EVALUATION OF A MARINE WATER CONCENTRATION METHOD FOR RECOVERY OF VIRUSES

By

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ABSTRACT

The goal of this project was to develop simple and rapid methods for virus detection in water to aid US marine mammal program veterinarians in caring for bottlenose dolphins (*Tursiops truncates*) and California sea lions (*Zalophus californianus).* These marine mammals need to be protected from viral diseases while they stay in a netted enclosure in San Diego Bay, California. The purpose of this study was to evaluate the Innovaprep Concentrating Pipette Select (CP Select) for the rapid concentration of viruses in environmental water samples. Bacteriophages MS2, P22, Phi6, and PhiX-174 were seeded in artificial seawater and tested for recovery after using the CP Select. These bacteriophages vary in characteristics and were selected to be surrogates for animal viruses (e.g., *Levivirdae, Podoviridae, Cystoviridae,* and *Microviridae*). Recovery for all bacteriophages was evaluated either using a plaque assay and/or Droplet Digital PCR (ddPCR). After a method was established using phages, three animal viruses: adenovirus (*Adenoviridae*), coronavirus OC43 (*Coronaviridae*), and morbillivirus canine distemper virus (CDV, *Paramyxovirus*) were included in this study using artificial seawater as well as water from the John Shedd Aquarium and San Diego Bay. These animal viruses were quantified using ddPCR. The culture assay average recovery for each phage was: 82.73 ±27.3% for P22 (n=6), 4.84 ±3.8% for MS2 (n=3), 32.77 ±19.3% for PhiX174 (n=6), and 71.48 ±42.5% for Phi6 (n=6). The ddPCR assay average recovery was 7.82 ±8.1% for MS2 (n=3), 34.72 ±19.7% for Phi6 (n=6), 39.31 ±26.6% for Adenovirus (n=9), 19.04 ±11.6% for OC43 (n=9), and 19.84 ±13.6% for CDV (n=9). In the future, this method will be shared with the US marine mammal program so that they may implement surveillance in their netted enclosure. This method could also be implemented in aquaria or at public beaches for water quality testing.

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CP Select	Innovaprep Concentrating Pipette Select
phage	bacteriophage
E. coli	*Escherichia coli*
hrs	hours
sec	second
ms	milliseconds
dsDNA	double-stranded deoxyribonucleic acid
ssRNA	single-stranded ribonucleic acid
PCR	polymerase chain reaction
qPCR	quantitative polymerase chain reaction
ddPCR	droplet digital polymerase chain reaction
µm	micrometer
nm	nanometer
KD	kilodalton
NTU	Nephelometric turbidity units
PFU	plaque forming units
GC/mL	gene copies per milliliter
BSL	biosafety level
ASW	artificial seawater
JSA	John Shedd Aquarium
SDB	San Diego Bay

CHAPTER 1: INTRODUCTION

1.1: Significance of the Study

Marine mammal populations are vulnerable to a diverse set of pathogens, including viruses, bacteria, or other organisms that cause disease (Waltzek et. al, 2012). Viruses are particularly worth investigating because they are the most prolific ocean taxa at an estimated viral load of ~4 x10^{30} virions (Wellehan & Cortes-Hinojosa, 2019; Suttle, 2005). Viruses exist in one or two orders of magnitude greater than bacterial and eukaryotic cells and if put end to end they would span ~10 million light years, wrapping around the universe (Wellehan & Cortes-Hinojosa, 2019; Suttle, 2005). Viral pathogens affecting marine mammals have been historically challenging to study and there is limited knowledge on their occurrence in marine environments, partly due to the absence of efficacious concentrating techniques for marine waters.

The United States of America Office of Naval Research in conjunction with the Department of Defense runs an U.S. Marine Mammal Program involving bottlenose dolphins (*Tursiops truncates*) and California sea lions (*Zalophus californianus*) (NIWC Pacific Public Affairs Office, n.d.). The program began in 1959 and since the U.S. Navy has trained dolphins and sea lions to be teammates against underwater threats (NIWC Pacific Public Affairs Office, n.d.). The animals go on missions that include recovering objects, searching, and marking underwater mines, as well as detecting and apprehending unauthorized swimmers attempting to harm harbor facilities (NIWC Pacific Public Affairs Office, n.d.). These marine mammals need to be protected from viral diseases while they stay in a netted enclosure in San Diego Bay, California. One of the overall goals of the program is to develop a method for surveillance of pathogenic viruses in marine waters. To achieve this goal this study sought to evaluate the Concentrating Pipette Select (CP Select; Innovaprep, Drexel, MO, USA) as an automated

approach for the recovery of mammalian viruses from marine waters. This was accomplished by using bacteriophages as well as select animal viruses in laboratory seeded suspensions to optimize and evaluate the CP Select. The ability for the CP Select to recover infectious viruses via plaque assay was tested as well as loads of nucleic acid with droplet digital PCR (ddPCR).

1.2: Introduction to Viruses

Viruses are infective agents, obligate parasites who need a host to replicate and are classified on the basis of their genomic material and divergent elements of structure and replication (Roossinck 2011). Not all viruses are pathogenic to humans, some infect other hosts including bacteria, insects, plants, and fungi (Roossinck, 2011). Knowledge of these replicating agents is useful for predicting disease risk and epidemiological characteristics (Wellehan & Cortes-Hinojosa, 2019). Contributing factors to the spread of viruses may be the social behavior of the host species, virus-host adaptations, habitat, and geographical barriers. In this case the marine habitat is of interest and there is consideration of spread of viruses to the marine mammals of interest located in an enclosure (Jo et. al., 2018). Animals that exist in enclosures and aquaria may be particularly susceptible to disease as these are sites where contact between humans and marine mammals occurs frequently which can lead to disease transmission, such as transferring localized skin infections from animals to humans or vice versa (Waltzek et. al., 2012). These infections have occurred in many recreational industries (whale-watching tours, oceanaria, and 'swimming with dolphins' programs) as well as during research, and interactions with rehabilitators, trainers, veterinarians, and volunteers (Waltzek et. al., 2012). Some viruses have the ability to host-switch, of which infamous examples include severe acute respiratory syndrome coronavirus (*Coronaviridae*), influenza (*Orthomyxoviridae*), and human immunodeficiency virus (*Retroviridae*) (Waltzek et. al., 2012; Woolhouse & Gowtage-Sequeria,

2005). There are also various drivers for emerging and reemerging viruses in marine mammals, including climate change, poor population health, pathogen evolution, contaminated food or water supplies, and international trade (Woolhouse & Gowtage-Sequeria, 2005).

In addition to viruses that infect animals and humans, there is a group of viruses known as bacteriophage (class *Caudoviricetes*) (Zhu et. al., 2022). These viruses infect bacteria (Zhu et. al., 2022). Bacteriophages are often used as surrogates for animal viruses in evaluating the performance of detection methods and virus inactivation studies. Key bacteriophages that have characteristics similar to pathogenic viruses of interest are often used to evaluate and optimize methods for their recovery and detection. Table 1 shows viruses that have been utilized for methods development and some of the important characteristics that may influence recovery, which include strandedness, nucleic acid make-up, and diameter.

Table 1: Bacteriophages and animal viruses used as surrogates for developing methods for virus recovery and detection.

Virus	American Type Culture Collection (ATCC) Number	Baltimore Classification	Diameter (nm)
Bacteriophages			
MS2	15597-B1	IV ((+) ssRNA)	24-27
P22	19585-B1	I (dsDNA)	52-60
Phi6	N/A*	III (dsRNA)	80-100
PhiX174	13706-B1	II (ssDNA)	23-27
Mammalian Viruses			
Adenovirus 10	VR1504	I (dsDNA)	70-100
OC43 Coronavirus	VR1558	IV ((+) ssRNA)	80-120
CDV Morbillivirus	VR1587	V ((-) ssRNA)	150-300

*Obtained from Dr. Wigginton, University of Michigan.
dsDNA is double-stranded deoxyribonucleic acid.
ssRNA is single-stranded ribonucleic acid.
(+) denotes positive-sense and (-) denotes negative-sense RNA.

1.2.1 Bacteriophage

In this study, bacteriophages (phages) were chosen to perform the initial testing due to their similarity in size, shape, surface properties, morphology, mode of replication, and environmental persistence to mammalian viruses of interest; yet are non-infectious to humans, animals, or plants (Gallandat & Lantagne, 2017). Some phages have historically been used as indicator organisms for water fecal pollution (Dawson et al., 2005) and are useful as surrogates in water treatment process research (Amarasiri et al., 2017; Grabow, 2001; Heffron & Mayer, 2016; Heffron et. al., 2019). Phages are also used as models for highly infectious pathogens because they can be readily and simply quantified using plaque assays, are relatively inexpensive to study, and can be rapidly propagated in vitro, as well as being safer to handle (Gallandat & Lantagne, 2017; McNully et al., 2018; Heffron et. al., 2019). Phages have also been used as tracers for fecal contamination in water because of their low detection limit using current methodologies. They have high host specificity (their ability to selectively invade specific bacterial cells), and key phage are present in areas associated with human fecal waste (Shen et al., 2008). Shortcomings of phages are that they experience natural inactivation as time progresses, can clump leading to settling out of the water, and can absorb onto surrounding particulate matter (Shen et al., 2008). Phages chosen for this study were culturable which allows for assessment of viability quantification through the cell culture techniques.

1.2.2.1 Bacteriophage MS2

The bacteriophage MS2, *Emesvirus zinderi* (Family *Levivirdae*), have ssRNA, a capsid and are non-enveloped and a small structure (~25 nm in diameter)(Bastin et. al., 2020). The phage is F-specific and associated with *Escherichia coli* (*E. coli*) and fecal pollution. MS2 is frequently used as a surrogate to assess pathogenic enteric viruses, for instance noroviruses

(Bastin et. al., 2020). MS2 can be grown with a bacterial culture and is safe to handle, unlike noroviruses and other viruses that affect human and animal health (Bastin et. al., 2020). MS2 can reproduce in human and animal gut microbia where *E. coli* their bacterial host also resides thus is often found in feces and sewage (Dawson et al., 2005; Bastin et. al., 2020).

In a study estimating inactivation rates of enveloped and non-enveloped viruses in unpasteurized wastewater, MS2 persisted for an average of 121 hrs at 25 ℃ (mimicking summer temperatures) and 175 hrs at 10 ℃ (mimicking winter temperatures) (Ye et. al., 2016). Ye et. al., 2016 also pasteurized their wastewater samples by heating to 70 ℃ for 3 hrs. In pasteurized wastewater MS2 was found to persist for an average of 121 hrs at 25 ℃ and 212 hrs at 10 ℃ (Ye et. al., 2016). In this same study, three different concentration methods were compared: polyethylene glycol (PEG) precipitation, ultracentrifugation, and ultrafiltration with centrifugal devices. Low mean recoveries were observed for MS2 with the ultracentrifugation method possibly due to virus inactivation by the high g-force (La Rosa et. al., 2020; Ye et. al., 2016). The optimized ultrafiltration method, first centrifuging the sample then passing it through 0.22 µm PES membrane filter and then concentrated with Centricon centrifugal filters (Milipore), had a 55.6 % recovery for MS2, which was the highest among all protocols assessed (Ye et. al., 2016; La Rosa et. al., 2020). MS2 may reflect the recovery percentage of norovirus, poliovirus, and influenza or other respiratory viruses.

1.2.2.2 Bacteriophage P22

The bacteriophage P22 is in the Genus *Lederbergvirus* in the family *Podoviridae* and infects *Salmonella enterica/ typhimurium* (McNully et al., 2018). P22 is a dsDNA tailed phage with an icosahedral shape that ejects its DNA into its host (McNulty et al., 2018). The size of P22 ranges between 52 to 60 nm with a short tail (Shen et al., 2008). In a study performed by

Shen et al. 2008, bacteriophage P22 was tested in a complex surface water system and was successfully traced traveling down a river. P22 is a candidate for phage therapy for the foodborne pathogen *Salmonella typhimurium* (Gildea et. al., 2022). This is due to P22's ability to withstand the variations of pH throughout the small and large intestines (Gildea et. al., 2022). This ability to endure low pH (4 to 8) and stationary conditions translates to resisting harsh environmental conditions. This larger dsDNA phage was studied due to its unique structure and ability to survive in the environment. P22 may reflect the recovery percentage of adenovirus (due to similarity in size and both are double-stranded DNA viruses) more than the other phages.

1.2.2.3 Bacteriophage Phi6

The bacteriophage Phi6 (Family *Cystoviridae*) is the only known enveloped bacteriophage (Gallandat & Lantagne, 2017). The phage is dsRNA and has an icosahedral capsid, the structure is about ~80-100 nm in size (Gallandat & Lantagne, 2017; Fedorenko, et. al., 2020). A study testing four phages' ability to be inactivated by various disinfection treatments in comparison to Ebola virus, found Phi6 was slightly more resistant (Gallandat & Lantagne, 2017). Phi6 was thus recommended to be a surrogate for Ebola virus chlorine-based disinfectants evaluations over MS2, M13, and PR772 (Gallandat & Lantagne, 2017). In a study testing inactivation rates of enveloped and non-enveloped viruses in unpasteurized wastewater, Phi6 was found to persist for 7 hrs at 25 ℃ and 28 hrs at 10ºC (Ye et. al., 2016). In pasteurized wastewater Phi6 persisted for an average of 53 hrs at 25 ℃ and 146 hrs at 10 ℃ (Ye et. al., 2016). The authors also compared various virus's recovery using ultrafiltration, first centrifuging the sample then passing it through 0.22 µm PES membrane filter and then concentrated with Centricon centrifugal filters (Millipore), method to have a 18.2% recovery for Phi6 (Ye et. al., 2016). In a study by Adcock et. al., 2009, Phi6 was also proposed as a potential surrogate for

H5N1 avian influenza virus when testing persistence and inactivation in fresh water. Phi6 was chosen because of its similarities to AIV such as size range, external lipid-protein envelope, and segmented genome (Adcock et al., 2009). In a news article about Peru, veterinarians confirmed H5N1 AIV in sea lions and a dolphin (Schnirring, 2023).

1.2.2.4 Bacteriophage PhiX174

The bacteriophage PhiX174 (Family *Microviridae*) has an icosahedral, non-tailed virus with a circular ssDNA, and 23 to 27 nm in diameter (Michel et. al., 2010; Heffron et. al., 2019). Since PhiX174 is a coliphage, it is often used as an indicator of fecal contamination in aquatic environments, per the International Organization for Standardization, ISO 10705-2 (Michel, et. al., 2010). PhiX174 has restrictions to which receptors it can attach on *E. coli* and related bacteria such as *P. aeruginosa* (Michel et. al., 2010). Bacteriophage PhiX174 was found to be comparable to mammalian viruses when testing removal from water matrices with an inactivator in Heffron et. al., 2019. This was concluded to be either due to electrostatic repulsion with ferrous iron (inactivator) or because it has a thicker capsid than other phages MS2, P22, and another coliphage known as fr (Heffron et. al., 2019). PhiX174, similar to MS2, can be a surrogate for enteric viruses.

1.2.2 Mammalian Viruses

1.2.2.1 Human Adenovirus Type 10

Adenoviruses belong to the *Adenoviridae* family, which is further divided into *Mastadenovirus* and *Aviadenovirus* genera (Mena & Gerba, 2008; van Regenmortel et. al., 2000). Adenoviruses are comprised of linear dsDNA, are non-enveloped, have an icosahedral virion enclosed by a capsid, and are 70 to 100 nm in diameter, see Table S1 in the appendix (Enriquez 2002; Mena & Gerba, 2008; Heffron et. al., 2019). Human adenovirus serotype 10 is within the *human mastadenovirus* subgroup D and can infect human as well as murine and porcine hosts (Mena & Gerba, 2008). Adenovirus infections are often subclinical, with the virus is still shed in the feces (Mena & Gerba, 2008; Foy 1997). Adenoviruses remain infectious in water longer than other enteroviruses because they are also thermally stable (Mena & Gerba, 2008; Thurston-Enriquez et al., 2003b). They are capable of surviving for months at low temperature waters because the genome is comprised of double-stranded DNA which may provide more stability as well as host cell repair enzymes to repair damaged DNA (Mena & Gerba, 2008; Thurston-Enriquez et al. 2003b). Adenoviruses appear to be more resistant to inactivation by ultraviolet (UV) light compared to other known waterborne pathogens (Mena & Gerba, 2008; Thurston-Enriquez et al. 2003b). Notably in the context of marine mammals, a case study of four captive bottlenose dolphins with self-limiting gastroenteritis were identified (Rubio-Guerri et al., 2015). A novel adenovirus that was seropositive for canine adenoviruses 1 and 2 (Rubio-Guerri et. al., 2015). The partial sequences of adenovirus were associated with fatal adenoviral hepatitis in sea lions (Rubio-Guerri et. al., 2015). The adenoviral infection was detected using PCR in fecal material (Rubio-Guerri et. al., 2015).

1.2.2.2 Human Coronavirus (HcoV-OC43)

Human Coronavirus HcoV-OC43 (OC43;Family *Coronaviridae*, Order *Nidovirales;* Dallner et. al., 2022) is an enveloped, positive ssRNA virus with a crown-like appearance, and 80 to 120 nm in diameter (Liu et. al., 2021; Dallner et. al., 2022). OC43 is a betacoronavirus that can cause mild common cold-like infections, the pandemic causing SARS-CoV-2 is also a betacoronavirus (Dallner et. al., 2022). Thus, OC43 is used as a surrogate for SARS-CoV-2, due to the similar physiochemical properties, receptor binding proteins, and genetic sequences (Dallner et. al., 2022). OC43 and SARS-CoV-2 both target the human respiratory system, transmit via respiratory droplets, and are comparatively resistant to disinfectants (Schirtzinger et. al., 2021). OC43 grows in human colon cancer (HRT-18) cells (Schirtzinger et. al., 2021).

Yang et. al., 2022 performed computational analysis of marine mammal's host cell ACE2 binding receptor, which allows the SARS-CoV-2 virus to enter the cell and infect it. Bottlenose dolphins and other cetacean species were predicted to be highly susceptible to the spike protein on SARS-CoV-2, according to Yang et. al., 2022. This implicates that since the marine mammals in the US Navy are predicted to be highly susceptible to coronavirus infections the need for an efficient water testing method for this family is vital.

1.2.2.3 Morbillivirus (Canine Distemper Virus)

Morbillivirus canine distemper virus (CDV) is in the Family *Paramyxovirus* and Genus *Morbillivirus* (Jo et. al., 2018; Duque-Valencia et. al., 2019). These viruses comprise of single-stranded, non-segmented, negative-sense, enveloped, RNA-genome viruses, and 150 to 300 nm in diameter, see Table S1 in the appendix (Jo et. al., 2018; Duque-Valencia et. al., 2019). This family causes neurologic, multisystemic, and respiratory diseases in mammals. Dolphin morbillivirus has been known to cause outbreaks in the bottlenose dolphin population since the 1980s (Sierra et. al., 2014). Examples of outbreaks include in 2013 along the Atlantic coast of

the United States and 2017 in southeast Brazil (Jo et. al., 2018). Outbreaks similar to these killed thousands of dolphins in the Mediterranean Sea (1990-92), northwestern Atlantic coast (1987-88), and Gulf of Mexico (1993-94; Jo et. al., 2018). Phocine distemper virus (Family *Paramyxovirus* and Genus *Morbillivirus*) has been recorded to cause infections in sea lions among other marine mammals along the North Pacific Ocean surveyed between 2001-2016 (VanWormer et. al., 2019). While difficult to detect, there are cases of animals being infected in the wild. The marine mammals in the US navy go out to perform Their tasks and then return to their bay. If an animal contracts this virus and brings it back to the bay the likelihood of them spreading it to other mammals nearby is increased. Canine distemper is a surrogate for the dolphin and cetacean morbilliviruses and measles viruses; it was chosen for this study because it is a model virus and safe for humans to handle in a BSL-2 lab (Diaz-Delgado et. al., 2019; Rendon-Marin et. al., 2019).

1.3: Methods of Detection

There are various methods of detection for viruses, but the ones focused on in this study are culture-based detection and polymerase chain reaction (PCR) based detection.

1.3.1 Culture-Based Detection

The ability of bacteriophages to infect specific bacteria down to the strain level makes the method very advantageous for seeded studies. Phages are also efficient at attaching to their bacterial host, even under harsh conditions (Meile et. al., 2020) and thus the use of plaque assays for viruses in various types of waters is fairly robust. All bacteriophages used in this study produce plaques in 24 hrs.

A disadvantage to culture-based detection is that it can take days to obtain results for animal viruses. It is a lengthy process particularly for animal viruses that requires relatively

precise timing for the assay to be successful. The tissues that animal viruses focused on in this study take roughly 4 to 8 days to propagate (Ammerman et. al., 2008).

1.3.2 Droplet Digital PCR

Polymerase chain reaction (PCR) was invented in 1983 and has advanced substantially (Zhu et al., 2020). The use of a heat stable polymerase from the bacteria *Thermophilus aquaticus* (Taq polymerase) which remains active at 94 ℃ allowed for the eventual development of PCR (Zhu et al., 2020). Advancements to the method included the addition of a denaturing step to split the dsDNA into ssDNA, thereby allowing for replication and reverse transcription (RT) to test for RNA viruses. Currently, there are three basic techniques: end-point PCR, quantitative PCR (qPCR) and digital PCR (dPCR;Zhu et al., 2020). Utilized in this study was the digital droplet PCR (ddPCR) (Bio-Rad, Hercules, California, USA) system passes the sample through a microfluidic chip forming segmented flow and splitting the sample into roughly 20,000 droplets with oil forming an emulsion (Zhu et al., 2020). The sample is then placed in a thermocycler and with the use of specific primers the targeted gene is replicated within the droplets, this separates the system from qPCR. The reader then quantifies using probe-based fluorescence using a most-probable-number (Zhu et al., 2020).

The qPCR method was the gold standard technique for quantifying DNA or RNA in both clinical and environmental samples (Taylor et. al., 2017). Taylor et. al., (2017) state that the fundamental cause of poor-quality data is associated with inhibition of the Taq polymerase and primer annealing due to inadequate dilution of residual protein and contaminates. Samples containing low abundance of the targets are often the least likely to pass quality assurance checks (Taylor et. al., 2017). They found ddPCR produced more precise results with samples that had low levels of nucleic acids or high levels of contaminates (Taylor et. al., 2017). In another study

comparing reverse transcription (RT for RNA Targets) RT-qPCR to RT-ddPCR, Flood et. al., 2021 found that RT-ddPCR had higher sensitivity and precision when testing for SARS-CoV-2 genetic markers. RT-qPCR theoretical detection limit is the lowest standard curve concentration while for ddPCR it is three positive droplets (Flood et. al., 2021).

The nucleic acid-based methods have the advantage of high specificity and potential automation, reproducibility, and can also detect non-culturable organisms (Meile et. al., 2020). Opposingly, specificity of results can be impacted by general targets common amongst varying species, generating false-positive results. In addition, PCR methods cannot distinguish between live and dead cells as nucleic acid-based methods amplify the genetic information from both viable and dead cells (Meile et. al., 2020).

1.4: Virus Concentration Methods for Water Samples

Early in the history of viruses it was known that they could be transmitted via water, roughly 80 years ago sewage and water testing began with poliovirus detection (Metcalf et. al., 1995). As science advanced, methods for virus recovery and detection were of great interest. There have been a number of concentrating methods developed to recover enteric viruses (Cashdollar & Wymer, 2013). Concentration of water samples is needed because the quantity of pathogenic viruses found in environmental samples are low and thus, they cannot be detected without concentration, which can be challenging. Three types of filters exist, centrifugation, adsorption/ elution, and ultrafilters.

Centrifugation uses centrifugal force applied to a sample to separate the components according to particle size, density, medium viscosity, and rotor speed (Taulbee and Maroto-Valer, 2000). This method of separation allows two cell types to be distinguished, the denser material becomes the pellet, and the remaining material remains the supernatant. In the literature,

there is data on centrifugal concentration for many viruses in many studies (Taulbee and Maroto-Valer, 2000; Boujnouni et. al., 2022). Disadvantages include a limited sample capacity, as the rotor in the centrifuge can only hold a certain type of bottle. The high speed of the centrifuge could lead to inactivation, which is a concern for plaque assay. The upfront cost of a centrifuge is also high (Taulbee and Maroto-Valer, 2000; Boujnouni et. al., 2022).

Adsorption and elution method uses electronegative or electropositive filters. Water is passed through these filters and the viruses are captured on the surface; an elution of these filters allows for concentration. Historically, beef extract has been used to elute viruses from the filters (Cashdollar & Wymer, 2013). This method can filter large amounts of sample volume and does not clog unless the sample is extremely turbid (Taulbee and Maroto-Valer, 2000). In the literature, there is data on adsorption/ elution concentration for many viruses in many studies (Taulbee and Maroto-Valer, 2000; Boujnouni et. al., 2022). This method tends to be more economical (Boujnouni et. al., 2022). Adsorption/ elution requires a certain level of expertise to perform the preconditioning of the filter or water samples (Boujnouni et. al., 2022).

The CP Select is an ultrafilter that uses size exclusion as the mechanism of concentration. When water samples pass through capillaries, hollow fibers, or flat sheets using tangential flow any nano particles are concentrated (Cashdollar & Wymer, 2013). An advantage of CP Select hollow-fiber ultrafiltration systems is that the filters cost less than other field sampling systems (Cashdollar & Wymer, 2013). The CP Select is also an automated and rapid compact device that requires little training. This device can concentrate multiple pathogens and does not require preconditioning of the filter or sample (Boujnouni et. al., 2022). Disadvantages include the pipette's tendency to clog and slow rate of filtration (Cashdollar & Wymer, 2013). This also means that smaller volumes of sample are concentrated, which is not ideal for environmental

samples. Since ultrafiltration is a newer technology, specifically the CP Select, there is limited knowledge on its effectiveness (Boujnouni et. al., 2022). Hollow-fiber ultrafiltration recoveries were generally greater than either electropositive or electronegative filters, but the results were not statistically significant (Cashdollar & Wymer, 2013).

Table 2: Comparison of some concentration methods.

Method Type	Advantages	Disadvantages
Centrifugation	• Data in literature with many viruses • Separation of 2 cell types (pellet vs supernatant	• Limited sample capacity • High speeds can lead to virus inactivation • Expensive upfront cost
Adsorption/ Elution	• Filters large volumes • Data in literature with many viruses • Economical	• Can clog with extremely turbid samples • Requires precondition of filter or water sample
Ultrafiltration	• Compact device • Multi-pathogen concentration • Economical • No preconditioning of water samples	• Tendency to clog • Slow filtration rate • Filters small volumes • Limited data available on effectiveness

Information from (Taulbee & Maroto-Valer, 2000; Cashdollar & Wymer, 2013; Boujnouni et. al., 2022).

There are 9 key studies that have evaluated the CP Select for concentrating viruses in environmental samples (Table 3). All the studies used wastewater or contaminated river water as the matrix for the seeded studies and concentrated between 40 and 500 mL. However, virus recovery in marine water has never been assessed.

Falman et. al., (2019), compared concentration systems such as beef extract-Celite, ViroCap flat disc filter, CP Select, PEG/NaCl precipitation, and skimmed-milk flocculation with poliovirus type 1 (PV1). The CP Select, along with the other methods, were tested as secondary concentration steps. They concentrated 10 L of the wastewater using ViroCap Filter and eluted 100 mL for the processing, of which a concentration range of 10^3 to 10^4 PFU PV1 were seeded

and mixed. This 100 mL was then concentrated using the CP Select. The manufacturer recommended settings were used (valve open, 35; valve closed, 100; pulse count, 2; flow buffer, 12; and extraction delay, 6). The CP Select was found to have the lowest recovery at 0.32 % (n=4), compared to skimmed-milk flocculation at 116 % (n=6), PEG/NaCl precipitation at 60.6 % (n=5), beef extract-Celite at 41.8 % (n=10), and ViroCap disc filter at 17.2 % (n=3). Falman et. al., 2019 found that the CP Select pipette tips tended to foul after concentrating 50 mL of their total 100 mL sample. Combined with the potential viral loss due to the increased surface area from using two pipette tips for one sample and the cost of the machine, the CP Select was not further evaluated in their study (Falman et. al., 2019).

McMinn et. al., (2021) tested coronavirus OC43 in wastewater and used RT-qPCR. They took 2 L wastewater samples into a dead-end hallow fiber ultrafilter (D-HFUF) as a primary concentration method, then the CP select was used as the secondary concentration method. As shown in Table 3, the recovery of the complete method for OC43 was 22 ±4%. McMinn et. al., 2021 compared differing elution approaches, hand-driven syringe elution against the CP Select automated elution system. They found a hand-driven syringe elution (48 ±2%) to be the best method (p=0.0299) over the CP Select elution (31 ±3%) (McMinn et. al., 2021).

Gonzalez et. al., (2020) used raw wastewater samples in their experiments, first centrifuging 125 mL to remove suspended solids (10,000 g force for 10 mins) and then using 100 mL of supernatant in their experiments. The recoveries for the CP Select and centrifugation method for bovine coronavirus (BCoV) was 5.5 ±2.1% and bovine respiratory syncytial virus (BRSV) was 7.6 ±3.0% (Gonzalez et. al., 2020). On later samples in the study, they used electronegative filtration for the raw wastewater samples and found the recovery for BCoV to be 4.8 ±2.8% and BRSV had a recovery of 6.6 ±3.8% (Gonzalez et. al., 2020). These recoveries

were lower than the samples they extracted without concentration (BCoV 59 ±14% and BRSV 75 ±13%) but they determined that concentration was needed to detect low viral concentrations from the environment (Gonzalez et. al., 2020).

Fores et. al., (2021) compared the CP Select using ultrafilter tips to the Centricon Plus-70 centrifugal ultrafiltration (CEUF) device. Two hundred mL of raw wastewater influent was spiked with MS2 and Murine Herpesvirus (MHV) and then centrifuged to remove suspended solids (Forés et. al., 2021). The same was divided into two aliquots of 100 mL and then underwent the differing concentration methods, RT-qPCR was used for quantification (Forés et. al., 2021). No statistical differences were observed between the two concentration methods for MS2 and naturally occurring viruses (Forés et. al., 2021). The mean recovery in the CP Select for MS2 was 27.72 ±24.46% and for MHV was 7.51 ±6.14% (Forés et. al., 2021). This study also added Tween-20 to some of the wastewater samples (Forés et. al., 2021). No statistically significant difference was observed between samples with or without Tween-20 (Forés et. al., 2021).

Juel et. al., (2021) compared the CP Select against the established electronegative membrane filtration (EMF) method and then preformed recovery assays for BCoV via RT-qPCR. They chose five wastewater samples that were turbid and five samples that appeared to be clear. They also filtered varying volumes (40, 60, 100 mL) and recorded the time each system took to concentrate. The average time for the CP Select was 9.25 minutes while EMF took over 30 minutes for samples over 60 mL (Juel et. al., 2021). There were also 25 % of samples that tested negative for SARS-CoV-2 in the wastewater for EMF method but positive for the CP Select (Juel et. al., 2021). They found that for the CP Select 60 mL was the ideal sample size, with a recovery of 36.81 % BCoV (Juel et. al., 2021).

Lu et. al., (2022) centrifuged sewer shed wastewater samples and then passed the supernatant through a 0.45 μm sterile filter unit. The samples were then filtered using the 0.05 μm PS hollow fiber concentrating pipette tip (Lu et. al., 2022). ddPCR was used for quantification of the SARS-CoV-2 genes and OC43 (Lu et. al., 2022). OC43 was spiked into the sample as a process control; the average recovery was 15.5 ±7.6% (Lu et. al., 2022).

Ahmed et. al., (2021) seeded gamma-irradiated SARS-CoV-2 into 50 mL of wastewater. The samples were then spun in a centrifuge (4000 g force for 30 mins) and the supernatant was passed through the CP Select and then quantified using RT-qPCR (Ahmed et. al., 2021). An unirradiated 0.05 μm PS hollow fibre filter CP tip was used along with the manufacturer's wastewater settings on the CP Select, the tip was eluted twice (Ahmed et. al., 2021). The two wastewater treatment plants (WWTP) tested had mean recovery efficiencies of 55.5 ± 11.8 % and 65 ± 23.6% using the CP Select method (Ahmed et. al., 2021). This produced higher recoveries than the adsorption-extraction (AE) supplemented with $MgCl_2$ method also tested (Ahmed et. al., 2021). The recovery efficiency of the CP Select method was not different among the 10 WWTPs tested, which was also advantageous to the AE method (Ahmed et. al., 2021).

Rusinol et. al., (2020) compared three different concentration methods (e.g., skimmed milk flocculation (SMF), Centrifugal ultrafiltration (CeUF), and the CP Select) for detecting MS2. The samples were centrifuged prior to concentration and there were varying volumes of wastewater for each concentration method (Rusinol et. al., 2020). qPCR was used to quantify the results. MS2 was utilized as a process control and surrogate for SARS-CoV-2 (Rusinol et. al., 2020). The CP Select had an average of 51 % recovery for MS2 while the SMF had 29 % and the CeUF had 16.5 % recoveries (Rusinol et. al., 2020).

Lee et. al., (2021) was trying to find hepatitis A (*Picornaviridae*) from sewage pipes that transport untreated sewage into river water surrounding a clam farm. They used norovirus (*Caliciviridae*) as their control to test for recovery efficiency (Lee et. al., 2021). The samples were collected at 15 separate locations, three times in November (Lee et. al., 2021). They first centrifuged the samples (6,000 rpms for 10 mins), then the supernatant was passed through a vacuum filter storage bottle system and then the supernatant was processed through the CP Select (Lee et. al., 2021). The samples were quantified using real-time RT-PCR, the average recovery of seeded norovirus was 4.5 % (Lee et. al., 2021). They state that the virus recovery rate was expected to be low due to the two suspended matter removal steps (centrifugation and vacuum filter) prior to CP Select concentration (Lee et. al., 2021).

Table 3: Review of viruses evaluated through the CP Select.

Pathogen Evaluated	Sample Type	Sample Concentrated (mL)	Number of Samples (n=x)	Average Recovery %	Reference
Poliovirus Type 1 (PV1)	Raw Wastewater Influent	100	4	0.32	Falman et. al., 2019
Coronavirus (OC43)	Primary Treated Wastewater	100	4	22 ±4	McMinn et. al., 2021
Bovine coronavirus (BCoV)	Raw Wastewater Influent	100	198	5.5 ± 2.1	Gonzalez et. al., 2020
Bovine Respiratory Syncytial Virus (BRSV)				7.6 ± 3.0	
MS2	Raw Wastewater Influent	100	22	27.72 ± 24.46	Forés et. al., 2021
Murine Herpesvirus (MHV)			10	7.51 ± 6.14	
BCoV	Wastewater from Sewer Shed	40 to 100	40	36.81	Juel et. al., 2021
OC43	Wastewater from Sewer Shed	100	101	15.5 ± 7.6	Lu et. al., 2022
Gamma-irradiated SARS-CoV-2	Raw Wastewater Influent	50	4	55.5 ± 11.8 and 65 ± 23.6	Ahmed et. al., 2021
MS2	Wastewater	80	4	51	Rusinol et. al., 2020
Norovirus	Untreated sewage that flows into river water surrounding farms	500	45	4.5	Lee et. al., 2021

1.5: Research Objectives

Bottlenose dolphins and California sea lions play a valuable role in the US Navy for national defense. Development of a non-invasive, early detection surveillance method for viral pathogens in marine environments to reduce health risks for marine mammals was a particular research gap. The Innovaprep Concentrating Pipette Select (CP Select) ultrafiltration system as a simple bench top system was evaluated in this study as the Navy needs a simple method for concentrating viruses that can be used with minimal training. The objective of this study was to evaluate the efficiency of the CP Select for viruses in marine waters and assess the time needed to concentrate the samples. The sub-goal was to compare recovery of phages (MS2, P22, Phi6, & PhiX174) and animal viruses (Adenovirus, OC43, & CDV) using both culture and PCR methods. Due to ddPCR being the approach used, this study also focused on developing the ddPCR protocols for MS2 and adenovirus detection.

CHAPTER 2: MATERIALS AND METHODS

2.1: Environmental Samples

The types of natural water samples included in this study were marine waters from the San Diego Bay Dolphin and Sea Lion enclosure and the Shedd Aquarium in Chicago, Illinois. The samples from San Diego were collected by Office of Naval Research Personnel Dayna Beagle. The samples from the Shedd Aquarium were collected by Dr. Frank Oliaro. One liter grab samples were shipped overnight on ice to the MSU laboratory and stored at 4 ℃ for further processing. Samples were either processed directly through the concentrating pipette (CP Select) (Innovaprep, Drexel, MO, USA) or seeded with key phages or animal viruses (see sections 2.3 and 2.4). After processing with the CP Select some of the concentrates were also sent to Dr. Tiong Aw Gim at Tulane University for further MinION (Oxford nanopore technologies, Oxford, UK) sequencing analysis.

2.2: Virus Preparation and Cell Culture

Stocks of bacteriophage MS2 (ATCC#15597-B1), a model for non-enveloped positive-sense RNA viruses, P22 (Dr. Felix D. Herelle, University of Laval) a model for non-enveloped DNA phage, Phi6 (Dr. Krista Wigginton, University of Michigan) a model for enveloped double stranded RNA viruses, and PhiX-174 (ATCC#13706-B1) a model for non-enveloped negative-sense single stranded DNA viruses, were propagated using the following protocols described below.

2.2.1: MS2

To propagate bacteriophage MS2 (ATCC#15597-B1), the host *E.coli* Famp (ATCC #700891) was grown in Tryptic Soy Broth (TSB) medium including 10 % volume ampicillin and streptomycin antibiotics at 37 ℃ for 4 hrs in stationary culture. Stationary culture is when

equilibrium was reached amongst dividing cells and dying cells, so the bacterial cell count remained constant. One milliliter (mL) of ATCC stock MS2 was added to the host and incubated under the same conditions for 16 to 18 hrs. Post incubation period, the bacterial host was lysed as the phage grew causing a clearing of the cell suspension. The debris was removed from the suspension via filtration using 0.22 µm polyethersulfone (PES) membrane (MilliporeSigma, St. Louis, MO, USA) and the filtrate containing the phage was collected. The MS2 stocks were stored at 4 ℃ and tittered using an agar overlay method (see below).

For the titer process, 0.5 mL of Famp *E.coli* host culture was added to a melted overlay tube containing 2.5 mL Tryptic Soy Agar (soft agar at 1.5 %; TSA) kept in a liquid state in a water bath at 50 ℃. Two mL of virus suspension were mixed with the host in the melted agar overlay and poured onto a plate containing solid TSA. Plates were allowed to solidify and were then incubated at 37 ℃ for 16 to 24 hrs after which the plaques were counted. Virus titers of approximately 10^6 plaque forming units (PFU) per mL were routinely obtained and were used as the seed and measured as the "Before Concentration" samples.

2.2.2: P22

The protocol to propagate bacteriophage P22 used the host *Salmonella typhimurium* LT2 (#1023 Dr. Felix D. Herelle, University of Laval). The method was similar to section 2.2.1 MS2 except no antibiotics were added. For the overlay process, 0.5 mL of LT2 host grown to stationary culture was added to a melted overlay tube containing 2.5 mL TSA. The protocol thereafter followed that of 2.2.1 MS2. Titers of 10^6 to 10^8 PFU/mL were achieved for P22.

2.2.3: Phi6

To propagate bacteriophage Phi6, *Pseudomonas syringae* bacterial host (Dr. Wigginton, University of Michigan) was grown in King's B medium at room temperature (20 ℃) for 6 hrs in stationary culture. The rest of the protocol followed the section as described in 2.2.2.

For the overlay process, 0.5 mL of *P. syringae* stationary culture host was added to a melted overlay tube containing 2.5 mL King's B Agar. This soft agar was kept in a liquid state in a water bath at 50 ℃. Two mL of the host was added to the melted agar overlay, then 0.5 mL of virus suspension was added and mixed. This was then poured onto a plate containing a solid King's B agar. Plates were allowed to solidify and were then incubated at room temperature (20 ℃) for 16 to 24 hrs. Plaques were then counted, virus titers of approximately 10^6 PFU per mL were routinely obtained and were used as the seed measured as the "Before Concentration" samples.

2.2.4: PhiX174

To propagate bacteriophage PhiX174 (ATCC#13706-B), the host *E.coli* CN-13 (ATCC#700609) was grown in TSB medium with 10 % volume of nalidixic acid antibiotic at 37 ℃ for 4 hrs until stationary culture was reached. The protocol was then the same as described in section 2.2.1. Titers of 10^6 PFU/mL were achieved for PhiX174.

2.2.5: Adenovirus Type 10

The human adenovirus 10 (ATCC#VR1504) was propagated by Dr. George Lukasik's laboratory (BCS Laboratories, Gainesville, FL). Briefly, virus suspension was inoculated onto monolayer of recently passaged A549 (ATCC CCL-185) in cell culture flasks. The cells were incubated with Dulbecco's Modified Eagle Medium (DMEM) containing 2 % Fetal Bovine Serum at 35.5 ℃. Following the development of 80 % cytopathic effect, the flasks containing cells were rapidly frozen at -80 ℃ . The flasks were then rapidly thawed in a warm water bath and then frozen again at -80 ℃. The flasks were rapidly thawed again. The liquid suspension was centrifuged at 10,000 x g for 20 minutes, the supernatant was filtered through 0.22 µm PES filter and aliquoted. The virus aliquots were immediately frozen and stored at -80 ℃. Titer quantification was performed via plaque assay at 10^6 PFU/mL, which was closer to 10^8 GC/mL.

2.2.6: OC43 Coronavirus HcoV

The human coronavirus OC43 (ATCC#VR1558) was propagated by Dr. George Lukasik's laboratory (BCS Laboratories, Gainesville, FL). Briefly, virus suspension was inoculated onto monolayer of recently passaged HRT-18G (ATCC CCL-244) in cell culture flasks. The protocol was then the same as described in section 2.2.5. Titer quantification was performed via plaque assay at 10^6 PFU/mL, which was closer to 10^8 GC/mL.

2.2.7: Morbillivirus (Canine Distemper Virus)

The Canine distemper virus (CDV; ATCC VR-1587) was propagated by Katie Vigil (Tulane University School of Public Health and Topical Medicine Department of Global Environmental Health). The protocol by Ammerman et. al., (2008) was replicated for the Vero cell line (ATCC CCL-81) propagation. The virus was inoculated onto a 24-hour old Vero cell monolayer with 70 to 90 % confluency. The cells were incubated with DMEM media containing 10 % Fetal Bovine Serum at 37 ℃ with 5 % CO_2. Flasks with >80 % confluency were freeze-thawed three times at -80 ℃ and room temperature to ensure cell lysis. The lysate was filtered through a 0.45 µm membrane filter and aliquoted. The virus aliquots were frozen and stored at -80 ℃. Titer quantification was performed via AbsoluteQ dPCR at 10^7 GC/mL, which is also 10^7 GC/mL on Bio-Rad's ddPCR system.

2.3: Artificial Seawater Preparation for Seeded Studies

Artificial seawater was prepared from sterile laboratory nanopure reagent water using Instant Ocean salts (Instant Ocean, Blacksburg, VA, USA). In the beginning of the study, the estimated salinity of natural seawater was predicted to be 29.16 mS/cm. Approximately 110 g of Instant Ocean salts were added to 5 liters of water in a plastic carboy, the salinity of this mixture was 29.16 mS/cm. After receiving environmental samples for the San Diego Bay and Shedd

Aquarium the conductivity was measured to be between 40.0 to 48.0 mS/cm. To examine different salinities, 40 g of Instant Ocean salts were added to 2 liters of autoclaved nanopure water in a glass PYREX media bottle (CORNING, Corning, NY, USA) then adjusted to the desired conductivity 40.0 to 48.0 mS/cm. For full absorption, the salts were added into the water while it was warm and then left to spin on a stir plate overnight to cool to room temperature. The conductivity was tested using a Russell RL060C Portable Conductivity/ Temperature Meter (Thermo Scientific, Waltham, MA, USA). Traceable conductivity standard certified reference material catalog number 23226-589 (VWR, Radnor, PA, USA) was used to calibrate the conductivity meter. The pH was tested using a Denver Instrument UltraBASIC UB-5 pH meter (Cole-Parmer, Vernon Hills, IL, USA) and the pH of artificial seawater was adjusted to 6.5 or 8.0 (see Table S2 & S3). pH adjustments were made to the water using 10 M NaOH and 3 M HCL solutions. Test dust ISO 12103-1, A2 fine (Powder Technology Inc., Burnsville, MN, USA) was added to increase turbidity in some of the early artificial seawater to be 3.0 mS/cm. Test dust was not routinely added, due to the turbidity of San Diego Bay and John Shedd Aquarium samples being less than 1.0 mS/cm.

2.4: Concentrating Pipette Select

The ability of the automated ultrafiltration CP Select (InnovaPrep, Drexel, MO, USA) was evaluated for concentrating viruses from marine waters. A 0.05 μm polysulfone hollow fiber was used as recommended by the manufacturer to capture viruses. The recommended instrument settings for 0.05 μm tip were used in the beginning of the project marked as Master 1: 100% pump rate with 0% Tween 80. Valve open, 800 milliseconds (ms); valve closed, 100 ms; pulse count, 2; foam factor, 10; flow start, 3.0 sec; flow end; 0.3 sec; extended delay; 3 sec; pump; 100%; and extended pump delay, 1 sec.

Valve open means the length of time that the elution valve is open per pulse, similarly, valve closed means the time in between pulses when the valve is closed (Birkenholz, 2020). Pulse count means the number of cycles that the elution valve will open and close (Birkenholz, 2020). Foam factor is the frequency of foam release during an elution, set at 10 means that the foam valve will power on for 5 ms and off for 5 ms, this repeats until the elution is completed (Birkenholz, 2020). Flow start determines the sensor's sensitivity of flow needed to continue processing, lower number means less liquid is required to establish flow, similarly flow end is the flow sensor sensitivity needed to determine that there is no flow and the CP Select can stop (Birkenholz, 2020). Extended delay sets the delay time between the vacuum relief and the foam valve opening. Pump power sets the flow of the liquid (Birkenholz, 2020). Extended pump delay is the delay after the foam valve open time before the permeate pump is turned on to remove residual fluid (Birkenholz, 2020).

When the ultrafilter pipettes were used Master 2 CP Select settings were used: 100% pump rate with 0% Tween 20. Valve open, 800 ms; valve closed, 100 ms; pulse, 2; foam factor, 10; flow start, 3.0 sec; flow end; 0.2 sec; extended delay; 3 sec; and extended pump delay, 1 sec (Birkenholz, 2020).

Once the CP Select system was turned on, the maintenance setting was used to prime the machine for use. The CP Select gave prompts on the screen which the user would follow. After this, a new pipette was placed into the machine and the sample was concentrated. For the spiked tests, certain variables were recorded, as shown in Table S5 in the appendix. The pipette was eluted using FluidPrep Elution Fluid Can- Tris 0.075% Tween 20/25 nM Tris (InnovaPrep, Drexel, MO, USA). The sample was eluted into a 50 mL tube and then measured using a 5 mL pipettor, then transferred into a cryovial for storage. This filter tip was eluted using the

automated wet foam elution process, the final elution volume for one pipette and one elution was on average 1.50 mL. The pipette was eluted again if the volume was below 400 microliters (µL) for analysis if necessary.

For each replicate test to evaluate virus recovery through the CP Select, 1 mL of the virus stock preparation between 10^6 and 10^8 PFU (depending on the virus) was spiked into 1000 mL of artificial seawater. Later in the study, when using the ultrafilter tips 0.50 mL of the virus stock was spiked into 500 mL of artificial seawater. Each test was run alongside a negative seawater sample that was not spiked, see section 2.7: Controls. The sample was then mixed, 10 mL was taken out for the "Before Concentration" sample for culture-based analysis for bacteriophages. One mL was then removed from the "Before Concentration" volume for molecular analyses for MS2, Phi6, Adenovirus, OC43, and CDV. The remaining 499 mL was aliquoted into a Whirl-Pak bag (Whirl-Pak, Fort Atkinson, WI, USA) which was placed inside of a 2 L beaker and then concentrated through the CP Select.

The "after concentration" sample was then compared to the "before concentration" with culture-based analysis for bacteriophages MS2, P22, Phi6, and PhiX174 and molecular assay for MS2, Phi6, Adenovirus, OC43, and CDV. The sample was extracted the same day as it was concentrated. The RNA extracted sample was kept at -80 ℃ until ddPCR was performed and then for long term storage; and the DNA extracted samples were stored at -20 ℃.

2.5: Virus Plaque Assay and Enumeration

A 10-fold dilution series was performed on the "before concentration" and "after concentration" samples in phosphate buffered saline (PBS). Bacteriophages were tested using the double agar overlay plaque assay using a modification of EPA method 1602 to assess the live virus in the samples, see section 2.2 (EPA, 2001). A host was created for the viruses being tested

the day prior to concentration of the samples and allowed to incubate for 16 hrs. That host was then used to create a new culture and incubated for 4 hrs in a 36 ℃ incubator until it was ready for use. For bacteriophages PhiX174, MS2, and P22, 0.5 mL of the corresponding host suspension was added to 2.5 mL TSA overlay, and then 2 mL of the sample being assayed, either "before concentration" or "after concentration". The overlay tube would be quickly mixed and poured into a 15 mL TSA plate, swirled, and then set to solidify. These plates were then placed into a 36 ℃-incubator, for 16 hrs and then the plaques were counted.

The procedure was slightly different for Phi6. The host was incubated at room temperature 20 ℃, for 4 hrs then used for plating with the agar overlay. Two mL of the bacteriophage Phi6's host *P. syringae*, was added to the 2.5 mL King's B overlay, and then 0.5 mL of the sample was added. The overlay was quickly mixed and then poured onto a 15 mL King's B agar plate and allowed to solidify. The plates were also incubated upside down at 20 ℃ for 16 to 18 hrs.

2.6: Digital Droplet PCR

The QXDx AutoDG ddPCR System and BIO RAD QX200 Droplet Digital PCR (ddPCR) Reader instruments were used for all molecular analyses. A C1000 Touch Thermal Cycler with 96–Deep Well Reaction 1851197 thermocycler (Bio-Rad, Hercules, California, USA) was used for all the samples. PX1 PCR Plate Sealer 1814000 was used to seal the plates closed with foil (Bio-Rad, Hercules, California, USA). A concentration gradient was performed to find the optimum reverse transcriptase (between 55 ℃ to 65 ℃) and annealing temperature thermocycler settings for all targets. Primers and probe sequences used are shown in Table 4.

Table 4: PCR primer and probe sequences for viruses tested.

Virus	Name	Sequence	Citation
MS2*	Forward	5'-TGCTCGCGGATACCCG-3'	Trojnar et. al., 2020
	Reverse	5'-AACTTGCGTTCTCGAGCGAT-3'	
	Probe	[6~FAM]ACCTCGGGTTTCCGTCTTGCTCGT-[BHQ1A~Q]-3'	
Phi6*	Forward	5' -TGGCGGCGGTCAAGAGC- 3'	Flood, et. al., 2021
	Reverse	5' -GGATGATTCTCCAGAAGCTGCTG- 3'	
	Probe	5'-FAM-CGGTCGTCGCAGGTCTGACACTCGC-BHQ1-3'	
OC43	Forward	5′-CGATGAGGCTATTCCGACTAGGT- 3′	Pecson, et. al., 2021
	Reverse	5' -CCTTCCTGAGCCTTCAATATAGTAACC-3'	
	Probe	5' -6FAM-TCCGCCTGGCACGGTACTCCCT-BHQ- 3'	
Adeno	JTVXF	5'-GGACGCCTCGGAGTACCTGAG- 3'	Jothikumar, et. al., 2005
	JTVXR	5'- ACIGTGGGGTTTCTGAACTTGTT- 3'	
	JTVXP	5'-CTGGTGCAGTTCGCCCGTGCCA- 3'	
CDV	CDV4.1-F	5' -CTGTCRGTAATCGAGRATTCGA- 3'	Halecker, et. al., 2021
	CDV3-R	5' -GCCGAAAGAATATCCCCAGTTAG- 3'	
	CDV3.1-FAMas	5' -FAM-ATC TTC GCC AGA RTC YTC AGT GCT-BHQ1- 3'	

*Phage

2.6.1: Bacteriophage MS2

The "before concentration" and "after concentration" samples, along with the negatives, were extracted using QIAamp Viral RNA Mini kit (QIAGEN, Hilden, Germany). The extraction protocol followed the manufacturer's protocol where 140 µL of sample was extracted and 80 µL of final eluant was produced. Of that final eluant, 5 µL was added to 15 µL of master mix, samples were run in triplicate. Molecular quantification of MS2 was conducted using the following thermocycler settings 25 ℃ for 3 min, 47 ℃ for 1 h, 95 ℃ for 10 min, followed by 40 cycles of 95 ℃ for 30 s and 55 ℃ for 1 min with ramp rate of 2 ℃ s^{-1} followed by a final cycle of 98 ℃ for 10 min then held at 4 ℃.

2.6.2: Bacteriophage Phi6

The “before concentration” and “after concentration” samples, along with the negatives, were extracted using the same procedure as listed in section 2.6.1. Molecular quantification of Phi6 was conducted using a method described by Flood et al. 2021. Primers and probe sequences used are shown in Table 3.

2.6.3: Adenovirus Type 10

The “before concentration” and “after concentration” samples, along with the negatives, were extracted using QIAamp DNA Mini kit 51306 (Qiagen, Hilden, Germany). The procedure from the QIAamp handbook was followed were 200 µL of sample was extracted and 200 µL of final eluant was produced. Of that final eluant 5 µL was added to 15 µL of master mix, samples were run in triplicate. Molecular quantification of Adenovirus was conducted using a qPCR method that was optimized for ddPCR. The protocol took approximately 2 hours to complete with the following PCR conditions: hot-start denaturation step at 95 ℃ for 10 min, followed by 39 cycles with a 94 ℃ denaturation for 30 s, 55 ℃ annealing for 1 min, and 98 ℃ elongation for 10 min (at a temperature transition rate of 2 ℃ s^{-1})(Jothikumar, N. et al, 2005). The BIO RAD QX200 Droplet Digital PCR (ddPCR) analysis was performed. A concentration gradient was preformed to find the optimum thermocycler settings.

2.6.4: OC43 Coronavirus HCoV

The “before concentration” and “after concentration” samples, along with the negatives, were extracted using QIAGEN Qiamp Viral RNA Mini kit following the manufacturer’s protocol. Molecular quantification of OC43 was conducted using the method described by Flood et al., 2021.

2.6.5: Morbillivirus (Canine Distemper Virus)

The “before concentration” and “after concentration” samples, along with the negatives, were extracted using Qiamp Viral RNA Mini kit (QIAGEN, Hilden, Germany). The extraction protocol followed the manufacture’s protocol where 140 µL of sample was extracted and 80 µL of final eluant was produced. Of that final eluant, 5 µL was added to 15 µL of master mix, samples were run in triplicate. Molecular quantification of CDV was conducted using the following thermocycler settings 25 °C for 3 min, 42 °C for 1 hr, 95 °C for 10 min, followed by 40 cycles of 95 °C for 30 s and 55°C for 1 min with ramp rate of 2 °C s^{-1} followed by a final cycle of 98 °C for 10 min then held at 4°C.

2.7: Controls

Controls were run at every step of the process. Unseeded controls were run through the CP Select after the seeded samples in case of contamination. These unseeded samples were plated in triplicate following the same protocol as seeded samples. The plaque assay also has media, overlay, PBS, positive and negative plate controls. Extraction controls were run with every set on ddPCR, along with positive and negative controls. There were negative controls from the concentration, extraction, and ddPCR steps.

2.8: Statistical Analysis

Recovery efficiency for the plaque assay was calculated by dividing the "after concentration" PFU by the "before concentration" PFU and then multiplied by 100. Similarly, for ddPCR assay recovery efficiency was calculated by dividing the total gene copies (GC) per 500 mL "after concentration" by the "before concentration" then multiplied by 100 (Flood et. al., 2021).

$$\frac{\text{After Concentrate}\left(\text{PFU or}\frac{\text{GC}}{\text{mL}}\right)}{\text{Before Concentrate}\left(\text{PFU or}\frac{\text{GC}}{\text{mL}}\right)} \times 100 = \text{Recovery Efficency \%}$$

All MS2, Phi6, Adenovirus, OC43, and CDV data was converted from GC per reaction to GC per volume of sample. Negative samples were accepted if less than three droplets were above the set threshold. The limit of detection was calculated identical to Flood et. al., 2021 but for 500 mL samples. The detection limit for RNA samples was 342.85 GC per 500 mL and DNA samples was 600 GC per 500 mL, when accepted droplets were 20,000.

$$\text{Virus GC per 500 mL} = \frac{\left(\frac{\text{GC per rxn}}{V_r}\right) \times V_e \times \left(\frac{V_f}{V_c}\right)}{V_i} \times 500$$

where V_i = Initial volume of sample concentration (mL), V_f = Final volume of sample after concentration (mL), V_r = volume of RNA template used per PCR reaction (µL), V_e = Final volume for DNA or RNA eluted from extraction (µL), V_c = Volume of concentrated sample used for extraction (mL) (Flood et. al., 2021).

Statistics and data visualization was executed using Graphpad Prism 9.5.1 (Graphpad Software, CA, USA). Results for Figures 1 to 3 were performed using Prism.

CHAPTER 3: RESULTS

3.1: Bacteriophages Recovery Results Using CP Select

During the beginning of the project, a virus concentration method for marine waters was created using bacteriophages because they are safe to handle and inexpensive. Initially, artificial seawater was made with parameters reported from the San Diego Bay between May 2020 and July 2020, as shown in Table S2 (Appendix) for 19 samples. Table S2 in the appendix includes the target test conditions and acceptable range for pH of 6.5, turbidity of 3 Nephelometric turbidity units (NTU), and conductivity of 29.16 mS/cm. The physiochemical parameters of the natural seawater samples in 2022 were slightly different when compared to 2020, possibly due to different sample locations. The study performed in 2020 was evaluating pier sites while this study sampled near the dolphin and sea lion enclosure. To better reflect the natural waters the artificial seawater tested parameters were a pH of 8.0, turbidity of 1.0 NTU, and salinity or conductivity of 40.0 mS/cm in Table S3. Four bacteriophages were tested: to avoid plating conflicts between MS2 and PhiX174, P22 and MS2 were run together and Phi6 and PhiX174 were run together. The sample information can be found in the appendix Table S5, showing the sample ID, viruses seeded, volume of the phage seeded into the starting volume of the sample, the volume that was concentrated, the time taken to concentrate, number of pipettes and elutions per sample, followed by the elution volume and CP protocol setting. The starting volume of samples was 500 mL but the CP Select processed an average of 133 mL. The range of elution volumes was between 0.65 to 1.9 mL. This would make the ideal concentration factor 500 times, but the actual between 60 to 200 times.

The recoveries using PFU culture assay results averaged 110.38 ±193.3% for P22, 7.40 ±13.4% for MS2, 7.56 ±7.0% for PhiX174, and 31.77 ±21.6% for Phi6, as shown in Tables 5

and 6. The recoveries using the ddPCR assay results for the beginning of the project averaged 3.01 ±2.3% for MS2 and 16.90 ±20.7% for Phi6 (Table 7). In the tables below, input is "before concentration" sample results and output are "after concentration" results. All calculated averages shown are arithmetic means.

Table 5: Bacteriophages P22 and MS2 recovery results based on culture assays results with ~29.1 mS/cm conductivity and 0.05 µm pipette tips in artificial seawater.

Sample ID	P22 input (PFU/mL)	P22 output (PFU/mL)	P22 recovery %	MS2 input (PFU/mL)	MS2 output (PFU/mL)	MS2 recovery %
PM210819	2.15E+05	1.34E+06	624.85	3.07E+05	1.27E+05	41.39
PM210914	2.46E+06	7.16E+05	29.08	2.53E+06	3.45E+05	13.66
PM210916	2.15E+05	1.53E+05	71.41	3.14E+06	1.73E+04	0.55
PM210928	2.05E+06	1.03E+06	50.29	2.13E+06	3.51E+04	1.65
PM211007	2.28E+06	9.35E+05	41.02	2.79E+06	4.86E+04	1.74
PM211117	1.71E+06	5.67E+05	33.06	1.93E+06	2.19E+04	1.14
PM220510-1	2.15E+07	1.00E+07	46.69	4.42E+06	9.10E+04	2.06
PM220510-2	2.15E+07	1.06E+07	49.33	4.42E+06	1.03E+05	2.33
PM220512	2.42E+07	1.16E+07	47.72	4.78E+06	1.34E+05	2.8
AVERAGE	8.46E+06	4.10E+06	110.38	2.94E+06	1.03E+05	7.48

Table 6: Bacteriophages PhiX174 and Phi6 culture assay results with ~29.1 mS/cm conductivity and 0.05 µm pipette tips in artificial seawater.

Sample ID	PhiX174 input (PFU/mL)	PhiX174 output (PFU/mL)	PhiX174 recovery %	Phi6 input (PFU/mL)	Phi6 output (PFU/mL)	Phi6 recovery %
PP210819	1.99E+05	3.47E+04	17.44	0.00E+00	0.00E+00	0.00
PP210826	8.40E+05	1.67E+04	1.98	1.06E+08	6.53E+05	0.62
PP210909	1.01E+06	3.30E+04	3.28	TNTC*	8.43E+07	NR^
PP210921	7.45E+05	1.57E+04	2.11	3.22E+08	2.45E+07	7.61
PP211005	1.27E+06	1.02E+05	7.99	1.76E+08	8.13E+07	46.28
PP211103	7.04E+05	1.80E+04	2.55	1.74E+08	2.28E+07	13.12
PP211117	7.25E+05	1.46E+04	2.01	1.74E+08	6.60E+07	37.94
PP220208	1.68E+07	3.58E+06	21.28	3.75E+07	1.47E+07	39.16
PP220505-1	1.32E+07	1.50E+06	11.37	4.93E+07	2.78E+07	56.48
PP220505-2	1.39E+07	7.82E+05	5.62	5.20E+07	2.75E+07	52.91
AVERAGE	4.94E+06	6.10E+05	7.56	1.21E+08	3.50E+07	31.77

*TNTC is too numerous to count
^No Results (negative control)

Table 7: Bacteriophages MS2 and Phi6 ddPCR results with ~29.1 mS/cm conductivity and 0.05 µm pipette tips in artificial seawater.

Sample ID	Virus	Input (GC/L)	Output (GC/L)	Recovery %
PM210819	MS2	3.68E+06	2.49E+05	6.76
PM210914	MS2	2.24E+06	2.79E+04	1.25
PM210916	MS2	3.92E+06	4.09E+04	1.04
PM210928	MS2	4.27E+06	1.95E+05	4.56
PM211007	MS2	3.20E+06	1.16E+05	3.64
PM211117	MS2	5.01E+06	5.12E+04	1.02
PM211227F	MS2	4.85E+07	1.57E+05	0.32
PM220510-1	MS2	4.57E+07	1.15E+06	2.52
PM220510-2	MS2	4.57E+07	1.05E+06	2.29
PM220512	MS2	4.11E+07	2.74E+06	6.68
AVERAGE		2.03E+07	5.78E+05	3.01
PP210819	Phi6	4.89E+06	2.45E+05	5.01
PP210826	Phi6	7.95E+08	6.05E+07	7.61
PP210909	Phi6	1.08E+12	1.77E+07	0.00164
PP210921	Phi6	1.82E+10	1.99E+09	10.89
PP211005	Phi6	1.65E+10	4.14E+09	25.11
PP211103	Phi6	7.06E+09	1.34E+09	18.96
PP211117	Phi6	7.32E+09	1.59E+09	21.74
PP220208	Phi6	1.41E+10	9.95E+09	70.75
PP220505-1	Phi6	4.92E+09	6.66E+07	1.35
PP220505-2	Phi6	4.92E+09	3.71E+08	7.55
AVERAGE		1.15E+11	1.95E+09	16.90

Artificial marine water aquarium samples were received from the Shedd Aquarium as a preliminary study to examine time taken to concentrate and the volume of the eluate. To increase the elution volume so that enough sample was available for testing the CP Select protocol was changed from Master 1 to Master 2. This was the recommended setting for ultrafilter pipette tips (section 2.4: Concentrating Pipette). This had a pulse of 2 which increased the testable elution volume but did not affect the recovery percentage, as shown in Table 8.

Table 8: Recovery results from elution study with culture assay for PhiX174 and ddPCR assay for Phi6 in John Shedd Aquarium water.

Sample ID	Volume of each Phage Seeded (µL)	Starting Sample Volume (mL)	Volume Concentrated (mL)	Elution volume (mL)	PhiX174 culture recovery %	Phi6 ddPCR recovery %	CP Protocol
NegOC*	-	250	195	0.25	NR	NR	Master 1
NegG3*	-	250	245	0.2	NR	NR	Master 1
OC220811-1	250	250	45	0.35	2.39	19.4	Master 1
OC220811-2	250	250	29	0.45	3.10	23.44	Master 1
G3220811-1	250	250	36	0.45	2.85	12.83	Master 1
G3220811-2	250	250	58	0.9	6.57	50.23	Master 1
G3220811-3	250	250	67	0.28	6.02	18.31	Master 1
G3220811-4	250	250	39	0.45	2.86	18.65	Master 1
AVERAGE			89	0.42	3.97	23.81	
Neg 8-16*	-	100	95	0.3	NR	NR	Master 2
OC220816	100	100	29.5	0.7	2.68	29.44	Master 2
G3220816	100	100	33	0.8	4.19	0.18	Master 2
AVERAGE			53	0.6	3.43	14.81	

*Unseeded control
^No Results

The conductivity of these samples averaged around 49 mS/cm which was almost double that of the conductivity used in the previous ASW experiments at 29.1 mS/cm. A salinity study was then performed using Phi6 (Table 9) and adenovirus (Table 14) to see if increased salt content influences recoveries. Table 9 shows that there was a difference in the average recovery when comparing triplicate experiments. The average Phi6 recovery at 29 mS/cm was 22.58 ±9.83% while at 49.8 mS/cm the recovery was 4.73 ±1.03%.

Table 9: Recoveries of Phi6 in water samples of different salinities (conductivity at 29 vs 49.8 mS/cm) as measured using ddPCR.

Sample ID	Conductivity (mS/cm)	Phi6 Input (GC/mL)	Phi6 Output (GC/mL)	Phi6 Recovery %
S1220901-1	29	6.28E+08	7.17E+07	11.42
S1220901-2	29	8.57E+08	2.57E+08	29.94
S1220901-3	29	1.80E+09	4.75E+08	26.39
AVERAGE		1.10E+09	2.68E+08	22.58
S2220901-1	49.8	8.01E+08	4.06E+07	5.07
S2220901-2	49.8	2.77E+09	9.89E+07	3.57
S2220901-3	49.8	8.68E+08	4.81E+07	5.55
AVERAGE		1.48E+09	6.25E+07	4.73

Innovaprep was contacted to discuss the impact of salinity. It was discovered that the 0.05 µm pipette tips were expired, although there was no expiration date on the box. Innovaprep suggested the use of ultrafilter tips compared to 0.05 µm tips for the bacteriophages. They also suggested the use of Tween-20. In Table 10, we see the comparison of newly ordered 0.05 µm pipette tips and ultrafilter tips (100 KD effective for particles ~20 nm and larger). The average MS2 recovery using the 0.05 µm pipette tips was 0.13 ±0.10% while for the ultrafilter tip it was 0.53 ±0.24%. The samples were re-analyzed on ddPCR due to suspected inhibition issues. This was done by being further diluted from 100-fold to 10000-fold to see if recoveries improved. The new MS2 recovery for 0.05 µm pipette tips was 0.22 ±0.05% while for the ultrafilter tip it was 1.09 ±0.54%. The project moved forward with utilizing ultrafilter pipette tips.

Table 10: Recovery of MS2 comparing two CP Select pipette tips using artificial seawater assayed with ddPCR.

Sample ID	Pipette tip	Conductivity (mS/cm)	Input (GC/mL)	Output (GC/mL)	MS2 Recovery %
5AM221010-1	0.05 µm	40	1.83E+07	4.88E+04	0.27
5AM221010-2	0.05 µm	40	1.69E+07	3.66E+04	0.22
5AM221010-3	0.05 µm	40	1.89E+07	3.28E+04	0.17
AVERAGE			1.80E+07	3.94E+04	0.22
UAM221010-1	ultrafilter	40	6.88E+06	1.18E+05	1.71
UAM221010-2	ultrafilter	40	9.29E+06	6.95E+04	0.75
UAM221010-3	ultrafilter	40	6.42E+06	5.22E+04	0.81
AVERAGE			7.53E+06	7.99E+04	1.09

*Master 2 setting

The method evolved as experiments were undertaken. The final procedure included the use of the ultrafilter tip with the recommended CP Select settings (see Master 2 setting in section 2.4) and 40 mS/cm salinity for the artificial seawater experiments. Using culture assays, the average recovery for P22 was 82.73 ±27.3% (n=6), for MS2 was 4.84 ±3.8% (n=3), for PhiX174 was 32.77 ±19.3% (n=6), and for Phi6 was 71.48 ±42.5% (n=6), as shown in Tables 11 and 12. In Figure 1, nested T-test found t= 0.1594, df= 2, p= 0.8880. This non-significant result confirms that the random pairing of the phages did not affect recoveries. The chi-square value was 7.769, df=1, P value= 0.0059, meaning that there was a difference in recovery between phages. The ddPCR assay average recovery for MS2 was 7.82 ±8.1% (n=3) and for Phi6 was 34.72 ±19.7% (n=6), Table 13. In Figure 2, an unpaired t test with Welch's correction resulted in a t= 2.893, df= 6.968, p= 0.0233, which showed a significant difference between the means of recovery between MS2 and Phi6 phages. The F test to compare variances of MS2 and Phi6 was not significant (p= 0.3052), meaning that did not significantly differ in standard deviation.

Table 11: Culture assay recovery of P22 and MS2 in artificial seawater using the ultrafilter tips and Master 2 setting.

Sample ID	P22 input (PFU)	P22 output (PFU)	P22 recovery %	MS2 input (PFU)	MS2 output (PFU)	MS2 recovery %
PM221120-1	5.09E+06	4.41E+06	86.63	NR	NR	NR
PM221120-2	2.88E+06	3.20E+06	111.14	NR	NR	NR
PM221120-3	2.22E+06	2.65E+06	119.12	NR	NR	NR
PM221220-1	9.56E+06	5.73E+06	59.94	4.70E+05	1.15E+04	2.45
PM221220-2	9.54E+06	5.57E+06	58.40	4.75E+05	1.38E+04	2.90
PM221220-3	1.68E+06	1.03E+06	61.17	1.04E+05	9.55E+03	9.18
AVERAGE	5.16E+06	3.77E+06	82.73	3.50E+05	1.16E+04	4.84

^No Results

Table 12: Culture assay recovery of PhiX174 and Phi6 in artificial seawater using the ultrafilter tips and Master 2 setting.

Sample ID	PhiX174 input (PFU)	PhiX174 output (PFU)	PhiX174 recovery %	Phi6 input (PFU)	Phi6 output (PFU)	Phi6 recovery %
AP221025-1	NR	NR	NR	6.56E+06	9.12E+06	139.02
AP221025-2	NR	NR	NR	3.27E+06	3.08E+06	94.29
AP221025-3	NR	NR	NR	4.15E+06	3.58E+06	86.06
PP221205-1	3.23E+05	2.09E+05	64.68	6.90E+06	2.81E+06	40.75
PP221205-2	1.62E+05	5.40E+04	33.33	4.13E+06	1.20E+06	29.07
PP221205-3	1.54E+05	6.98E+04	45.29	3.78E+06	1.50E+06	39.72
AX221212-1	2.95E+06	5.09E+05	17.29	NR	NR	NR
AX221212-2	3.11E+06	6.21E+05	19.98	NR	NR	NR
AX221212-3	3.55E+06	5.69E+05	16.03	NR	NR	NR
AVERAGE	1.71E+06	3.39E+05	32.77	4.80E+06	3.55E+06	71.48

^No Results

Table 13: ddPCR assay recovery of MS2 and Phi6 in artificial seawater using the ultrafilter tips and Master 2 setting.

Sample ID	Virus	Input (GC/mL)	Output (GC/mL)	Recovery %
PM221220-1	MS2	1.44E+07	5.40E+05	3.74
PM221220-2	MS2	1.54E+07	3.92E+05	2.54
PM221220-3	MS2	2.80E+06	4.81E+05	17.19
AVERAGE		1.09E+07	4.71E+05	7.82
AP221025-1	Phi6	2.31E+08	9.99E+07	43.33
AP221025-2	Phi6	1.10E+08	7.67E+07	69.63
AP221025-3	Phi6	1.91E+08	4.38E+07	22.98
PP221205-1	Phi6	2.47E+09	8.34E+08	33.82
PP221205-2	Phi6	1.17E+09	1.84E+08	15.76
PP221205-3	Phi6	1.19E+09	2.73E+08	22.82
AVERAGE		8.93E+08	2.52E+08	34.72

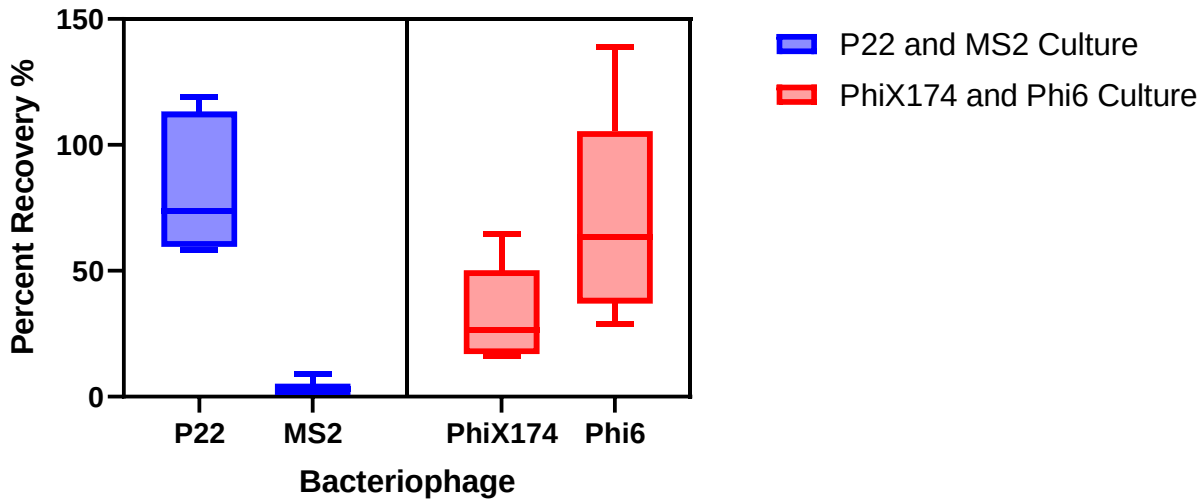

Figure 1: Comparison of P22, MS2, PhiX174, and Phi6 recovery using the ultrafilter tips and Master 2 setting with culture assay.

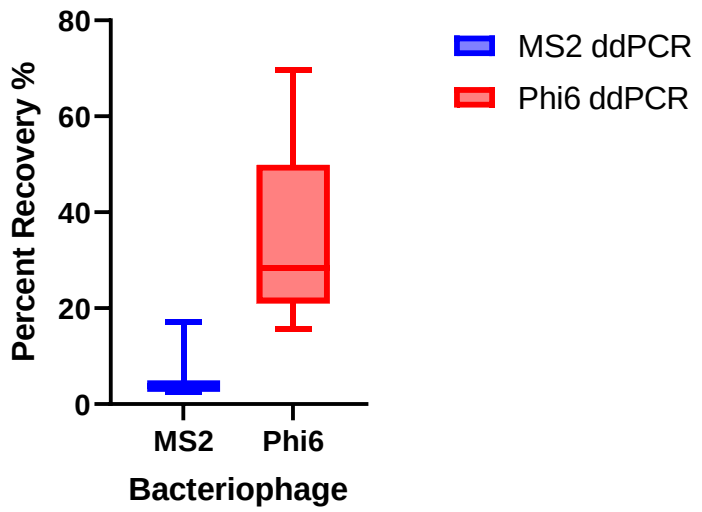

Figure 2: Comparison of MS2 and Phi6 recovery using the ultrafilter tips and Master 2 setting with ddPCR assay.

3.2: Animal Virus Results

Comparison of varying salinities on the recovery of adenoviruses using the CP Select was undertaken. As shown in Table 14 the adenovirus recovery at 29.8 mS/cm was 18.12 ±6.89% while for 49.2 mS/cm it was 1.54 ±1.41%. The average recovery when the samples were seeded in 250 mL then diluted to 500 mL, was an average of 0.17 ±0.2%. To rule out ddPCR inhibition, the samples concentrated were diluted after extraction and prior to PCR (Table 15) and the average recovery was 0.34 ±0.25%. Adenovirus was also used to compare the 0.05 µm and ultrafilter tips. The average recovery of 0.05 µm pipette was lower at 0.11 ±0.08% compared to using the ultrafilter tips with a recovery of 2.48 ±2.15%, shown in Table 16.

Table 14: Percent recovery of Adenovirus (Adeno) using ddPCR with varying salinities (conductivity) of artificial seawater.

Sample ID	Conductivity (mS/cm)	Adeno Input (GC/mL)	Adeno Output (GC/mL)	Adeno Recovery %
AA220913-1	29.8	7.42E+08	1.09E+08	14.72
AA220913-2	29.8	1.03E+09	2.67E+08	26.05
AA220913-3	29.8	1.18E+09	1.60E+08	13.61
AVERAGE		9.84E+08	1.79E+08	18.12
SAA220913-1	49.2	1.43E+09	3.99E+07	2.79
SAA220913-2	49.2	8.49E+08	2.57E+07	3.02
SAA220913-3	49.2	2.18E+08	5.79E+06	2.66
SAA220929-1	49	1.87E+09	1.82E+06	0.10
SAA220929-2	49	2.45E+09	5.87E+06	0.24
SAA220929-3	49	3.58E+09	1.56E+07	0.44
AVERAGE		1.73E+09	1.58E+07	1.54
SAA220929-1 D	49 diluted to 26	6.42E+09	2.40E+06	0.04
SAA220929-2 D	49 diluted to 26	7.14E+09	4.12E+06	0.06
SAA220929-3 D	49 diluted to 26	3.44E+09	2.09E+07	0.41
AVERAGE		5.67E+09	9.14E+06	0.17

Table 15: Comparing dilution of artificial seawater samples after extraction for ddPCR inhibition.

Sample ID	Conduc. (mS/cm)	Diluted BC* after extraction	Diluted AC* after extraction	Adeno input (GC/mL)	Adeno output (GC/mL)	Adeno recovery %
SAA220929-1	49	100-fold	100-fold	1.87E+09	1.82E+06	0.10
SAA220929-2	49	100-fold	100-fold	2.45E+09	5.87E+06	0.24
SAA220929-3	49	100-fold	100-fold	3.58E+09	1.56E+07	0.44
AVERAGE				2.63E+09	7.76E+06	0.26
SAA220929-1	49	100-fold	10000-fold	2.30E+09	3.10E+06	0.13
SAA220929-2	49	100-fold	10000-fold	1.30E+09	7.96E+06	0.61
SAA220929-3	49	100-fold	10000-fold	1.03E+09	2.70E+06	0.26
AVERAGE				1.54E+09	4.59E+06	0.34

*BC means “before concentration” and AC means “after concentration.”

Table 16: Comparison of pipette tips used for recovery of adenovirus in artificial seawater using ddPCR.

Sample ID	Pipette tip	Conductivity (mS/cm)	Adeno Input (GC/mL)	Adeno Output (GC/mL)	Adeno Recovery %
5AM221010-1	0.05 µm	40	9.59E+09	1.84E+07	0.19
5AM221010-2	0.05 µm	40	1.12E+10	1.35E+07	0.12
5AM221010-3	0.05 µm	40	8.84E+09	2.54E+06	0.03
AVERAGE			9.88E+09	1.15E+07	0.11
UAM221010-1	ultrafilter	40	1.65E+09	1.68E+07	1.02
UAM221010-2	ultrafilter	40	2.53E+09	3.72E+07	1.47
UAM221010-3	ultrafilter	40	1.85E+09	9.14E+07	4.95
AVERAGE			2.01E+09	4.85E+07	2.48

The method was then finalized to use the ultrafilter pipettes, conductivity range 40.0 to 48.0 mS/cm, and pH 8.0. Each sample was concentrated using one pipette until it fouled. The remaining volume was measured for calculations. Table 17 shows adenovirus in artificial seawater had an average recovery of 11.58 ±5.3%. In water from the dolphin enclosure at the J. Shedd Aquarium the average recovery was 59.81 ± 20.6% and in San Diego Bay water the average recovery was 46.53 ±22.5%. The overall average recovery of adenovirus was 39.31 ±26.6%. OC43 and CDV had comparable results to each other, shown in Tables 18 and 19. The artificial seawater tests resulted in 12.75 ±7.6% recovery for OC43 and 11.91 ±7.0% for CDV. The Shedd Aquarium samples had an average of 19.16 ±16.0% for OC43 while CDV had 21.28 ±21.3% recovery. Finally, San Diego Bay water had 25.22 ±10.4% for OC43 and 26.32 ±8.8% for CDV. The overall recovery of OC43 was 19.04 ±11.6% and for CDV was 19.84 ±13.6%. In Figure 3, a nested one-way ANOVA p= 0.2190, there was no significant difference in recovery between the different water matrixes ASW, JSA, and SDB. The chi-squared 3.274, df= 1, p= 0.0704, showed non-significant differences between Adenovirus, OC43 and CDV recovery.

Table 17: Comparison of adenovirus recovery in artificial seawater, Shedd aquarium, and San Diego Bay water using ddPCR.

Sample ID	Date	Matrix	Adeno input (GC/mL)	Adeno output (GC/mL)	Adeno recovery %
ASW221215-1	12/15/2022	Artificial Seawater	1.09E+09	1.79E+08	16.45
ASW221215-2	12/15/2022	Artificial Seawater	6.68E+08	3.95E+07	5.91
ASW221215-3	12/15/2022	Artificial Seawater	5.71E+08	7.07E+07	12.38
AVERAGE			7.76E+08	9.64E+07	11.58
JSA221221-1	12/21/2022	Shedd Aquarium	4.17E+08	2.42E+08	58.04
JSA221221-2	12/21/2022	Shedd Aquarium	3.58E+08	2.91E+08	81.27
JSA221221-3	12/21/2022	Shedd Aquarium	2.65E+08	1.06E+08	40.13
AVERAGE			3.47E+08	2.13E+08	59.81
SDB221221-1	12/21/2022	San Diego Bay	5.63E+08	3.45E+08	61.29
SDB221221-2	12/21/2022	San Diego Bay	5.56E+08	3.20E+08	57.65
SDB221221-3	12/21/2022	San Diego Bay	1.56E+09	3.23E+08	20.66
AVERAGE			8.93E+08	3.29E+08	46.53
TOTAL AVE.					39.31±26.6

Table 18: Comparison of OC43 coronavirus recovery in artificial seawater, Shedd aquarium, and San Diego Bay water using ddPCR.

Sample ID	Date	Matrix	OC43 input (GC/mL)	OC43 output (GC/mL)	OC43 recovery %
ASW221215-1	12/15/2022	Artificial Seawater	2.04E+09	4.37E+08	21.46
ASW221215-2	12/15/2022	Artificial Seawater	1.25E+09	1.01E+08	8.08
ASW221215-3	12/15/2022	Artificial Seawater	1.11E+09	9.71E+07	8.72
AVERAGE			1.47E+09	2.12E+08	12.75
JSA221221-1	12/21/2022	Shedd Aquarium	3.50E+08	6.00E+07	17.11
JSA221221-2	12/21/2022	Shedd Aquarium	3.53E+08	1.27E+08	36.09
JSA221221-3	12/21/2022	Shedd Aquarium	7.31E+08	3.13E+07	4.28
AVERAGE			4.78E+08	7.28E+07	19.16
SDB221221-1	12/21/2022	San Diego Bay	3.89E+08	9.43E+07	24.23
SDB221221-2	12/21/2022	San Diego Bay	3.47E+08	1.25E+08	36.11
SDB221221-3	12/21/2022	San Diego Bay	8.63E+08	1.32E+08	15.31
AVERAGE			5.33E+08	1.17E+08	25.22
TOTAL AVE					19.04±11.6

Table 19: Comparison of CDV morbillivirus recovery in artificial seawater, Shedd aquarium, and San Diego Bay water using ddPCR.

Sample ID	Date	Matrix	CDV input (GC/mL)	CDV output (GC/mL)	CDV recovery %
ASW221215-1	12/15/2022	Artificial Seawater	1.78E+07	3.52E+06	19.74
ASW221215-2	12/15/2022	Artificial Seawater	7.53E+06	4.59E+05	6.10
ASW221215-3	12/15/2022	Artificial Seawater	6.80E+06	6.72E+05	9.88
AVERAGE			1.07E+07	1.55E+06	11.91
JSA221221-1	12/21/2022	Shedd Aquarium	9.37E+06	1.18E+06	12.57
JSA221221-2	12/21/2022	Shedd Aquarium	6.83E+06	3.11E+06	45.52
JSA221221-3	12/21/2022	Shedd Aquarium	4.99E+06	2.87E+05	5.76
AVERAGE			7.06E+06	1.53E+06	21.28
SDB221221-1	12/21/2022	San Diego Bay	6.77E+06	2.34E+06	34.50
SDB221221-2	12/21/2022	San Diego Bay	9.64E+06	2.64E+06	27.43
SDB221221-3	12/21/2022	San Diego Bay	1.55E+07	2.64E+06	17.03
AVERAGE			1.06E+07	2.54E+06	26.32
TOTAL AVE.					19.84 ±13.6

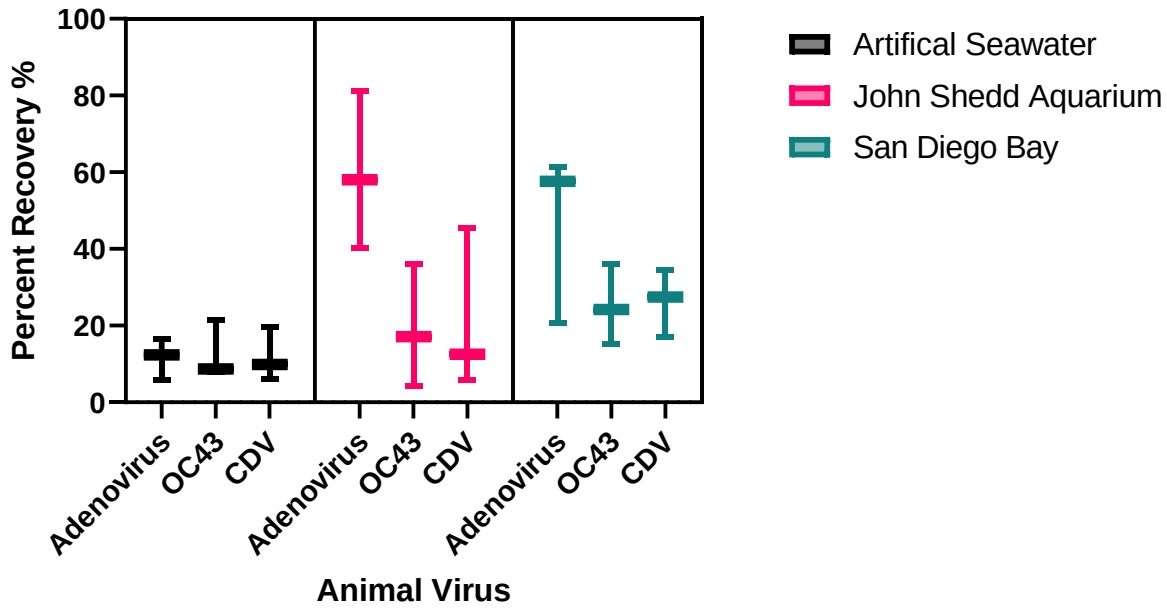

Figure 3: Comparison of Adenovirus, OC43, and CDV recovery in artificial seawater, John Shedd aquarium, and San Diego Bay water samples with ddPCR assay.

Table 20: Summary of results from the complete method.

Sample type	Virus and Type	Assay type	Recovery (%)	n=
Artificial seawater	Salmonella virus P22 (double stranded DNA)	Culture	82.73 ±27.3	6
Artificial seawater	Pseudomonas virus Phi6 (double stranded RNA)	Culture	71.48 ±42.5	6
J. Shedd Aquarium	Adenovirus (double stranded DNA)	ddPCR	59.81 ±20.6	3
San Diego Bay	Adenovirus (double stranded DNA)	ddPCR	46.53 ±22.5	3
Artificial seawater	Pseudomonas virus Phi6 (double stranded RNA)	ddPCR	34.72 ±19.7	6
Artificial seawater	Coliphage PhiX174 (single stranded DNA)	Culture	32.77 ±19.3	6
San Diego Bay	CDV Morbillivirus (- single stranded RNA)	ddPCR	26.32 ±8.79	3
San Diego Bay	OC43 Human Coronavirus (+ single stranded RNA)	ddPCR	25.22 ±10.4	3
J. Shedd Aquarium	CDV Morbillivirus (- single stranded RNA)	ddPCR	21.28 ±21.27	3
J. Shedd Aquarium	OC43 Human Coronavirus (+ single stranded RNA)	ddPCR	19.16 ±16.0	3
Artificial seawater	OC43 Human Coronavirus (+ single stranded RNA)	ddPCR	12.75 ±7.6	3
Artificial seawater	CDV Morbillivirus (- single stranded RNA)	ddPCR	11.91 ±7.04	3
Artificial seawater	Adenovirus (double stranded DNA)	ddPCR	11.58 ±5.3	3
Artificial seawater	Coliphage MS2 (+ single stranded RNA)	ddPCR	7.82 ±8.1	3
Artificial seawater	Coliphage MS2 (+ single stranded RNA)	Culture	4.84 ±3.8	3

CHAPTER 4: DISCUSSION

Virus surveillance of water is a rapid non-invasive option to monitor and protect captive marine mammals' health associated with infectious diseases. The health of the bottlenose dolphins and California sea lions in the U.S. Marine Mammal Program are a high priority however a robust environmental surveillance program has yet to be implemented. There is no one concentration method that is effective at recovering all types of viruses which is partly due to their diversity in size and structure (Petrova et. al., 2020). Creating a method that would concentrate many viruses adequately would be helpful for the surveillance of diseases that affect mammals in the U.S. Marine Mammal Program. The goal of this project was to create a method to concentrate viruses from marine water using a compact device towards virus detection. Varying families of viruses were tested through the Innovaprep Concentrating Pipette Select (CP Select). Historically, filtration has been used to concentrate enteroviruses from differing water matrixes, mainly wastewater (Petrova et. al., 2020).

An optimized protocol for the CP Select was developed in this study. The data suggests that the ultrafilter pipette tip with the recommended manufacturer settings works best for viruses in marine water. Concentrating the water until the pipette tip fowls and eluting the pipette once resulted in the highest recoveries for seeded experiments. There was no addition of Tween-20 into the samples because there was no statistical proof that it increased virus recovery, see Table S7.

The CP Select method culture assay recovery outcomes for all viruses ranged from 2.45 % to 119.12 % and averaged 54.12 %. A summary of the results for the complete method can be shown in Table 19. The lowest culture assay average recovery was 4.84 ±3.8% for MS2 phage and the highest was with P22 phage at 82.73 ±27.3% . The CP Select ddPCR assay recovery

outcomes ranged from 2.54% to 81.27% and averaged 25.99%. The lowest ddPCR average recovery was 7.82 ±8.1% for MS2 and the highest was with Adenovirus at 39.31 ±26.56%.

One of the goals of this study was to develop a simple and rapid method that could be used by Navy personnel. PCR is faster than culture and while this study focused on CP Select recoveries, the complete method included extraction of the nucleic acid for PCR analysis. A small test was done to compare extraction efficiency using the QIAamp Powerwater kit (QIAGEN, Hilden, Germany) to the QIAamp Viral RNA Mini Kit, see Table S8.This kit did have slightly higher recoveries but due to the complicated protocol it is not recommended for use at marine facilities, aquaria or on board a ship. One of the reagents in the powerwater kit must also be kept in a chemical fume hood especially while in use, which is not available in navy vessels or the navy personnel ONR lab. The QIAamp Viral RNA Mini Kit was used for all of the final results.

The time taken for the process from beginning to end generally ranged from 5 to 7 hrs depending on the number of samples and target viruses. The CP select on average took 17 mL per minute to concentrate the marine water samples. Extraction with the QIAamp Viral RNA Mini Kit took a minimum of 35 minutes for four samples, additional samples added time. The ddPCR set up which includes reagent thaw, making the mastermix, and setting up the auto-droplet generators as well as the reader templates took a minimum of 20 minutes. Plating the mastermix and samples ranged from 5 to 20 minutes depending on number of samples. Auto-droplet generators took an average of 4 minutes per column. Thermocycling the samples took roughly 3 hrs for RNA viruses and 2 hrs for DNA viruses. The droplet reader took 3 minutes per sample or 24 minutes per column.

4.1: Virus Characteristics influencing recovery

The viruses tested in the study can be used as surrogates for other viruses with similar characteristics. There were three single-stranded RNA viruses, one double-stranded RNA virus, two double-stranded DNA viruses and one single-stranded DNA virus tested. Two were enveloped viruses and four were non-enveloped viruses. All the viruses had varying sized diameters. The size range of the DNA viruses, in this study, were between 23 to 100 nm while the RNA viruses were 24 to 300 nm in diameter. The differences in nucleic acid, DNA viruses had a greater recovery than the RNA viruses, was more influential than size of the virus. It is not clear why but potentially this is due to the stability of the target.

Based on the CP Select's mechanism of concentration via size exclusion, the hypothesis was that Phi6 (80 to 100 nm diameter) would have the highest percent recovery out of all the bacteriophages and CDV morbillivirus (150 to 300 nm diameter) would have the greatest recovery overall. However, P22 (52 to 60 nm diameter) had the highest recovery percent. The DNA viruses had a significantly higher recovery than RNA viruses ($t=3.725$, $df=27.34$, $p=0.0009$). The DNA viruses were P22, PhiX174, and Adenovirus. P22 can endure a wide range of pH (4 to 8), and the pH in the water matrices of this study ranged from 6.5 to 8.0. PhiX174 has a thicker capsid than MS2 and P22 (Heffron et. al., 2019). As stated in section 1.2.2.1, Adenoviruses with double stranded DNA are more thermally stable, have host cell repair enzymes, and are more UV inactivation resistant (Mena and Gerba, 2008; Thurston-Enriquez et al. 2003b). MS2 had the lowest recovery among all the viruses tested in this study. This could be due to small size, capsid thinness, or instability of the target under high pH or salinity.

The three viruses with the highest average recoveries were P22, Phi6, and adenovirus. These viruses all have double-stranded nucleic acid. An unpaired t test with Welch's correction

comparing all single-stranded viruses to double-stranded viruses resulted in a significant difference (t=3.899, df=27.04, p=0.0006). There was also a significant difference in the F test to compare variances (F=4.407, DFn=20, DFd=26, p=0.0005). Double-stranded viruses had greater variance between sample recovery, whereas single-stranded viruses produced more consistent recovery results.

Size is one of the virus characteristics that could influence recovery. Falman et. al., 2019 used poliovirus type 1 (PV1) in wastewater and reported a low recovery of 0.32%, which is lower than the recovery of MS2 in this study. PV1 is a diameter of 25 to 30 nm which is close to MS2's diameter range of 23 to nm (Simonet and Gantzer, 2006). They are both non-enveloped single-stranded RNA viruses (Simonet and Gantzer, 2006). In a study by Simonet and Gantzer, 2006, MS2 was the more resistant virus to genome degradation than PV1 with UV radiation. This stability of the RNA may explain why MS2 had higher recoveries than PV1 although they were of similar physical characteristics including size, which is the basis of the exclusion filtration of the CP Select. Lee et. al., 2021, used norovirus as an efficiency control in untreated sewage that flows into river water surrounding farms. MS2 and norovirus are similar in size, both have positive sense single stranded RNA, and an icosahedral capsid (Dawson et. al., 2005). MS2 is often used as a surrogate for norovirus (Dawson et. al., 2005). In Lee et. al., 2021, the recovery of norovirus was 4.5% which is similar to the MS2 recovery in this study 4.84%.

In Fores et. al. 2021 the CP Select recovery for MS2 was 27.72 ± 24.46%, in Rusinol et. al., 2020 was much higher at 51% recovery, it was 7.82 ±8.1% for the ddPCR in this study. Fores et. al., 2021 had turbid wastewater samples that were seeded then centrifuged to remove suspended solids. The artificial seawater samples in this study had no organic matter, MS2 may

have higher recovery when it is allowed to attach to matter. The seeded MS2 was found to be 49 % in the pellet and 51 % in the supernatant (Fores et. al., 2021).

The ultrafilter pipette had higher recovery average than the 0.05 µm pipette tip. The bacteriophages are smaller viruses, and this may be why MS2's recovery improved, along with a more optimized ddPCR setting. The ultrafilter pipette did also increase adenovirus's recovery in a side-by-side comparison. The salinity/ conductivity of natural seawater was discovered to be 48 mS/cm around the same time the switch was made from 0.05 µm pipette tips to ultrafilter. The higher salinity samples had lower recoveries, but the ultrafilter tip increased the recovery to be similar to samples tested earlier in the study. Overall, the ultrafilter increased recovery for all seeded viruses but that change came with trade-offs. The smaller pipette tip (ultrafilter) lead to less volume of sample passing though.

The type and configuration of the nucleic acid and the capsid are other factors that may influence recoveries. McMinn et. al., 2021 reported OC43 recovery at 22 ±4% and Lu et. al., reported 15.5± 7.6% through the CP Select, this study found comparable results with an average recovery of 19.04 ±11.6%. Although the sample matrix differs, the range of OC43 is similar across methods. This is expected because it is the same virus, thus has the same configuration of nucleic acid. Ahmed et. al. 2021 showed the recovery of Gamma-irradiated SARS-CoV-2 in raw wastewater influent to be between 55.5 and 65%. Since OC43 and SARS-CoV-2 are both coronaviruses, it is hypothesized that their recoveries would be similar due to their type and configurations. The recovery of OC43 found in this study may be less than SARS-CoV-2, again, due to the difference in water matrix and dissolved organic matter in the samples. In Fores et. al., 2019, when testing for SARS-CoV-2 in a centrifuged sample, about 77% of the virus was found in the supernatant and 23% was found in the pellet. The recovery of bovine coronavirus (BCoV)

in Gonzalez et. al., 2020 was 5.5% and in Juel et. al., 2021 was 36.81%. Both are close to the recovery range of OC43 in this study, 19.04 ±11.6%. Since these are all coronaviruses, an estimated range of recovery through the CP Select can be deduced.

4.2: Matrix effects on virus recovery

This study is the first to test marine water through the CP Select and it was found to have differing recovery than wastewater. Several studies compare the CP Select to other concentration methods such as skim milk flocculation, ViroCap, PEG/NaCl, and others. The performance of the CP Select varied across these studies due to the methods used. Many studies used the CP Select as a secondary concentration system or concentrated the supernatant after centrifugation to prevent filter clogging. For the purposes of this study, the goal was to keep the method simple for use on a US Navy ship if necessary. Having another concentration steps may be needed for turbid samples or those with higher suspended solids such as wastewater. The average turbidity of natural marine and aquarium water samples used in this study was 0.19 NTU. In addition, the more steps in the procedure of sample concentration could cause greater virus losses.

In this study, the salinity or conductivity of the sample affected the recovery more so than pH, which did not have a significant effect at all. This may be due to the mechanism of the CP Select pipette tips. The 72 hollow fibers in each ultrafilter tip have 100 kilodaltons (KD) sized pores, trapping the particles ~ 20 nm or larger and allowing the water to pass through the machine. Once the water has been fully concentrated or the pipette becomes clogged, the wet foam elution pushes the particles in the fibers out for the filter. The small pore sizes led to increased recovery of the seeded tests but also increased clogging issues.

The artificial seawater made in the lab had difficulty dissolving the higher salt content. When these samples were concentrated there was increased cloudiness in the eluted sample.

Occasionally the salt would clog the CP Select pipette tip, this is a methods issue that was mitigated by adding salt while the nanopure water was hot and allowing it to mix overnight to get to room temperature. This did not completely solve the turbidity issue but there were fewer issues concentrating the unseeded environmental samples from San Diego Bay or John Shedd aquarium. The seeded sample was not fully processed by the ultrafilter pipette, something to consider if future studies with the CP Select use a phage tested in this study as a process control. Even though the environmental samples had higher salinity than the artificial seawater samples the recovery average was higher. While the turbidity of the environmental samples was similar or slightly lower than the artificial seawater samples these samples were associated with waters containing animals and plants and the organic matter in the environmental samples was known to be higher although not measured in this study. There were minimal clogging issues filtering 1000 mL and it was completed in an average of 22 minutes. However, with the lower recoveries the ultrafilter is recommended until further studies evaluate the 0.05 µm pipettes with natural marine waters.

4.3: Ease of Use

The CP Select is fairly user friendly and training someone new may take a few hours. The system runs itself generally taking an average of 17 mL per minute to concentrate marine water. The longer elution setting (2 pumps) was needed for adequate testing volume. There were no observed contamination issues throughout the study.

The pipette tips do clog easily which can be unfavorable when testing environmental samples where the concentrations of naturally occurring viruses are extremely low. This also adds to the cost of the consumables if multiple pipette tips and elution canisters are used to concentrate one sample. Certain seeded viruses caused the pipette tip to clog faster than others.

This may be due to higher bacterial debris in the stock virus prepared by lysis even with filtering the preparation. The stock was filtered again using a 0.22 µm membrane, but this did not mitigate the issue. There was no issue using the 0.05 µm tips to concentrate 1000 mL of unseeded natural seawater. Although, with a seeded surrogate virus process control, the amount of volume concentrated was not consistent.

4.4: Conclusion

This study furthered the scope for virus concentration method knowledge. The key finding was that the CP Select system is adequate for concentration and recovery of viruses in marine waters. DNA viruses had higher recoveries than RNA viruses and double-stranded viruses had higher recoveries than single-stranded viruses. The diameter size of the virus had less of an influence but was still notable. Salinity/conductivity affected virus recovery; the greater salt percentage correlated with lower recovery. A new ddPCR method was developed for MS2 and adenovirus.

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APPENDIX

Table S1: Characteristics of the viruses used in this study.

Virus	**MS2**	**P22**	**Phi6**	**PhiX174**	**OC43**	**Adenovirus**	**Morbillivirus**
Nucleic acid	RNA	DNA	RNA	DNA	RNA	DNA	RNA
Stranded	Single	Double	Double	Single	Single	Double	Single
Segmented	Yes	Yes	Yes	Yes	No	No	No
Sense	Positive	N/A	Positive	N/A	Positive	N/A	Negative
Enveloped	No	No	Yes	No	Yes	No	No
Diameter (nm)	23-28	60	80-100	25	80-120	70-100	150-300

Table S2: Artificial Seawater Target Test Conditions in 2020.

Physiochemical condition	**Target test condition**	**Acceptable range based on seawater condition**
pH	6.5	5.82-7.92
Conductivity (mS/cm) % Salinity	 29.16	20.04-29.79 35-36%
Turbidity (NTU)	3	0.82-3.78

Table S3: Artificial Seawater Target Test Conditions in 2022.

	Test condition
pH	8.0 ±0.2
Salinity (Converted to conductivity mS/cm)	40.0 mS/cm ±0.1
Turbidity (NTU)	1.0±0.2

Table S4: Physiochemical parameters of samples in the pilot study, culture assay results.

Sample ID	Date	Conductivity (mS/cm)	pH	Turbidity (NTU)
PM210819	8/19/2021	29.5	6.70	5.84
PM210914	9/14/2021	29.1	6.50	8.69
PM210916	9/16/2021	29.2	6.63	1.61
PM210928	9/28/2021	29.1	6.54	1.12
PM211007	10/7/2021	29.2	6.66	2.56
PM211117	11/17/2021	29.1	6.70	0.50
PM220510-1	5/10/2022	29.1	6.50	1.42
PM220510-2	5/10/2022	29.1	6.50	1.42
PM220512	5/12/2022	29.3	6.70	2.10
PP210819	8/19/2021	29.5	6.70	5.84
PP210826	8/26/2021	29.0	6.61	3.05
PP210909	9/9/2021	29.1	6.44	3.70
PP210921	9/21/2021	29.5	6.55	2.44
PP211005	10/5/2021	29.2	6.67	1.40
PP211103	11/3/2021	29.4	6.66	0.30
PP211117	11/17/2021	29.1	6.70	0.50
PP220208	2/8/2022	29.2	6.65	2.12
PP220505-1	5/5/2022	29.7	6.60	4.21
PP220505-2	5/5/2022	29.7	6.60	4.21

Table S5: Sample logistics for artificial seawater experiments run from August 2021 to May 2022.

Sample ID	Viruses seeded	Vol. Phage Seeded (uL)	Starting Sample Vol. (mL)	Vol. Concentrated (mL)	Time Take to Concentrate (mins)	Number of Pipettes Used	Number of Elutions	Final Elution Volume (mL)
PP210819	PhiX174/Phi6	1000	1000	960	35.50	1	1	0.80
PP210826	PhiX174/Phi6	1000	1000	900	26.00	3	5	2.00
PP210909	PhiX174/Phi6	1000	1000	991	39.00	2	4	1.10
PP210921	PhiX174/Phi6	1000	1000	991	48.00	2	3	1.60
PP211005	PhiX174/Phi6	1000	1000	991	57.00	2	2	1.00
PP211103	PhiX174/Phi6	1000	1000	991	61.00	3	4	0.20

Table S5 (cont'd)

PP211117	PhiX174 /Phi6	1000	1000	906	63.00	3	3	1.50
PP220208	PhiX174 /Phi6	500	500	469	55.00	4	4	5.80
PP220505-1	PhiX174 /Phi6	500	500	468	35.00	2	2	0.58
PP220505-2	PhiX174 /Phi6	500	500	494	36.00	3	3	1.00
PM210819	P22/MS 2	1000	1000	991	8.00	1	3	0.70
PM210914	P22/MS 2	1000	1000	991	53.00	3	3	1.90
PM210916	P22/MS 2	1000	1000	991	30.00	1	3	0.80
PM210928	P22/MS 2	1000	1000	991	30.00	1	2	0.60
PM211007	P22/MS 2	1000	1000	991	44.00	1	2	0.55
PM211117	P22/MS 2	1000	1000	989	55.00	1	2	0.40
PM211227F	P22/MS 2	1000	1000	854	46.00	1	2	0.30
PM220510-1	P22/MS 2	500	500	494	22.00	1	3	0.70
PM220510-2	P22/MS 2	500	500	494	17.00	1	3	0.60
PM220512	P22/MS 2	500	500	494	33.00	2	2	1.10

*All samples were in artificial seawater matrix and Master 1 CP Select setting.

Table S6: Shedd aquarium sample physiochemical data.

Sample ID	Shedd Sample ID	Animals Present	Date of Collection	Temp at time of collection (°C)	pH at time of Collection	Turbidity (NTU)	Conduct.(mS/cm)
NegOC 8-11	Oceanarium Misty Whale	dolphins and whales	7/28/2022	16.9	7.6	0.3	47.2
NegG3 8-11	Oceanarium Secluded Bay	dolphins and whales	7/28/2022	16.9	7.6	0.39	45
OC220811-1	Oceanarium Misty Whale	dolphins and whales	7/28/2022	16.9	7.6	0.3	47.2
OC220811-2	Oceanarium Secluded Bay	dolphins and whales	7/28/2022	16.9	7.6	0.39	45
G3220811-1	Ocean Gallery G3R38	no dolphins	7/28/2022	13.5	8.2	1.48	58.2
G3220811-2	Ocean Gallery G3E57	no dolphins	7/28/2022	13.5	8.2	0.36	48.6
G3220811-3	Ocean Gallery G3E60	no dolphins	7/28/2022	13.5	8.2	0.22	49.5
G3220811-4	Ocean Gallery Kelp Forest	no dolphins	7/28/2022	13.5	8.2	0.58	49.4
Neg 8-16	Ocean Gallery G3E59	no dolphins	7/28/2022	13.5	8.2	0.12	NA
OC220816	Oceanarium Grainge (GE)	dolphins and whales	7/28/2022	16.9	7.6	0.27	45.7
G3220816	Ocean Gallery G3E59	no dolphins	7/28/2022	13.5	8.2	0.12	NA

*All samples were Lake Michigan water combined with Instant Ocean salts.

Table S7: Comparison of MS2 recovery in artificial seawater, with and without 10% Tween-20.

Sample ID	10% Tween-20 added?	Vol Conc. (mL)	Elution Vol. (mL)	Input (GC/mL)	Output (GC/mL)	MS2 Recovery %
TM221130-1	Yes	500	1	3.75E+07	1.58E+05	0.42
TM221130-2	Yes	500	0.9	3.77E+07	1.11E+05	0.30
TM221130-3	Yes	500	0.85	4.25E+07	2.54E+05	0.60
AVERAGE				3.92E+07	1.74E+05	0.44
UAM221010-1	No	170	1.75	6.88E+06	1.18E+05	1.71
UAM221010-2	No	233	1.3	9.29E+06	6.95E+04	0.75
UAM221010-3	No	192	1.8	6.42E+06	5.22E+04	0.81
AVERAGE				7.53E+06	7.99E+04	1.09

*Master 2 setting and ultrafilter tip used for all samples