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Wastewater Assessment Based Viral Epidemiology (WAVE)

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MICROBIOLOGY AND ENVIRONMENTAL TOXICOLOGY

by

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Abstract

Wastewater Based Assessment Viral Epidemiology

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Currently, the entire globe is affected by the COVID-19 pandemic caused by the novel coronavirus, SARS-CoV-2(SCV2). The ability to detect, monitor and assess the spread of the virus is imperative to controlling the effects of the pandemic. In order to meet the need for global testing, several methods have been developed. As a complement to individual testing, wastewater-based epidemiology represents a low-cost way to estimate the prevalence of the virus in a community. This information can be used to influence public policy regarding viral mitigation measures. This thesis documents our efforts at implementing wastewater testing in Santa Cruz County over the past 18 months. We sampled from the Watsonville Wastewater Treatment Plant on a weekly basis and quantified the presence of SCV2 using reverse transcriptase digital PCR normalized to Pepper Mild Mottle Virus (PMMV). Our data showed a large discrepancy between reported case counts and SCV2 in wastewater. This data also revealed seasonal variation in PMMV, potentially hindering it as a reliable normalizer. In addition to our sewage work, we performed masked-based sampling for Pacific Elementary School and documented a probable positive. This work demonstrates the potential of sewage sampling for SARS-CoV-2 and some of the current unmet challenges in both sewage sampling and pandemic response as a whole.

Acknowledgements

First and foremost, I want to thank my mentor, David Bernick, who has supported me throughout this project and past projects over the last few years. I would also like to thank Ryan Modlin for his work on troubleshooting and sampling for this project. To everyone in the Bernick Lab who has supported this project, and myself; I give my deepest gratitude. Without their support I would not have made it through this difficult time. Thank you to my advising committee, Dr. Fitnat Yildiz and Dr. Michael Stone. Thank you to Rob Lin, Adam Langston, Christina Bouwens and the entire team at Combinati. Finally, I would like to thank Dr. David Ghilarducci, Dr. Gail Newel, Mike Crane, Brian Condy, Eric Gross, George Spix, John MacDonald and Scott Brown for their support. Without their support this work would not be possible.

Introduction

In March of 2020, the World Health Organization (WHO) classified COVID-19 as a pandemic[45]. As of February 2022, the causative agent of COVID-19, SARS-CoV-2 (SCV2), has killed at least 5.9 million worldwide and over 940,000 in the United States alone [58]. SCV2, is a single stranded, positive sense RNA virus of the coronaviridae family, that causes a wide variety of symptoms, most commonly fever, cough and shortness of breath [13]. Less commonly, SCV2 can also cause gastrointestinal issues[13]. Individuals infected with the virus shed it primarily through respiratory droplets and approximately 66% of patients shed the virus via feces[9].

In order to limit the spread of SCV2 it is essential to know who is infected with it. Infected individuals can then be quarantined to prevent further spread. Several individual testing methods have been developed in the past two years such as RT-LAMP, rapid antigen tests, and qRT-PCR[31]. Of these methods, saliva or nasopharyngeal swabbing followed by qRT-PCR has emerged as the gold standard for testing[6],[34]. While qRT-PCR has high specificity and sensitivity, issues remain. Individual testing has uneven test accessibility, return times for results may be long, and individuals may not get tested for a variety of reasons[34],[48],[38]. In addition, testing is biased towards symptomatic individuals. A significant portion of individuals infected with SCV2 are asymptomatic or presymptomatic and may unknowingly contribute to the spread of the virus[22],[28]. One study proposes that asymptomatic transmission may account for more than half of the total[28]. Finally, there are disparities in testing itself across socio-economic and socio-geographic lines[36],[32]. Rural communities and minority populations tend to get less tests per person when compared to white suburbanites. This can lead to an underestimation of cases in a given area and disparities in health related outcomes between communities [37]. To better protect our community, we sought to use a different testing

methodology to estimate the prevalence of SCV2.

Problem Statement and Goals

Wastewater-based epidemiology (WBE) represents a low-cost way to estimate the prevalence of the virus in a community[35]. WBE has been used previously to some success for polio virus in the 1990's and the original SARS virus was also detected in hospital wastewater[35],[2]. Wastewater sampling of SCV2 has been shown to be a leading indicator of cases and hospital admissions[50],[47]. Currently, wastewater sampling is being widely used across the United States in response to the ongoing pandemic[62]. It does not suffer from the same biases associated with individual testing methods and has much faster turnaround time. To assist with the pandemic response, and provide a compliment to individual testing efforts, we employed WBE in Santa Cruz County.

Wastewater itself is composed of two materials: blackwater and greywater. Blackwater is any waste from urinals or toilets while greywater is wastewater that has been used for showering, laundering or bathing[3]. Greywater typically composes the majority of wastewater, though the composition varies based on area and on what day. The sum of all the wastewater in a given system is referred to as the sewershed which flows to a wastewater treatment plant. Because all sewage in the sewershed coalesces at a single point, the wastewater treatment plant provides an ideal location for surveying a large population. However, mounting a defined response to a sizable population is logistically difficult, if not impossible. Smaller, well-defined, populations or "response clusters" such as college dormitories or assisted living facilities would permit a direct response to detection of SCV2 RNA. The sampling location for these response clusters must be chosen as close to the response cluster as possible, ideally containing only wastewater from the location in question to avoid false positives from upstream sewage. This past year we

monitored both city wide and response clusters.

The main goal of this project was to monitor for the presence of SCV2 in the wastewater of Santa Cruz County. This data would then be used to implement mitigation measures at either the county or cluster level. For response clusters, detection of any SCV2 would result in mitigation measures, while changes in prevalence at the community level will be relayed to county health to inform county wide measurements. Finally, we tested the use of pepper mild mottle virus as a normalizer to account for the variation of fecal material in any given sample.

Research Plan and Methods

Establishing Sampling Locations

Towards the beginning of the pandemic we reached out to Santa Cruz Public County Health Division and coordinated with county epidemiologist Dr. David Ghillarducci for where sewage sampling would be best deployed. The city of Watsonville was identified as a hot spot in the county. Watsonville is a primarily agricultural worker community that accounted for the majority of known cases, at the time, despite constituting only 29% of Santa Cruz County's population[23]. We worked diligently with the treatment plant and Watsonville became our main sampling location for the duration of the project.

After establishing ourselves in Watsonville, and verifying the effectiveness of our protocols, we attempted to expand to more sewersheds in the county. The lab reached out to the Santa Cruz Wastewater treatment plant, but were not able to establish a regular sampling plan. We also attempted to sample from numerous more defined populations such as a school in Scotts Valley, multiple dorms at UCSC, and an assisted living facility in Watsonville.

Later in the pandemic Dr. David Ghillarducci put us in contact with the superintendent of schools, Dr. Farris Sabbah, to establish wastewater sampling in schools when they reopened. A meeting with all 7 school districts in the County was held, and both Davenport and Pajaro Valley school districts expressed interest. Pajaro Valley contains 50 schools, while Davenport contains a single elementary school. Because of logistical limitations, we decided to work with Pacific Elementary School in Davenport. We submitted details to the Office of Research Compliance who stated that because the project focused on public health a review by the Institutional Review Board (IRB) was not necessary. A parent information night was held on April 14th and sampling started soon after on the 19th.

Sampling Methods

Twenty four hour composite samples were taken from the influent of the Watsonville Wastewater Treatment Plant on a weekly or biweekly basis and stored at 4°C for 1-3 days before being processed. Composite samples utilize an auto-sampler which samples small amounts of sewage at regular intervals over a defined set of time and combines them into a single sample. This method was chosen to minimize variability in the sewage sample itself. While composite samples were used for informing community spread, we chose to use grab samples to survey UCSC dorms which involves dipping a sampling bucket directly into the sewage to generate a sample. Grab samples were chosen in this case due to cost and because not all sampling locations could accommodate the auto-sampler itself.

At Pacific Elementary, it was determined that wastewater sampling would prove less useful. Due to reduced school days and the difficulty in accessing the sewer we decided to take a different approach: masked-based sampling. Masks have been shown to reduce

viral spread by trapping the virions normally shed by an infected individual in a filter[25]. By removing the viral RNA from this filter and quantifying it, one could determine if anyone was infected with the virus. Single use masks were provided to the students to wear for their school day. At the end of the time period(roughly 4 hours), students cut out the filter paper of their masks and pooled the filters in a 1.5M salt solution to lyse the virions. The resulting solution was concentrated and quantified using the 4S protocol and digital PCR, respectively.

RNA Extraction Methods

Sewage samples were processed using a variety of RNA extraction and concentration methods. Methods were assessed by quantifying the total nucleic acid content in the purified sample, purity, and volume of sewage processed. The initial method chosen was an adaptation of Thermo Fisher's Trizol cleanup protocol[27]. While this protocol did yield moderate levels of RNA (30-50 ng/uL), we ultimately abandoned it because of high phenol and guanidinium salt contamination. Cleaning up samples with additional chloroform steps did reduce the aforementioned contaminants, but this was a tedious process that did not scale well with multiple samples. In addition, the relatively low volume of wastewater (5mL) and high reagent cost pushed us to explore other methods.

We next adapted the Zymo Quick-DNA/RNA Viral MagBead Kit with additional ultrafiltration[52]. 40mL samples were spun down to concentrate solid particulate matter before processing with the MagBead Kit. Samples generally yielded between 50-150 ng/uL of nucleic acids with no phenol or guanidinium salt contamination. While we were able to detect a surrogate virus that we used as a control, we were unable to quantify SCV2. This could be due to the low case counts in the county, at the time, or the binding capacity of the magnetic beads (10ug). We attempted to concentrate the RNA further

using 10kD molecular weight cutoff (MWCO) columns. Unfortunately, no SCV2 was observed with this method either. We suspect that other bacterial nucleic acids present in the sample saturated the column and prevented SCV2 from binding. Despite the improvements in processing volume, nucleic acid yields, and purity, the process was time consuming. To meet our goal of sampling at multiple sewersheds we needed a protocol that provided additional throughput.

Finally, we used the 4S protocol developed by the Nelson Lab at UC Berkeley[59]. Figure 1 describes the general workflow. Samples were shaken thoroughly to ensure homogeneity. 40 mL of sewage was added to 9.5g of salt (final conc. 1.5M) to lyse virions and other pathogens in the sample. Solid particulate was removed using a 5 micron filter and samples were mixed with equal volume of 70% ethanol to precipitate nucleic acids. The solution was then vacuumed through a silica column to concentrate the nucleic acids before being washed. The concentrated nucleic acids were then eluted using 100 uL of MilliQ water. The increased binding capacity of the silica column (300 ug) allowed us to concentrate more nucleic acids with an average yield of 500 ng/uL. The vacuum manifold allowed us to concentrate roughly 20 samples in parallel and the protocol relied on low cost reagents that were easily accessible despite supply chain issues.

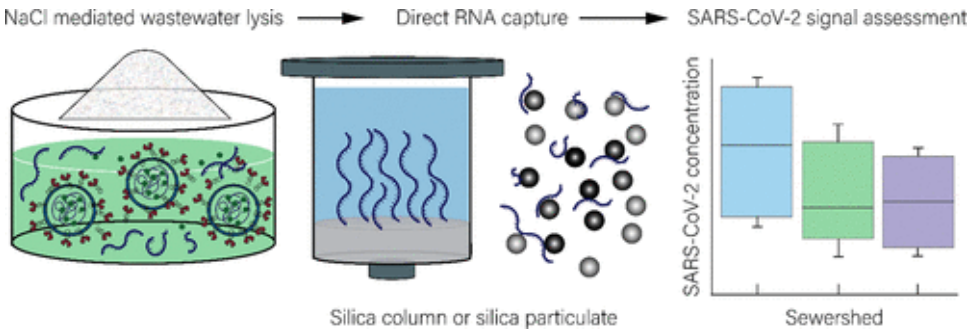

Figure 1: General workflow of the 4S protocol. Samples are lysed using high concentrations of salt before being concentrated on either a silica column or silica gel for future quantification. Figure adapted from[59].

Normalization

SCV2 is found alongside feces in blackwater. However, greywater significantly dilutes this material and that dilution may vary on any given day. To account for the varying dilution of sewage, it was important that we normalized our SARS-CoV-2 counts to a fecal indicator. This fecal indicator would tell us how roughly how much blackwater we captured in our sample. For our normalization we used another positive sense RNA virus, PMMV[53]. The virus normally infects pepper plants, causing mottling, but has recently emerged as an indicator of human fecal pollution[53],[21]. PMMV is found in processed pepper products (eg. hot sauce) and was the most abundant RNA virus in a metagenomic analysis of human feces[63]. The virus is also remarkably stable, demonstrating infectivity after passage through the human gut[63]. Current data suggests that PMMV concentrations in wastewater do not show significant seasonal changes, and PMMV is currently being used as a normalizer by multiple research groups[62],[33],[55].

Digital PCR Quantification

For our quantification method we chose dPCR for its sensitivity, and PCR-inhibition resistance which is important due to the low number of virions and inhibitors in sewage[14],[43],[54]. dPCR is a refinement of typical qPCR based methods that allows quantification of a given DNA sequence without the need for generation of a standard reference curve. The key feature of dPCR is that the sample is diluted and partitioned, typically using a fixed array or a droplet emulsion to the level of single molecules. Each individual partition then undergoes PCR amplification and an all or none digital signal is obtained for each partition. The number of target molecules is then calculated using the Poisson Distribution to account for partitions that would be expected to have one or more molecules present as initial templates [40].

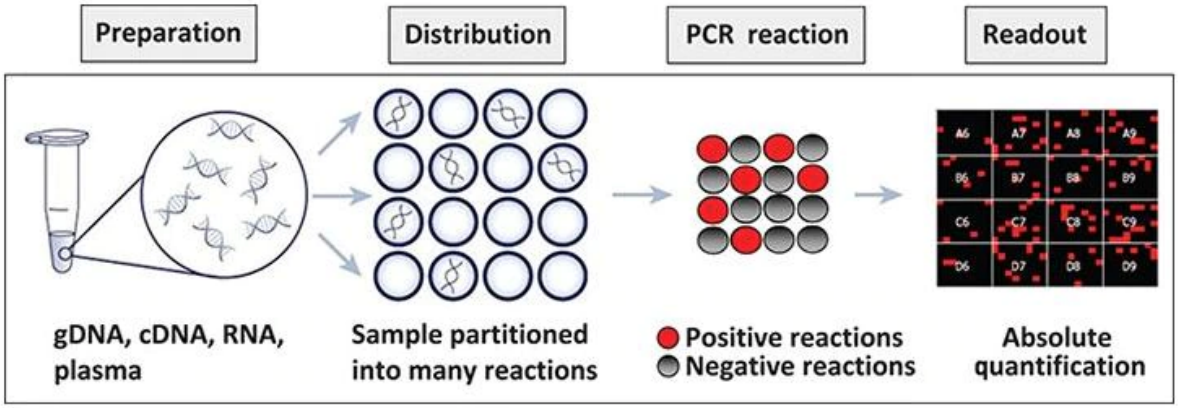

Figure 2: The figure above depicts the general workflow of dPCR[5]

Wastewater samples were analyzed using a 3-plex assay on the Combinati Absolute Q dPCR machine with 4 technical replicates. We used 4 samples to make sure that we would have sufficient replicates despite technical issues with the fixed array. FAM and HEX channels were used to quantify the N1 and N2 genes, respectively, using research-grade CDC standard primers and probes for SCV2[19]. The third channel was used to determine the amount of PMMV in the sample with primers that bound the Replication Associated Protein (RAP)[63]. The early version of the assay also contained primers and probes for RNAseP, but we did not use this data. Five microliters of purified and concentrated nucleic acids were mixed with the 3 plex-primer probe mix and a master-mix containing the reverse transcriptase and DNA polymerase necessary to perform the reaction. After amplification, fluorescent thresholds for each channel were adjusted and samples that displayed irregularities in the fixed array were discarded. Primer and probe sequences can be found in Table 1.

Gene Name	Sequence
COVID 19 N1 Forward Primer	GACCCCAAAATCAGCGAAAT
COVID 19 N1 Reverse Primer	TCTGGTTACTGCCAGTTGAATCTG
COVID 19 N1 Probe	FAM-ACCCCGCATTACGTTTGGTGGACC-BHQ1
COVID 19 N2 Forward Primer	TTACAAACATTGGCCGCAAA
COVID 19 N2 Reverse Primer	GCGCGACATTCCGAAGAA
COVID 19 N2 Probe	HEX-ACAATTTGCCCCCAGCGCTTCAG-BHQ1
PMMV RAP Forward Primer	GAGTGGTTTGACCTTAACGTTGA
PMMV RAP Reverse Primer	TTGTCGGTTGCAATGCAAGT
PMMV RAP Probe	Cy5-CCTACCGAAGCAAAT-BHQ1

Table 1: This table depicts the gene targets and the respective sequences for the primers and probes used in the dPCR quantification assay

Results

Sampling Locations

After verifying a successful RNA extraction and concentration method, we sampled from the Watsonville Wastewater Treatment Plant for approximately one year. A single grab sample was taken from an Assisted living Facility at Watsonville, but no SCV2 RNA was detected in the sample. This was likely due to issues with sampling as the sample had a much clearer color when compared to samples from the wastewater treatment plant. Shortly after, we were asked to discontinue sampling by the assisted living facility stating that they did not wish to know if SCV2 was in their wastewater.

Multiple samples were taken from various UCSC dormitories, but none tested positive for SCV2. Notably, some samples did test positive for trace amounts of RNAse P, showing we were likely getting some amount of human fecal material in the sample. Some samples also registered low levels of PMMV. A sampling plan was proposed to the University, but due to lack of financial and logistical support, no sampling plan was implemented.

Watsonville Results

After running each weekly sample using dPCR, counts (cp/uL) were averaged across successful replicates and plotted overtime to observe trends of SCV2 RNA in Watsonville. We made the assumption that we captured 100% of the virions present in our 40 mL sewage sample and multiplied our average counts by our concentration factor (400) before converting to cp/L. As seen in Figure 3, N1 and N2 counts trended in the same direction and were similar to each other, giving us confidence that we were acquiring SCV2. N1 consistently tested higher than N2 with an average ratio of 1.24 to 1. Though the reason for this is unknown, it is consistent with other literature[26],[17].

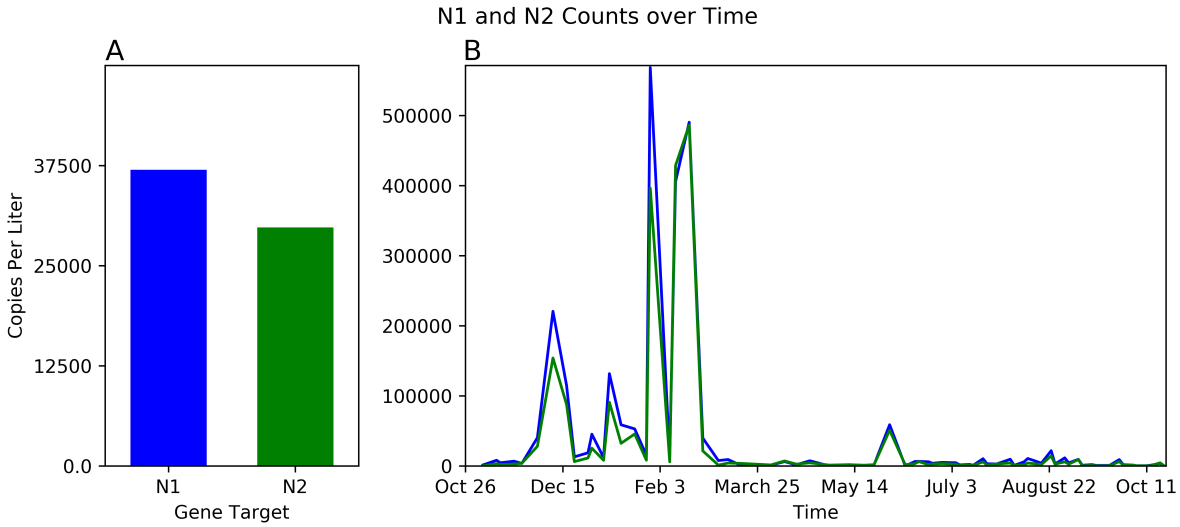

Figure 3: **A**: Shown are average counts for N1 and N2 over the entire sampling period for the Watsonville Wastewater Treatment Plant. N1(blue) averaged 3.70×10^4 cp/L while N2 averaged 2.98×10^4 cp/L. On average N1 was 1.24 times higher than N2.
B: Shown are the measurements of N1 and N2 over the entire sampling period. Each data point is the average of all replicates for a given sample.

Comparison To Case Counts

Daily numbers of known cases in Santa Cruz County were taken from the Santa Cruz County Covid Dashboard[23]. Importantly, the cases are from the entire county, not just those in Watsonville, which can make interpretation more difficult. Multiple attempts were made to contact Dr. David Ghillarducci of Santa Cruz County Public Health to acquire the raw case data representing solely Watsonville, but there was no response.

From the case counts, a 7 day rolling average was generated (Figure 4). For SCV2, the larger of N1 and N2 from a given day was displayed along with the average amount of PMMV across replicates. For almost all data points this was N1. To normalize the data, we first calculated a baseline value of PMMV over the sampling period by finding the median cp/ul. This was then assigned as 1 unit of PMMV. All PMMV values are

then scaled to this. Due to the differences in the raw values of SCV2 and PMMV we scaled SCV2 by 3X for easier visualization.

Over our entire sampling period the raw values of SCV2 varied significantly from week to week and over three orders of magnitude. Figure 4A shows that while raw counts did increase during peak cases in Santa Cruz, they don't appear to track particularly well with clinical cases. Early in our sampling, between mid-November and early January, it appeared that our normalization strategy was tracking with case counts (Figure 4B). This was confirmed upon personal communication with Dr. David Ghillarducci and, according to him, helped accelerate the shutdown of the county in December of 2020. Exceptions to our normalization strategy occurred when there was very little PMMV and SCV2 in the sample, such as in November of 2020 and March of 2021 (Figure 4B). Because there was so little fecal content in the sample, it resulted in a much higher normalized peak.

On January 28th we experienced an unexpected spike in SCV2. The raw SCV2 count was roughly 2 times higher than our previous highest sample, and we expected a corresponding increase in PMMV, showing that this sample had simply captured more fecal material than others. PMMV was instead much lower, resulting in an even larger spike when normalized, approximately 1080 units. As seen in Figure 6B, this dwarfs all of our other data points. In addition, this spike occurred at a time when cases in the county were declining from their peak on January 8th. We initially thought that this was an anomaly as the subsequent week had very little SCV2 in it, but the following week we saw a second large spike in SCV2 counts. If we interpret this peak as a non-anomaly it appears to be lagging new case counts by 20 days. We then attempted to assess the lag in cases compared to hypothetically active cases.

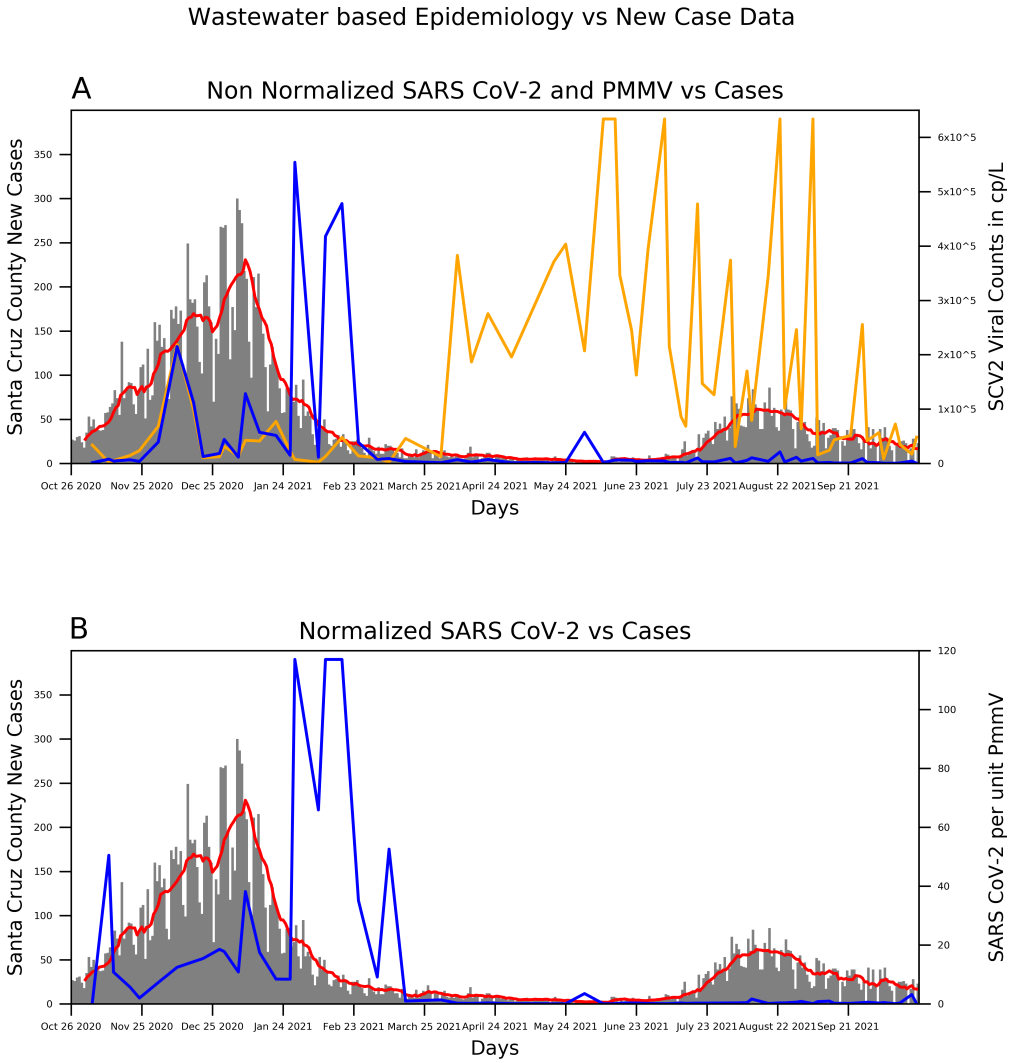

Figure 4: Gray bars represent the new known cases on a given day in the County of Santa Cruz. The red line is the resultant 7 day average from the new cases.
A: Shows our non normalized counts for both SCV2 and PMMV compared to new known cases in the county. SCV2 (blue) is taken as the larger of N1 or N2 for a given sample. PMMV is shown in orange and is the average count from all replicates of a given sample. Because PMMV was almost always larger than SCV2, the data was scaled by 3X for easier visualization.
B: Displayed are our results of SCV2 normalized to PMMV(blue). To better visualize the data, SCV2 normalized values were limited to 115 units of SCV2/PMMV.

Active case data was generated from known case data by assuming a 10 day infectious period after the case was documented. This is likely an overestimation as individuals may not get tested until they are multiple days into their infectious window. Regardless, Figure 6 still shows that even when you account for an infectious period of 10 days, our SCV2 counts still lag case counts. Adding on a 10 day infectious case window only shifts the peak of cases from January 8th to January 11th. This shrinks our gap of 20 days to 17 days. So clearly, simply account for a delay in shedding after reporting was not sufficient to explain this spike. This potentially runs counter to reports of sewage sampling as a predictive indicator of COVID cases[61].

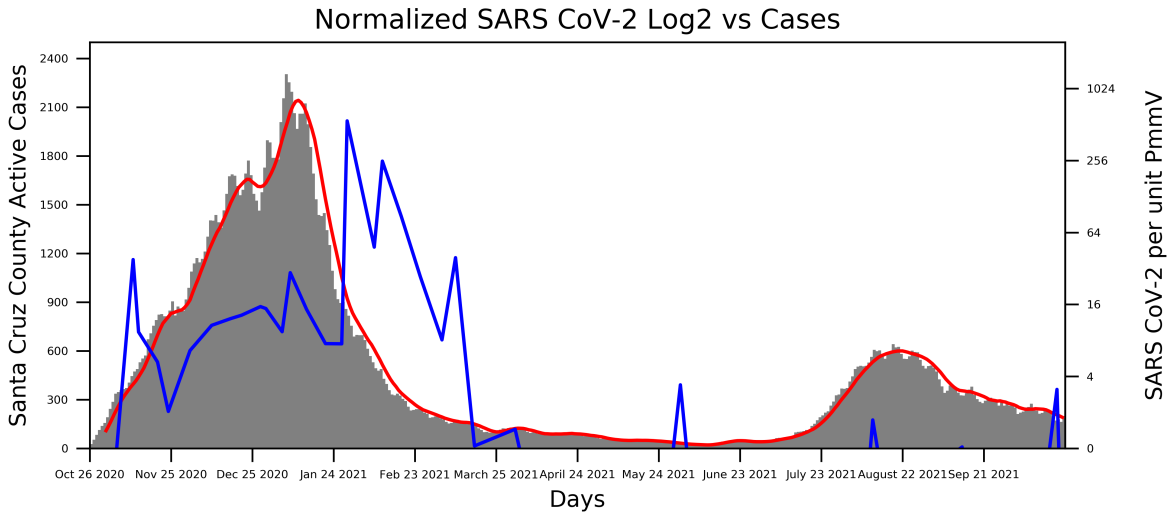

Figure 5: Shown here is a log base 2 transformation of our normalized SCV2 data(blue). Grey bars represent the hypothetically active cases in the county, while the red line is a rolling 7 day average.

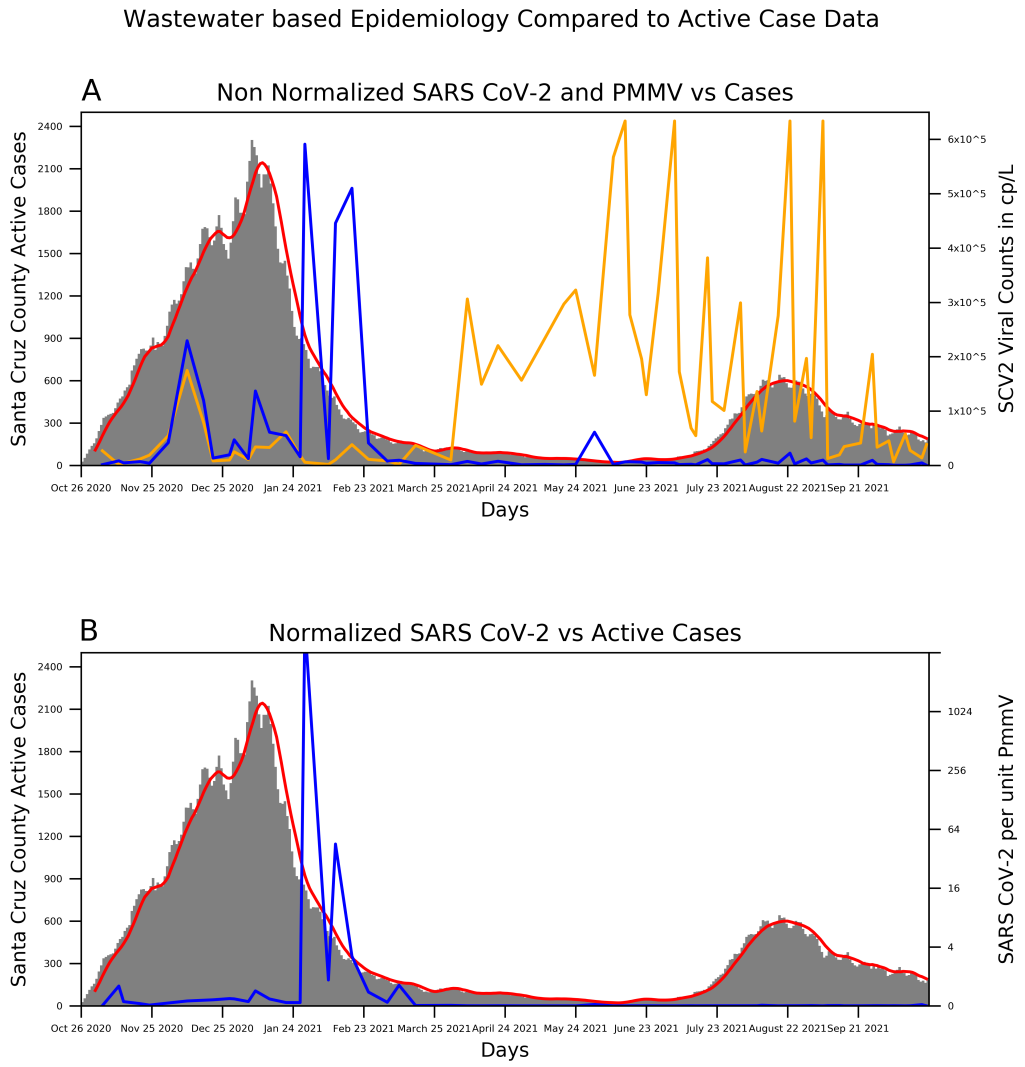

Figure 6: Comparison of SCV2 counts normalized/non-normalized with hypothetically active cases based on a ten day infectious period. Grey bars represent the active cases in the county, while the red line is a rolling 7 day average.
A: Displays SCV2 g with PMMV counts (orange).
B: Displays our normalized SCV2 (blue) counts compared with hypothetically active cases. On this figure there is no limit on the top of the graph, so the full size of the peak on Janary 28th can be visualized.

Upstream Analysis

In response to this large peak we attempted to sample further upstream to try and isolate where the SCV2 was coming from. In Watsonville there are three major lines that feed the sewage treatment plant. Earlier, we had sampled from a single upstream location(data not shown) and did not find any SCV2 or PMMV so we reduced our sampling system to 3 points. We sampled from 2 individual lines which we labeled as Pajaro and Riverside, and the combined influent of the plant using 24 hour composite samplers. Riverside contained the most SCV2, roughly one third compared to our non normalized peak in January (5.7×10^5 cp/uL vs 1.76×10^5 cp/uL). Pajaro tested much lower at 1.3×10^4 cp/uL and almost no SCV2 was detected at the treatment plant. We do not believe that it was an issue with sampling or RNA extraction given that we saw significant PMMV in the sample. Despite our novel discovery that PMMV varies seasonally, detecting it showed us that we had successfully captured some amount of RNA in the samples.

When normalized, Figure 7B shows that Pajaro tested higher than Riverside. We decided to stop sampling at Riverside and continued to sample at Pajaro to track the decrease in normalized counts for the next two weeks. This was before we fully understood the seasonal variation of PMMV and in retrospect the main driver for the large increase we saw was most likely from the Riverside line, though more data would be needed to confirm this. Importantly, this data shows heterogeneity between sampling locations. The treatment plant showed strikingly different levels of SCV2 despite the 2 upstream lines showing noticeable concentrations. Knowing the volumnetric flow rate of each of the respective lines would help inform us if our observation at the treatment plant was due to a dilution effect. Potentially, some SCV2 could have been degraded as it flowed along the pipe or there could be potential issues with the reliability of auto-samplers. Regardless, further study of the underlying dynamics of the sewage system are needed

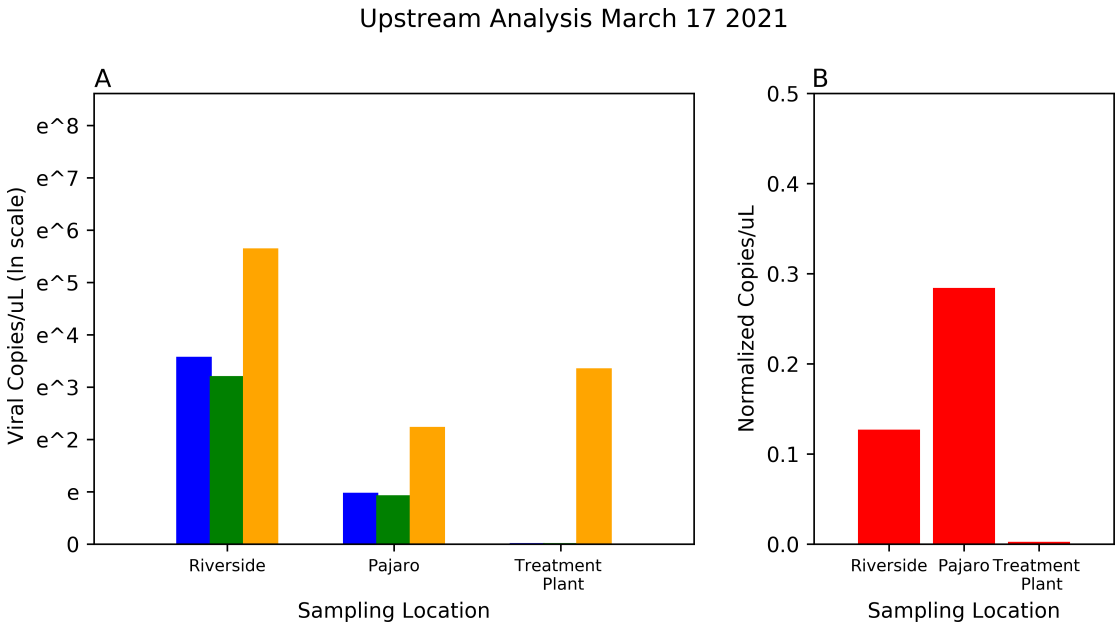

Figure 7: This figure shows the results of our sampling from multiple sampling locations on March 17, 2021.
A: Shown is the non-normalized results of our experiment. Bars are the averages of 4 technical replicates. N1 counts are displayed in blue, N2 counts are in green and PMMV counts are in orange.
B: Normalized counts were generated by dividing the higher of N1 and N2 (in this case N1) by the counts of PMMV and are displayed in red.

to understand the causes of this heterogeneity.

Variation in PMMV

Based on previous literature it was thought that PMMV would remain relatively stable over the sampling period, however this was not the case with our data[33][62]. Beginning in April 2021 we saw a statistically significant ($p<0.001$) spike in PMMV (Figure 8) that continued into the summer months, peaking in June. We then saw a general decline from June to October though the difference was not statistically significant due to the large variances between week to week samples (Figure 4). The decline from September to October was not statistically significant ($p = 0.064$), we suspect that given the trends,

additional sampling would confirm this.

During this time we also tried an additional normalizer, known as crAssphage, to see if it might be a better normalizer than PMMV. CrAssphage is a double stranded DNA bacteriophage that infects the human gut symbiote Bacteroides[15]. The virus accounts for 1.68% of all human faecal metagenomic sequencing reads in the public database[15]. Primers and probes were designed by Combinati (sequences not shown) and tested on a sewage sample, but crAssphage exceeded the limit of the Absolute Q at approximately 100,000 cp/uL. To accurately quantify crAssphage a serial dilution would be necessary alongside our SCV2 sample to make sure we had values within our measurable range. This would have increased operating costs so we ultimately decided to not investigate it further.

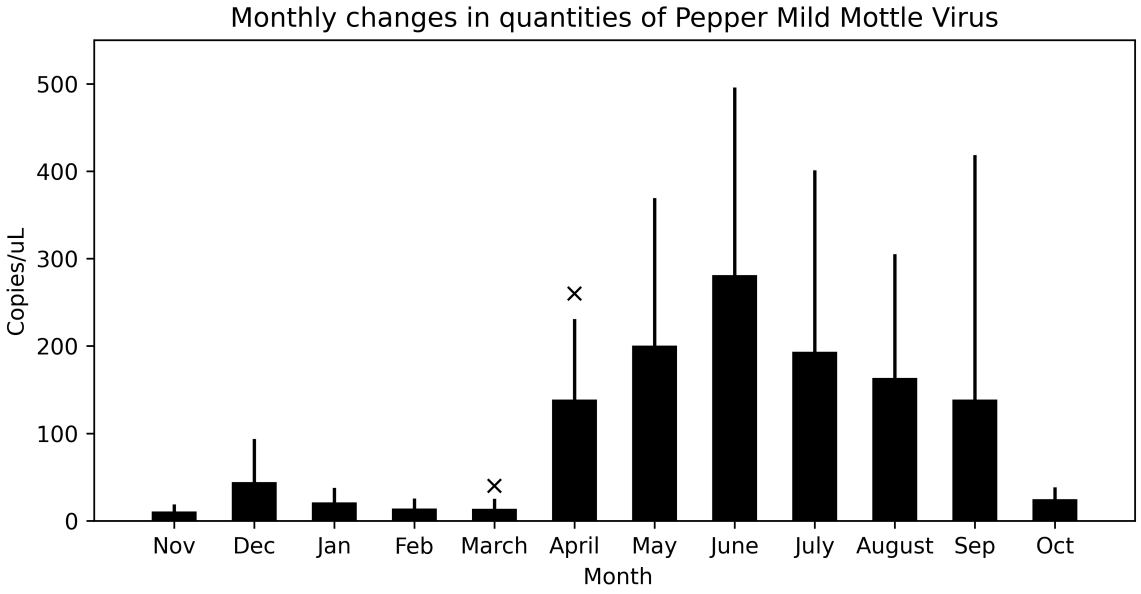

Figure 8: Shown above are the avg PMMV counts from each month of sampling. Errors bars represent the standard deviation of the sample. (X: $p<0.001$) Statistical significance was generated using a t-test.

After confirming that seasonal variation of PMMV was likely, we opted to end sampling in October of 2021. We had not seen significant variation in our SCV2 counts for roughly 6 months and we did not anticipate that we would see them going forward given the vaccination rate of Santa Cruz County. Our assumption was proven wrong when the Omicron variant emerged causing a new wave of cases in the winter of 2021. Due to logistical difficulties we did not restart sampling.

Davenport Results

Professor David Bernick performed training with the students and teachers on sample preparation and storage during the 16th of April before sampling proceeded on the 19th. The pooled sample from 12 students tested significantly ($p < 0.0001$) above the negative control Figure 9. After rerunning the sample an additional two times and achieving similar results(data not shown), the superintendent of Pacific Elementary School, Eric Gross, was informed. Parents were then informed and class was suspended for ten days and were advised to get their students tested. We could not compel parents to get their children tested nor could we control the method of testing. We suspect that some parents used rapid tests for their children given the less than 12 hour turn around. A single parent stated that they would not be getting their child tested.

On the 23rd of April, a parent contacted principals expressing concern that a consent form had not been signed stating that it was human subject research. Though we had already been reviewed, and found that no IRB approval was necessary, we decided to put everything on hold to maintain trust with the parents. After re-review by The Office of Research Compliance we were again found exempt from IRB approval. We reopened discussion with the parents with an additional parent meeting over Zoom and the response was overwhelmingly positive. We decided to generate a permission slip, despite the fact

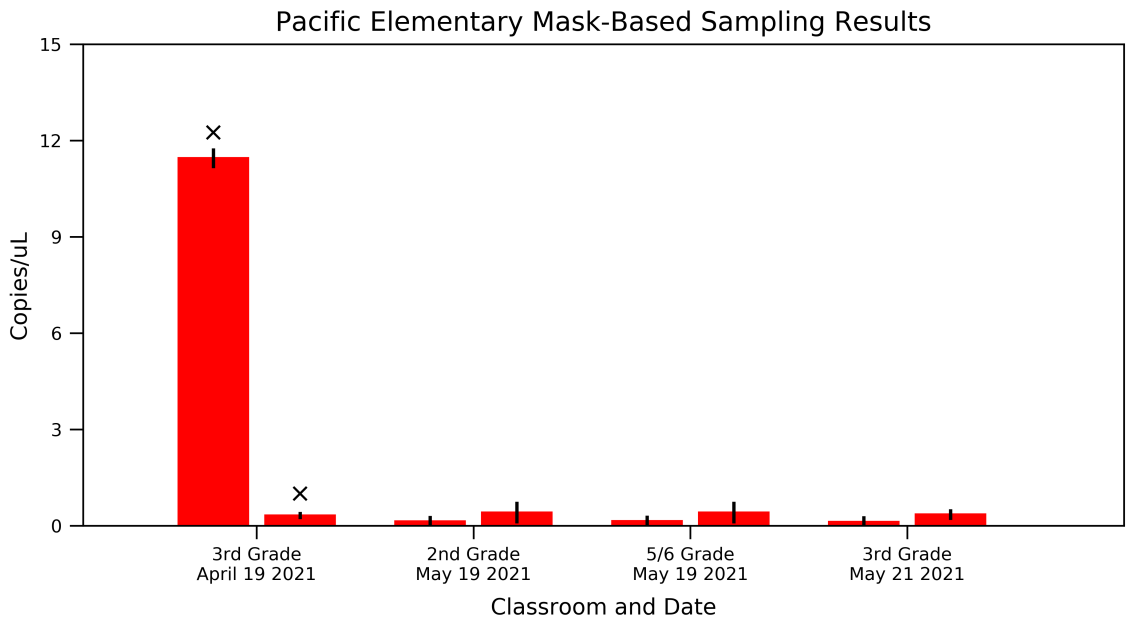

Figure 9: This figure depicts the results of mask based sampling at Pacific Elementary. Bar heights are the average of N1 and N2 across replicates. Error bars represent the standard deviation of the samples. An X denotes a significant difference between the sample and the negative control(X : $p < 0.0001$) Statistical significance was generated using a t-test.

that we did not need to. Responses to said permission slip took two weeks and we did not get returns for more than half of the students.

The project was expanded to include additional classrooms which all tested negative (Figure 7). The students then broke for summer break during which time we discontinued testing. For fall, we had planned to restart the same testing strategy, however the County of Santa Cruz required that all schools be tested through a third party company and so sampling was ceased.

This work demonstrates the potential of mask-based sampling as a tool in detecting SCV2. While we could not clinically confirm the positive, it seems very likely that there was a positive student, given the stark difference between the negative and positive control. This potentially prevented further spread in the classroom. This study also

demonstrated the importance of ethical performance of research. Despite not needing re-review, we opted to so that we could maintain trust. The response from the parents, was overwhelmingly positive and was critical to our continued sampling efforts. Mask-based sampling has been expanded to include diagnostics within the mask using lyophilized CRISPR circuits[42]. This technique may become invaluable in the next stages of the pandemic to detect infection.

Discussion

Results: Alternative Hypotheses

Our results show that N1 and N2 counts did correlate with one another throughout the sampling duration. This gives us assurity that we were detecting SCV2. Early in the pandemic, we thought that our normalization strategy was effectively tracking case counts, however a later January spike followed by seasonal variation in PMMV raised significant questions. Due to this variation, we conclude that PMMV is likely not a good normalizer for SCV2 in Watsonville. This may not be true for all sewersheds and further investigations into different sewersheds in the county could reveal this. The cause of the January peak still remains an open question, for which there are two main hypothesis.

The first is that the peak we saw in sewage did not reflect the true abundance of SCV2 in Watsonville. What we saw could have been due to a sampling or biasing error. This study implicitly assumes that all wastewater inputs contribute equally to the wastewater treatment plant. In other words, we assume that a SCV2 positive individual ten miles upstream contributes equally compared to someone 1 mile upstream. However, in reality virus may be lost traveling through the sewage pipes. This would mean that those closer to the sewage plant contribute much more than those further away. If there was a large

outbreak in close proximity to the plant, we would detect a large spike of SCV2 that would not reflect the abundance of SCV2 in the entire population. This is supported by Figure 7A which showed significant heterogeneity in the the streams feeding the wastewater treatment plant. This could be investigated by establishing multiple sampling locations, and investigating spatio-temporal dynamics of viruses and bacteria in sewage.

The second hypothesis is that the peak we saw in sewage did reflect the true abundance of SCV2 in Watsonville. This would run contrary to clinically reported cases at the time. However, clinical testing is not perfect. It is possible that individuals in Watsonville weren't getting tested at the time. Given the proximity to the holiday season it is possible that people were returning from vacations and were bringing back SCV2 with them. Potentially this spread in the area over the next few weeks resulting in the peak measured in Figure 4.

Improvements to the Study

In retrospect, additional elements could have been added to the study to improve its robustness. Firstly, we did not use a standard to compare the efficiencies of the various RNA extraction protocols we used. While we did look at raw nucleic acid count, cost, and scalability, a direct quantitative measure of a protocol's efficiency would have been useful. We could then definitively say which protocol is better, and we could better estimate the cp/L present in wastewater instead of assuming 100% capture. Besides an initial efficiency test, we should have added in a regular process control, to verify our RNA extraction protocol was successful. Both of these could be addressed by spiking in a known quantity of nucleic acid or surrogate virus. For example, bovine coronavirus vaccine (BCOV) has been used to some success as a spike in virus [30].

Logistical and financial support would have greatly improved the scope of the project.

The sampling and experimental procedures of this project was handled by a group of less than 10 people and our budget was roughly $65,000. This made it very difficult to expand our operations. Sampling multiple times a week would have helped smooth out some of the noise present in the sewershed and helped us detect increases in SCV2 faster. Sampling at multiple different locations in the sewershed could have helped us understand and model the underlying sewage dynamics and allowed us to mount isolated responses to increases in SCV2. Specifically, sampling at isolated sewersheds, be they high-risk care facilities or low-risk dormitories, would have allowed us to mount a direct response to detection of the virus and hopefully prevent spread. Additional funding would have allowed us to purchase more reagents, auto samplers, and allowed us to develop fully automated sample processing like those at UC San Diego[24].

Future Studies

Further studies should focus on understanding the underlying dynamics of the sewage system and further assessing normalizers. Sampling at multiple locations utilizing different bacteria or viruses, as well as metrics such as flow could help us understand how the sewershed behaves and build predictive models of SCV2 outbreaks. In addition, sequencing of highly concentrated samples will provide insight into circulating variants and detect when new variants of concern arrive in sewersheds.

Further research needs to be performed to understand how PMMV performs as a normalizer. In our case, we saw clear seasonal variation, but it may still be a reasonable normalizer in some cases. Multiple groups have seen success [60],[12] while others report crAssphage as a more promising normalizer[20]. Importantly the two studies who found PMMV as an effective normalizer measured from sludge rather than wastewater. Every sewershed is different and the variation found in Watsonville may not be present in other

sewersheds in the county or state. Other physiochemical parameters such as total suspended solids, biological oxygen demand, and daily flow could be useful in normalization as well. Further communication within the WBE community is imperative to generate standardized protocols and allow accurate comparisons between sewersheds.

Wastewater Based Epidemiology Outlook

While we did not sample past October, a new variant, labeled Omicron, emerged in South Africa in November and subsequently spread around the world causing record high cases[8],[51]. WBE was used to great effect. Biobot Analytics, among many others, gathered data from different sewersheds across the country and showed a large increase in normalized cp/mL. Of note this increase was well beyond what was clinically recorded (Figure 10). This large discrepancy is likely a result of cases exceeding testing capacity[44]. This demonstrates WBE success at detecting outbreaks on a national scale.

In addition to monitoring changes in SCV2 prevalence, WBE has seen success with monitoring changing variant populations[7],[4] including variants of concern such as Alpha and Delta[11]. Sequencing wastewater samples can also discover novel variants that are not captured by sequencing clinical samples[56]. This indicates potential gaps in clinical sequencing, or the potential presence of a non-human animal reserve that could generate new variants of concern.

WBE has received attention at both the international and national level. Across the globe there are over 3300 monitoring sites[41]. In the US data is now being shared and displayed using the National Wastewater Surveillance System (NWSS) generated by the CDC in February[18]. This page allows hundreds of communities to share their data and monitor changes in viral populations. However, only a handful of states are reporting their data[29]. Further expansion of WBE is necessary to generate a comprehensive re-

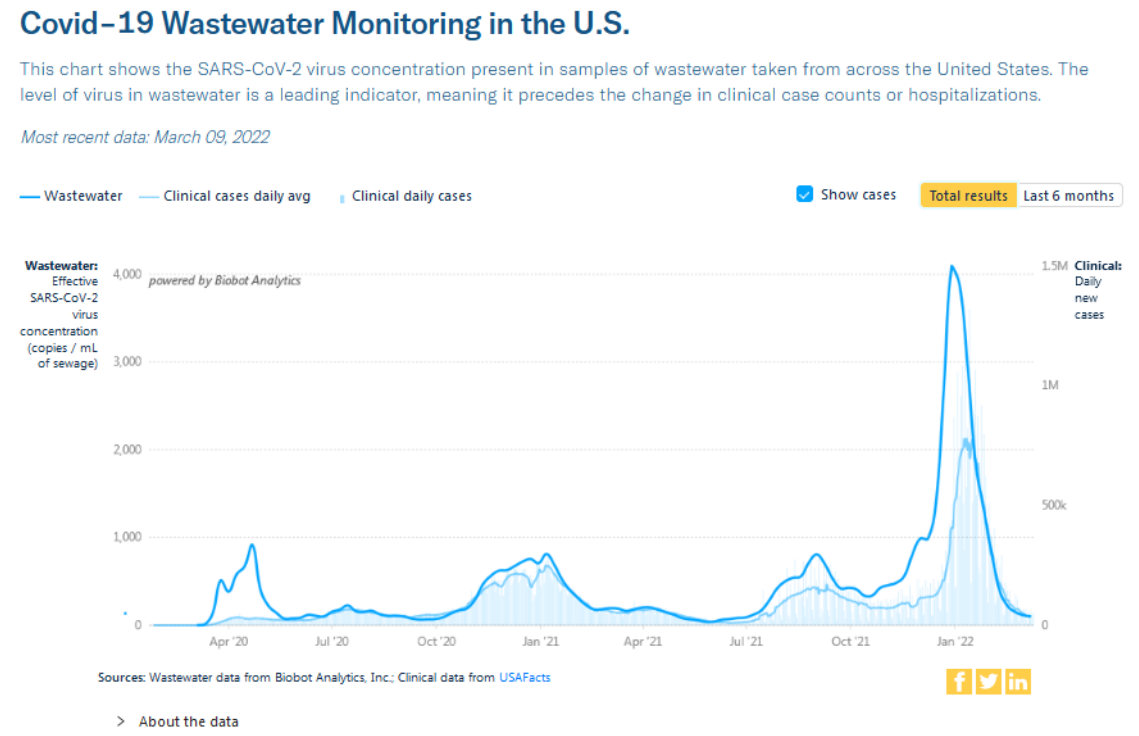

Figure 10: Shown is the data gathered by Biobot Analytics. The solid blue line denotes cp/mL of SCV2 in wastewater normalized for factors including population size, wastewater flow rates, and dilution. The data is generated by taking the mean of all samples taken that week in the country and weighted by the population size of each sewershed. This data is then used to generate a 3-sample rolling average which produces the data shown in the visualization. Clinical data is shown in light blue. Bars represent daily cases, while the line represents a rolling 7 day average. Figure was adapted from[1]

porting system and clear communication between wastewater facilities and public health leaders is imperative to respond to changes in SCV2.

While national level data is useful for detecting new variants or implementing policy changes, it is not useful for mounting direct responses. Even at the city level, it's incredibly logistically complicated to respond to an increase in SCV2. That is why, we believe an excellent use of WBE is to implement it for defined populations where a direct response is feasible. Any detection of SCV2 would result in immediate testing of the population

and isolation of positive individuals. Universities dorms, and healthcare facilities are two locations where we believe WBE would be most useful. Over 276 universities worldwide are sampling for SCV2 so far and hopefully more will join[41].

While WBE has remained focus on SCV2, it still will have a use after the end of the COVID-19 pandemic. Many pathogens such as *V. Cholerae, C. difficile, H. pylori, C. trachomatis, N. gonorrhoeae,* and many more all generate discharges that can be detected in sewage. These pathogens can be tracked at response clusters, or larger levels, and used to inform responses. National investment into a cohesive WBE platform could be an incredibly powerful tool for protecting public health in the future.

Conclusion

This project's goal was to evaluate the effectiveness of weekly sewage sampling of SCV2 normalized to PMMV during the course of the COVID-19 pandemic. While we were able to find an effective RNA extraction protocol that allowed us to rapidly generate results (<24 hour turn around), we saw significant discrepancies between our normalization strategy and clinically reported cases. Initially, we suspected our normalization with PMMV successfully related SCV2 prevelance to clinical cases, but a large unexpected spike and seasonal increases in PMMV show that the sewage system did not behave as expected. Based on our data, we conclude that PMMV is not a good normalizer for Watsonville, CA. Seasonal variation, combined with anomalies in normalization make it difficult. Importantly, PMMV may still be a reasonable normalizer for other sewer sheds. Additional sampling in Santa Cruz, and the surrounding area is needed to determine if this is the case.

WBE is now more important than ever to detect and monitor outbreaks. As mask mandates and restrictions are lifted across the country, people are less likely to get tested

thinking that the pandemic is reaching an end. Many areas are scaling down testing, due to costs, and the growing use of home testing leads to under reporting of cases[10],[39]. WBE could effectively fill those gaps. In addition, a new omicron subvariant, BA.2, has emerged and is likely driving case increases in both the United Kingdom and Europe[46]. BA.2 has also been detected in New York City and is an increasing proportion of new cases in the US[49]. SCV2 levels in wastewater have recently increased and while more data is necessary, it certainly does not bode well for the future[16]. This comes at a time when Congress declined to add $22.5 billion in funding for testing, treatments and vaccines[57]. WBE could give us the early warning signs we need to protect people from an encroaching BA.2 wave in the US. However, if politicians and public health leaders are not willing to implement politically unpopular measures to save lives, then it will be for naught. Even if we are done with the virus, it is clearly not done with us.

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