13). Modeling the fate of pathogens, including shedding rates, transport, growth and inactivation processes in the environment, are critical for the effectiveness of wastewater surveillance. The integration of animal and environmental samples into this work is crucial to get a well rounded picture of the status of health in an area (Figure 7).

Wastewater testing has been particularly powerful in tracking SARS-CoV-2 variants. Genomic surveillance has been critical for this sort of tracking (38)(39). Globally, only 13 countries or territories have been able to traditionally sequence and publicly share more than 5% of COVID-19 cases using laboratory and medical samples. The result is a spotty and geographically skewed picture of variant presence, evolution, and spread. By turning to wastewater, a non-traditional data source that functions independent of medical systems, public health officials and researchers around the world have filled critical gaps in pathogen genomic surveillance (38). In a recent study a cryptic COVID-19 variant was found in the New York City subway system that research suggests came from local rat populations (40). This study claimed that these “cryptic lineages” in the sewage system were different from all other COVID-19 variants and these could potentially be transferred through the rodent population. Though this research is still developing, it is an important example of how wastewater surveillance in animal populations can lead to novel detection of these variants (40). Advances in wastewater testing for COVID-19 can also facilitate surveillance for many pathogens, including respiratory pathogens like respiratory syncytial virus (RSV) and influenza virus. Wastewater can also be used to monitor other global threats, like antimicrobial resistant bacteria in disease-causing organisms (38)(26). With the technology to monitor wastewater in place, communities will be better able to quickly identify and track new disease threats as they surge and wane.

More specifically in regard to wastewater surveillance data normalization, with the standardization of protocol and method for this practice there will be a more reliable and robust framework for sharing and comparing wastewater data worldwide. This standardization will also provide a greater opportunity for improving the accuracy of overall research, inform risk/benefit analysis of treatment options, strengthen collaborations, accelerate viral research, and restore trust in the research enterprise as a whole.

Figure 7: One Health and waterborne disease exposure pathways



(A Water-Focused One-Health Approach for Early Detection and Prevention of Viral Outbreaks, 2019)

4.9 Conclusion

This study utilizes popular wastewater data normalization indicators as a surrogate for flow and population normalization to assess the utility of each method. Looking at the correlation coefficients it can be concluded from both the Department of Health and Seattle King County datasets that the COD normalization method has the strongest correlation to our comparative normalization method of flow and population. TSS as a method also looked promising as a normalization method for wastewater surveillance with a moderate correlation for the DOH dataset. PMMoV had the lowest correlation considerably. These results can help facilitate future wastewater surveillance method standardization by informing data analysis techniques utilized as well as illustrate variability between methods in general. Though these results have utility it is important to note that there just isn't enough wastewater surveillance data in respect to SARS-CoV-2 to use PMMoV, TSS, and COD to normalize data yet. Instead these measures should be used as indicators of performance to flag methods or samples in comparison. Finally, by including an evaluation of the interconnected nature of wastewater surveillance as part of the One Health framework, this water based research can grow in utility to include all facets of our natural world to help detect and prevent pathogenic diseases on a grander scale.

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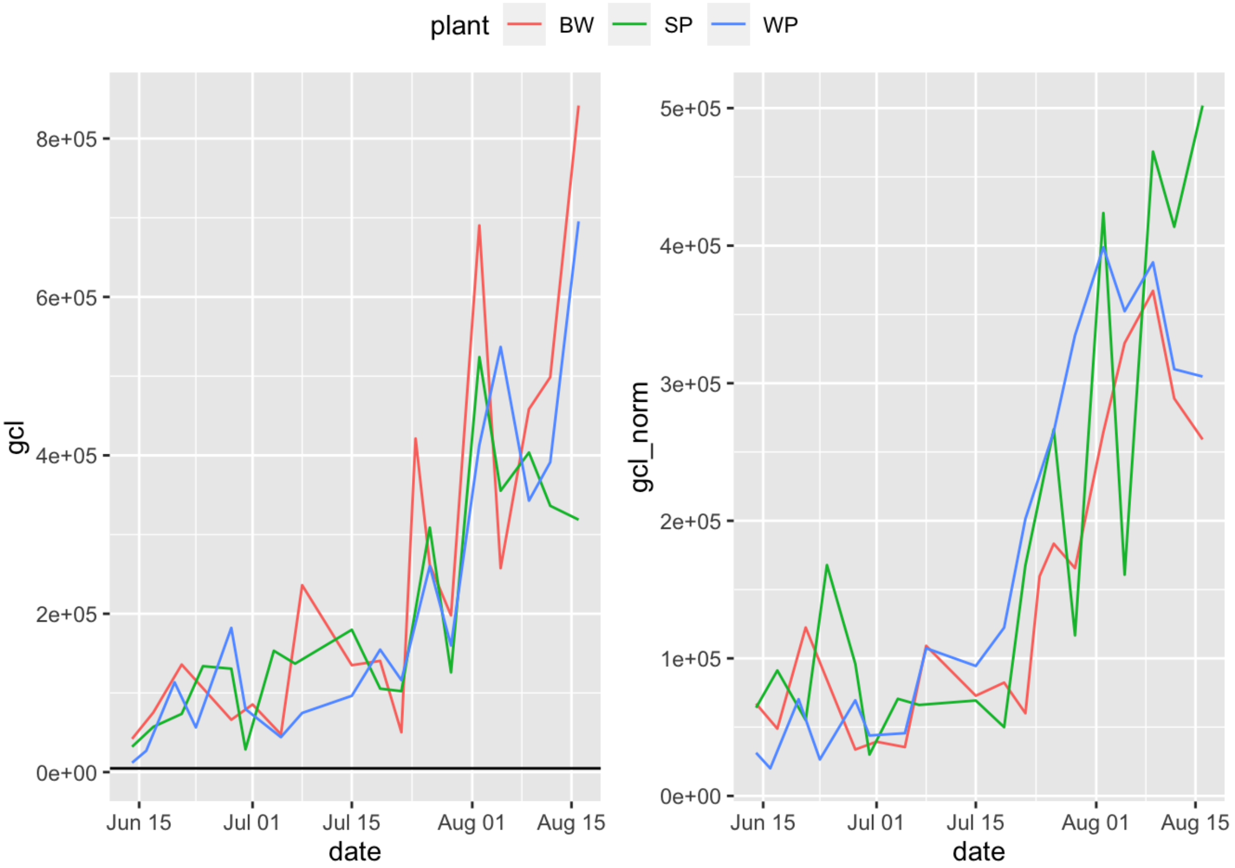
Appendix A: Supplementary Data

Seattle King County Data Data

The plotting for the Seattle King County Data measurements (gc/L and pmmov-normalized gc/L) span mid-June to mid-August 2021.

Figure 8: SARS-CoV-2 GC/L spanning from mid-June to Mid- August 2021

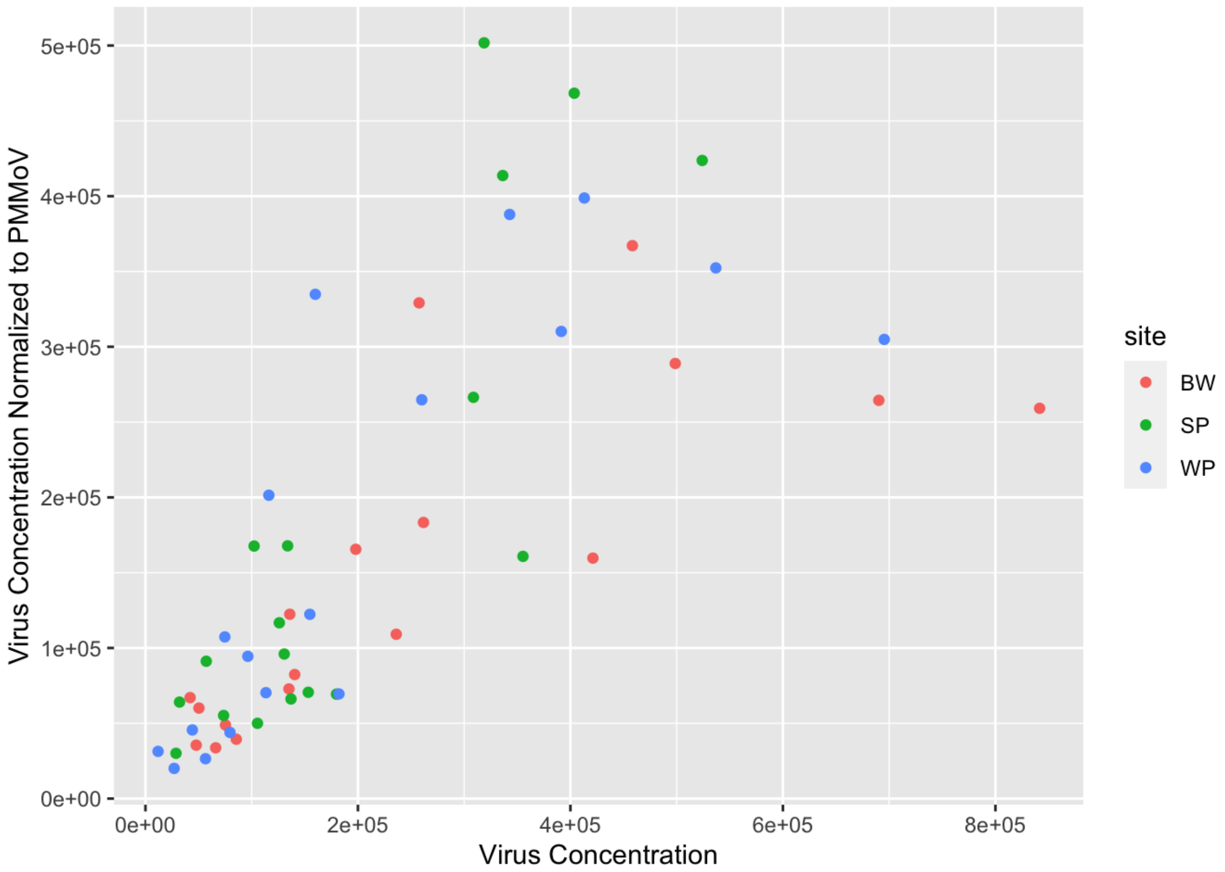
Figure 9: SARS-CoV-2 GC/L normalized to PMMoV spanning from mid-June to Mid-August 2021 (Seattle King County Data)



Here, the horizontal black line corresponds to the limit of detection (LOD) stated in the Seattle King County Data data documentation: *The LOD for lab protocol is 4,800 copies/L of sewage (see more details in Release Notes below). In terms of cases, we reliably detect the virus (>99%) when there is at least 1 infected person in a population of 6,500 people.

Seattle King County Data Additional Residual Plots and Correlations

Figure 10: SARS-CoV-2 GC/L vs GC/L normalized to PMMoV Concentrations (Seattle King County Data)



Correlation:

```
##                           PMMOV Normalized* SARS-CoV-2 genome copies/L sewage
"Virus Concentration"
## N1 and N2 Sar-CoV-2 Conc averaged (GC/L)
0.7263636
```

Figure 11: TSS mg/L vs TSS normalized GC/L (Seattle King County Data)

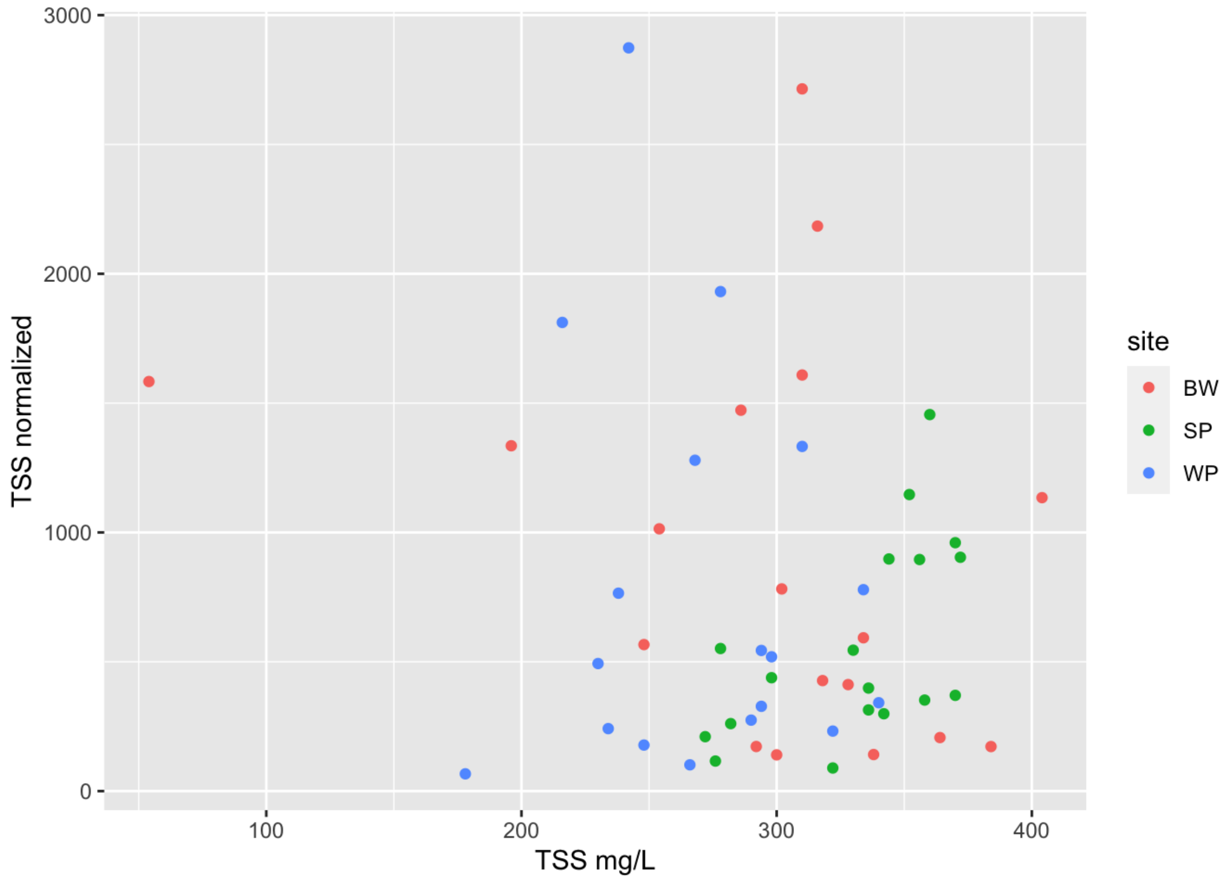


Figure 12: Flow Rate vs. PMMoV multiplier (Seattle King County Data)

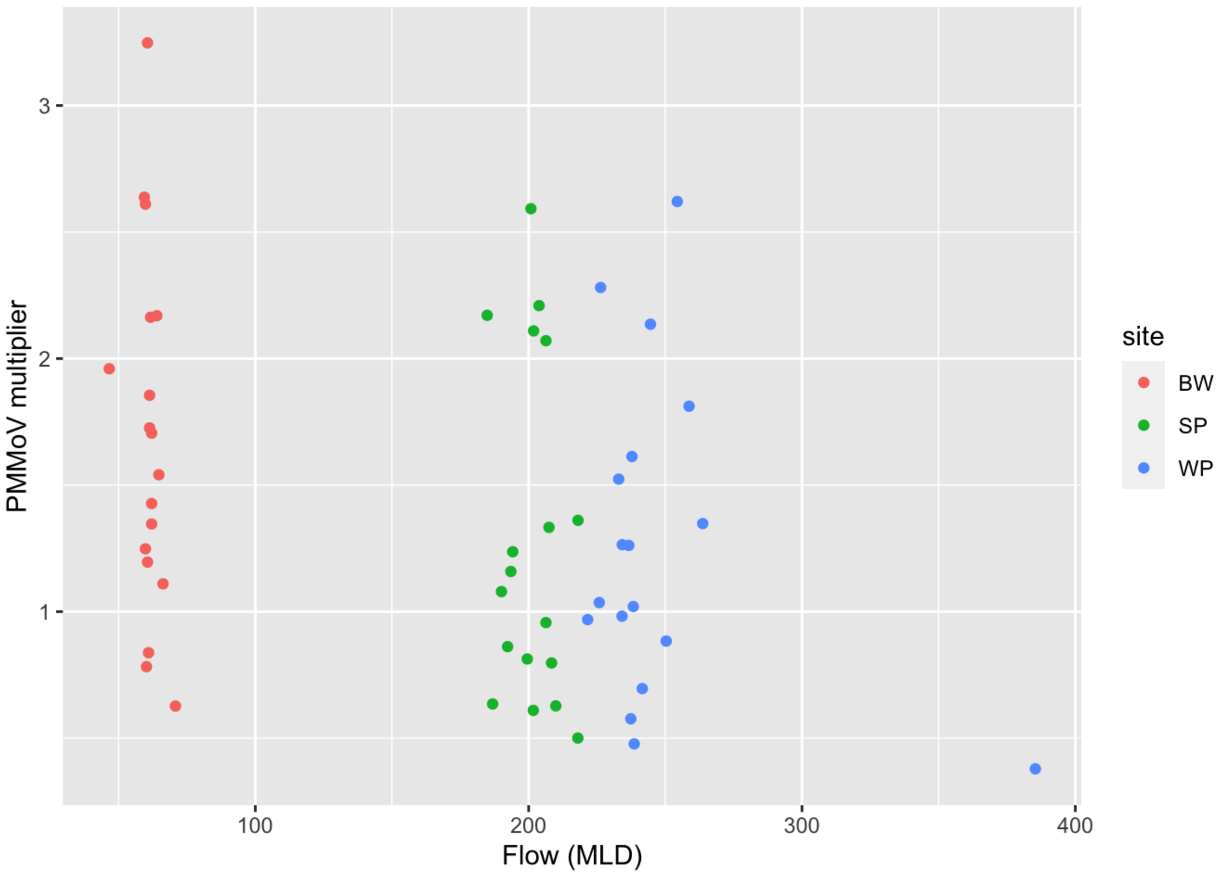
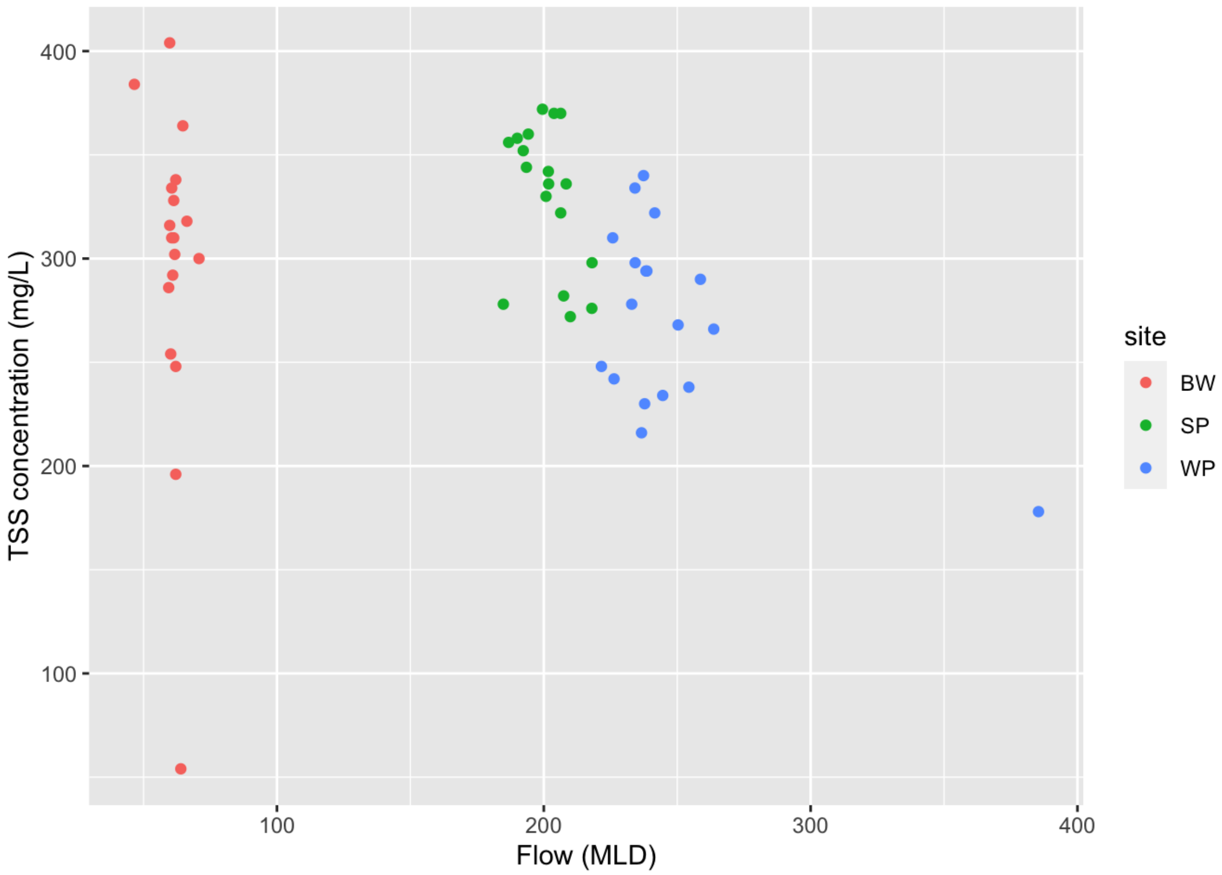


Figure 13: Flow Rate vs. TSS concentration (Seattle King County Data)



DOH Data

Data Processing Code

Below is the following R code created for comparative analysis.

```
# load in DOH data
doh_Seattle King County Data <- read_excel("DOH Data Normalized .xlsx",sheet = 1)
doh_benton <- read_excel("DOH Data Normalized .xlsx",sheet = 2)
doh_franklin <- read_excel("DOH Data Normalized .xlsx",sheet = 3)
doh_spokane <- read_excel("DOH Data Normalized .xlsx",sheet = 4)

# standardize column names
doh_Seattle King County Data <- doh_Seattle King County Data %>% rename(date =
`Sample collection date`,
                              totalpop = `Estimated Population`,
                              `N1 and N2 Sar-CoV-2 Conc averaged (GC/L)` =
                                `SARS-CoV-2 genome copies/L sewage \"Virus Concentration\"`,
                              `flow_rate (MGD)` = `Flow MGD (Millions of Gallons per day)`,
```

```
                          `Flow (MLD)` = `Flow MLD`,
                          `tss (MG/L)` = `TSS  ( MG/L)`,
                          site = `Treatment Plant`)
doh_Seattle King County Data$`Service Area Reporting County` <- "King"
doh_benton <- doh_benton %>% rename(`COD Normalized (gc SARS-CoV-2 per mg
COD)` =
                           `COD Normalized (gc SARS-CoV-2 per mg TSS)`)
doh_benton$`Service Area Reporting County` <- "Benton"
doh_franklin <- doh_franklin %>% rename(`COD Normalized (gc SARS-CoV-2 per mg
COD)` =
                           `COD Normalized (gc SARS-CoV-2 per mg TSS)`)

# make sure all numeric columns are stored as numeric
for(j in 3:(ncol(doh_Seattle King County Data)-1)){
  doh_Seattle King County Data[,j][(1)] <- as.numeric(doh_Seattle King County
Data[,j][(1)])
}
for(j in 3:(ncol(doh_benton)-1)){
  doh_benton[,j][(1)] <- as.numeric(doh_benton[,j][(1)])
}
for(j in 5:ncol(doh_franklin)){
  doh_franklin[,j][(1)] <- as.numeric(doh_franklin[,j][(1)])
}
for(j in 4:ncol(doh_spokane)){
  doh_spokane[,j][(1)] <- as.numeric(doh_spokane[,j][(1)])
}

# combine data frames
df <- bind_rows(doh_Seattle King County Data,doh_benton,doh_franklin,doh_spokane)

# correct normalization
df$`TSS Normalized (gc SARS-CoV-2 per mg TSS)` <-
  df$`N1 and N2 Sar-CoV-2 Conc averaged (GC/L)`/df$`tss (MG/L)`
df$`Flow (L/day)` <- df$`Flow (MLD)` * (10^6)
df$`Flow Normalized  (gene copies per day)` <-
  df$`N1 and N2 Sar-CoV-2 Conc averaged (GC/L)` * df$`Flow (L/day)`
df$`Normalized by Flow and Population (gene copies per person contributing to the
sewershed per day)` <-
  df$`Flow Normalized  (gene copies per day)`/df$totalpop
df$`COD Normalized (gc SARS-CoV-2 per mg COD)` <-
```

```
  df$`N1 and N2 Sar-CoV-2 Conc averaged (GC/L)`/df$`cod (MG/L)`
df$pmmov <- df$`N1 and N2 Sar-CoV-2 Conc averaged (GC/L)`/
  df$`PMMOV Normalized* SARS-CoV-2 genome copies/L sewage "Virus Concentration"`
```

DOH Additional Residual Plots and Correlation

Figure 14: TSS mg/L vs TSS normalized GC/L (DOH)

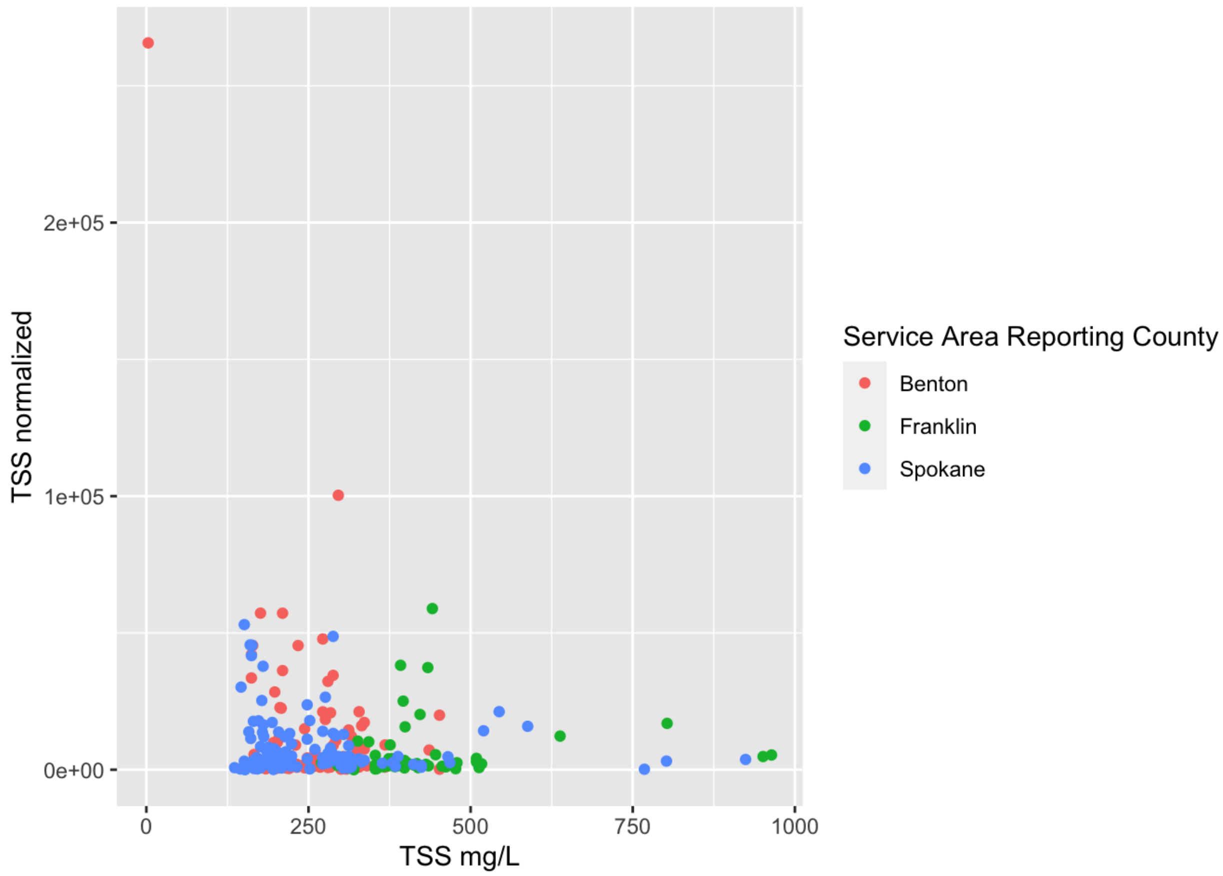


Figure 15: COD mg/L vs COD-normalized GC/L (DOH)

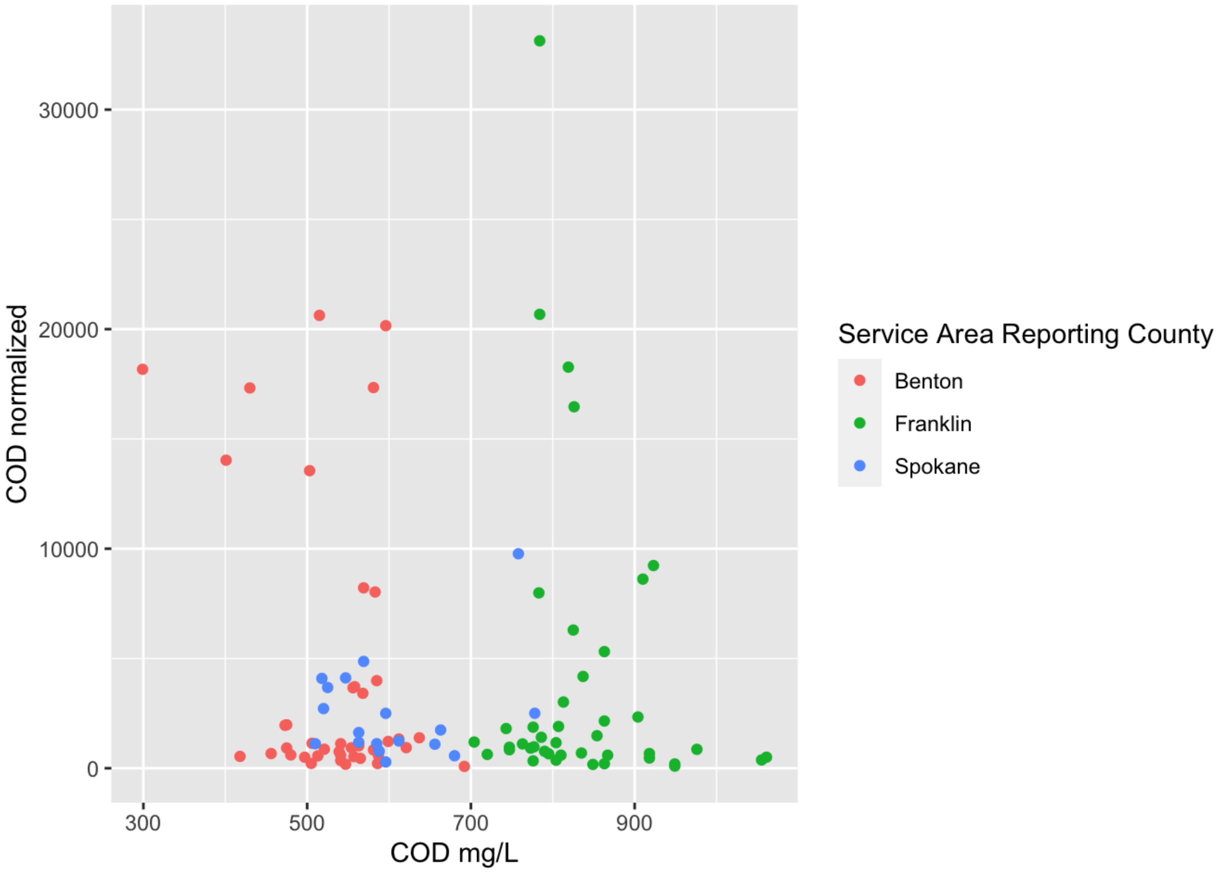


Figure 16: Flow rate vs. TSS concentration (DOH)

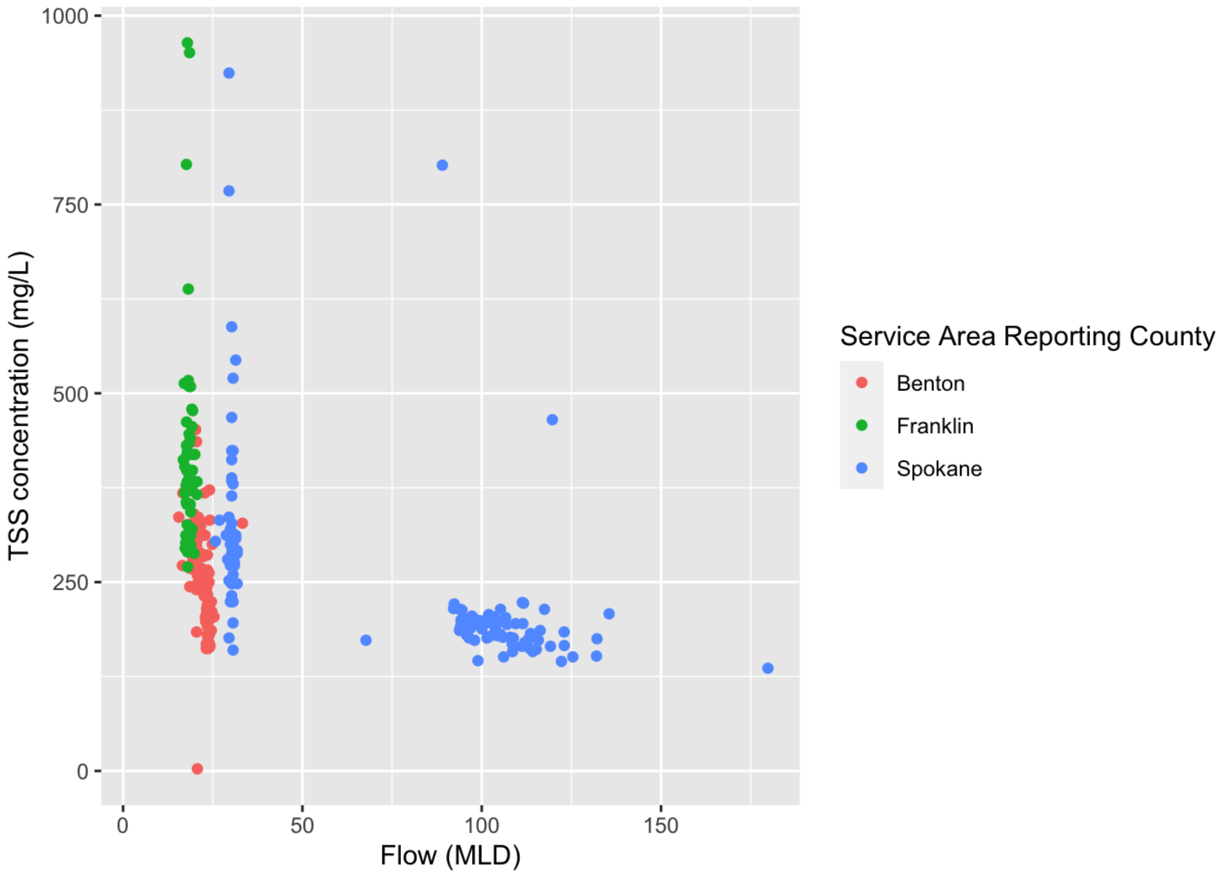
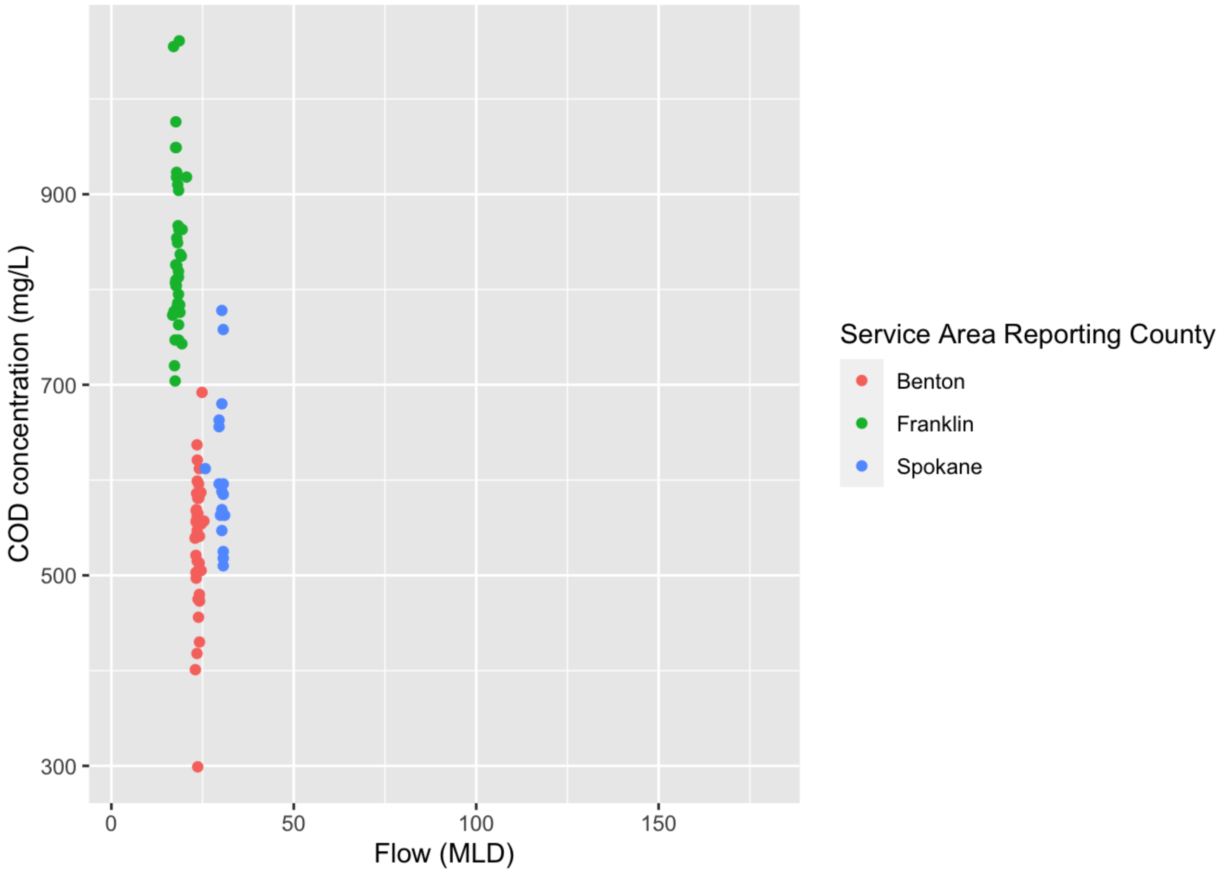


Figure 17: Flow rate vs. COD concentration (DOH)



Combined (DOH + Seattle King County Data)

Table 3: Correlation coefficient comparison chart for both Biotbot and DOH Data

	Unnormalized	**TSS**	**COD**	**PMMoV**
Unnormalized				
TSS	**0.58******			
COD	**0.95******	**0.95******		

PMMoV	0.73****	0.63****	NA	
Flow/Population	0.95****	0.60****	0.98****	0.50***

p<.0001=****,p<.001=***,p<.01=**,p<.05=*