Using SYBR Stain in the Assessment of Removal Efficiency and Continuous Detection of Viruses in Membrane Bioreactors

Team Name Deleted

Team University Deleted

Team Members Deleted

Faculty Advisor Deleted

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1.0 EXECTIVE SUMMARY

Mustang Matrix has developed a novel measurement device to rapidly determine viral removal efficiency and concentration. After considering ease of detection, economics, and practicality, Mustang Matrix developed a continuous sampling system with an in-line fluorometer that uses SYBR Gold Nucleic Acid Gel Stain (SYBR Gold) added to a sample stream concentrated by ultrafiltration. The viral DNA and RNA bind with a buffered solution of SYBR and emit fluorescence when excited to indicate virus concentration. A standard curve was developed to determine a relationship between fluorescence and virus concentration.

The bench scale process applied the SYBR method to analyze membrane bioreactor (MBR) influent and effluent. A sample of each was filtered (0.2 µm membrane) and then centrifuged with membrane tubes to concentrate the viruses. SYBR stain was added to each sample's concentrate and then the fluorescence was measured. The permeate from the membrane for each sample was used as a background measurement. The fluorometry results for the MBR influent and effluent correspond to a log removal value (LRV) of 2.3.

The large-scale process will use single-pass tangential-flow ultrafiltration to concentrate the viruses, dose the sample stream of MBR effluent with the SYBR solution, and then pass it through an in-line fluorometer to analyze the concentration of virus. This system will be connected to a SCADA system to facilitate automation and notify operators if virus concentrations are beyond a set threshold. The construction cost of the process is projected to be $300,000 with an annual operating cost of $190,000. Most of the operating expense is the purchase of chemicals for the analysis.

The sampling train avoids the production of hazardous waste as SYBR Gold is not hazardous and the remaining chemicals within the stain are present only in trace concentrations and can be safely broken-down during wastewater treatment. Additionally, Mustang Matrix has reviewed health and safety guidelines to ensure compliance and safe working conditions.

2.0 INTRODUCTION

Wastewater reuse has become essential to the well-being and sustainability of communities as water demand increases. Wastewater contains many viruses and the ability to reduce and monitor the concentration of viruses is imperative for human health. From 2000 to 2020, it was estimated that, worldwide, between 34 and 76 million people died from water related diseases [1]. Both untreated and treated wastewater can spread viruses because they are often released into lakes, rivers, the ocean, or other bodies of water. Advanced water treatment processes can reduce this spread because they offer a high level of treatment and are able to reduce the viral load. A membrane bioreactor (MBR) is a type of advanced wastewater treatment system which reduces viral content through biological treatment followed by filtration. An MBR system is an effective technology for use in wastewater treatment because of its small footprint, ease of automation, and production of high-quality effluent [2]. However, the challenge of frequently monitoring viral content of MBR effluent limits viability in wastewater reuse applications.

Mustang Matrix has developed a rapid monitoring system to verify that an MBR removes sufficient viral content. Six technologies were considered to determine an optimum rapid and reliable method. Based on the analysis of available technologies, Mustang Matrix created the Mustang Matrix-Gold Monitoring System (MM-Au), which is composed of ultrafiltration and the application of a fluorescent nucleic acid stain (SYBR Gold). The ultrafiltration system concentrates the viral content in the sample and the SYBR Gold binds to viral RNA and DNA. The resulting complex fluoresces and is detected by a fluorometer.

3.0 PROPOSED SITE BACKGROUND

The proposed pilot location for installing the detection method is the Las Cruces Wastewater Treatment Plant (LCWWTP) in Las Cruces, New Mexico, which discharges into the Rio Grande River. The Rio Grande provides drinking water and water for irrigation to the surrounding area, which has suffered from drought for fifteen years [3]. Monitoring virus detection is a crucial step in treating wastewater for reuse, a viable solution for drought. With the development of the COVID-19 virus, New Mexico created a Wastewater Surveillance System to monitor the

COVID-19 virus in sewage and observe the amount and spread of the virus [4]. In 2021, the Wastewater Surveillance System was able to prevent a COVID-19 outbreak based on wastewater samples from the juvenile center in Las Cruces, New Mexico [5]. The existing concern and spread of viruses through wastewater renders the location a good contender for the detection system. The LCWWTP has a MBR treatment system in place, and Mustang Matrix would seek an agreement with the LCWWTP to further develop the monitoring and detection system.

4.0 TECHNOLOGY BACKGROUND RESEARCH

Six technologies were analyzed during the research phase of this study and a decision matrix was used to determine the optimum strategy.

4.1 ENZYME-LINKED IMMUNOSORBENT ASSAY

Enzyme-linked immunosorbent assay (ELISA) is a method for detecting and measuring soluble substances using a liquid mixture of specific antibodies that bind to the target antigen. This involves four steps: coating, conjugate, incubation, and measuring the enzymatic activity of the bound enzyme [6]. ELISA is able to accumulate the detection of the generated fluorescence as incubation time increases and is sensitive to disease-related proteins and biomarkers. Two issues with this method are that ELISA is time consuming and the enzyme can denature leading to method failure [6].

4.2 PAPER ASSAY

Paper-based assays, or micropads, are a convenient method of identifying the presence of targeted substances. Paper assays can be done using lateral flow immunoassays (LFIAs), which utilize capillary action to transport sample fluid along a strip, consisting of a sample pad, conjugated pad, nitrocellulose membrane, and absorbent pad [7] . As the fluid moves through the conjugate pad, if specific biological molecules are present, they will be captured by the conjugate antibodies and a colored band will form across the strip. However, most paper assays are qualitative, and the quantitative methods usually have low specificity. This method would be challenging to apply to wastewater and lacks the ability to quantify virus presence.

4.3 BIOSENSOR

Biosensors are used to measure the concentration of an analyte present in a solution. In a biosensor, a biological material, such as an enzyme or antibody, would interact with the virus to generate a physical or chemical change that is read by a transducer. The transducer signal is amplified and relayed to a data display [8]. This process can be applied to a sample or as a continuous monitoring system. Positive aspects of biosensors include their relatively inexpensive cost, portability, size, and fast detection time [9]. However, these vary greatly depending on the type of biosensor and the specific virus configuration used, making generalizing the field of biosensors difficult. Since biosensors are configured for detecting a specific virus, their ability to quantify the wide range of potential viruses present in wastewater is limited. Additionally, there is limited literature and knowledge characterizing the use of biosensors for wastewater treatment. This makes biosensors less desirable when compared to more promising alternative technologies.

4.4 REAL-TIME POLYMERASE CHAIN REACTION

Real-time polymerase chain reaction (qPCR) copies specific DNA fragments repeatedly over many cycles [10]. A fluorescent dye generates a fluorescent signal which becomes detectable once a certain amount of copies have been created. While highly accurate and sensitive, qPCR requires time-consuming preliminary steps and high-throughput qPCR thermal cyclers have a large capital and operating cost. These challenges make scaling up qPCR to frequently measure viruses in treated wastewater unrealistic [11].

4.5 CARBON QUANTUM DOTS

Carbon quantum dots are semiconductor nanoparticles made of carbon that are 10 nanometers. They hold extremely accommodating specific photo-luminescent and optoelectronic properties due to their size. When stimulated by UV-light, quantum dots produce a glowing light that can be quantified using a spectrometer. Specific to wastewater treatment, carbon quantum dots are ideal due to high stability, good biocompatibility, high water dispersibility, great photo-stability, and overall low fabrication cost [12]. Although there are many functions to carbon quantum dots, within this project the potential function was to apply the quantum dots for fluorescent imaging

by manipulating them to adhere to viruses. However, carbon quantum dots would be difficult to work with because of the complexity of creating carbon quantum dots that would specifically bind to particular target viruses. The accessibility of equipment required within this process versus the budget made this method infeasible.

4.6 ULTRAFILTRATION AND SYBR GOLD

Ultrafiltration and SYBR Gold are used in conjunction to concentrate and stain viruses in wastewater. The SYBR Gold binds to viral RNA and DNA and fluoresces upon excitation at 495 nm [13]. The measured fluorescence intensity can be correlated to the concentration of viruses in the sample by generating a standard curve. The process can be done rapidly and requires minimal sample concentration, making it conducive to frequent monitoring. The SYBR stain is sensitive to light and should avoid bright areas. Nonviral material in wastewater can distort the fluorescence signal, necessitating that a background measurement be performed with the filtrate of the ultrafiltration system to maintain accurate results [13].

4.7 DECISION MATRIX

A decision matrix was used to compare six different methods to monitor and detect viruses (Figure 1). Methods were evaluated based on ability to evaluate LRV indicator and integrate a rapid, continuous viral monitoring process after the MBR step in the treatment train. The ratings were based on speed of detection, concentration detection accuracy, on-site practicality, capital cost, operating and monitoring cost, safety, and scalability weighted from 1-5 with 5 as the most important factor. Each method was scored from 1-4 with 4 as the highest mark. The analysis showed that the SYBR method has the highest favorability, due to its potential for quantification of detected viruses, low footprint and ability to be automated.

Criteria	Speed of Detection	Concentration Detection / Accuracy	On-Site Practicality	Capital Cost	O&M Cost	Safety	Scalability	Totals	Scale
Weighting	5	5	3	3	3	3	4		1-5
ELISA	3	4	2	1	2	4	2	70	
Biosensors	4	3	2	3	2	4	2	76	
Paper Assays	4	2	3	3	3	4	2	77	
PCR Testing	2	3	1	3	2	4	2	63	
Carbon Dots	1	3	1	3	2	4	2	58	
SYBR	3	4	3	3	3	3	3	83	

Figure 1. Decision Matrix

5.0 DESCRIPTION OF MONITORING METHOD

5.1 SELECTED CHOICE OF SURROGATE

The surrogate measurement chosen for MBR modeling was a bulk measurement of all viruses in the effluent. Measuring all viruses rather than a single microorganism helps to alleviate several challenges associated with MBR monitoring. For instance, attempting to measure a single microorganism in MBR effluent is difficult without extensive sample preparation given the low bacteria and virus concentrations. By considering all viruses, the number of measurable entities increases to a level that is easier to detect in a rapid fashion.

5.2 MEASUREMENT METHOD

Viruses in MBR effluent can be enumerated with the use of a nucleic acid fluorescent gel stain, SYBR Gold. The stain binds to viral DNA and RNA and, upon excitation at a wavelength of 495 nm, fluoresces at an intensity that correlates with viral abundance. SYBR Gold is among the most sensitive fluorescent stains on the market, and the staining process is as simple as adding it to a sample and allowing it to incubate for 10-15 minutes. These properties keep sample preparation to a minimum.

5.3 MBR EFFLUENT SAMPLE PREPARATION

A procedure to concentrate the viruses present in MBR effluent using ultrafiltration was developed to increase detection with SYBR Gold. The extent of concentration required to reach an acceptable level of sensitivity is feasible at a large scale. Wastewater contains some nonviral material that generates fluorescence upon mixing with SYBR Gold, interfering with the signal [13]. To obtain a fluorescence measurement that is closely associated with viral abundance, the fluorescence of this nonviral material is subtracted from the total fluorescence. The material, generally smaller than 10 nm, permeates the filter during the concentration step, allowing for easy collection and fluorescence analysis.

6.0 BENCH SCALE TESTING AND DESIGN

To assess the effectiveness of the proposed method, a bench-scale experiment was designed to test the key phases of the proposed method. Membrane failures of varying degrees were simulated to determine the method's detection ability.

6.1 METHODS AND MATERIALS

6.1.1 Wastewater Sampling and Effluent Spiking

MBR samples were collected from Cayucos Wastewater Treatment Plant in Morro Bay, CA. Effluent samples were taken from the post-membrane stream and influent samples were taken from the activated sludge compartment, directly before the membrane. Therefore, only virus removal due to the membrane is considered in this experiment. To simulate membrane failures, four 50 mL effluent samples were spiked with varying amounts of influent and compared to pure influent and effluent samples (Table 1).

Table 1. Influent Volumes Added to 50 mL Effluent Samples

Name	Influent Volume Added (µL)	Influent:Effluent Ratio
Spike #1	15.81	1:3163
Spike #2	50	1:1000
Spike #3	158	1:316
Spike #4	500	1:100

6.1.2 Sample Filtration and Concentration

The samples were filtered to separate nonviral particles and concentrated to increase the virus concentrations to levels detectable by the fluorescence assay.

Each sample was first prefiltered using 0.2 µm Thermo Fisher Titan3™ Cellulose Acetate Syringe Filters to remove most bacteria and large particles (Figure 1). 12 mL of the prefiltered effluent samples were placed into a 100 K MWCO Millipore Amicon Ultra-15 Centrifugal Filter Unit and centrifuged at 5000 x g for 15 minutes. 200 µL of each virus-free filtrate was collected for subsequent fluorescence analysis. The filtrates should not contain viruses, but contain particles smaller than 10 nm that generate additional fluorescence when mixed with SYBR Gold

[13]. To complete the concentration step, the effluent samples were centrifuged for 3 more rounds, adding 10-12 mL of pre-filtered sample each round. 200 µL of the concentrated retentates were collected (Figure 2). Slight leakage occurred in the centrifugal filters during the second round of experiments, so one less round of centrifugation was performed. Sample losses (up to 3 mL) were recorded and factored into the calculations.

Figure 2. Prefiltration through 0.2 µm filters

Figure 3. Recovery of concentrated sample

200 µL of the pre-filtered influent sample were collected and did not require concentration. 5 mL of the influent sample were centrifuged and 200 µL of filtrate was collected. All 200 µL samples were stored in autoclaved 1.5 mL black microfuge tubes at 4 °C.

6.1.3 Fluorescence Assay

To test the fluorescence assay, a SYBR Gold staining solution was made, consisting of 10,000x stock SYBR Gold diluted 1:4735 in 21x Tris-EDTA buffer. 10 µL of SYBR Gold staining solution was added to each of the 200 µL MBR samples. After incubating at room temperature for 15 minutes, the samples were transferred to a 96-well plate and the fluorescence was measured at excitation and emission wavelengths of 495 nm and 540 nm, respectively, by a Spectramax Spectrofluorometer (Figures 3 and 4). The fluorescence of the filtrates was subtracted from that of their respective retentates to achieve a value more closely related to viral abundance.

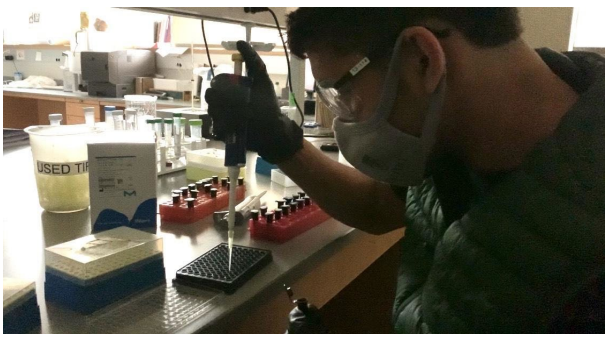


Figure 4. Pipetting stained samples into fluorometer plate

Figure 5. Placing samples into fluorometer

6.1.4 Standard Curve to Relate Fluorescence Intensity to Virus Concentration

To relate fluorescence intensity values to virus concentrations, a standard curve was generated by following the same staining and measurement with samples of known T4 bacteriophage concentrations. The method relies on the assumption that SYBR Gold stains T4 phages in a manner consistent with the diverse set of viruses present in wastewater samples.

6.2 RESULTS AND DISCUSSION

6.2.1 Standard Curve

A standard curve was generated with a concentration range of 1 x 10^7 to 3 x 10^8 viruses/mL (Figure 5).

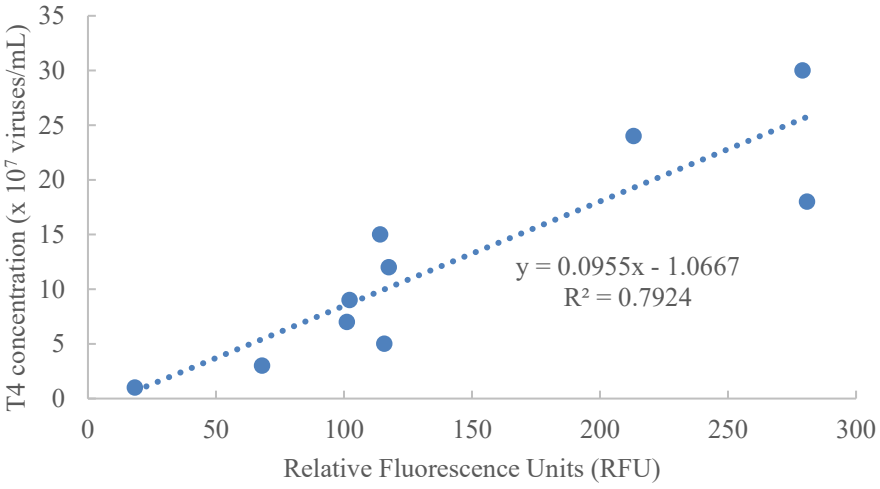


Figure 6. Fluorescence intensities of samples containing known T4 bacteriophage concentrations.

The linear regression equation relates fluorescence intensity values to T4 phage concentrations and was used to calculate the total virus concentrations of the wastewater samples. The limit of detection (LOD) of the fluorescence assay was determined to be 1 x 10^7 viruses/mL.

6.2.2 Wastewater Testing

Testing was performed twice with two sets of wastewater samples obtained one week apart (Tables 2 and 3). The reduction in virus concentration resulting from MBR treatment was quantified by a log removal value (LRV) for each sample. LRV is the logarithm of the influent pathogen concentration divided by the effluent pathogen concentration (1).

Log removal value: $$LRV = log_{10}\left[\frac{Influent\ virus\ concentration}{Effluent\ virus\ concentration}\right] \quad (1)$$

Based on their influent:effluent ratios, the LRVs of spiked samples #1 to #4 should approach but not exceed 2, 2.5, 3, 3.5, respectively. The measured LRV values do not consider virus removal due to pre-membrane biological treatment due to the location of influent sampling.

Table 2. Experiment 1 Results

Sample	Influent:Effluent Ratio	Concentration Factor Achieved With Centrifugal Filters	Fluorescence Intensity (RFU)	Estimated Virus Concentration of Concentrated Samples (x10^7 viruses/mL)	Estimated Virus Concentration of Original Samples (x10^5 viruses/mL)	Estimated Log Removal Value
Influent	—	30x	271	25	83	—
Spike #1	1:100	203x	80.5	6.6	3.3	1.4
Spike #2	1:316	218x	52.6	4.0	1.8	1.7
Spike #3	1:1000	219x	38.2	2.6	1.2	1.9
Spike #4	1:3163	220x	66.3	5.3	2.4	1.5
Effluent	—	219x	11.7	ND*	< 0.46	2.3

* ND: Not detected, below limit of detection (LOD). The value was adjusted to the LOD, 1 x 10^7, for subsequent calculations

Table 3. Experiment 2 Results

Sample	Influent:Effluent Ratio	Concentration Factor Achieved With Centrifugal Filters	Fluorescence Intensity (RFU)	Estimated Virus Concentration of Concentrated Samples ($x10^7$ viruses/mL)	Estimated Virus Concentration of Original Samples ($x10^5$ viruses/mL)	Estimated Log Removal Value
Influent	—	1x	47.5	—	350	—
Spike #1	1:100	145x	134	12	8.1	1.6
Spike #2	1:316	146x	24.2	1.2	0.85	2.6
Spike #3	1:1000	146x	29.5	1.8	1.2	2.5
Spike #4	1:3163	136x	15.9	ND*	< 0.74	2.7
Effluent	—	140x	38.7	2.6	1.9	2.3

* ND: Not detected, below limit of detection (LOD). The value was adjusted to the LOD, 1 x 10^7, for subsequent calculations

The LRV values in experiment 1 are consistently lower than those in experiment 2. This is due to the difference in estimated influent virus concentration between experiments. In experiment 1, the influent sample was concentrated 30x before staining. In experiment 2, it was found that the unconcentrated viral abundance was sufficiently high for the fluorescence assay, and that concentrating the sample reduced the estimated viral abundance by a factor of 0.24. This was likely due to virus loss in the concentration step and/or the viruses being over-concentrated beyond the range of accurate detection of the fluorescence assay. Further testing should assess the magnitude of virus loss due to the concentration step and determine the upper range of the fluorescence assay.

All samples, except for the effluent in experiment 1 and Spike #4 in experiment 2, were above the assay's limit of detection. This shows that there is a strong ability for the method to detect membrane failures. Figure 6 shows the estimated virus concentrations of the pure and spiked effluent samples.

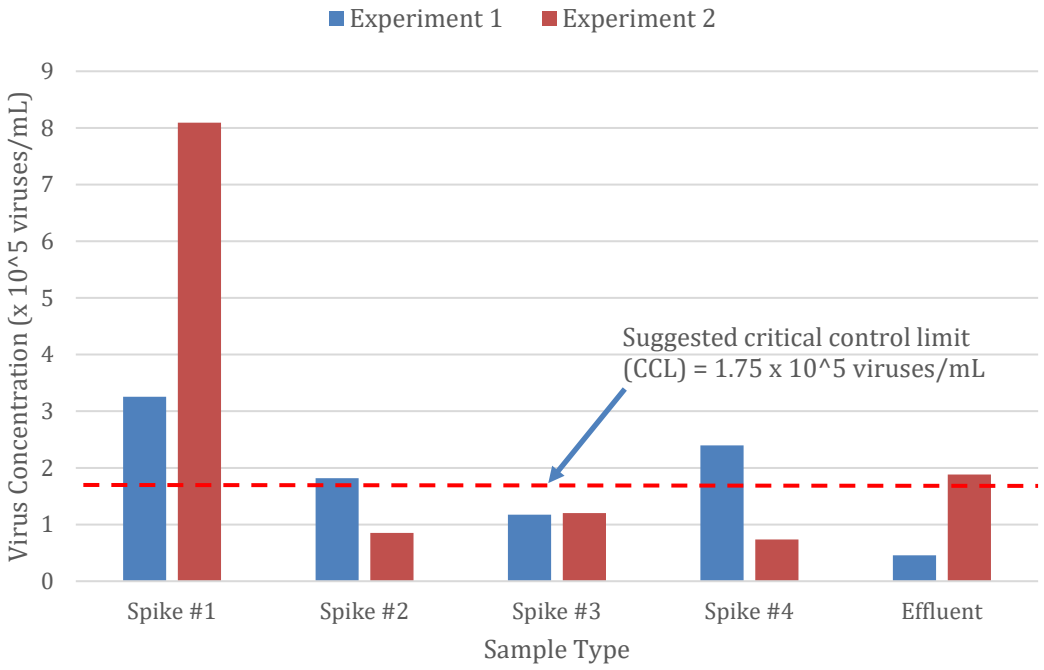


Figure 7. Virus concentration estimates of pure and spiked effluent samples from both experiments

While the method exhibits high sensitivity, there is also unwanted variability indicated by several deviations from expected values. For example, virus estimations of the pure effluent in experiment 2 and Spike #4 from experiment 1 are higher than samples with greater influent:effluent ratios from their respective experiments. Unexpected values were likely due to the limited amount of trials. Due to a shortage of centrifugal filters, only one trial could be performed per sample. Further testing should seek to reduce the method's variability which would bolster its potential for monitoring virus removal.

Based on these results, a critical control limit (CCL) of 1.75 x 10^5 viruses/mL in MBR effluent is proposed. Using the influent virus concentration from experiment 2, this CCL corresponds to an LRV of approximately 2.3, which is considerably higher than the default virus LRV of MBRs, 1.5. Figure 6 shows which samples would be deemed a membrane failure using this CCL. The effluent from experiment 2 would trigger a false alarm, which indicates the need to reduce variability of the method. Mustang Matrix believes that variability can be significantly reduced with further testing and that a lower CCL is attainable.

6.3 BENCH SCALE CONCLUSIONS

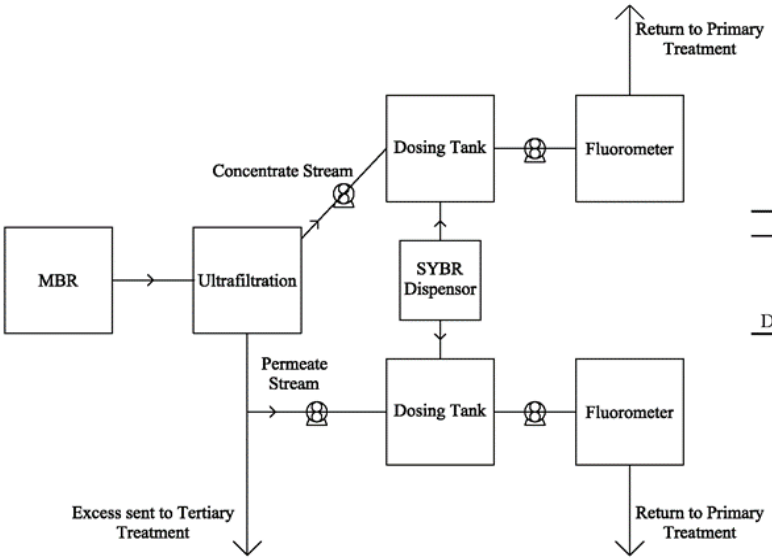
The results of the bench scale experiment identify the strengths of the proposed method and the areas in need of improvement. In under 2 hours in a lab setting, the method was able to detect total virus concentrations as low as 4.6×10^4 viruses/mL in MBR effluent, but considerable measurement variability creates a risk of triggering false alarms. Further study should conduct more trials to see if experimental errors are mitigated. In addition, to obtain a more accurate relationship between fluorescence intensity and viral abundance, the standard curve should be generated using spiked MBR effluent samples whose total virus contents are enumerated by epifluorescence microscopy. Generating a standard curve using T4 bacteriophage samples may not have yielded a fluorescence–viral abundance relationship that accurately applies to MBR samples which contain a diverse array of viruses. Furthermore, measurements obtained by this method should be compared to LRVs of viral pathogens or pathogen indicators. Mustang Matrix attempted to determine the LRV of an MBR by enumerating somatic coliphages with plaque-forming assays, but results deviated by several orders of magnitude from expected values.

While the sensitivity of the method was sufficient for detecting virus concentrations associated with LRVs much higher than the MBR default of 1.5, there are opportunities for improvement. For example, one study found that the staining efficiency of SYBR Gold was significantly increased when incubated at 80 °C as opposed to room temperature [14]. Other techniques such as sonication and optimizing the SYBR Gold staining concentration should be explored as a means of increasing the sensitivity of the fluorescence assay. This would reduce the extent to which MBR effluent samples would need to be concentrated at full-scale. While not yet conclusive, the bench-scale results show that the proposed method has the potential to effectively monitor virus removal of MBRs at full-scale.

7.0 FULL SCALE DESIGN

The full-scale will be constructed as a separate system that will tap effluent from the mainline MBR treatment process at the Las Cruces Wastewater Treatment Plant (LCWWTP). It is

designed to test at an hourly interval but can be adjusted for quicker time intervals if desired (Figure 7).



Flow Stream	Rate (L/day)
MBR Sample Stream	240
Concentrate	2.4
Permeate	2.4
Diversion to Tert. Treatment	235.2

Figure 8. Process flow diagram of monitoring system integrated into an operational membrane bioreactor treatment process.

The detection train proceeds as follows. A 240 liters per day (lpd) sample stream of MBR effluent will be diverted and passed through a single-pass tangential-flow ultrafiltration system that contains a 100k molecular weight cut-off (MWCO) membrane to concentrate viral material. After ultrafiltration, 2.4 lpd of concentrate are produced. The remaining sample leaves the system as treated water, 2.4 lpd of ultrafiltration permeate are kept, and the remaining flow is sent to tertiary treatment. Depending on expected initial virus concentrations, the degree of concentration of the ultrafiltration system can be changed to adjust the sensitivity of the system. Peristaltic pumps inject 25.3 mL/d of buffered SYBR solution to the separate streams of ultrafiltration concentrate and permeate, which sit in small 100 mL batch tanks for mixing. The SYBR concentration is maintained at 1:10,000 during fluorescence measurement. The sample must mix with the SYBR solution for a period of at least 15 minutes for binding, after which it is sent one batch at a time to an in-line fluorometer to measure viral concentration. A SCADA system controls timing of the batch system and collects data, relaying it to an operator. Once measured, the fluid streams are returned to primary treatment. The ultrafiltration permeate's

background fluorescence is subtracted from the concentrate's to determine the viral abundance in the concentrate. If it is above a set limit appropriate to plant conditions, an operator will be notified to investigate the issue.

8.0 ECONOMICS

The largest market for this system is direct potable reuse, which is the sector most likely to require continuous monitoring of viruses in future legislation. Therefore, companies searching for a continuous virus monitoring system will be looking for the lowest cost vendor that can meet regulatory requirements. Mustang Matrix has made efforts to reduce the cost of the MM-Au system, such as installing a supervisory control and data acquisition (SCADA) system to allow operations to run automatically and reduce labor costs. The cost expenditure (CAPEX) includes any equipment, materials, and labor required to construct and install the MM-Au system. It is estimated that installation of the MM-Au unit would take two months to complete with three workers working eight hours a day, five days a week. The low volume of water required for the system will cause minimum disruption to normal operations at a plant and installing a new connection will take only a few hours, during which flow can be redirected to a basin. During the two-month construction period, operators can receive the required training for the system that will be integrated into their workday. The capital cost is $300,000 (Table 4).

The operating expenditure cost (OPEX) considers the required SYBR Gold, electricity, filter disposal, maintenance, replacements, and labor needed to operate MM-AU on an annual basis. The average industrial electricity rate of 9 cents per kilowatt in Las Cruces, NM was used to determine the cost of electricity. A rate of $70 for an operator was used to determine labor costs. Labor required was determined using values from similar lab tasks typically completed at wastewater treatment plants [15]. All estimated costs include sales tax and values have been rounded to the nearest hundred. The operational cost is $190,000 per year (Table 5).

Table 4. Capital Expenditure for the Proposed MM-Au System

Item	Cost per Unit	Quantity	Total Cost
Dosage Tanks[a]	$200	4	$800
Fluorometer[b]	$1,800	4	$7,200
Ultrafiltration System[c]	$68,000	2	$140,000
Peristaltic Pump[d]	$1,400	4	$5,600
Microdosing Pump[e]	$1,700	4	$6,800
Piping[f]	$13	50	$650
SCADA[g]	$11,000	1	$11,000
Construction[h]	$5,900	1	$5,900
Labor	$25,000	3	$75,000
Contingency		20%	$35,000
Engineering[i]		5%	$8,800
Total Capital Cost			**$300,000**

[a]Obtained from Baoshishan
[b]Obtained from Turner Systems
[c]Obtained from EMD Millipore
[d]Obtained from Grainger
[e]Obtained from Quantex
[f]Obtained from JM Eagle
[g]Obtained from IndustLabs
[h]Assumed to be 50% of the capital cost
[i] Assumed to be 5% of capital cost

Table 5. Operating Expenditure for the Proposed MM-Au System

Item	Unit Cost	Unit	Cost
SYBR Gold Stock[a]	$416	mL	$77,000
Buffer Stock[b]	$2.61	mL	$48,000
UF Replacement Filters[c]	$3,500	Cartridge	$42,000
Electricity	$0.09	$/kWh	$850
Waste Disposal	$50	$/ton	$50
Labor	$70	$/hr	$4,100
Maintenance[d]	10%		$13,000
Total O&M Cost			**$190,000**

[a] Obtained from Thermofischer
[b] Obtained from Sigma Aldrich
[c] Obtained from EMD Millipore
[d] Based on 10% of CAPEX

The largest system cost is the purchase of SYBR Gold and stock buffer solution. Individual unit purchase rates were assumed for SYBR Gold and buffer stock prices, therefore bulk order may result in lower operating costs than the given value. The price of operating the MM-Au system will be most sensitive to the price of SYBR Gold as shown in Figure 8. As part of a DPR system, the ability to absorb the costs of running an MM-Au system will be dependent on the size of the plant and the local rate for water, improving as both increase.

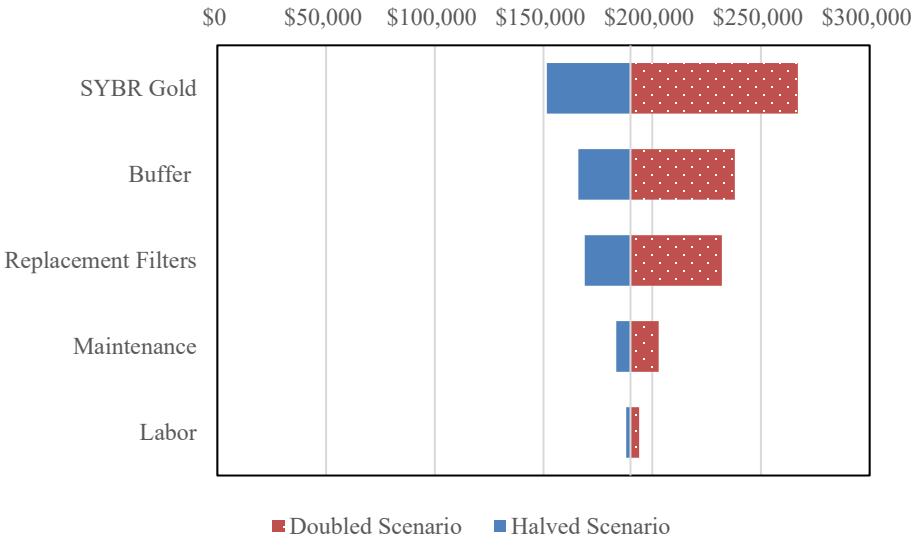


Figure 9. Sensitivity tornado chart for the five largest operating expenses based on scenarios where each specific item is halved or doubled in cost.

9.0 REGULATIONS

Regulations for monitoring enteric viruses in wastewater and drinking water are in the process of being established by the USEPA. Within the last three years, the Center of Disease and Control (CDC) and United States Department of Human and Health Services (USHHS) established the National Wastewater Surveillance System (NWSS), which will test for the detection of diseases in wastewater [16]. The regulations that New Mexico follows are governed by the USEPA and follow the Clean Water Act (CWA) and Safe Drinking Water Act (SDWA).

9.1 DRINKING WATER REGULATIONS

The LCWWTP effluent is discharged into the Rio Grande River which will be used for agricultural and drinking water use downstream. The strictest regulations regarding viral concentrations in drinking water come from the EPA National Primary Drinking Water Standards (NPDWS) which require enteric virus concentrations to be minimized by the treatment technique (TT) of each facility. Enteric viruses are those that are transmitted through the fecal-oral route and can be passed through ingesting contaminated water [17]. At this time, there is no specific treatment required as long as 99.99% of the viruses are removed or inactivated. The public health goal for virus concentrations is 0 (mg/L) but this is unenforceable since public health goals do not consider the limits of detection and treatment technology are often set at levels that technology cannot achieve [18]. The EPA establishes these goals based on the maximum level where no harmful effects would occur to humans and to allow a buffer of safety. The goal for all biological contaminants, including viruses, is set at zero since the presence of even one bacteria or virus can cause adverse health effects in humans [19].

9.2 WASTERWATER REGULATIONS

The effluent created by LCWWTP must meet the industrial effluent guidelines of Title III standards of the CWA. These regulations outline pollutant discharge and the water quality criteria required to maintain surface water quality standards. The LCWWTP must meet 20.6.4. New Mexico Standards for Interstate and Intrastate Surface Waters (NMAC) because the Rio Grande River exceeds New Mexico state limits [20]. The system must also meet the NPDWS set by the SDWA for drinking water use. The LCWWTP must also follow its National Pollutant Discharge Elimination System (NPDES) permit required when a system discharges pollutants into water in the United States, which does not require a minimum virus concentration [21].

9.3 DISPOSAL REGULATIONS

The disposal regulations for SYBR staining solution may vary depending on the local municipality or institution because it contains SYBR Gold and Tris EDTA buffer. Some have approved of it to be disposed of in the wastewater system [22]. Under the U.S. federal

regulations, the SYBR gel stain is classified as a non-hazardous material. The SYBR stain is typically stored with dimethyl sulfoxide (DMSO) as a solvent for storage stability (SDS). The proposed disposal procedure of the SYBR staining solution varies based on regional and national hazardous waste regulations [23]. For the purposes of Mustang Matrix, the solution will be rerouted to primary treatment because it is classified as a non-hazardous material.

10.0 HEALTH AND SAFETY

10.1 BENCH- SCALE SAFETY

Mustang Matrix understood that bench-scale safety was imperative to performing the experiment. The main safety concerns were regarding the chemicals and machinery required for the bench scale.

10.1.1 Chemical Safety

For the required chemicals, the material safety data sheets (MSDS) were reviewed and located in an accessible location. The SDS required the SYBR Gold and wastewater samples to be stored under appropriate temperatures and light exposure [24]. The main chemical concern was the use of SYBR Gold because of the vague disposal requirements and potential health risks. Consequently, further study was performed to better understand the requirements for SYBR Gold. When in the lab, Mustang Matrix determined the proper personal protective equipment (PPE) guidelines to be the following: safety goggles or glasses, close-toed shoes, long pants, lab coats, and 9mm nitrile gloves. PPE was worn when handling any material for the bench scale including wastewater samples. Further studies should be done to confirm that the SYBR Gold has little to no genotoxicity and acute toxicity [25]. The SYBR Gold stain contains dimethyl-sulfoxide (DMSO), which may facilitate the absorption of other chemicals if absorbed into the skin [26]. If ingested, DMSO may cause gastrointestinal irritation with nausea, vomiting, diarrhea, and may cause nervous system effects [26]. The SYBR Gold stain required the buffer solution, Tris-EDTA, which is a non-hazardous substance. The solution containing SYBR Gold and buffer solution will require proper disposal per the SDS (varies by local municipality) and at the competition will be in accordance with the guidelines established by the state of New

Mexico. The wastewater samples will also need to be disposed of following the New Mexico guidelines.

10.1.2 Machinery Safety

To test the bench-scale design, Mustang Matrix was required to use two machines: an autoclave and a centrifuge. Mustang Matrix was trained by a *university*-certified lab technician to learn how to operate the centrifuge and autoclave. To gain access to the machines in a lab, an online lab safety training course was completed by all group members. All testing and machine operation trainings were completed with multiple members present because a minimum of two members of the team are required to be in the lab when working. Before using the lab machines, the operators were required to read the instructions to ensure that they were being used correctly and reduce the risk of accidents.

When operating the autoclave, heat resistant gloves and aprons were worn in addition to PPE to protect from heat related injury. Internal pressure and temperature gauges were checked twice during the entire process to prevent injury of team members operating the autoclave: before activating a new cycle and once before opening the door after the cycle was finished. The autoclave and the objects within were left to cool for 30 minutes prior to extraction.

To operate the centrifuge, the centrifuge pins, spindle, and rotor were inspected for damage prior to each use and safety glasses were worn to protect researchers in case of a machine malfunction. Centrifuge tubes properly sized for the rotor were filled no more than two-thirds full to prevent leakage. The masses of the tubes were within 0.5 g of one another and placed opposite of each other in the rotor to avoid imbalance. Rotor speeds never exceeded 80% of the maximum speed specified by the manufacturer to account for centrifuge age.

10.2 FULL-SCALE SAFETY

The Occupational Safety and Health Act (OSHA) for construction of the WWTP expansion falls under 29 CFR 1910 standard for General Industry [27]. MM-Au involves a wet process, so it is crucial to maintain a clean and dry workspace [28]. To have a safe workspace, it is the project

manager's job to follow the OSHA standards for general industry to avoid injuries during construction or operation of the upgrade. The project manager needs to check that the operators have proper training when operating the machinery required for MM-Au. Mustang Matrix is dedicated to ensuring worker safety and requires that a job hazard analysis (JHA) is completed when applicable in accordance with OSHA regulations for each operator.

Once the upgrade is running, the operators need to follow 29 CFR 1926 for the proper disposal of SYBR, buffer solution, and wastewater samples and will follow the established guidelines by the relevant local municipalities [29]. The chosen waste facility, LCWWTP, discharges effluent into the Rio Grande River that is used for agricultural and drinking water use. Effluent must meet 20.6.4 New Mexico Standards for Interstate and Intrastate Surface Waters. Based on the Resource Conservation and Recovery Act (RCRA), SYBR Gold is not classified as hazardous waste [25]. Although for full scale implementation, Mustang Matrix recommends that secondary containment is used for the containers holding SYBR. If a spill occurs, the SYBR can be cleaned up using an inert absorbent material [24]. The SYBR Gold stain is a combustible liquid and MM-Au recommends the following preventative safety measures: avoid heat, hot surfaces, open flames, ignition sources, strong acids, and strong oxidizing agents [24].

11.0 PUBLIC INVOLEMENT

The public should be informed and involved when the LCWWTP is being renovated. Effectively informing local residents can be accomplished by utilizing an action plan aimed at educating residents of the importance of viral content monitoring and applying new monitoring technology, such as the SYBR Method. The distribution of the action plan will begin with informational pamphlets that will be mailed to local residences in Las Cruces, New Mexico six months prior to the installment of the new viral monitoring system. The pamphlets will notify residents of the innovation and of the dates of informational meetings. An online public forum will also be launched on the Las Cruces City website. This forum will have much the same information as the pamphlets but will be more accessible. Both the pamphlets and the forum will have contact information for citizens to contact the WWTP with questions.

Three months prior to the installation, a public meeting will be coordinated with the city of Las Cruces and be displayed on their webpage. Additionally, information regarding the implementation of the SYBR Gold method will be featured in Las Cruces's regularly held public utility meetings [30]. The meetings will prioritize and address the public's concerns by responding to questions and providing further details about potential impacts from the innovation.

Finally, a satisfaction survey will be conducted three months after the installation to ensure that there are no issues within the community because of the new virus measurement process. Advertisements for this survey will be put up in the local newspaper and on the City's website, encouraging people to contact the city or the WWTP with any water-related concerns.

12.0 CONCLUSION

Mustang Matrix's research, bench-scale experiments, and full-scale analysis suggest that a bulk virus measurement using a fluorescent nucleic acid stain is a promising method for rapidly monitoring virus removal of an MBR. Using all viruses as a surrogate instead of a single microorganism greatly increases the number of quantifiable entities, simplifying the sample preparation process. Using single-pass tangential-flow ultrafiltration, viruses in MBR effluent samples can be sufficiently concentrated for the fluorescence assay in about 30 minutes. After an additional 15 minutes required for the fluorescent stain to mix with the sample, the fluorescence can be measured, which is related to virus concentration by a standard curve. The measurement can therefore be taken at a frequency of under 1 hour, in a fully automated fashion. The bench scale results suggest that a critical control limit of 1.75×10^5 viruses/mL, corresponding to an LRV of about 2.3 (depending on influent virus concentrations), is detectable by our method. With further optimization that decreases measurement variability and increases the sensitivity of the fluorescence assay, this method has the potential to detect membrane failures that correspond to LRVs much higher than the MBR default of 1.5, improving the viability of MBRs for direct-potable reuse applications.

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Acknowledgements

Mustang Matrix would like to thank to the individuals and organizations that helped make this report possible:

List deleted

MEMORANDUM

CENTRAL COAST REGIONAL WATER QUALITY CONTROL BOARD

To: Mustang Matrix Team

From: *Name Deleted*, Water Resource Control Engineer

Date: 3/30/2022

Per your request on March 8th, 2022, I have reviewed the Regulatory Requirements Section of your report and listed recommendations below:

1. I recommend going into more detail on the precise regulations that are referenced here. For example, "who is developing these regulations (State of NM? USEPA?) and what is the scope and intent of this regulation".
2. It looks like the acronyms in the parentheses for certain parts of the section are not defined anywhere.
3. Separate the drinking water and wastewater regulations as they seem to be combined. I recommend a paragraph to discuss the wastewater regulations for treatment, discharge into the surface water, and reuse (NPDES, Title III, etc). I recommend starting off very high level here with a few sentences containing a background of wastewater regulations including the Clean Water Act and NPDES program. Mentioned how sludge disposal is incorporated into NPDES and associated requirements. Then have a second paragraph discussing just the drinking water side of things (NPDWS), which do have specific limit for virus. In both sections discuss where (if at all) the state of NM has promulgated their own requirements that are more stringent than the federal requirements set by USEPA.
4. Define the municipal code referenced in the report and expand upon how it relates to the Clean Water Act. Discuss exactly what does the discharge need to apply to within this regulation. I am not seeing virus-specific requirements. Is this perhaps a narrative requirement related to public health? If so, call it out.
5. Make the sentences pertaining the specific effluent limits their own paragraph and expand. This seems to me to be conveying an important part of this section namely: (1) what do the regulations mean for this facility in terms of effluent limits and monitoring, (e.g. there are no numeric limits for virus) and (2) what does the project team recommend in the short and long term to improve water quality, public health and compliance with federal regulations. (e.g. update monitoring requirements for NM facilities in the short term, long term consider state-level or federal action by working with USEPA to promulgate virus limits.). As aside, and I may be missing something, but I am not seeing the obvious tie in with the ecoli reference. Maybe a little more discussion is needed.
6. It is good to include the solid waste removal of the testing materials. Consider giving this section (and the wastewater and drinking water) sections their own subheadings.

Signature and Name Deleted
X _____

MEMORANDUM

To: Mustang Matrix Team

From: *Name Deleted*, PhD, PE
Title Deleted

Date: 3/27/2022

Subject: Review of the Health and Safety section

Per you request on February 21, 2022, I have reviewed the Health and Safety section of your report on monitoring viruses using the application of a fluorescent DNA gold stain. This memo is a professional review and provides recommendations for implementation in the final report. Overall, the Health and Safety section is unclear, lacking key information, and technical flow. Recommendations to create a complete meaningful section is listed below.

Recommendations:

- The Health and Safety field uses an informal convention on formats and vocabulary, which are designed to help convene information effectively. The *University* Illness and Injury Prevention Plan is a good example that could be emulated, in condensed form, for your report. Available on the *University* website.

- Distinguish clearly between discussion of laboratory and industrial safety. The specifics of the industrial process you propose are yet to be developed, but OSHA industrial safety guides would provide general advice.

I am happy to address any questions. Best wishes on your project.

MEMORANDUM

NAVAL FACILITIES ENGINEERING AND EXPEDITIONARY WARFARE CENTER

To: Mustang Matrix Team

From: *Name Deleted,* MS, FE
Environmental Engineer
Affiliation Deleted

Date: 3/25/2022

This memo critiques and provides recommendations for the Economics section discussed in the draft report authored by the Mustang Matrix team. Overall the Economics' section is missing some key components necessary for a complete economic assessment.

I advise the team address and consider the following:

- Critical market costs include manufacturer titles, however these references were not enough for me to track down what materials were being referenced.
- Most of the costs are itemized in a sufficient manor, however construction costs do not seem to include labor or overheard. In addition, there are no costs attributed to engineering development that I could readily find.
- The COPEX analysis predicts an estimated cost that is far below anything I have ever seen in industry. I would reconsider assumptions that are being made and readdress what costs are included. I would recommend comparing the team's costs to an existing baseline technology.
- In its current, state the economic analysis fails to consider and evaluate how regulatory considerations may impact, cost, schedule, negotiations, redesign and overall feasibility of their proposed process.
- The economic analysis fails to show the relationship between cost and the potential for implementation.
- ROI is missing.
- Project and construction schedules are missing. In addition, I predict the construction completion will take much longer than predicted.

More work is needed to complete a meaningful economic analysis. Place holders or reasonable estimates can be used for data that will be obtained through bench scale testing and additional literature review. Overall, it is important that the team is able to describe where costs are coming from and how they are being calculated using references and illustrating assumptions. Any further questions can be sent to *deleted*

Signature Deleted

Name Deleted
Environmental Engineer
Signed by: WHITE.MADELEINE.M I.1557896968