Testing of methods to isolate live-infectious viruses from seawater

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Abbreviations

ASK	Atlantic Salmon Kidney cell line
cDNA	complementary deoxyribonucleic acid
CHH-1	Chum Salmon Heart cell line
CHSE-214	Chinook salmon embryo cell line
CPE	Cytopathic effect
Ct-value	Cycle threshold value
DNA	Deoxyribonucleic acid
FBS	Fetal Bovine Serum
g / RCF	The relative centrifugal force
gr	Grams
HPR	Hypervariable region
IFAT	Indirect fluorescent antibody technique
IMR	Institute of Marine Research
IPN	Infectious Pancreatic Necrosis
IPNV	Infectious Pancreatic Necrosis Virus
ISA	Infectious Salmon Anaemia
ISAV	Infectious Salmon Anaemia Virus
L15	Leibovitz´s L-15 Medium
NaCl	Sodium Chloride
NTC	No template control
OIE	The World Organization for Animal Health
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase Chain Reaction
PD	Pancreas disease
PEG	Polyethylene glycol
PFA	Paraformaldehyde
QTL	Quantitative trait locus
Real-time RT-PCR	Real time reverse transcriptase polymerase chain reaction
RNA	Ribonucleic acid
RT	Room temperature
SAV	Salmonid alphavirus
SPDV	Salmon Pancreas disease virus
SW	Seawater
UF	Ultrafiltration membrane
VHS	Viral Hemorrhagic Septicemia
VHSV	Viral Hemorrhagic Septicemia Virus

Abstract

Viral infections are a major issue in Norwegian aquaculture and are the cause of mortalities, reduced slaughter quality and great economic losses. Some of the viruses of concern in Norway are infectious salmon anemia virus (ISAV), salmonid alphavirus (SAV), infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV). Current detection of viruses is largely dependent on tissue and organ sampling from fish followed by molecular analysis. The existing methods for virus detection are resource-demanding and time-consuming, so easier methods for detection of virus from the seawater would be beneficial. Several filtration-based methods for detection of aquatic viruses from seawater, like the Virus-absorption-elution (VIRADEL) technique, chemical flocculation, and the use of ultrafiltration membranes, has been described, but most of these methods only used RT-qPCR to detect viral RNA, so it is unknown if the detected virus is infectious.

The SARS-CoV-2 pandemic has led to the development of cheaper, easier, and faster methods for virus isolation and detection from wastewater, including bead-based methods. There has also been a development in the use of polyethylene (PEG) based methods. In this project, one bead-based method, Nanotrap Magnetic Virus Particles, and two PEG-based methods, Intact Virus Precipitation Reagent) and Lentivirus Concentrator Solution, were tested in their ability to isolate virus from medium or seawater spiked with ISAV, SAV, IPNV or VHSV by RT-qPCR. The Lentivirus and the Nanotrap method were also tested to isolate live-infectious virus (ISAV and IPNV) by infecting cells with isolated virus from both small and larger volumes of seawater and analyzing them by the Indirect Fluorescence Antibody Technique (IFAT) or cytopathic effect (CPE).

This project show that virus was detected by RT-qPCR from both medium and seawater using all three methods. Both the Lentivirus and the Nanotrap method were able to isolate live infectious ISAV and IPNV from small and larger volumes of seawater. Both these methods can be used for detection of live infectious virus from environmental and fish lab samples, but further testing is still needed.

1 Introduction

1.1 Norwegian aquaculture

The Norwegian fish farming industry has expanded rapidly since the 1970s and has now become one of the world's leading producers of Atlantic Salmon (*Salmo Salar*), trout (*Salmo trutta)* and rainbow trout (*Oncorhynchus mykiss*). Due to the rapid growth of the industry in Norway, there has also been an increase in the challenges regarding fish health and welfare. These challenges can lead to great economic losses due to mortality, reduced growth, expensive treatments, and reduced quality of the fish produced (Oliveira *et al.*, 2021). In 2021, 338 million Atlantic salmon were transferred to the sea phase of the production (Data smolt sea transfer 1994-2021[1]). 60 million fish died in the same phase (Data Production loss 1994-2021[2]) (The Norwegian Directorate of Fisheries, 2022). To further expand the Norwegian aquaculture industry and to ensure sustainable development of production, it is crucial to reduce fish mortality and improve fish health and welfare by finding solutions to some of the most challenging pathogens and other welfare issues. According to the annual Fish Health Report from the Norwegian Veterinary Institute, sea lice infestations, bacterial diseases, injuries due to handling and mechanical treatments, and viral diseases are some of the major health issues in salmon today (Sommerset *et al.*, 2023).

1.2 Viral pathogens affecting aquaculture

Some of the pathogens causing problems in aquaculture are marine viruses. Several viruses cause severe diseases in aquaculture today, and virus-related diseases are considered to be one of the major causes of mortality in the marine phase of farmed Atlantic salmon (Sommerset *et al.*, 2020). Virus infections can also lead to great economical losses due to increased mortality, reduced quality, and need for implementation of biosecurity measures. Some of the major viral diseases today are pancreas disease caused by salmonid alphavirus (SAV), infectious salmon anemia (ISA) caused by salmon anemia virus (ISAV), Cardiomyopathy syndrome (CMS) caused by Piscine myocarditis virus (PMCV) and heart and skeletal muscle inflammation (HSMI) caused by Piscine Orthoreovirus (PRV) (Sommerset, *et al.,* 2023). Other viruses that can be a potential threat to aquaculture are infectious pancreatic necrosis virus (IPNV) and viral hemorrhagic septicemia virus (VHSV).

[1] Data smolt sea transfer 1994-2021
[2] Data Production loss 1994-2021

1.2.1 Infectious salmon anaemia virus (ISAV)

Infectious salmon anemia virus (ISAV) is an aquatic orthomyxovirus, structurally similar to the influenza virus (Díaz *et al.*, 2014). The virus is helical and enveloped and consists of 8 segments of negatively charged single stranded RNA (Figure 1.1) (Plarre, 2011; Rimstad *et al.*, 2011). The first six segments contain one open reading frame (ORF) each, while segment seven and eight contains at least two ORFs each (Plarre, 2011; Rimstad and Markussen, 2020). The genome codes for at least ten proteins; four main structural proteins (Matrix protein (M), nucleoprotein (NP), hemagglutinin-esterase (HE) and fusion protein (F)), four minor structural proteins and two non-structural proteins (Plarre, 2011).

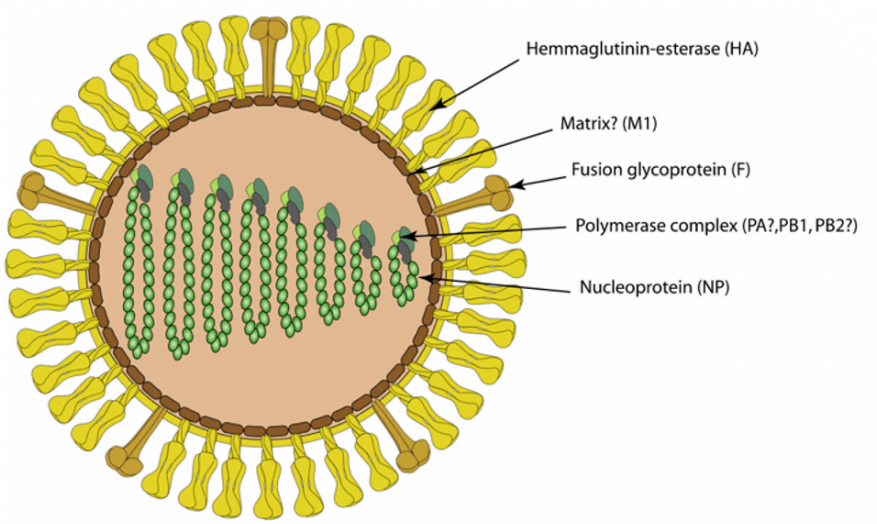

Figure 1.1: Illustration of the structure of the ISA virus and the main proteins; Matrix protein, Fusion glycoprotein, nucleoprotein, Hemmaglutinin-Esterase, and the polymerase complex. The illustration is reproduced from: https://viralzone.expasy.org/95

There are two forms of ISAV; a virulent form, called HPRΔ, that causes severe disease; and a low virulent form, called HPR0, which is more widespread and prevalent but does not cause obvious disease. The ISAV HPR0 has been detected in both brood stock, fry, parr, smolt and adult salmon, and is found in both fresh water and seawater (Nylund *et al.*, 2019). ISAV HPRΔ is proposed to develop from HPR0 through mutations in the hypervariable region (HPR) of the hemagglutinin-esterase protein and the fusion protein, which means that the low virulent HPR0 is a continuous source to the virulent HPRΔ ISAV (Nylund *et al.*, 2019).

ISAV HPRΔ causes the disease infectious salmon anemia, which is a notifiable, serious, and contagious disease in Atlantic salmon and trout. The virus first establishes in the fish´s gills and skin, before an infection emerges and attacks the circulatory system, which can lead to serious anemia and other signs of infectious salmon anemia. The target cells for the infection are endothelial cells in the blood vessels, endocardial cells in the heart, leucocytes, and gill epithelial cells (Plarre, 2011; Rimstad and Markussen, 2020). Most ISA outbreaks happen in the seawater phase during the spring or late fall, most often in periods with temperatures between 10 and 15 degrees (Plarre, 2011; Rimstad and Markussen, 2020), but the disease appears throughout the year as well (Rimstad and Markussen, 2020).

ISA cause increased mortality, serious anemia with thin and watered-down blood and various signs of circulatory failure. Some common clinical signs are pale gills due to anemia, exophthalmia, eye-bleedings, and bleedings in the skin and internal organs. Infected fish are also often lethargic (Rimstad and Markussen, 2020). During dissection of infected fish, common signs are oedema and ascitic fluids in the visceral cavity and congestion of liver, spleen, and gut. The liver is often dark, pale, or yellowish and the kidneys and spleen swollen in the more serious cases (Plarre, 2011; Rimstad and Markussen, 2020). Necrosis and petechia in the visceral fat are also common findings.

Salmonids are the only known reservoir species for ISAV, and Atlantic salmon and trout are the only natural hosts where replication of the virus can happen (Plarre, 2011; Nylund *et al.*, 2019). The virus is transmitted both horizontally and vertically (Nylund *et al.*, 2019) and is mostly a problem in the salmonid farming industry. Outbreaks has been reported from most of the salmon producing countries in Northern Europe and North and South America (Rimstad *et al.*, 2011; Dean *et al.*, 2022).

In Norway, ISAV was first detected from an outbreak in a hatchery in Bremnes in 1984 (Marshall *et al.*, 2014; Dean *et al.*, 2022). After this outbreak, there was a steady increase in detections until they reached a peak in 1991, when 80 outbreaks were reported (Dean *et al.*, 2022). In 1991 several measures were initiated to reduce the number of outbreaks (Rimstad *et al.*, 2011). These measures included the implementation of combat zones, increased distance between farming locations, regulations that made sure that there was only one generation of fish in each farm and fallowing of farms that had a disease outbreak (Plarre, 2011; Rimstad *et al.*, 2011; Nylund *et al.*, 2019). There was also an increased focus

on hygiene, risk assessments and disinfection of water used in production. All these measures resulted in a considerable decrease in annual outbreaks to around 10 to 20 (Plarre, 2011). An overview of the Norwegian outbreaks of ISAV in the period 2018 to 2022 is given in Figure 1.2.

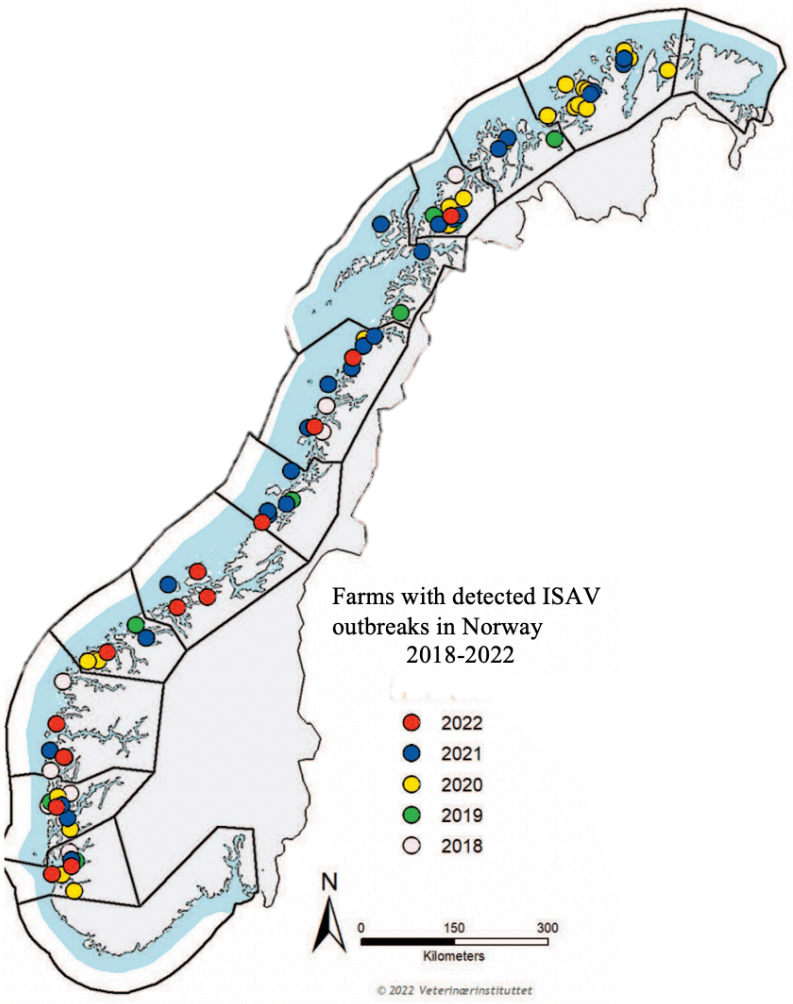

Figure 1.2: A map of Norway showing the seawater sites that had outbreaks of ISAV in the period 2018-2022. The figure is reproduced from the Fish Health Report 2022, from the Norwegian Veterinary Institute.

A primary diagnosis is based on clinical symptoms and by observations from the facility, combined with characteristic histological and histopathological changes, but due to several differential diagnoses, the agent must be detected through virus detection in tissue samples from various organs by real-time RT-PCR or other methods like IFAT, immuno-histochemistry or *in situ* hybridization (Dean *et al.*, 2022). Outbreaks of ISAV are regulated with strict measures, such as establishment of control zones and observational zones with systemic monitoring and frequent sampling immediately around the location with the detected

outbreak. The disease is usually treated by extermination of the entire population in the farm and fallowing of the location to limit the virus spreading to other facilities (Rimstad and Markussen, 2020). An important preventive measure is to remove ISAV HPR0 from the brood stock (Nylund *et al.*, 2019).

1.2.2 Salmonid alphavirus (SAV)

Salmonid alphavirus (SAV), which is also called Salmon Pancreas disease virus (SPDV) belongs to the family Togaviridae and genus Alphavirus (Jarungsriapisit *et al.*, 2016a; Mendes and Kuhn, 2018). SAV is a small, spherical, icosahedral, enveloped, and single stranded positive sense RNA virus (Strauss and Strauss, 1994; Jansen *et al.*, 2010; Graham and Mcloughlin, 2011). An illustration of the structure of the virus is shown in Figure 1.3.

The genome has two open reading frames (ORFs) that encodes capsid proteins and structural glycoproteins (E1-E3 and 6K) and four non-structural proteins (nsP 1-4) (Weston *et al.*, 1999; Mcloughlin and Graham, 2007; Fringuelli *et al.*, 2008; Karlsen *et al.*, 2015). The virus enters the cells via receptor mediated endocytosis, and replication occurs in the host cells cytoplasm. During the process, they acquire a lipid envelope developed from glycoproteins E1 and E2, arranged in spikes used for budding through host plasma membrane when they egress after replication (Mcloughlin and Graham, 2007). The interactions between the capsid and the glycoproteins are probably how budding occurs (Karlsen *et al*. 2015). Six subtypes of SAV, SAV 1-6, have been identified based on the nucleic acid sequences of the nsp3 and E2 gene (Jarungsriapisit *et al.*, 2016a; Jansen *et al.*, 2017; Gallagher *et al.*, 2020). SAV subtypes 1, 4, 5 and 6 have caused outbreaks of disease in Ireland or Scotland (Mcloughlin and Graham, 2007; Jansen *et al.*, 2010; Gallagher *et al.*, 2020). Only two of them, SAV2 and SAV3, is known to cause disease in Norway (Hjortaas *et al.*, 2016). PD was first described in Scotland in the end of the 1970s (Jansen *et al.*, 2010; Soares *et al.*, 2019) and from Norway in 1989 (Poppe, Rimstad & Hyllseth, 1989).

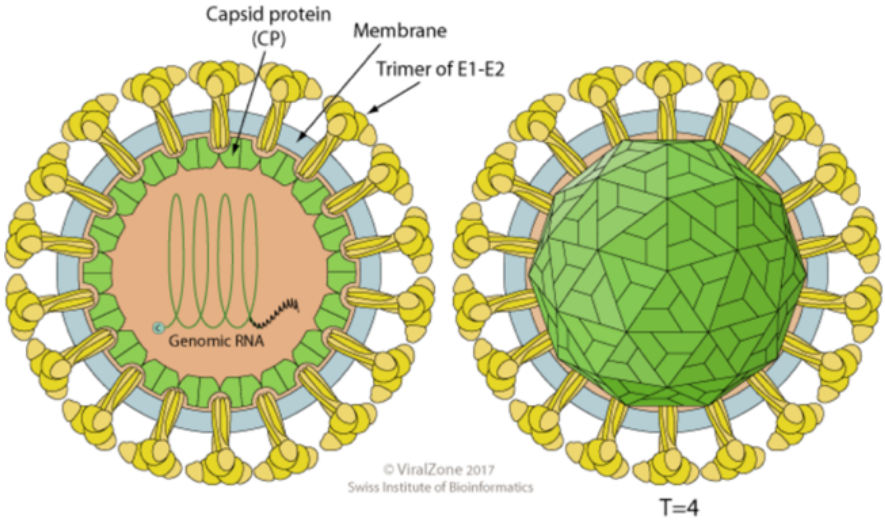

Figure 1.3: Illustration of the general icosahedral structure of the salmonid alphavirus and some of the proteins involved. Reproduced from: https://viralzone.expasy.org/3

Before 2011, only SAV3 was isolated from PD outbreaks in Norway (Jansen *et al.*, 2010; Hjortaas *et al.*, 2016; Jarungsriapisit *et al.*, 2016a) and SAV3 has so far only been detected in Norway, from Atlantic salmon and Rainbow trout in seawater. In 2011, the first case of SAV2 was detected in Atlantic salmon in Nord-Trøndelag, Norway at a marine site (Hjortaas *et al.*, 2016). A second outbreak of SAV2 was shortly after the first outbreak detected in Møre og Romsdal (Hjortaas *et al.*, 2016). SAV2 had earlier only been found causing sleeping disease in rainbow trout reared in fresh water in continental Europe and the UK (Hjortaas *et al.*, 2016; Jansen *et al.*, 2017). Today, SAV2 and SAV3 are both present in distinct geographic regions of Norway and spread along the Norwegian coastline. SAV2 is present in Trøndelag, while SAV3 is present in Rogaland and Vestland (Gallagher *et al.*, 2020). Møre og Romsdal have outbreaks of both subtypes. A small number of SAV2 outbreaks have also occurred in Vestland (Gallagher *et al.*, 2020). Sporadic outbreaks of PD have also been recorded from the northernmost counties in Norway (Karlsen *et al.*, 2006; Hjortaas *et al.*, 2016). An overview of the geographic distribution of the PD outbreaks in Norway (SAV2 and SAV3) is given in Figure 1.4.

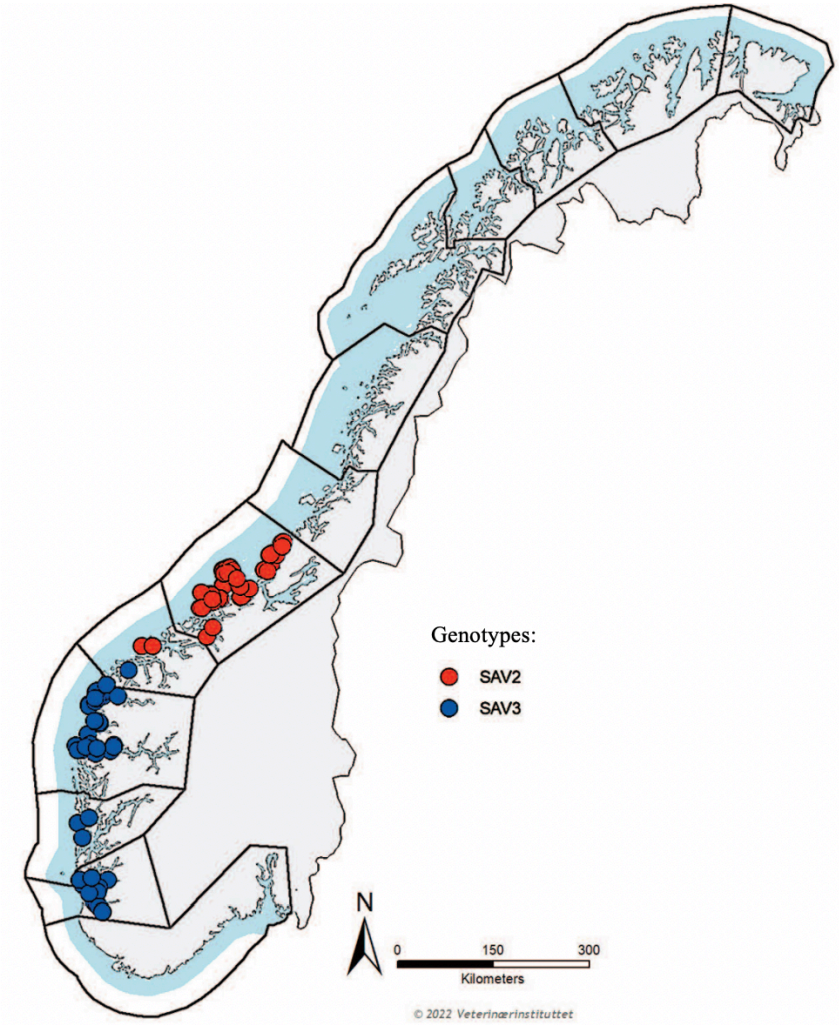

Figure 1.4: A map over the detected PD outbreaks (SAV2 and SAV3) in Norway in 2022. The figure is reproduced from the Fish Health Report 2022, from the Norwegian Veterinary Institute.

Pancreas disease is a serious and economically damaging disease that causes huge problems in the seawater phase of salmon farming industry in Northern Europe, especially in Norway, Ireland, and Scotland. The disease cause damage to the pancreas and leads to inflammation in the heart and skeletal muscles (Graham and McLoughlin, 2011). Typical signs of disease in affected fish are lethargy, loss of appetite and reduced growth due to pancreas failure and change in swimming pattern due to muscle damage (Mcloughlin and Graham, 2007; Jansen *et al.*, 2017). Petechial haemorrhages can also be seen over the pyloric caeca and surrounding fat (Mcloughlin and Graham, 2007). Typical internal signs of disease are empty intestines or yellow mucoid gut content and signs of circulatory failure as well as faecal casts (Mcloughlin and Graham, 2007; Graham and Mcloughlin, 2011; Jansen *et al.*, 2017). Common histopathological findings are necrosis and often complete loss of exocrine pancreatic acinar tissue, inflammation and necrosis in the heart and inflammation and degeneration of skeletal

muscles (Mcloughlin and Graham, 2007; Karlsen *et al.,* 2015; Hjortaas *et al.*, 2016; Jansen *et al.*, 2017). Kidney lesions have also been described from affected fish in late stages of PD in Norway (Mcloughlin and Graham, 2007). The distribution and severity of lesions depend on the stage of infection (Mcloughlin & Graham, 2007).

Mortality is highly variable and can range from insignificant to up to 63% (Jansen *et al.*, 2017; Soares *et al.*, 2019). Norwegian outbreaks caused by SAV3 have significantly higher mortality levels compared to outbreaks caused by SAV2 (Jansen *et al.*, 2017). PD can lead to great economic losses and welfare problems in aquaculture due to mortality, poor growth in surviving fish and reduced slaughter quality because of the muscle damage and damaged pancreas (Graham and Mcloughlin, 2011; Hjortaas *et al.*, 2016).

Most outbreaks are diagnosed in the period between May and October, but disease outbreaks can occur at any time of the year, in all stages of the marine production cycle (Mcloughlin and Graham, 2007; Jansen *et al.*, 2017). A diagnosis of PD can be made based on clinical signs and characteristic histopathology combined with detection of the virus using PCR, immunohistochemistry, real time RT-PCR or cultivation of virus in cell culture (Mcloughlin and Graham, 2007; Jansen *et al.*, 2017). The gills, pseudobranch and the heart are the most suitable tissues for SAV-detection in all stages of the disease (Andersen *et al.* 2007*;* Andersen, 2012).

SAV spreads through seawater actively through human activity, like movement of infected fish, or passively through natural water currents. PD occurs mainly through horizontal transmission (Jarungsriapisit *et al.*, 2016a) and the main reservoir of SAV is infected farmed salmonids (Jarungsriapisit *et al.*, 2016a; Gallagher *et al.*, 2020). PD is a stress related disease, which means that fish can be infected with SAV with no signs of disease prior to a stressful event. A stressful event, like handling, movement, treatments or change in water quality, can trigger the infection to develop into serious disease (Jansen *et al.*, 2017). To reduce the risk of PD it is important to avoid contact between infected and uninfected populations, and to ensure good biosecurity in transport and use of personnel, boats, and equipment. It is also important with good hygiene and careful management to avoid stress and to improve fish health. Dietary management is also used to reduce stress (Graham & Mcloughlin, 2011), and special feeds to strengthen the immune system during PD outbreaks are available, but the effect is not documented (Jansen *et al.*, 2017). Other factors that contribute to a reduced risk for PD

outbreaks is fallowing between cohorts and production cycles, biosecure slaughter of infected fish, low stock densities in pens and vaccination (Jansen *et al.*, 2017).

A monovalent PD vaccine based on inactivated SAV1 has been available since 2007. In 2015 a seven-component vaccine, AquaVac® PDt, MSD Animal Health, became commercially available in Norway (Jansen *et al.*, 2017). In 2018, a 1-component DNA vaccine for PD, called CLYNAV™ from Elanco, was approved for sale in Norway. CLYNAV™ consists of a recombinant DNA-plasmid that is taken up in the cells around the place of intramuscular injection and encodes for proteins from SAV3. The purpose of this vaccine is active immunization of Atlantic salmon to reduce mortality, damage to pancreas and heart and skeletal muscles, and reduce inhabitation of growth due to PD caused by SAV3. Vaccination against PD reduces the risk of PD and the disease severity by reducing the number of outbreaks, mortality and the number of fish discarded at slaughter (Jansen *et al.*, 2017). Studies have also shown that infected PD vaccinated salmon shed lower levels of SAV into the water compared to unvaccinated fish from feces and mucus, but complete efficiency and immune response is still not known entirely. (Skjold *et al.*, 2016; Jansen *et al.*, 2017). A high vaccination coverage could contribute to reduction in the infection pressure in endemic areas (Jansen *et al.*, 2017).

In Norway, PD was made a notifiable disease in 2007, and a large part of western Norway became defined as an endemic PD zone for SAV3 (Jansen *et al.*, 2017). A second endemic zone for marine SAV2 was established in 2012, north of the SAV3 zone (Jansen *et al.*, 2017). There have been sporadic outbreaks of PD north of the zones, but they have been controlled by slaughter or relocation to within the endemic zone, which have prevented further spread (Jansen *et al.*, 2017). Sites within a 10 km radius of an infected site will be sampled monthly over a period to reduce risk of local spread (Jansen *et al.*, 2017). Most of the SAV2 outbreaks occur in Nordmøre and Sør-Trøndelag, while the SAV3 outbreaks are registered mostly between Ryfylke and Stad (Hjortaas et al., 2016; Sommerset, *et al.*, 2023). In 2022, 98 new outbreaks were registered in Norway. 49 of the outbreaks were SAV2 and 49 SAV3. While there has been a significant decrease in the number of total outbreaks of PD the last two years, there has been an increase in the number of marine SAV2 outbreaks (Sommerset, *et al.*, 2023). The annual numbers of outbreaks in Norway in the period 2007-2022 are given in Figure 1.5 (SAV2) and Figure 1.6 (SAV3). The figures are reproduced from the Norwegian Veterinary Institute´s Fish Health Report 2023 (Sommerset, *et al.*, 2023).

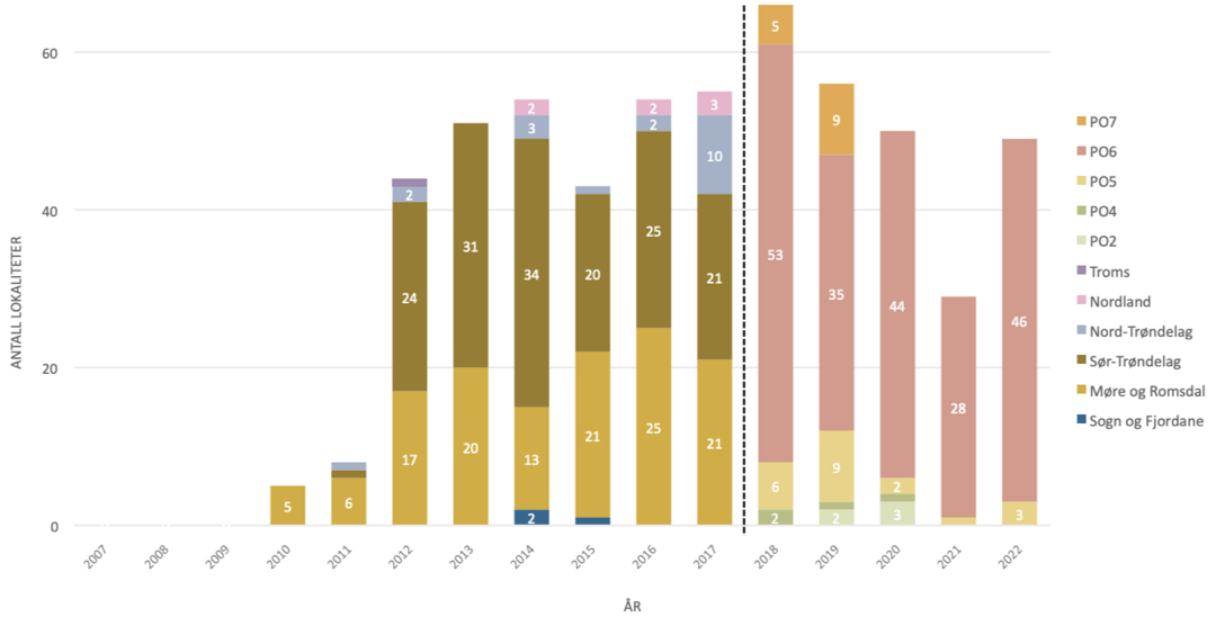

Figure 1.5: The number of new outbreaks of SAV2 in each county in Norway in the period between 2007 and 2017 and the number of new outbreaks in each production zone between 2018 and 2022. The figure is reproduced from the Fish Health Report 2022, from the Norwegian Veterinary Institute

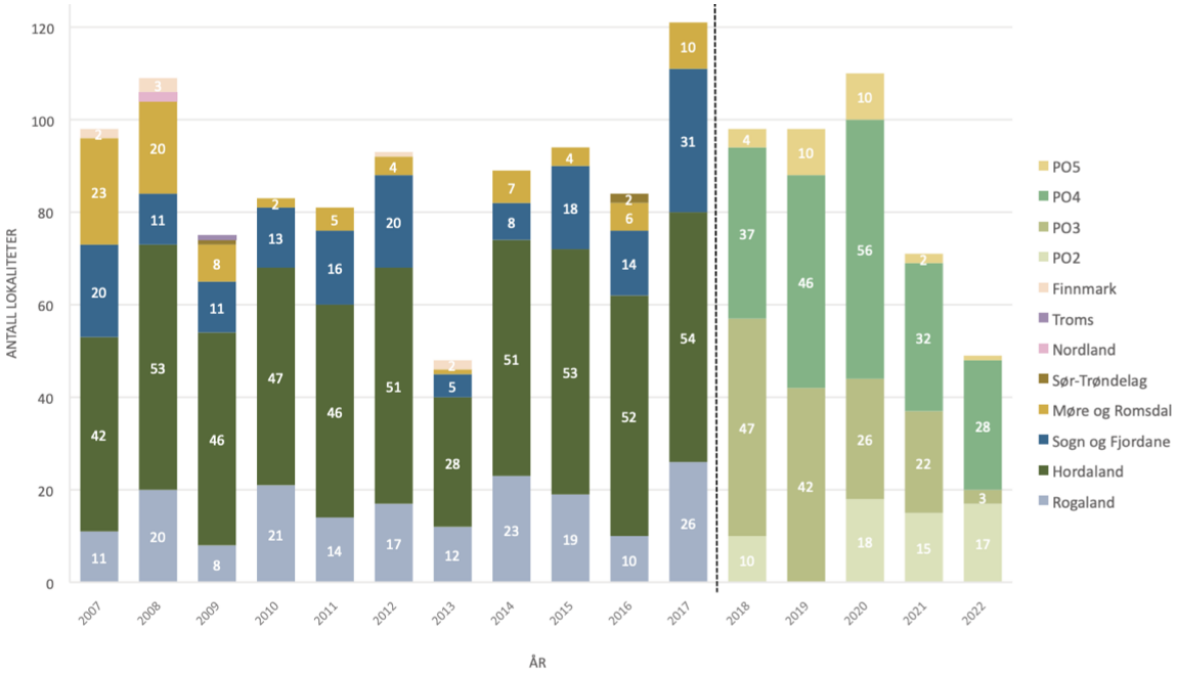

Figure 1.6: The number of new outbreaks of SAV3 in each county in Norway in the period between 2007 and 2017 and the number of new outbreaks in each production zone between 2018 and 2022. The figure is reproduced from the Fish Health Report 2022, from the Norwegian Veterinary Institute

1.2.3 Infectious pancreatic necrosis virus (IPNV)

Infectious pancreatic necrosis virus (IPNV) is a non-enveloped icosahedral virus with two segments (A & B) of double stranded, linear RNA genome (Cohen, Poinsard and Scherrer, 1973; Dopazo, 2020; Tapia *et al.*, 2022). The structure of the virus is illustrated in Figure 1.7. The genome encodes five viral proteins, named Vp 1-5. Three of the proteins are structural, and the other two are an RNA-dependent RNA polymerase and a non-structural protein (Woo et al., 2011; Dopazo, 2020; Tapia et al., 2022). Segment A of the genome contains two open reading frames (ORFs) that encodes for a polyprotein that comprises most of the viral proteins (VP 2, 3, 4 and 5). Segment B has one ORF that encodes the RNA-dependent RNA-polymerase, VP1, needed for genome replication and transcription (Santi, Vakharia and Evensen, 2004; Woo *et al.*, 2011; Dopazo, 2020).

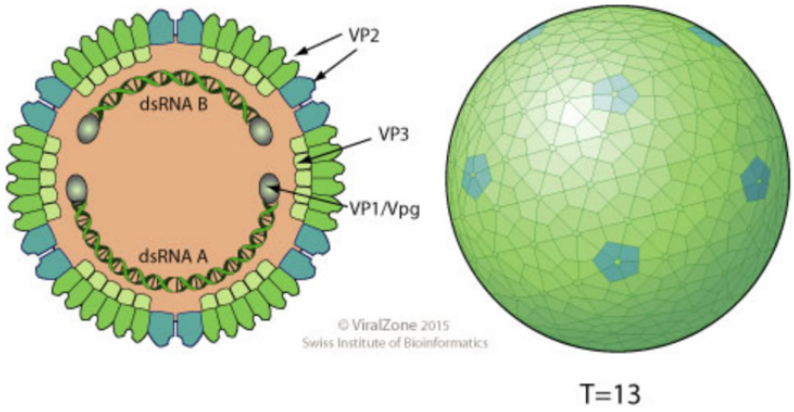

***Figure 1.7*:** Illustration of the general structure of the IPN virus and some of the viral proteins. The figure is reproduced from: https://viralzone.expasy.org/571

The virus belongs to the family Birnaviridae and the genus Aquabirnavirus (Woo *et al.*, 2011; Dopazo, 2020). IPNV is very abundant and found in almost every salmon producing country all over the world in both fresh water and sea water, in wild population and in aquaculture. IPNV was also the first virus to be isolated from teleosts, from brook trout in the US in 1957 (Wolf and Quimby, 1971; Santi, Vakharia and Evensen, 2004; Tapia *et al.*, 2022). The virus is the causative agent of a contagious disease, infectious pancreatic necrosis (IPN), considered to be one of the most important diseases in salmon production in Europe (Roberts and Pearson, 2005; Dopazo, 2020). It can lead to high mortality in first-feeding fry and post-smolts after sea-transfer in Atlantic salmon (*Salmo salar*), rainbow trout (*Oncorhynchus mykiss)* and other salmonid species (Roberts and Pearson, 2005; Woo *et al.*, 2011). IPNV is most infectious to salmonid species, and the term IPNV is only used for the virus strains that

affects salmonids and cause specific symptoms. The virus has been isolated from other species as well, but when the virus affects non-salmonids, it is called IPN-like disease (Woo *et al.*, 2011; Dopazo, 2020).

Individuals that survive an infection can persist as asymptomatic carriers of the virus, and function as a reservoir for the virus by spreading the virus via feces and gonadal fluids, especially during stressful periods (Bowden, Smail and Ellis, 2002; Woo *et al.*, 2011; Dopazo, 2020). IPNV is transmitted efficiently both vertically and horizontally, through the gills, intestinal epithelium or through the skin into the water (Dopazo, 2020; Tapia *et al.*, 2022). The virus can enter blood leucocytes and spread to the main target organs which are the pancreas, liver, and kidney, but also to the spleen, heart, brain, skin, and reproductive cells (Dopazo, 2020).

Typical signs of IPN disease, other than increased mortality, are a combination of behavioral changes and macroscopic and internal lesions. The main behavioral change is abnormal erratic corkscrew swimming (Woo *et al.*, 2011; Dopazo, 2020). Anorexia, exophthalmia, petechial hemorrhages, and skin darkening in post-smolts are also common signs of disease. Distended abdomen due to accumulation of ascitic fluids are also common (Santi, Vakharia and Evensen, 2004; Roberts and Pearson, 2005; Woo *et al.*, 2011; Dopazo, 2020). Histopathological signs include focal necrotic lesions in the exocrine pancreas, due to acinar cell necrosis, necrosis of intestinal mucosa, liver necrosis and leukocyte infiltration, and damage of hepatic tissue (Woo *et al.*, 2011; Bruno, Noguera and Poppe, 2013; Dopazo, 2020).

Due to the significant economic impact of the disease in salmonid farming, major taken efforts have been made to control IPN (Dopazo, 2020), which have led to reduced losses, but the disease is still a considerable problem in aquaculture, both in hatcheries and after sea-transfer (Roberts and Pearson, 2005). Because of the vertical transmission via the eggs, brood stocks are routinely tested for the presence of the virus in kidney or gonadal fluids and are not used for egg production if they test positive. Eggs are also disinfected. Hatcheries use IPNV-free water when possible (Roberts and Pearson, 2005). Control of movements and better transfer systems of the fish, specific diets, risk assessments and vaccines have also contributed to the reduced losses (Woo *et al.*, 2011; Dopazo, 2020). Improved biosecurity strategies have also been implemented to reduce horizontal transmission and stress factors for the fish

(Dopazo, 2020). Genetically resistant fish have been developed through identification of a quantitative trait locus (QTL) for resistance to IPN in Atlantic salmon. Breeding of brood stock and roe with this trait has led to a considerable reduced number of infected fish (Roberts and Pearson, 2005; Tapia *et al.*, 2022).

1.2.4 Viral hemorrhagic septicemia virus (VHSV)

Viral hemorrhagic septicemia virus (VHSV) belongs to the genus Novirhabdovirus and family Rhabdoviridae (Einer-Jensen *et al.*, 2004; Duesund *et al.*, 2010). The VHS virus genome is enveloped, and consists of a non-segmented, negative-sense, linear and single stranded RNA (Figure 1.8). The genome consists of six open reading frames encoding a non-structural protein (NV) and five structural proteins; nucleoprotein (N), phosphoprotein (P), Matrix protein (M), glycoprotein (G) and RNA-polymerase (L) (Schutze, Mundt and Mettenleiter, 1999; Einer-Jensen *et al.*, 2004; Duesund *et al.*, 2010; Smail and Snow, 2011; He *et al.*, 2014; Walker *et al.*, 2018). VHSV has been isolated from more than 80 fish species globally in both freshwater and seawater, in wild fish populations and in aquaculture at the Northern Hemisphere (Munro et al., 2015, OIE, 2021). The large distribution both globally and in susceptible species shows a high ability of evolution of new strains with higher virulence and host adaption, which makes it a risk in fish farming (Sandlund *et al.*, 2014).

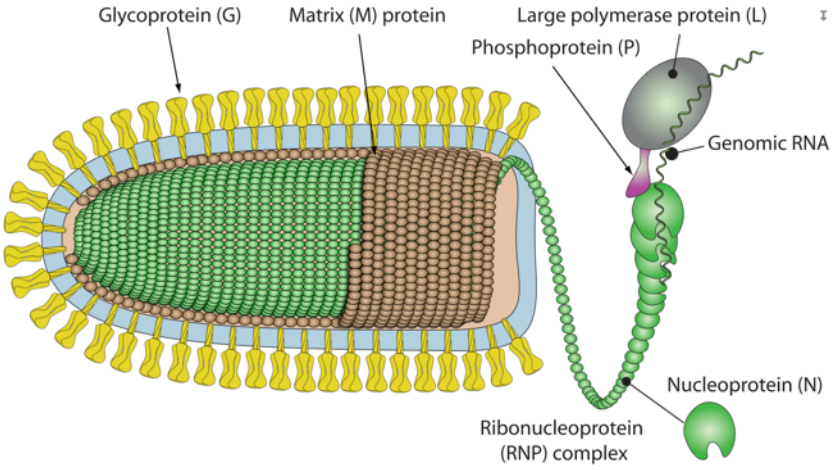

Figure 1.8: Illustration of the general structure of the VHS virus and the five structural proteins the genome encodes for. Reproduced from: https://viralzone.expasy.org/76?outline=all_by_species

The VHSV isolates have been divided into four main genotypes; I, II, III and IV, based on phylogenetic studies of the nucleoprotein and glycoprotein encoding genes (Einer-Jensen *et al.*, 2004; Dale *et al.*, 2009; Smail and Snow, 2011; Munro *et al.*, 2015). The distribution of the genotypes is based on geographical origin more than host specificity (Snow *et al.*, 2004; Smail and Snow, 2011). Some of the genotypes are known to cause disease in one species, while others have a broad host register. Genotype Ia is highly virulent for rainbow trout and is the genotype that causes the biggest worries in freshwater farms in Europe (Smail and Snow, 2011). Genotypes I, II and III are found in Europe and genotype IV includes isolates restricted to the North American and Northern Pacific waters. The seawater isolates are represented in all genotypes, while the freshwater isolates all belong to genotype I or IV (Snow *et al.*, 2004; Dale *et al.*, 2009; Duesund *et al.*, 2010; Sandlund *et al.*, 2014; OIE, 2021).

The virus can cause severe infectious and deadly disease outbreaks, especially in rainbow trout (*Oncorhynchus mykiss*) in freshwater farms in western Europe. The disease, which can lead to great economic losses, is declared a notifiable disease by the World Organization for Animal Health (OIE) (Einer-Jensen *et al.*, 2004; Smail and Snow, 2011; Munro *et al.*, 2015). VHS is still considered to be one of the most serious viral diseases in aquaculture, causing both an acute phase of the disease characterized by haemorrhagic septicaemia, and a chronic phase characterized by nervous symptoms (Skall, Olesen and Mellergaard, 2005; Sandlund et al., 2014; Guðmundsdóttir et al., 2019; OIE, 2021). In the acute phase, the affected fish display abnormal swimming behaviour like spiral swimming or flashing, darkening of the skin, pale gills due to anaemia, exophthalmia, and haemorrhages at the base of the fins and on the gills, eyes, and skin (Skall, 2005, OIE). The fish is also often slow and lethargic (Skall, Olesen and Mellergaard, 2005). The necropsy findings often include petechial bleedings in internal organs and extensive haemorrhages in muscles. A grey, pale liver and a swollen, dark spleen are also common findings (Skall, Olesen and Mellergaard, 2005; Guðmundsdóttir et al., 2019). There is currently no available vaccine, and the disease is controlled by "stamping out", which means that the whole population that is infected is slaughtered (Sommerset, *et al.*, 2022).

Mortality depends on environmental and physiological conditions, but also the age of the fish. It may be up to 100% in fry, but typically between 30 and 70% in older fish. VHS is a cold-water disease, so mortality is highest at temperatures around 9-12 °C (Skall, Olesen and

Mellergaard, 2005). Transmission of the virus primarily occurs horizontally through water, excreted in the urine and ovarian fluids, or directly from the skin (Smail and Snow, 2011). VHS outbreaks mainly occur in freshwater rainbow trout farms, but there have also been a few outbreaks in European sea farms (Sandlund *et al.*, 2014). The last outbreak of VHS in Norway occurred in a seawater site rearing rainbow trout in Storfjorden, Sunnmøre in November 2007, caused by genotype III (Dale *et al.*, 2009; Sandlund *et al.*, 2014). The outbreak in Storfjorden was the first in Norway since a control program was implemented and the disease was eradicated in 1974 (Dale *et al.*, 2009; Sandlund *et al.*, 2014).

1.3 Benefits of developing new methods for virus isolation

The current method for detection of presence of viruses, including ISAV, SAV, IPNV and VHSV, is largely dependent on fish sampling for immunohistochemistry analysis and nucleic acid isolation from tissue samples (Bernhardt, 2021). Detection of viruses depend on laboratory tests like cultivation of virus in cell culture, real time RT-PCR, immunohistochemistry, or microscopic pathology. This is often combined with gross signs of disease and histopathological examinations (OIE, 2021).

The control of PD requires monthly sampling and testing by PCR of fish from all seawater sites in the PD zones, according to the revised legislation from 2017 (Weli *et al.*, 2021a; Sommerset *et al.,* 2023). This method requires sacrifices of large amounts of fish and is both an economical and animal-welfare issue in salmonid aquaculture.

The existing methods for detection of viruses and disease in fish are resource-demanding and time-consuming, so easier methods for virus detection from seawater would be beneficial both in research facilities and in aquaculture. Compared to using the more traditional methods of sampling and detection of disease, virus detection from seawater could make earlier detection possible. Earlier detection could in turn work as a warning system, and lead to earlier implementation of disease control measures (Bernhardt *et al.*, 2021). Seawater sampling and detection is a non-invasive procedure that would reduce the need for sampling of fish, so it would be both cost-efficient and more animal welfare-friendly (Bernhardt, 2021). Seawater sampling is also less selective and might be more representative of the infection status of the fish population at site, compared to detection by sampling of fish (Bernhardt *et al.*, 2021), and

would be useful in monitoring of viruses from water in surveillance programs. Sampling of fish to detect and confirm disease would still be needed, but water sampling could be a useful method to monitor and test if virus is present in the surrounding environment.

1.4 Previously described methods for detection of aquatic viruses in seawater

1.4.1 Virus-absorption-elution (VIRADEL) technique

A commonly used method for concentration and detection of virus from seawater or wastewater is based on the virus-absorption-elution (VIRADEL) technique (Goyal and Gerba, 1983). This technique uses 1MDS electropositive microfilters specifically designed for capture and recovery of virus from large volumes of water by electrostatic attraction (Polaczyk, Roberts and Hill, 2007). The method differs from other filtration techniques, like ultrafiltration, because virus is not filtered based on size exclusion. 1 MDS filters typically have pores larger than 1 µm, so virus captured by these filters are instead retained because of hydrophobic and electrostatic interactions (Polaczyk, Roberts and Hill, 2007). After absorption, the attached virus can be eluted by exposing the filter to an alkaline, proteinaceous solution containing amino acids to decrease the charge attraction (Polaczyk, Roberts and Hill, 2007).

The VIRADEL technique has been used for concentration of viruses in several studies. One of them was published by Andersen, Hodneland and Nylund in 2010. In this study, the VIRADEL method was used for concentration of SAV from seawater samples by using one-layer of electropositive ZetaPlus® Virosorb® 1 MDS filters (Andersen, Hodneland and Nylund, 2010a). This filter is composed of cellulose medium and an electropositive glass filtration system, held in a polypropylene casing (Polaczyk, Roberts and Hill, 2007). The seawater was vacuum filtered through the 1 MDS filter with a water flow of 0.2-0.5 liters per minute, after adding 20 µl of H. salinarum (Andersen, Hodneland and Nylund, 2010a). Then, the filters were placed upside down in lysis buffer and shaken for 10 minutes at room temperature. Finally, ethanol was added to the samples, and then they were vortexed and frozen, ready for RNA extraction (Andersen, Hodneland and Nylund, 2010b).

VIRADEL was also used in studies published by Jarungsriapisit *et al.* in 2016. In one of these studies, SAV3 was concentrated from seawater samples to monitor virus shedding during a bath immersion challenge model for SAV3 in seawater to investigate if the fish were more

susceptible to infection at either three or nine weeks after seawater-transfer (Jarungsriapisit et al., 2016b). The concentration of virus was performed by using the VIRADEL technique with some modifications. Electropositive glass and cellulose Zeta Plus™ 1 MDS filters were used, and the water samples were vacuum filtered at a flow of 70-100 ml per minute. After filtration, the filters were placed upside down in a petri-dish containing lysis buffer and agitated for 15 minutes on an orbital shaker. Finally, 400 µl of eluant was transferred into a tube and stored at –80 °C for RT-qPCR. In a different article published by Jarungsriapisit *et al.* in 2016, the VIRADEL method was also used to concentrate SAV3 in a bath-challenge study on the relationship between viral dose and outcome of SAV3 infection in Atlantic salmon (Jarungsriapisit *et al.*, 2016a), Electropositive 1 MDS filters were also used in this study. After filtration, the filters were placed upside down in petri dishes containing L15 + 10% FBS and agitated for 15 minutes in a shaker. The eluent was then collected and passed through a 0.22 µm syringe filter unit. 100 µl of the eluant was finally transferred into a microtube with lysis buffer.

The VIRADEL technique is an effective method for concentration of virus from large volumes of seawater, but there are some drawbacks, like limited volume capacity, variable recovery of viruses and selective absorption of viruses to filters (John *et al.*, 2011). The method is also relatively time-consuming and costly (John *et al.*, 2011). It is also difficult to test if the concentrated virus is live-infectious or not because the lysis buffer added to the eluant makes it not possible to infect cells with the isolated virus.

1.4.2 Chemical flocculation

In 2011, John et al published an article named "A simple and efficient method for concentration of ocean viruses by chemical flocculation". In this study, a new technique to recover viruses from wastewater by using iron-based flocculation and large-pore-size-filtration followed by an optimized resuspension method of the virus-containing precipitate in a buffer was tested. Flocculation is when a chemical coagulant, in this case iron chloride ($FeCl_3$), is added to the solution to facilitate bonding between particles, creating larger aggregates to make it easier to separate from the rest of the solution by large-pore-size filtration. Iron-based flocculation was used because it is inexpensive, non-toxic, and effective (John *et al.*, 2011). Biologically benign solvents were then used to redissolve the flocculants. In this method, a two-component mixture was used. The first component dissolute solid iron hydroxides and the second one chelates iron in solution to prevent re-precipitation. The iron-

based flocculation method is a more efficient, reliable, and inexpensive method than the more traditional ultracentrifugation methods (John *et al.*, 2011). This method has been tested at IMR, and it worked well for detection of virus RNA by PCR detection. However, it didn't work for isolation of live virus when the eluted virus was used to infect cells because of the elution buffer used for isolation. The buffer used was hard to make and relatively unstable, so it had to be made fresh each time. It also showed toxicity with the ASK cells.

1.4.3 Ultrafiltration membranes

Through several articles, Weli and Bernhardt *et al* have developed a method for easier concentration and detection of SAV from seawater than the more traditional methods. This was done by evaluating two types of filters and four different buffer solutions (eluents) for concentration of SAV3 from seawater (Weli, *et al.*, 2021a). The filter and buffer that gave the highest recovery percentage of SAV3 and was the most efficient was further used to isolate SAV3 and ISAV from both natural and artificial seawater samples (Bernhardt *et al.*, 2021; Weli, *et al.*, 2021a; Weli, *et al.*, 2021b; Weli, *et al.*, 2021c). The most efficient method tested concentrates SAV from seawater by filtration through a 47 mm MF-Millipore™ electronegative membrane filter into a 47 mm in-line filter holder using a peristaltic pump. After filtration, the filter was placed upside down in a petri dish containing lysis buffer and put on an orbital shaker for 30 minutes. Then, the seawater concentrate was aliquoted and stored at –80 °C until RNA extraction and RT-qPCR (Bernhardt, 2021; Bernhardt *et al.*, 2021).

Because seawater contains salt and other RT-PCR inhibitors that can influence virus detection and quantification, reverse transcriptase droplet digital PCR (RT-dd PCR) was used instead of regular RT-PCR to get improved detection and quantification of RNA from the target virus (Weli, *et al.*, 2021b; Weli, *et al.*, 2021c).

All these articles only use PCR-based methods for detection, so it is unknown if the detected virus is infectious or not. This filtration method also needs some equipment for setting up and preforming the filtration, and the reverse transcriptase dd PCR used in this method has a limited availability and is quite expensive to run.

In 2022, Mota *et al*. published an article that evaluates the use of ultrafiltration (UF) membranes to remove fish viruses and bacteria from water from aquaculture (Mota *et al.*, 2022). This method has already been frequently used to remove virus, bacteria and other microorganisms from drinking water and wastewater, but the use in aquaculture has been limited, so the removal efficiency of fish viruses by UF membranes needed to be evaluated (Mota *et al.*, 2022). The performance of a capillary polyether sulfone UF membrane to remove IPNV and the bacteria Aeromonas salmonicida from natural seawater spiked with the pathogens was tested in the study. This was done by pumping 10L of the spiked seawater through an UF membrane bench-scale unit consisting of cylindrical tank to add the pathogen solution, a magnet gear pump, a waterflow meter, a temperature sensor, a water pressure sensor, a UF membrane and a cylindrical tank to collect the membrane permeate. When the water was filtrated, the membrane permeate was collected and filtered under vacuum through an electropositive charge filter disc by using a filtration pump (Mota *et al.*, 2022). The filter was then placed in lysis buffer and incubated for 30 min for RNA extraction. This study showed that the removal efficiency of both IPNV and A. salmonicida was 100%, which indicates that was acting as a physical barrier and that the membrane completely removed the microorganisms (Mota *et al.*, 2022).

1.5 New developments in virus isolation methods

Cost-effective, rapid, and efficient methods for concentration and isolation of viruses are needed for efficient surveillance of viruses in both wastewater and other water samples worldwide (Ahmed *et al.*, 2023). The SARS-CoV-2 pandemic have led to an increased interest in the development of cheaper, easier, and faster methods for virus isolation and detection from wastewater samples and clinical diagnostics, and several articles have been published during the last couple of years. There has been an increased use of polyethylene-based methods for isolation (Alexander *et al*., 2020; Torii *et al*., 2022). Some bead-based methods have also been published (Andersen *et al., 2023*).

1.5.1 Polyethylene glycol (PEG)

Polyethylene glycol (PEG) is one of the conventional methods for concentration of viruses and bacteriophages (Alexander *et al.*, 2020) from environmental samples. The PEG precipitation method has been used to concentrate virus samples for decades and has been

adapted and optimized to many RNA viruses in the later years (Alexander *et al.*, 2020). Recently the method has also been used to detect SARS-CoV-2 RNA in wastewater (Torii *et al.*, 2022).

The PEG precipitation method is a relatively simple method, which can precipitate virus in low-temperature, high-salt environment that stabilizes viral particles and produces a carrier medium isotonic to cells (Alexander *et al.*, 2020). PEG traps solvents and sterically excludes proteins, like virions, from the solvent phase, which makes it possible to concentrate the proteins and their precipitation after centrifugation (Torii *et al.*, 2022). This method has relatively low running costs compared to other methods like ultracentrifugation and can be performed with basic laboratory equipment (Torii *et al.*, 2022).

Torii *et al.* published a study in 2022 that compares different procedures of PEG precipitation with different operational conditions to evaluate the recovery efficiency of viruses by using procedures described in various published articles. This study showed that a shorter incubation time (2 hours) had better recovery of virus compared to longer incubation time.

1.5.2 Magnetic particles (beads)

Several methods based on beads to capture and concentrate virus from samples has been developed for a wide range of viruses, like Influenza virus, Zika and coronavirus from wastewater and clinical samples (Ahmed *et al.,* 2023). One of these methods, which is also verified for SARS-CoV-2 viral samples, are the Ceres Nano Manual Nanotrap® Wastewater protocol. This method isolates virus by using Nanotrap® Magnetic Virus Particles which has high affinity for the analyte (virion), so it can attract, capture, and concentrate the viruses from a large solution volume (Ahmed *et al.,* 2023; Andersen *et al.,* 2023). When the viruses are bound to the magnetic particles, the virions can be concentrated and removed from the samples by a separation step in a magnet (Andersen *et al.*, 2023). After concentration, the virions are tightly bound to the magnetic particles, so a lysis step is needed to purify the viral nucleic acids (Andersen *et al.*, 2023).

1.6 Aims of study

The aim of this study is to test and compare three different methods in their ability to precipitate different aquatic viruses from medium and seawater spiked with the viruses ISAV, SAV, IPNV and VHSV by RT-qPCR. The methods tested are the Intact Virus Precipitation Reagent (Thermo), Nanotrap Magnetic Virus Particles (Ceres Nanoscience) and Lentivirus Concentrator Solution (MD Anderson).

The most promising methods will then be used to isolate virus from a larger volume of seawater. Aliquots of isolated virus from both small and larger volume will also be used to try to infect cells to test if the isolated virus is live-infectious by the Indirect Fluorescence Antibody Technique (IFAT) or cytopathic effect (CPE).

2 Material and methods

2.1 Seawater

The seawater used for dilution of the virus stocks in all experiments was collected directly from the seawater tap (SV20) in wet lab 6 at the Institute of Marine Research (IMR), Nordnesgaten 50 at the same day as the dilutions were made for testing each of the methods. 100 ml of seawater was aliquoted into two 50 ml tubes and stored in the fridge at 4°C until it was used.

Detection of viruses in seawater by using a qPCR-based method consists of three steps: concentration of the virus, extraction of RNA, and performance of RT-qPCR.
Detection of live infectious virus is done by putting the isolated virus onto cells.

2.2 Virus stocks

Stocks of the four viruses used in this study were prepared at Institute of Marine Research (IMR) by Dr. Craig Morton in the period 2017-2019. Virus cultivation was performed with suitably permissive fish cell lines. SAV was grown in the CHH-1 cell line; ISAV was grown in the ASK cell line; and IPNV and VHSV were grown in the CHSE-214 cell line.

The ISAV virus stock had a titre of 3.1 x 10^5 TCID50/ml, and the SAV3 virus stock had a titre of 3.9 x 10^6 TCID50/ml. The virus titrations were performed at the Norwegian Veterinary Institute in Oslo.

2.2.1 Dilution series of the viral stocks

The virus stocks were used to make dilution series (10^{-1} - 10^{-9}) in both sterile tissue culture media (L15 + 2% FBS) and in seawater.

The dilution series was made by adding 100 µL from the viral stocks (ISAV, SAV, VHS or IPNV) to 900 µL of medium/seawater, making a 1:10 (10^{-1}) dilution. Then 100 µL was taken out from this tube and added into the next, making a 1:100 (10^{-2}) dilution. This was done to all the tubes to make a dilution series with dilutions from 10^{-1} to 10^{-9} (Figure 2.1) for each of the four viruses.

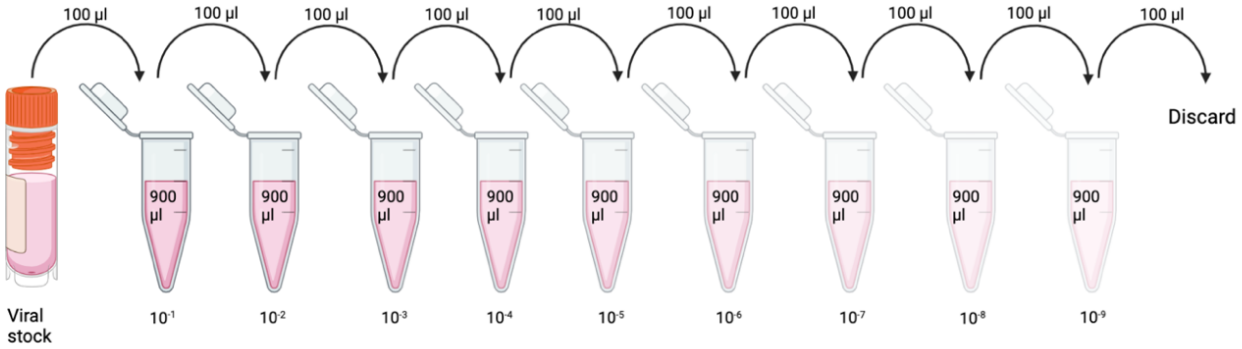

Figure 2.1: Illustration of how the dilution series were made in medium and seawater. The illustration was made in BioRender

For RNA isolation and RT-qPCR, 100 µL from each dilution was removed and mixed with 350 µL RLT-buffer and the viral RNA was isolated using the Qiagen RNeasy Mini kit. The RNA-isolation was followed by RT-qPCR. This step was done to measure the Ct-values of the whole dilution series to use as a reference when testing the different methods for virus isolation, and to decide which dilutions to test in the different isolation methods.

2.3 Virus isolation methods

Three virus isolation methods were tested in this study:

- Intact Virus Precipitation Reagent
- Lentivirus concentrator solution
- Manual Nanotrap® Wastewater protocol

2.3.1 Intact Virus Precipitation Reagent (Invitrogen)

This method isolates virus from various samples, such as water or cell free medium by removing water molecules from solution to force less soluble components like viral particles out of the solution, allowing them to be precipitated by centrifugation. The protocol is optimized for SARS-CoV-2 but could be optimized for other enveloped viruses as well.
To test the Invitrogen Intact Precipitation Reagent (Thermo Fisher Scientific cat # 10720D), from here called Invitrogen reagent, the manufacturer`s recommended protocol was followed. (Appendix 7.2)

For testing this method, the desired dilutions of all four viruses were made in 2 ml tubes. The dilutions used were 10^{-2}, 10^{-4} and 10^{-5}. The method was tested for stock virus samples diluted in both medium (L15 + 2 % FBS) and seawater (Figure 2.2).

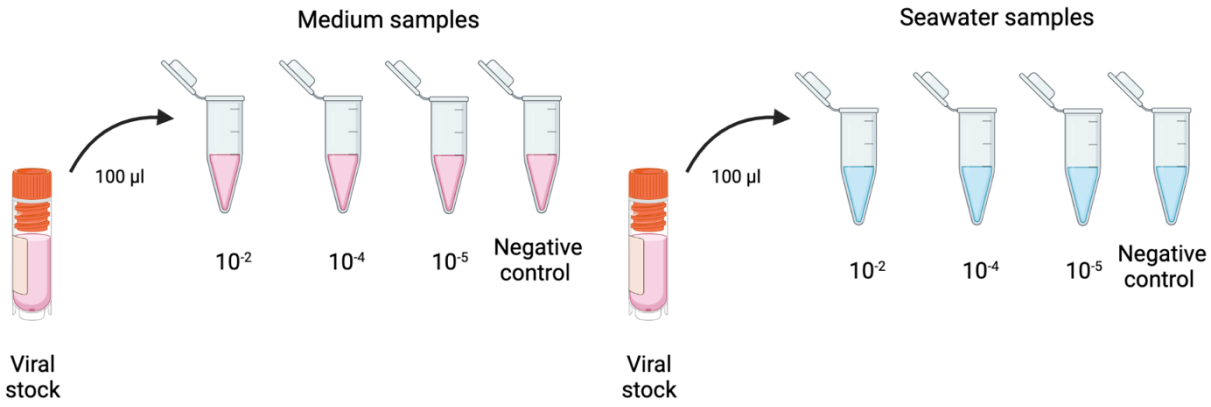

Figure 2.2: Illustration of the samples used for each virus in both medium and seawater to test all three methods. The illustration is made using BioRender.

500 µL of Invitrogen reagent was added to all samples (three dilutions of each virus + one negative control without virus) and mixed by vortex and pipetting. The samples were then incubated overnight in a rotary mixer in the cold room at 4°C.

The following day, the samples was centrifuged at 10 000 x g at 4 °C for 30 minutes in a precooled micro-centrifuge (Mikro 200R Hettick Zentrifugen). When this method was tested the first time, the samples were made with medium with 2% FCS and spun at 3,200 x g, which is recommended in the protocol when the intended downstream use is RT-qPCR. However, there was no pellet in any of the samples in the first round of testing, so all subsequent samples were spun at 10,000 x g and with 10% FCS in the medium instead.

After centrifugation, the supernatant was removed, and the pellet was dissolved in 100 µL PBS. The 100 µL virus suspension was then mixed with 350 µL RLT-buffer and stored at -80 °C, prior to RNA extraction and RT-qPCR

2.3.2 Lentivirus concentrator solution (MD Anderson Cancer Center)

The Lentivirus concentrator solution method has been published previously by the MD Anderson Cancer Center at the University of Texas. This method uses a 10% w/v polyethylene glycol (PEG) solution, which binds up the water molecules and forces the less soluble components, like viral particles, out of the solution so they can be taken out of the solution after centrifugation.

The PEG solution was made by following the Lentivirus concentrator protocol. 80 g of PEG-8000 and 14 g of sodium chloride (NaCl) was dissolved in 20 ml of 1xPBS with gently stirring on a magnetic stirrer with heat. The pH was adjusted to 7.3-7.3. The solution was then stored in the fridge at 4°C. (Appendix 7.3)

For testing the methods, one negative control without any virus and 3 different dilutions of all four viruses was made. The dilutions used were 10^{-2}, 10^{-4} and 10^{-5}. The method was tested for stock virus samples diluted in both medium (L15 + 2 % FBS) and seawater.

333 µL PEG solution was added to all the samples of 1 ml each and mixed by shaking. The samples were incubated overnight at 4°C in a rotor. The samples were then centrifuged (Mikro 200R Hettick Zentrifugen) at 10,000 x g for 60 minutes the following day. The supernatant was then removed, and the pellet resuspended in 100µL PBS. 350 µL RLT was added, and RNA was isolated using the Qiagen RNeasy Mini kit. The RNA was then stored in the -80 °C freezer, prior to RNA extraction and RT-qPCR.

2.3.3 Manual Nanotrap® Wastewater protocol (CERES NANO)

Manual Nanotrap wastewater protocol using QIAGEN QIAamp Viral RNA mini kit

The Ceres NANO Nanotrap method is based on adding Nanotrap Magnetic particles to the samples to capture and concentrate viruses. The method is verified for SARS-CoV-2 viral wastewater samples (Ceres Nanoscience).

The Nanotrap method was tested on the same virus dilutions (10^{-2}, 10^{-4} and 10^{-5} + one negative control without virus) as the other methods. All four viruses, in both medium and seawater, were tested.

The method was tested by following the Ceres Nano Manual Nanotrap Wastewater protocol (Appendix 7.3).

20 µL of magnetic virus particles were added to each sample and mixed by inverting the tubes. The samples were then incubated at room temperature for 10 minutes. After 10 minutes, the samples were put in a magnetic rack (Invitrogen) to isolate the beads to the wall of the tube. The supernatant was removed, and the beads washed with molecular grade water (VWR Life Science). Then they were put in the magnet again, and the supernatant was once again removed. The beads were then resuspended in PBS and lysis buffer (buffer AVL-carrier RNA) was added. The samples were incubated at room temperature for 10 minutes, before they were put back on the magnetic rack to remove the beads by transferring the supernatant into new tubes. Finally, the RNA isolation was done using the protocol for QIAGEN QIAamp Viral RNA mini kit (as recommended by Ceres Nanoscience). The isolated RNA was then stored in the -80°C freezer, ready to run RT-qPCR.

2.4 RNA isolation

RNA isolation is a method used to separate pure RNA from mixtures of DNA or proteins. This technique is necessary to do prior to many molecular procedures, like cDNA synthesis and RT-qPCR. There are various approaches to do RNA purification, depending on the starting sample. Total RNA purification involves lysis of cells or tissues, and extraction and purification of RNA from the sample. The first step of the RNA isolation is the sample collection and protection. It is critical to find the most appropriate method of cell or tissue lysis for maximizing the quality and yield of RNA. The second step is RNA preparation. The different RNA preparation methods can be classified into four general techniques: direct lysis, magnetic particles, spin basket and organic extraction methods (Thermo Fisher Scientific). The last step of the RNA isolation is to resuspend the purified RNA in RNAse-free water.

The RNA isolation of the samples from all methods tested was done by following the manufacturers' protocol for the QIAGEN RNeasy kit prior to running the real-time RT-PCR. The only exception was for the samples from the Ceres Nano method, where the QIAGEN QIAamp Viral RNA Mini kit was used.

2.4.1 QIAGEN RNeasy mini kit

The QIAGEN RNeasy mini kit can be used for fast and efficient purification of high-quality total RNA from yeast, animal tissues or cells. It can also be used for cleanup of RNA from enzymatic reactions like DNase or proteinase digestion. This method combines microspin technology with the selective binding properties of a silica-based membrane with a specialized high-salt buffer that allows RNA to bind to the membrane.
The samples are first lysed and then homogenized with a highly denaturing guanidine-thiocyanate-containing buffer (RLT buffer) that inactivates RNAses to ensure purification of intact RNA. Ethanol is added to make sure that the conditions are ideal for RNA to bind to the membranes in the RNeasy Mini spin columns. Then, the samples are transferred to the columns, where the RNA binds to the membrane, making it possible to wash away the contaminants efficiently by using different washing buffers (buffer RW1 and buffer RPE). All the binding, washing and elution steps are performed by centrifugation. Finally, the pure, concentrated RNA is eluted in 30-50 µl RNAse free water. This procedure purifies all RNA molecules longer than 200 nucleotides.

2.4.2 QIAGEN QIAamp Viral RNA Mini Kit

The QIAGEN QIAamp Viral RNA Mini kit is a procedure to purify viral RNA from cell-free body fluids, plasma, serum, or other samples containing virus with fast spin-columns. The isolated viral RNA can be used in a wide range of downstream applications, like viral genotyping, epidemiology, or research on infectious diseases.

140 µl of PBS is first added to each sample due to a small volume. Then, the samples are lysed under denaturing conditions by adding a lysis buffer, which inactivates RNases and makes it possible to isolate intact viral RNA. The lysis buffer used in this method is made by mixing buffer AVL with carrier RNA-buffer AVE. The carrier RNA-buffer AVE is made by adding buffer AVE to lyophilized carrier RNA to obtain a solution of 1 µg/µL. The carrier RNA improves binding of the viral RNA to the membrane, and limits degradation of the viral RNA caused by residual RNase activity. Buffer AVE is RNase-free water with sodium azide that prevents microbial growth and contamination with RNases.

Ethanol is then added to the samples, before they are loaded into the QIAamp Mini spin columns placed in 2 ml collecting tubes. Buffering conditions are adjusted to give optimum

binding of the RNA to the silicia membrane in the spin-columns. The RNA binds specifically to the membrane, making it possible to efficiently wash away contaminants using wash buffers in two steps separated by brief centrifugation steps. The two different washing buffers used, AW1 and AW2, improve the purity of the eluted RNA. Finally, pure RNA is eluted in a low-salt RNase-free buffer provided with the kit, ready for direct use or storage. Purified viral RNA is free of nucleases, proteins, salt and other contaminants and PCR inhibitors. There is no need for the use of phenol or chloroform extraction by using this kit.

2.5 Real-time RT-PCR

The extracted RNA from all samples was analyzed by using the AgPath-ID™ One-Step RT-PCR kit (Applied biosystems®) and QuantStudio™ Real-Time PCR System (Applied biosystems®) to detect RNA from specific pathogens (ISAV, SAV, IPNV or VHSV).

Real-Time Reverse Transcription Polymerase chain reaction (Real-Time RT PCR) is a reliable and sensitive molecular biological technique for detection and quantification of specific nucleic acids like RNA. The method is also known as quantitative PCR (qPCR). The RNA is transcribed into complimentary DNA (cDNA) by reverse transcriptase, which is then used as a template for the PCR. The AgPath-ID™ One-step RT-PCR kit does not require cDNA synthesis of the template in advance which makes it possible to run both reverse transcription and the PCR-reaction in the same process and in the same tube. The method is based on detection and quantification of fluorescence emitted from a reporter molecule during accumulation of PCR product in each amplification cycle.

The mastermix for the different viruses was made by mixing sequence specific forward and reverse primers and probes (TaqMan®), 25 X RT-PCR enzyme mix (Applied Biosystems®), 2X RT-PCR buffer (Applied Biosystems®) and RNase-free water (Sigma-Aldrich) in tubes. The components with their associated volumes of the RT-PCR reaction mixes are given in Table 2.1 and 2.2. After adding the components, the tubes were vortexed and spun down. Then, 8 µL of mastermix was added to each well in a 96 well reaction plate (Quant Studio Applied Biosystems). 2 µL of RNA-template was also added to the wells. ROX™ is the passive reference dye used and is included in the RT-PCR buffer. A passive reference is used to normalize the fluorescent receptor signal by removing fluorescence variations and variations in instrument scanning and provide a stable baseline. The baseline represents the background signals like the low-level fluorescence emitted during early PCR cycles.

***Table 2.1*:** The components of the RT-PCR reaction mix (mastermix) for SAV

Reagents	**Volume (µL) per sample**
RT-PCR buffer	5
RT-PCR Enzyme mix	0.4
Forward primer	0.4
Reverse primer	0.6
TaqMan® probe	0.16
RNA	2
RNAse-free water	1.44
Total	10

***Table 2.2*:** The components of the RT-PCR reaction mix for ISAV, IPNV and VHSV

Reagents	**Volume (µL) per sample**
RT-PCR buffer	5
RT-PCR Enzyme mix	0.4
Forward primer	0.4
Reverse primer	0.4
TaqMan® probe	0.16
RNA	2
RNAse-free water	1.64
Total	10

Positive controls (POS) and Non-template Control (NTC) (nuclease-free water) for each virus was also included in all runs. The NTCs were used to detect any contaminations in the Real-time RT-PCR reagents, and the positive controls were used to ensure that the mastermix was working as expected. All samples were analyzed by qPCR in triplicates. The wells were finally sealed with an adhesive sealing sheet (Thermo Scientific), before the plates were spun down for approximately 20 seconds, and then inserted in the QuantStudio™ 5 Real-Time PCR System (Applied biosystems, Thermo Fisher Scientific) and analyzed. The plate setup and temperature and time settings for the run was set in QuantStudio™ Design & Analysis Software v1.5.1.

Reverse transcription is the first step of the real time RT-PCR, and happens at 45°C for 10 minutes, where the RNA is transcribed to cDNA. Then the initial denaturation starts at 95°C for 10 minutes. The reverse transcriptase is inactivated, and the Taq DNA-polymerase activated. Finally, the amplification starts. In this stage, the DNA dissociation happens for 15 seconds at 95°C, followed by annealing and elongation for 45 seconds at 60°C. The amplification step is repeated 40 times (cycles). The ramp rates (heating and cooling) are set to 1.6°C/sec. The fluorescent signal is measured and registered at the end of each cycle in real time.

The results from the Real time RT-PCR were analyzed by using the QuantStudio™ Design & Analysis Software 5 by Applied Biosystems®. While the Real-time RT-PCR is running, the fluorescent signal generated through amplification of the target template in each PCR cycle is detected. The results show how many amplification cycles it takes for the fluorescence signal of the reaction to cross the target threshold value (0.2 in this analysis). This is where the fluorescence signal exceeds the background fluorescence. The amplification cycle where the amplification curve crosses the target threshold is given as a Cycle threshold (Ct)-value. The Ct value can be used to calculate the initial RNA copy number because the Ct value is related to the starting amount of target. A high Ct-value indicates low amount of target template in the sample, and a low Ct-value indicates a high amount of target template because as the template amount decreases, the cycle number where an amplification is seen increases. The data can then be analyzed to determine absolute or relative quantity of the target sequence in the original sample.

2.6 Virus isolation from small volumes of seawater

The Lentivirus concentrator solution method and the Ceres nano Manual Nanotrap Wastewater protocol was tested to isolate ISAV and IPNV again. Moving forward, only ISAV and IPNV was tested due to limited time. This time, the isolated virus was put onto cell plates in the end, to see if the isolated virus was able to infect the cells. Both methods were used to isolate virus from seawater spiked with virus in the concentration 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} for both viruses, and from samples with just seawater and PBS. All samples were in duplicates, marked A or B (Figure 2.3). The A samples were analyzed by using Real-time RT-PCR, while the B samples were added to plates of cells and incubated for seven days before they were analyzed by CPE or IFAT.

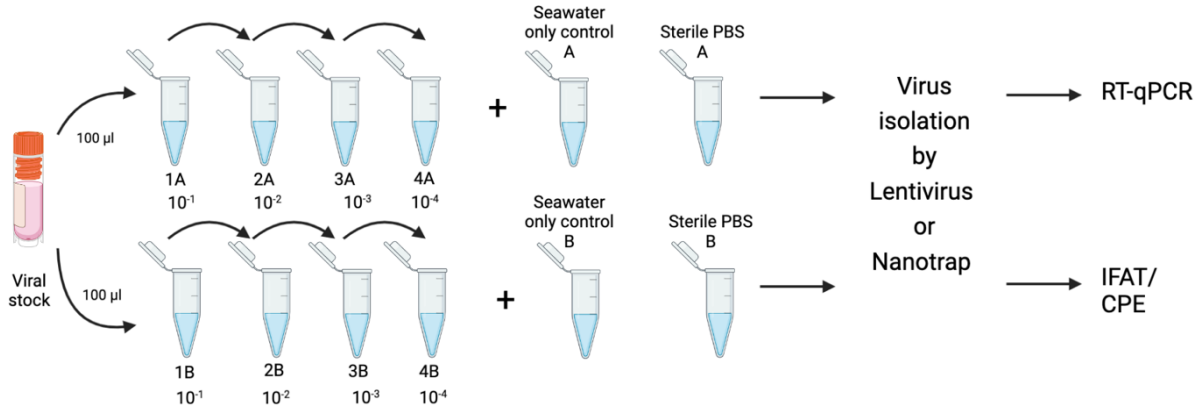

Figure 2.3: Overview of the samples of each virus analyzed in the Lentivirus and Nanotrap magnetic beads methods. Illustration made using BioRender.

2.6.1 Lentivirus concentrator solution

The Lentivirus protocol was performed as previously described in section 2.3, except for the B samples in the last step. The B samples that later were put on cells, were resuspended in 500µL of medium (L15 + 2% FBS + 2% antibiotics) after the centrifugation instead of PBS and split into two tubes for each sample. One of the tubes for each sample were boiled before all B samples were put on cells. The A samples was resuspended in 100 µL PBS, like previously.

2.6.2 The Ceres nano Manual Nanotrap® Wastewater protocol

The Nanotrap protocol was also followed for this experiment, described in section 2.3. Like for the Lentivirus method, the only difference was in the last steps for the B samples, where the solution with the beads still in it were resolved in 500 µL of medium (L15 + 2% FBS + 2% antibiotics). No lysis buffer was added, and the beads stayed in the solution when it was added to the cells.

The A samples was done as before, where both 150 µL of PBS and 560 µL of QIAGEN Virus Lysis Buffer (Buffer AVL) from the Viral RNA Mini Kit was added to the samples containing the beads. The samples were then incubated for 10 minutes at room temperature, before they were put back on the magnet for the beads to separate from the lysate solution. The supernatant was then RNA isolated and analyzed by Real-time RT-PCR.

2.7 Virus isolation from larger volumes of seawater

The Lentivirus concentrator solution method and the Ceres nano Manual Nanotrap Wastewater protocol was also tested to isolate ISAV and IPNV spiked in a larger volume of seawater (30 ml). After the virus was isolated, it was put on cells to see if it was infectious to cells in addition to isolate RNA and run RT-qPCR from the samples.

2.7.1 Lentivirus Concentrator Solution

The lentivirus method was tested for isolating both ISAV and IPNV from 30 ml samples. For each virus, there were two tubes (A and B) of 30 ml seawater spiked with 100 µL virus each, two tubes of just seawater and two tubes of PBS (Figure 2.4). The A samples were analyzed by RT-qPCR and the B samples were put on cells after the virus isolation was completed.

After the virus was added to the first two tubes, 100 µL was removed from all the tubes and kept for RT-qPCR. Then, 10 ml of the lentivirus concentrator solution was added to each tube and mixed by shaking. The tubes were then incubated on a rotory mixer in the cold lab at 4°C overnight.

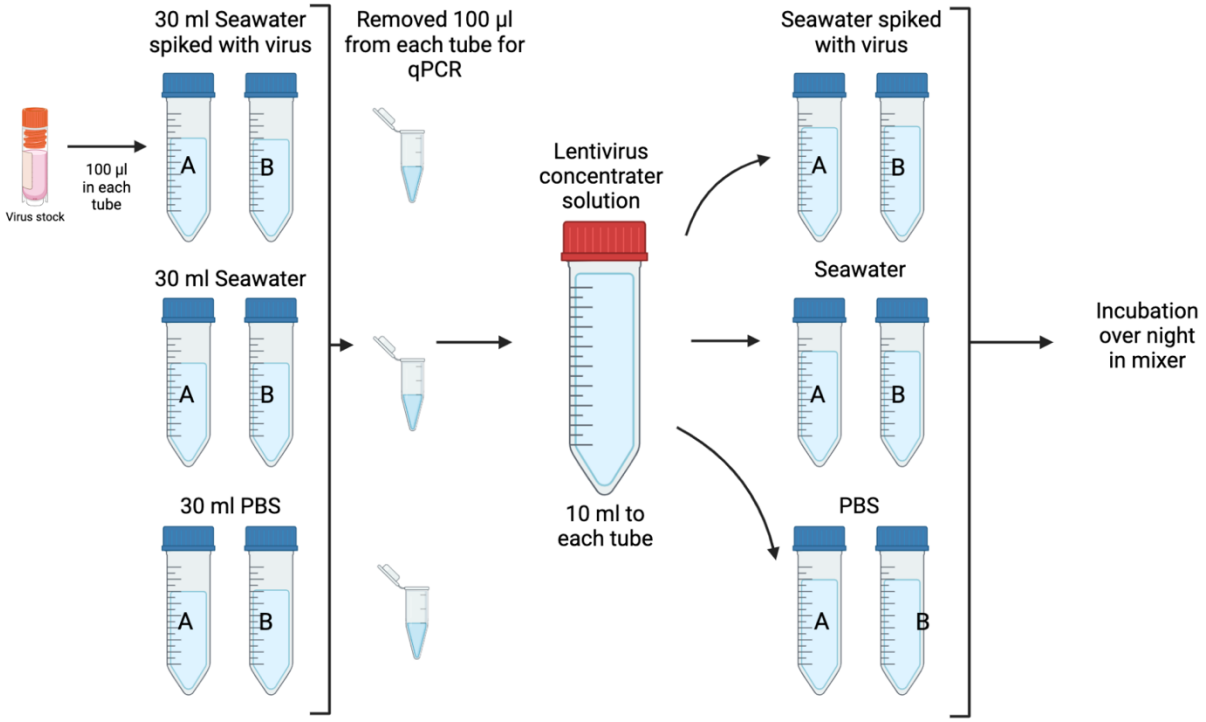

Figure 2.4: Setup for testing the lentivirus method on larger volumes of seawater. Illustration made using BioRender.

The following day, the tubes were spun down at 1600 x g for 60 minutes. The supernatant was then removed, and each pellet in the A samples was resuspended in 1 ml PBS. Then they were stored in the fridge until RNA isolation was done by using the QIAGEN RNeasy kit later the same day.

The pellets in the B samples were resuspended in 500 µL of medium and split into two 1,5 ml tubes each. Half of the tubes were then boiled at 95°C in a heating block. Finally, the B samples were put on cells, along with a positive control (virus diluted 1:100). 100 µL of the samples was added to each of the wells. The ISAV samples were put on a plate of ASK cells and the IPNV samples on a plate of CHSE cells. When the virus samples were added to the plates, the plates were incubated for 6 hours, before the virus was removed and replaced with 200 µL of medium (L15 with 2% FBS and 2% antibiotic) before they went back in the incubator for seven days. At day seven, the ISAV plates were analyzed by using the indirect fluorescent antibody technique (IFAT), while the IPNV plates were analyzed by plate reading in the microscope.

2.7.2 Manual Nanotrap® Wastewater protocol

The Ceres Nanotrap wastewater protocol was also tested for isolating ISAV and IPNV from 30 ml samples. The setup was the same as for the lentivirus method, with two tubes (A and B) with seawater spiked with 100 µL virus, two tubes with just seawater and two tubes of PBS for each virus (Figure 2.5).

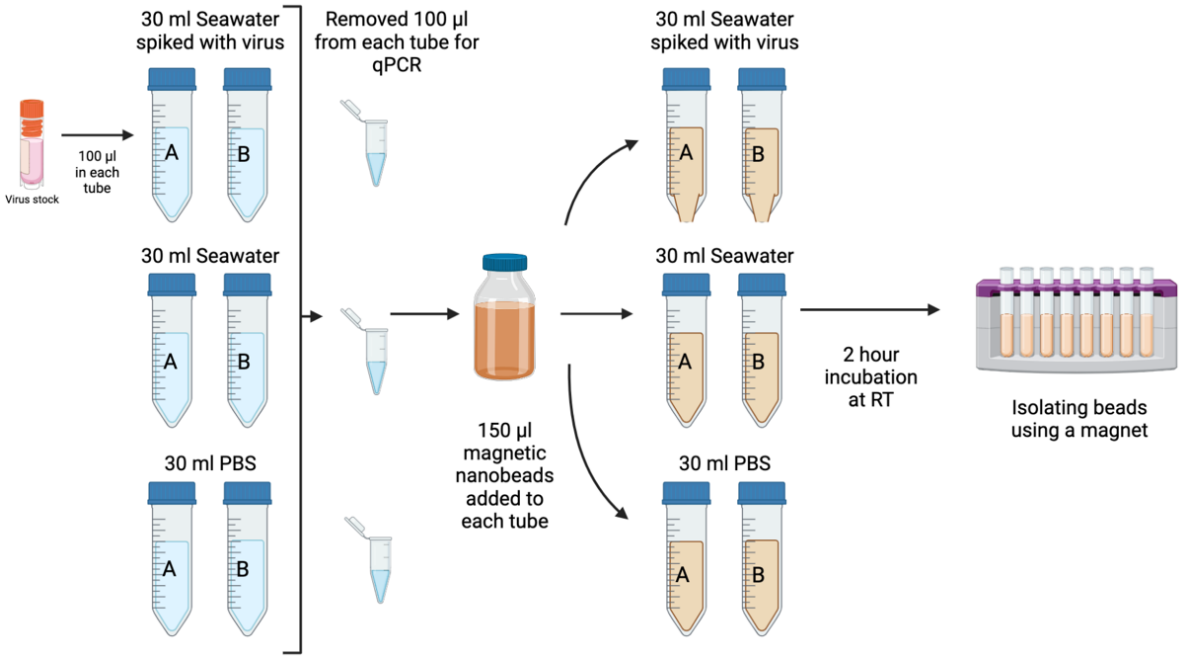

***Figure 2.5**:* The setup for the Nanotrap magnetic beads protocol for larger scale samples. Illustration made in BioRender.

100 µL was removed from each tube for RT-qPCR. Then, 150 µL of magnetic beads was added to each tube and mixed by inversion. The tubes were then incubated for 2 hours at room temperature (RT) but inverted every 15 minutes during the incubation time. After incubation, the tubes were put in a magnet to isolate the beads. The supernatant was removed and then the beads were washed two times in molecular grade water. The water was removed and the pellet in the A tube was resuspended in 150 µL PBS for RT-qPCR and 560 µL virus lysis buffer (buffer AVL). The samples were incubated for 10 minutes, before they were put back in the magnet to separate the lysate solution from the beads. The A samples were then RNA isolated using the QIAGEN QIAamp Viral RNA Mini kit.

The beads in the B tubes were resuspended in 500 µL medium (L15 + 2% FBS + 2% antibiotics), and then split into two tubes per sample. A positive control for each virus (diluted 1:100 in seawater) was also made. Half of the samples were boiled for 10 minutes at 95 °C in a heating block. Then, all samples were put on cells on plates, ISAV on ASK cells and IPNV on CHSE cells, and incubated for 6 hours before the virus was removed and replaced with 200 µL of medium. The plates were incubated again for 7 days, before the cells were analyzed with IFAT or plate reading in microscope.

2.8 Infection of cell lines with isolated virus

Cell plates were made to be able to infect cells with the isolated virus. The plates were made by removing the medium from flasks containing cells. Then, trypsin was added to release the cells from the bottom of the cell flask. When the cells were free, medium was added to stop the trypsinization. The medium containing the cells were transferred to a 50 ml tube and spun down for 5 minutes at ~190 x g (1000 RPM). The cells were then counted in the microscope, using a counting chamber, to get the average amount of cells per ml. The amount of cells were then used to calculate how much cell suspension and medium to add to each well.

The number of cells per ml was then calculated by:

$$the\ average\ number\ of\ cells * 90\ (constant) * 1000\ (ml)$$

To calculate the amount of cell suspension to add to each well

$$\frac{Desired\ number\ of\ cells\ per\ well}{number\ of\ cells\ per\ ml} * number\ of\ wells$$

200 µL of medium and cell suspension was added to each well, so the amount of medium and cell suspension was given by the number of wells used multiplied by 200µL.

CHSE cells were added to the plates used for analyzing IPN samples, while ASK cells were used for the ISAV samples. For the small volume sample trial, cells were added to 8 rows (64 wells). One row was used as a positive control, and some were used for negative controls. For the large-scale trial, cells were added to 6 rows (48).

Half of the samples were boiled to have a negative control for virus replication and as a test to kill or deactivate everything the seawater could contain that might be cytotoxic to the cells. PBS and seawater were also added to the cells to see if it was cytotoxic to cells or not.

The plates containing the cells were then incubated for a few days, until the samples containing isolated virus were added. The virus samples were added to the plate by first removing the existing medium by pipetting. Then, 100 µL of each sample was added to the wells. Positive controls (virus diluted 1:100 in medium) and negative controls (PBS) were also added. The plates were then incubated for 6 hours, before the samples were removed and replaced with 200 µL medium. The plates were then incubated for 7 days in the incubator at 15 °C before they were analyzed by plate reading in the microscope or by IFAT.

2.9 Indirect Fluorescent Antibody Technique (IFAT) – ISAV staining

The indirect fluorescent antibody technique is a sensitive and semi-quantitative method to detect specific antigens or antibodies in samples, like viral proteins in infected cells.
This method is based on forming an antigen-antibody complex in samples where the specific antigen is present. It is a two-step procedure involving two specific antibodies. The primary antibody, which is virus-specific, binds to a specific virus protein, while the secondary antibody is labeled with fluorophore and specifically binds to the primary antibody bound to the antigen (illustrated in Figure 2.6). Multiple secondary antibodies can bind to each primary antibody-antigen complex, which makes the indicator signal strong. The cells will only be visible in the fluorescent microscope if they are infected and have been bound by antibodies.

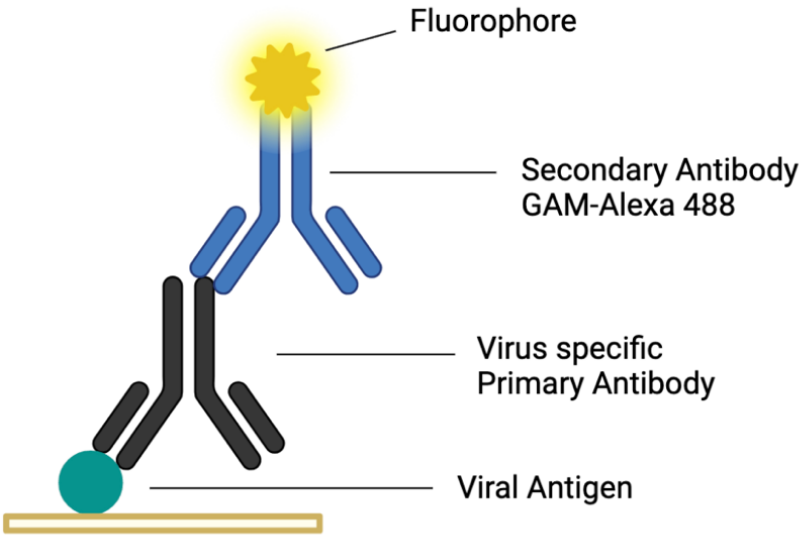

Figure 2.6: Illustrating the principle of IFAT. The illustration was made using BioRender.

The Indirect Fluorescent Antibody Technique was performed on the cell plates containing ISAV from the small volume and larger volume samples to see if the cells were infected. The cells were first fixed to preserve cell morphology, using paraformaldehyde (PFA). First, the medium was carefully removed from all the wells by pipetting. The cells were then washed two times in 100 µL PBS (Dulbecco´s Phosphate Buffered Saline, Sigma). 100 µL of 4% PFA was added to each well. After 10 minutes of incubation at room temperature (RT), the PFA was removed, and the cells washed three times in PBS. After fixation, the cells were permeabilized by adding 100 µL of PBS + 0.5% Tween-20 to each well and incubated at room temperature for 15 minutes. The cells were washed two times in PBS. Next, the murine anti-ISAV primary antibody (clone P10; Aquatic Diagnostics) was diluted 1:500 in PBS. 100

µL was added to each well, and then the plate was incubated for 1 hour at RT. After 1 hour, the primary antibody was removed, and the cells washed two times with PBS. Then, the secondary antibody (Goat Anti-Mouse IgG – Alexa 488; Thermo Fisher Scientific) was diluted 1:400 in PBS. 100 µL was added to each well, and the plate was incubated again for 1 hour at RT covered in aluminum foil because the secondary antibody is light sensitive. The secondary antibody was removed, and the excess antibodies were removed by washing the cells two more times with PBS. Finally, 100 µL PBS was added to the wells, and the plate was ready for examination by fluorescence microscopy.

2.10 Cytopathic effect (CPE)

Because we didn't have a specific antibody for IPN, we weren't able to analyze the plates containing IPNV with IFAT. These plates were thereby analyzed by plate reading 7 days after the wells where infected. The plate reading was done under the microscope, looking for cytopathic effect or lysis of the cells in each well. Cytopathic effect is the changes in cell morphology caused by viral infections.

2.11 Data collection and processing

The Ct results from the Real-time RT-PCR was transferred from QuantStudio™ Design & Analysis Software 5 to Microsoft® Excel to retrieve the Ct-values and make tables of the values. The values were then transferred to GraphPad Prism 9.5.0.

GraphPad Prism was used to make all graphs with the Ct results.
The graphs were made by inserting the Ct values and samples names into column tables with one grouping variable, with each group defined by a column. The replicate values were entered stacked in columns. The plot was then made by choosing the graph type "scatter dot plot" plotted from min to max values, with a line at mean Ct value for each sample. The values on the Y-axis (Ct-values) were reversed, and the range was set to minimum 15 and maximum 40. Ct values over 40 were considered as undetermined.

The illustrations were made using BioRender, available at https://www.biorender.com/.

3 Results

3.1 Ct results from dilution series

Dilution series of ISAV, SAV, IPNV and VHSV was made in the dilutions 10^{-1} to 10^{-9} from virus stock in medium (L15 + 2% FBS) and in seawater. 100 µL was taken out from each dilution from every virus and analyzed by RNA isolation followed by RT-qPCR. The results from the dilution series in medium are given in Figure 3.1 and in seawater in Figure 3.2.

3.1.1 Dilution series in tissue culture medium (L15 + 2% FBS)

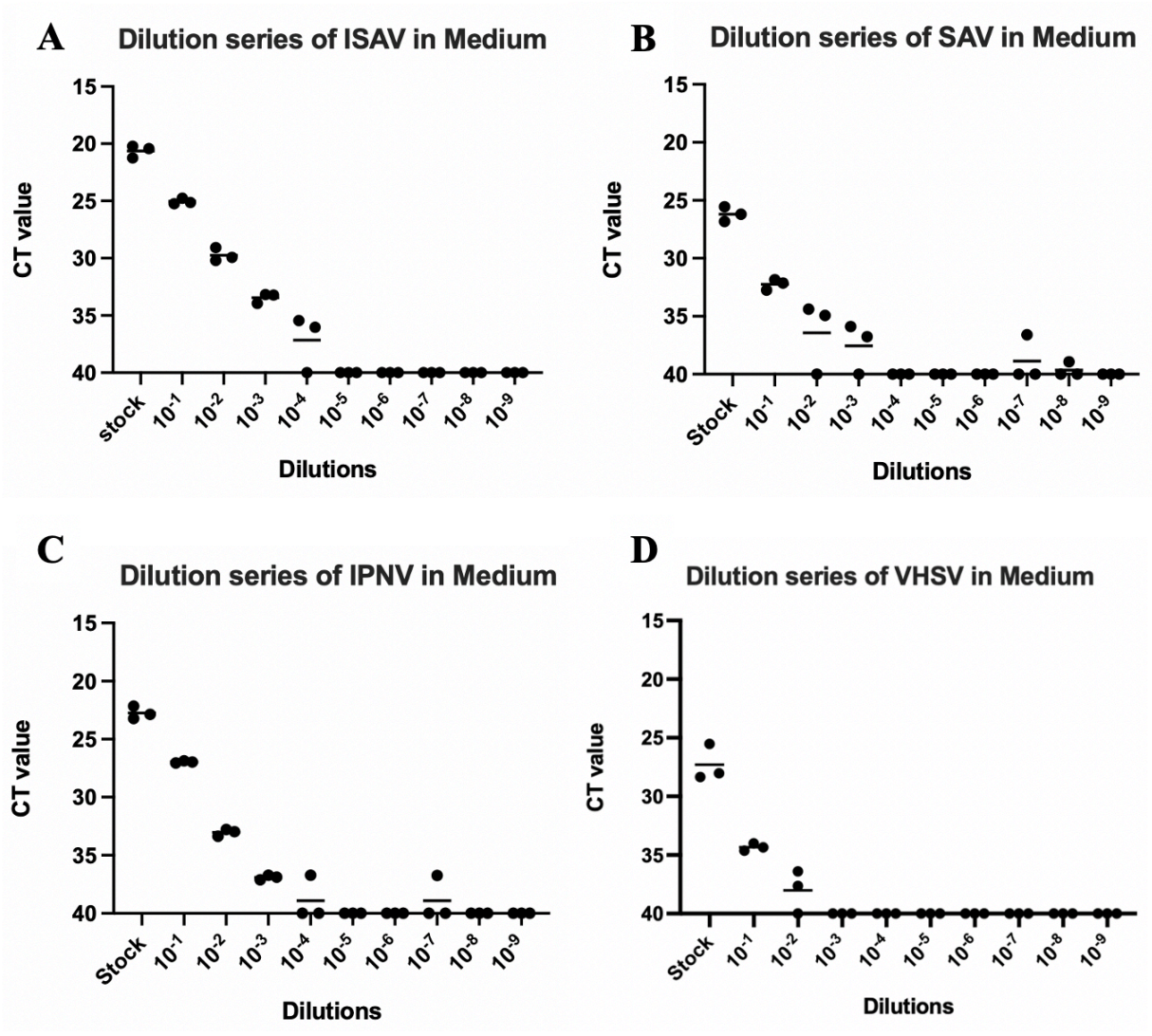

Figure 3.1*:* All samples from the dilution series were analyzed by RT-qPCR. The Ct results from the dilution series in medium spiked with ISAV are given in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates. The mean Ct value for each sample is given by the line.

For ISAV, the results show that virus is detected in the dilutions 10^{-1} to 10^{-4}. In all the rest of the dilutions, with a lower concentration of virus, no viral RNA is detected, and the Ct value came out as undetermined. The results from the SAV dilution series show that virus is detected in dilutions 10^{-1} to 10^{-3}. One replicate of each of the dilutions 10^{-7} and 10^{-8} also came out as positive, while the two other tests were negative. For dilutions 10^{-4} to 10^{-6} and 10^{-9}, all triplicates came out negative. For IPNV, viral RNA was detected in all triplicates in the dilutions 10^{-1} to 10^{-3}. One of the triplicates was positive for the dilutions 10^{-4} and 10^{-7}. The results from the VHSV dilution series show that viral RNA was detected from the stock sample and the dilutions 10^{-1} and 10^{-2}. The rest of the dilutions were negative.

3.1.2 Dilution series in seawater (SW)

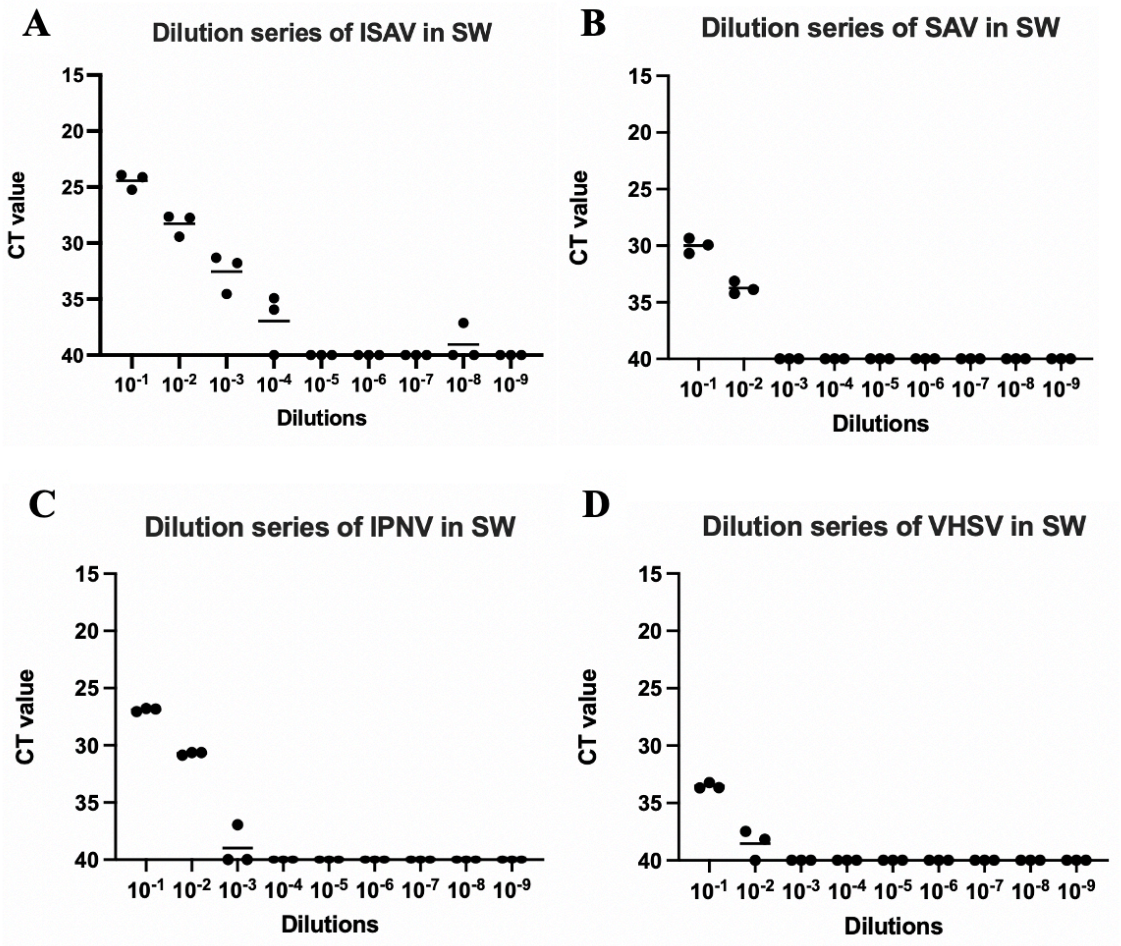

Figure 3.2: All samples from the seawater dilution series were analyzed by RT-qPCR. The Ct results from the ISAV samples are given in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates. The mean Ct value for each sample is given by the line.

The results from the dilution series in seawater show that viral RNA was detected from the 10^{-1} to 10^{-4} dilutions of ISAV. One of the triplicates in the dilution 10^{-8} was also positive. In the rest of the dilutions, no viral RNA was detected. For the SAV and VHSV dilution series, only the 10^{-1} and 10^{-2} dilutions were positive. The results from the IPNV dilutions show that virus was detected in dilutions 10^{-1} to 10^{-3} and in one of the triplicates in dilution 10^{-9}.

3.2 Ct results Invitrogen Intact Virus Precipitation Reagent

The Invitrogen reagent was used to isolate virus from samples of virus spiked in tissue culture medium (L15 + 2% FBS) and in seawater in three different dilutions. The dilutions used to test the methods were 10^{-2} which had a positive Ct value for all four viruses, 10^{-4} which sometimes had a positive RT-qPCR result and 10^{-5} which always came out undetermined (Figure 3.1 and 3.2).

The Invitrogen reagent was added to each sample, incubated overnight in a mixer, and spun down. Then, the supernatant was removed, and the pellet resuspended in 100µL PBS. 100 µL was taken out from each sample and analyzed by RNA isolation and RT-qPCR. The results from the RT-qPCR of the medium samples are given in Figure 3.3 and the results from the seawater samples are given in Figure 3.4.

3.2.1 Tissue culture medium (L15 + 2% FBS)

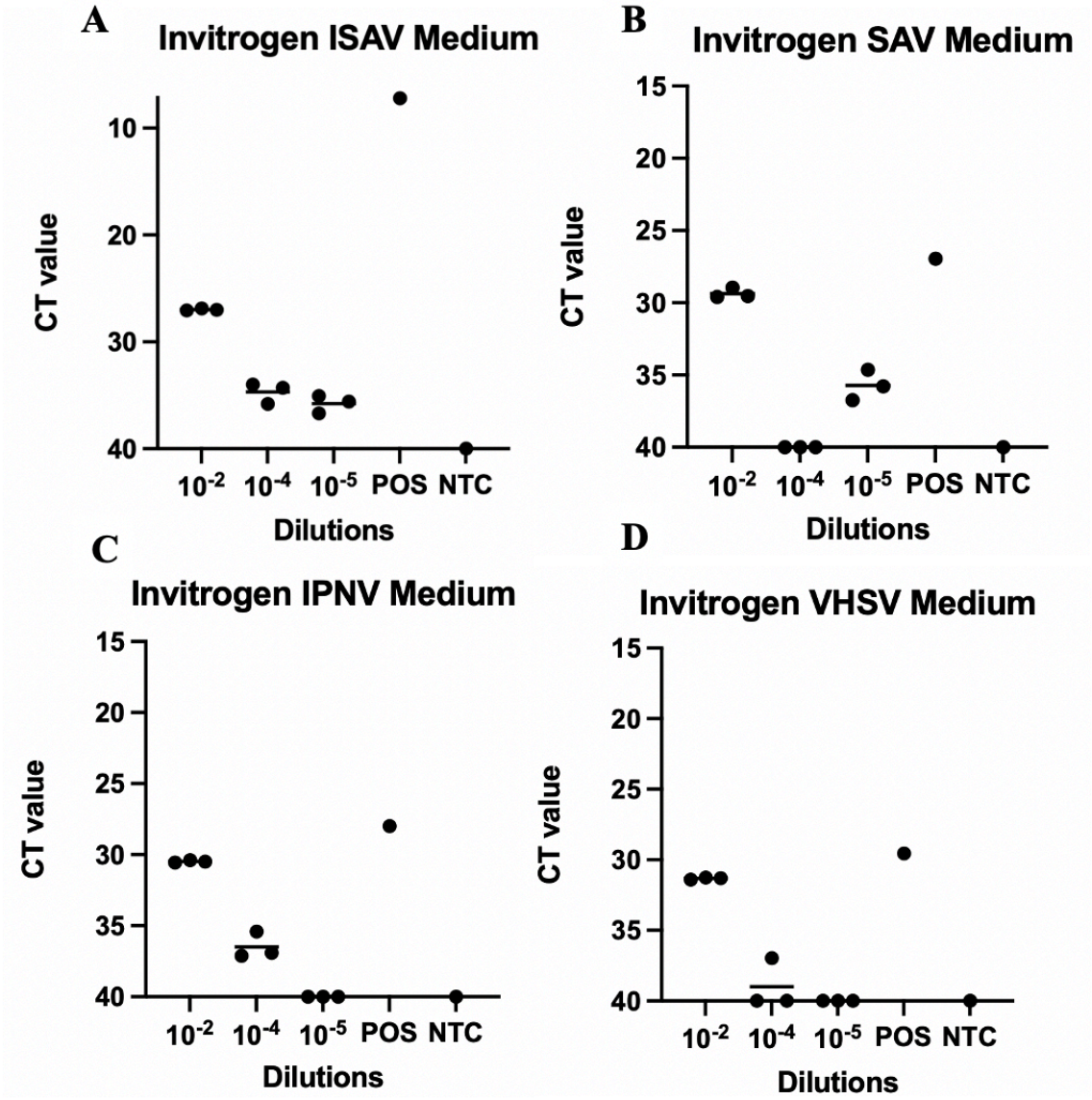

Figure 3.3: The Invitrogen Intact virus precipitation reagent was used to isolate virus from different dilutions of virus spiked in medium. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples are shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the RT-qPCR of the Invitrogen method show that viral RNA is detected for all dilutions in the ISAV samples. For the SAV samples, the results are positive for the 10^{-2} and the 10^{-5} dilutions, while the Ct value for the 10^{-4} dilution was undetermined. For IPNV and VHSV, the samples with the highest concentrations of virus (10^{-2} and 10^{-4}) were positive, while the 10^{-5} samples were negative for all triplicates for both viruses.

3.2.2 Seawater

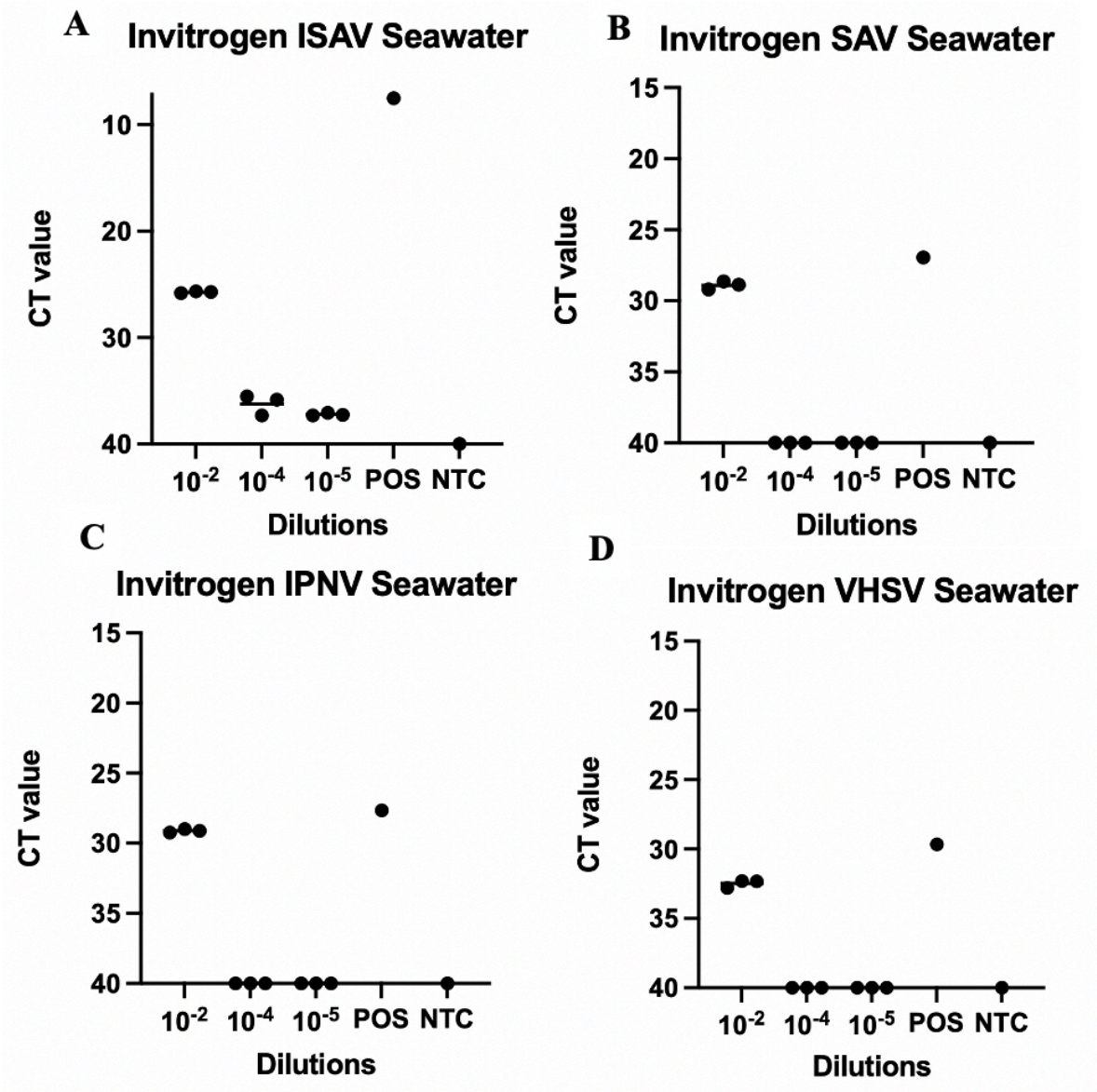

Figure 3.4: The Invitrogen Intact virus precipitation reagent was used to isolate virus from different dilutions of virus spiked in seawater. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples are shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the seawater samples show that from the ISAV samples, viral RNA was detected from all dilutions. From the SAV, IPNV and VHSV samples, viral RNA was only detected from the sample with the highest virus concentration (10^{-2}).

3.3 Ct results Lentivirus concentrator solution

The Lentivirus concentrator solution was also used to try to isolate virus from samples of virus spiked in tissue culture medium (L15 + 2% FBS) and in seawater in three different dilutions. The dilutions used were 10^{-2}, 10^{-4} and 10^{-5} like for the Invitrogen method. The lentivirus concentrator solution was added to each sample, incubated overnight, and spun down, before the supernatant was removed and the pellet resuspended in 100 µL PBS. 100 µL was taken out from each sample and analyzed by RNA isolation and RT-qPCR. The Ct results for the samples in medium are given in Figure 3.5, and the results from the seawater samples in Figure 3.6.

3.3.1 Tissue culture medium

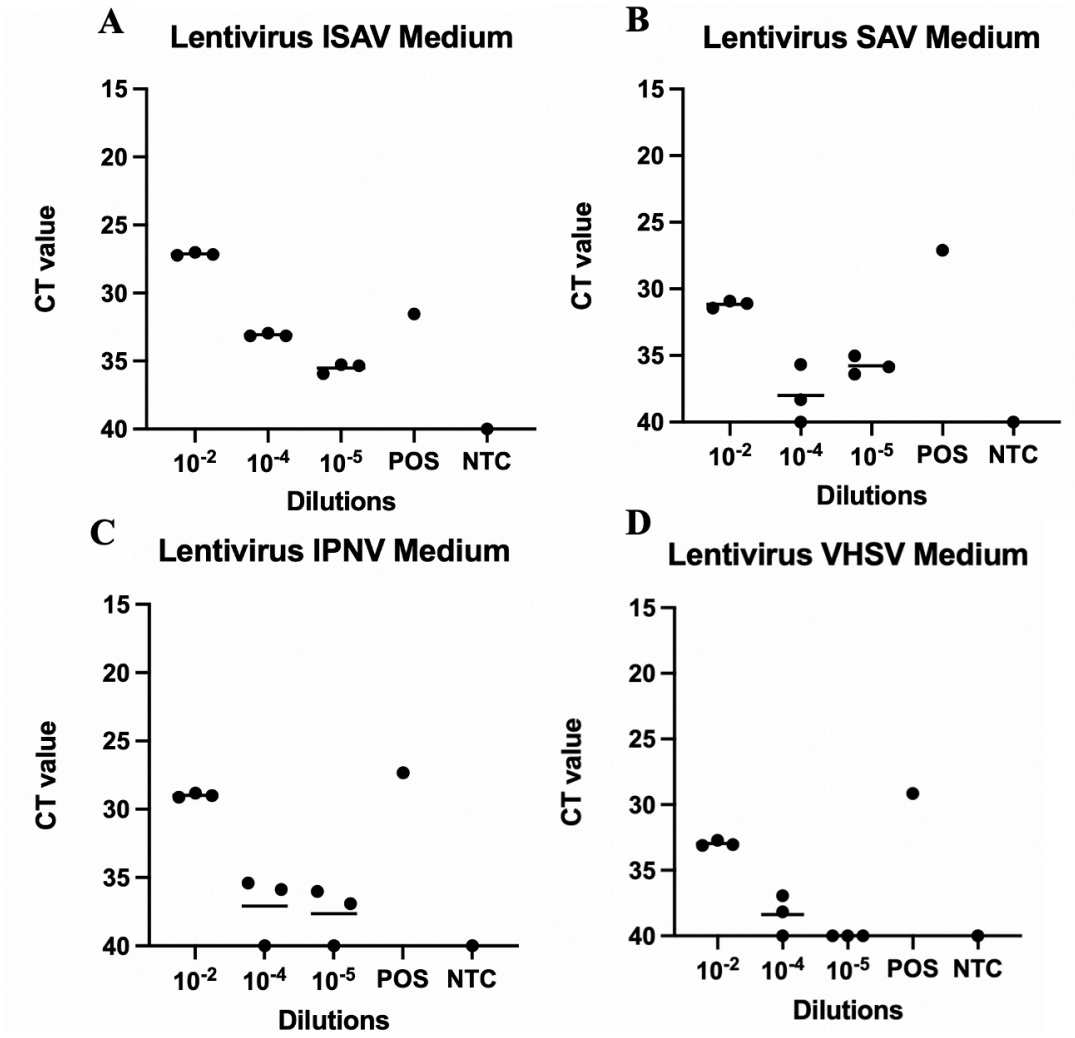

Figure 3.5: The lentivirus concentrator solution was used to isolate virus from medium spiked with virus in the dilutions 10^{-2}, 10^{-4} and 10^{-5}. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples are shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the RT-qPCR of the samples with isolated virus in medium from using the Lentivirus concentrator solution shows that for ISAV, SAV and IPNV, viral RNA is detected from all samples. For VHSV, the results are positive for the samples with the highest concentrations, but negative for the 10^{-5} sample.

3.3.2 Seawater

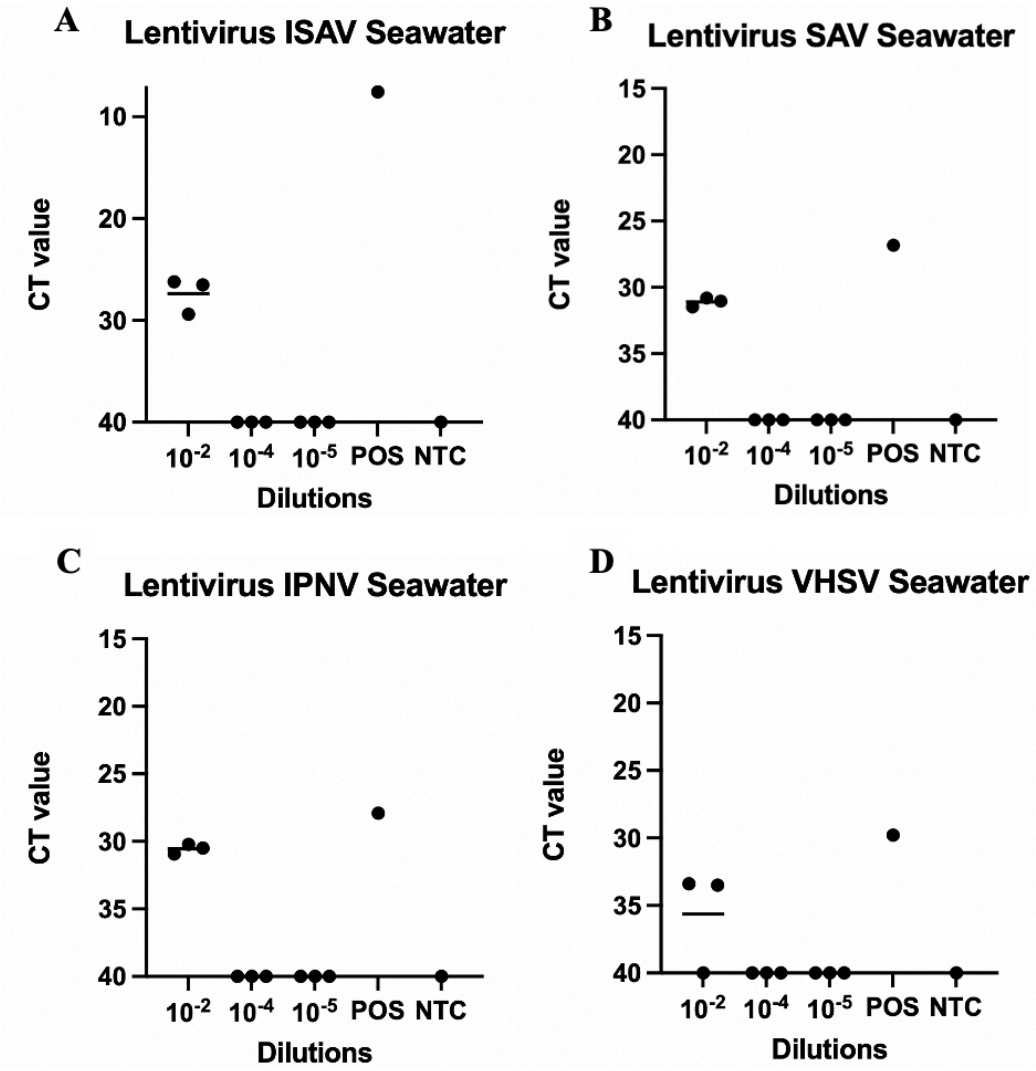

Figure 3.6: The lentivirus concentrator solution was used to isolate virus from seawater spiked with virus in the dilutions 10^{-2}, 10^{-4} and 10^{-5}. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples are shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the sweater samples with virus isolated using the lentivirus concentrator solution, show that viral RNA is only detected in the 10^{-2} dilutions for all four viruses. For VHSV, only two of the triplicates were positive.

3.4 Ct results CERES NANO Nanotrap virus capture kit

The CERES NANO Nanotrap virus capture kit was also used to try to isolate virus from samples of virus spiked in tissue culture medium (L15 + 2% FBS) and in seawater in the dilutions 10^{-2}, 10^{-4} and 10^{-5} as for the two other methods. Magnetic beads were added to each sample and incubated for 10 minutes so the viral particles could bind to the beads, before the samples were put in a magnet to isolate the beads from the solution. After washing, the viral particles were removed from the beads using a lysis buffer. RNA isolation and RT-qPCR were preformed to analyze the samples. The results from the medium samples are given in Figure 3.7 and the seawater samples in Figure 3.8.

3.4.1 Medium

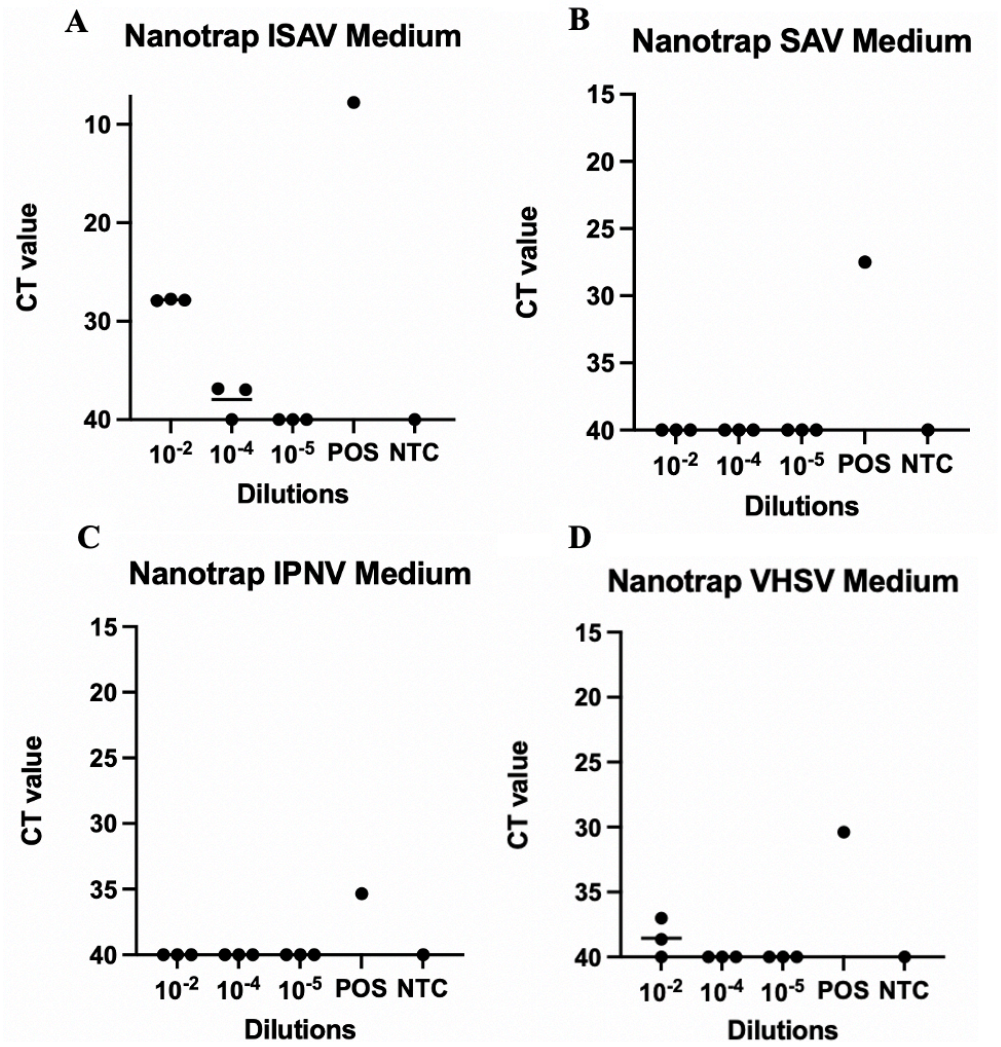

Figure 3.7: The Nanotrap kit was used to isolate virus from samples with medium spiked with virus in the dilutions 10^{-2}, 10^{-4} and 10^{-5}. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples is shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the RT-qPCR of the medium samples from testing the CERES NANO Nanotrap virus capture kit show that no viral RNA was detected in any of the dilutions of SAV and IPNV. For the ISAV samples, the 10^{-2} is positive for all triplicates, while two of the triplicates are positive for the 10^{-4} dilution. For VHSV, only two of the triplicates of the sample with the highest concentration (dilution 10^{-2}) was positive. The other dilutions were negative.

3.4.2 Seawater

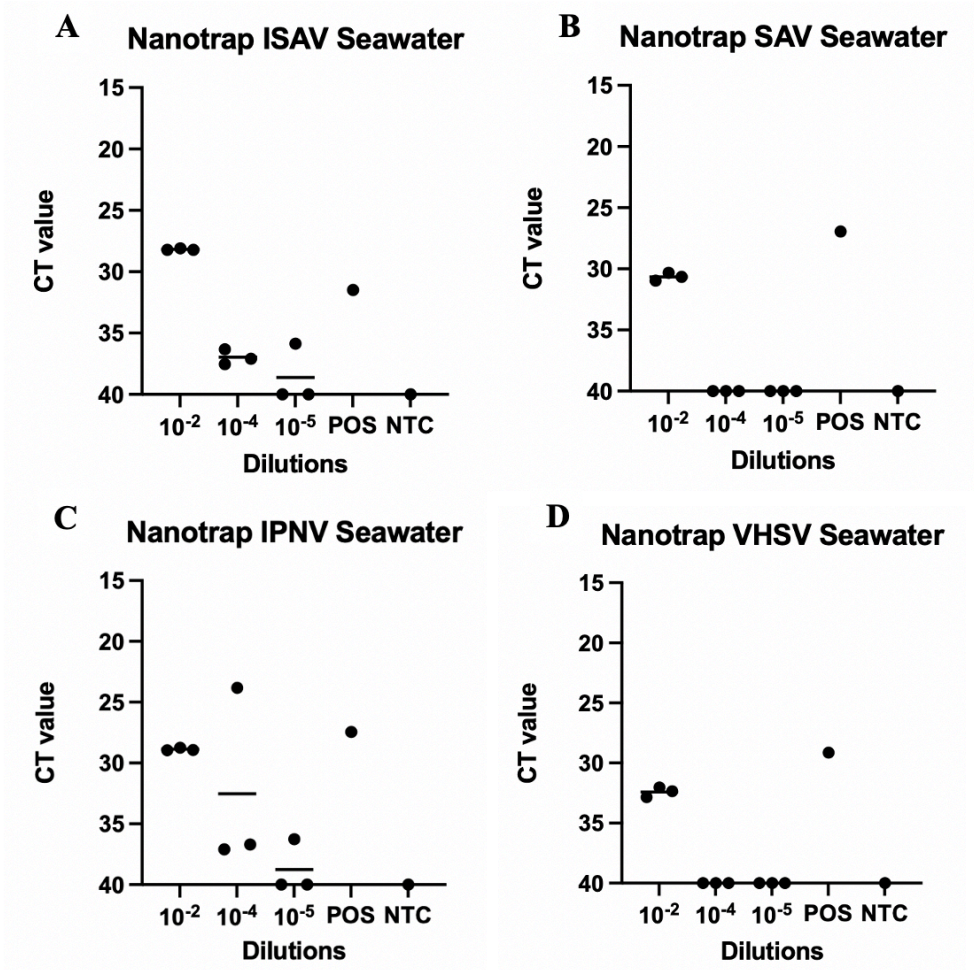

Figure 3.8: The Nanotrap kit was used to isolate virus from samples with seawater spiked with virus in the dilutions 10^{-2}, 10^{-4} and 10^{-5}. 100 µL was taken out of the samples with isolated virus and analyzed using RT-qPCR. The Ct results from the ISAV samples are shown in panel A, SAV in panel B, IPNV in panel C and VHSV in panel D. All samples were tested in triplicates.

The results from the RT-qPCR of the seawater samples from testing the CERES NANO Nanotrap virus capture kit show that for ISAV, viral RNA was detected in all dilutions. For

SAV and VHSV, only the 10^{-2} dilution was positive. For IPNV, viral RNA was detected in dilutions 10^{-2} and 10^{-4}. For the 10^{-5} dilution, only one of the triplicates was positive.

3.5 Virus isolation from a small volume of seawater results

When the initial testing of the methods was completed, and all methods seemed to be able to precipitate virus, two of the methods were tested again for isolation of ISAV and IPNV to see if they could be used to precipitate live virus as well. This was first tested for seawater samples with the same small volume as for the previous testing. The two methods used for this part were the Lentivirus concentrator solution and the Nanotrap magnetic beads.

Both methods were tested for four different dilutions of virus spiked in 1 ml of seawater: 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4} and for 1 ml PBS and 1 ml seawater samples. All samples were in duplicates (A and B). 100µL was taken out of each of the A samples before the virus isolation methods were preformed and analyzed by RT-qPCR for both methods. The A samples were analyzed by RNA isolation and RT-qPCR, and the B samples were put onto cells and incubated for 6 hours, before the virus were removed and replaced with medium. The cell plates were analyzed after seven days by IFAT or CPE.

3.5.1 Lentivirus concentrator solution

The virus isolation from a small volume of seawater by using the Lentivirus concentrator solution was done by adding 333 µL of the PEG solution to all the diluted viral samples and to the PBS and SW control samples. The samples were then mixed by shaking and incubated in a mixer overnight. The following day, the samples were spun down and the supernatants were removed. For the A samples, the pellet in each sample was then resuspended in 100 µL PBS and analyzed by RT-qPCR. The pellet in the B samples were resuspended in 500 µL of medium (L15 + 2% FBS + 2% antibiotics) and split into two tubes, where one of them were boiled before all the B samples were put on cells and incubated for 7 days. At day 7, the plates were analyzed by IFAT or plate reading to look for CPE.

The Ct results for the A samples of both ISAV and IPNV are given in Figure 3.9. The IFAT results from the ISAV infected cells are given in Figure 3.10, the ISAV CPE results in Figure 3.11 and the IPNV CPE results in Figure 3.12 (B samples).

CT results

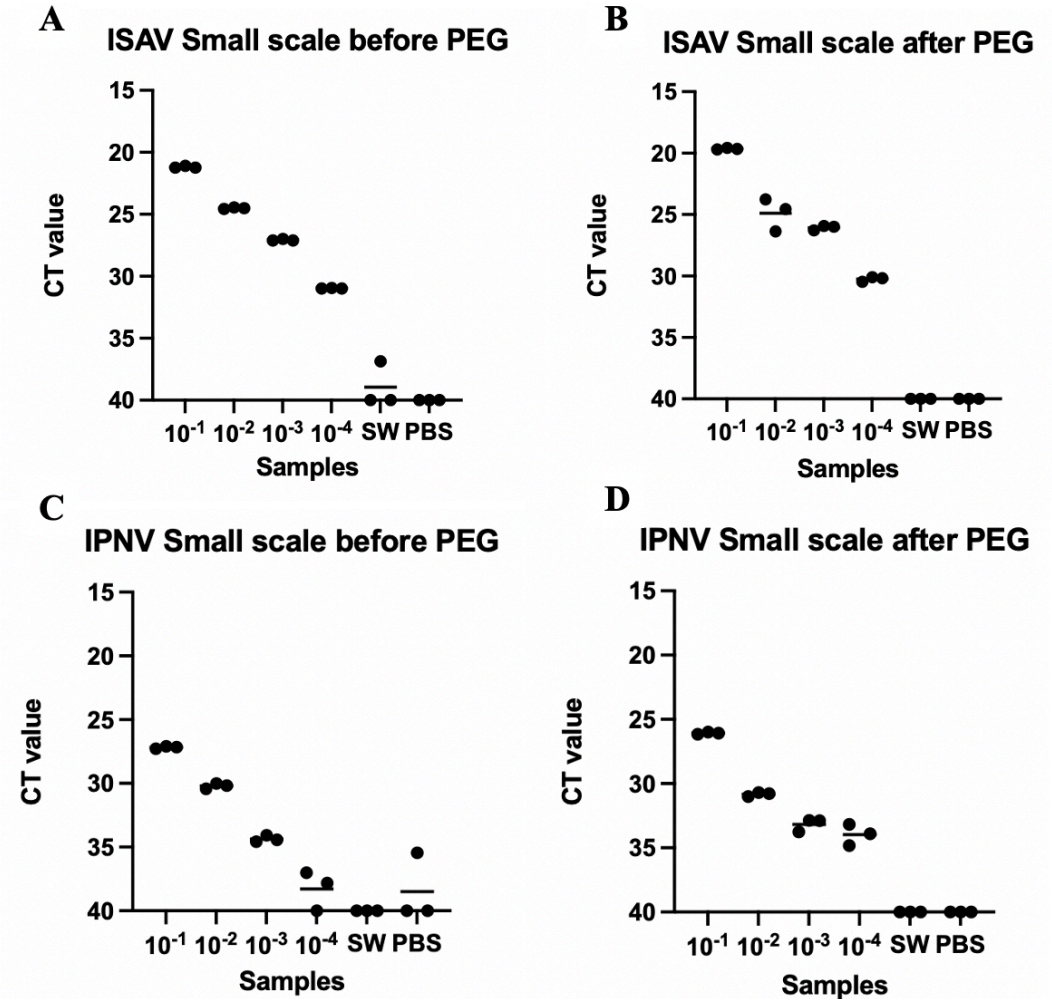

Figure 3.9: The Lentivirus concentrator solution method was used to isolate virus from samples with seawater spiked with virus (ISAV and IPNV) in the dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, SW samples and PBS samples. All samples were tested in duplicates (A and B). The A samples were analyzed by RT-qPCR. 100 µL was taken out of each of the A samples for both viruses before the PEG solution was added and analyzed by RT-qPCR. For ISAV, the Ct results from the samples before PEG was added are given in panel A, and the results after the virus isolation in panel B. For IPNV, the before PEG samples are given in panel C and the results after virus isolation in panel D. All samples were tested in triplicates.

The Ct results from the A samples show that viral RNA was detected from every sample containing virus. The Ct results are lowest for the 10^{-1} dilution, and gradually increasing for the lower concentrations, both before and after the virus isolation was performed. The Ct results are generally a bit lower for all samples after isolation, than before. For ISAV, one of the triplicates was positive in the SW control sample before PEG was added, but not after virus isolation. For IPNV, one of the triplicates from the PBS sample was positive before PEG, but not after isolation.

IFAT results ISAV

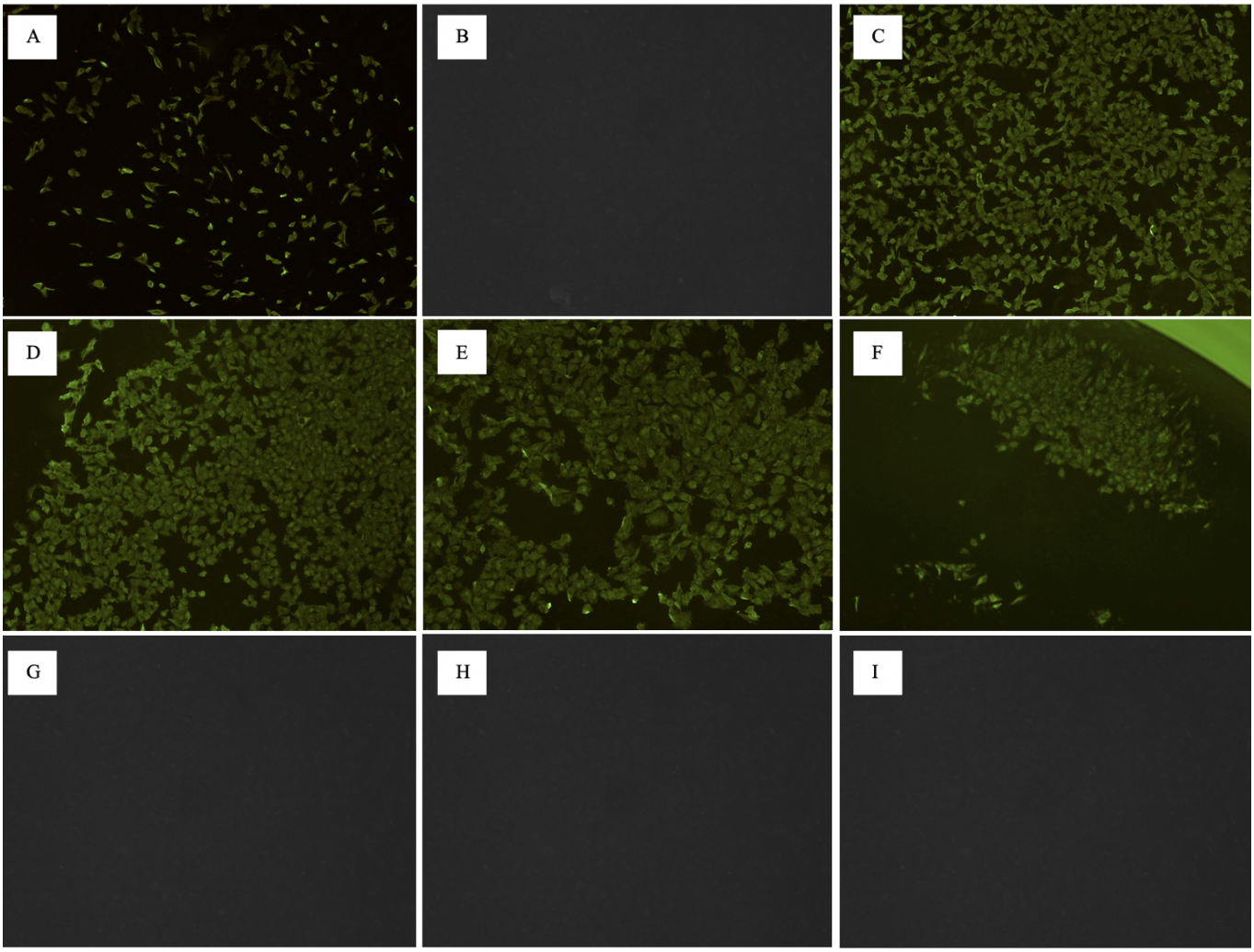

Figure 3.10: IFAT staining and fluorescence microscop*y* at day 8 after incubation were used to analyze the ASK-cells infected with the samples with ISAV isolated using the Lentivirus solution on small scale samples of different dilutions of virus spiked in seawater. Panel A shows the positive control (virus spiked in medium 10^{-2}), panel B the Negative control (PBS), panel C virus diluted 10^{-1}, panel D virus diluted 10^{-2}, panel E virus diluted 10^{-3}, panel F virus diluted 10^{-4}, panel G *boiled sample of* virus diluted 10^{-1}, panel H SW sample and panel I PBS sample. All samples are shown at 4x magnification.

The results from the IFAT staining of the cells infected with virus isolated by the lentivirus concentrator solution show that the cells were infected by all the virus dilution samples and in the positive control. No cells were infected by the negative controls, the PBS or SW samples, or by the viral samples that were boiled before they were put on cells.

CPE results (plate reading) ISAV

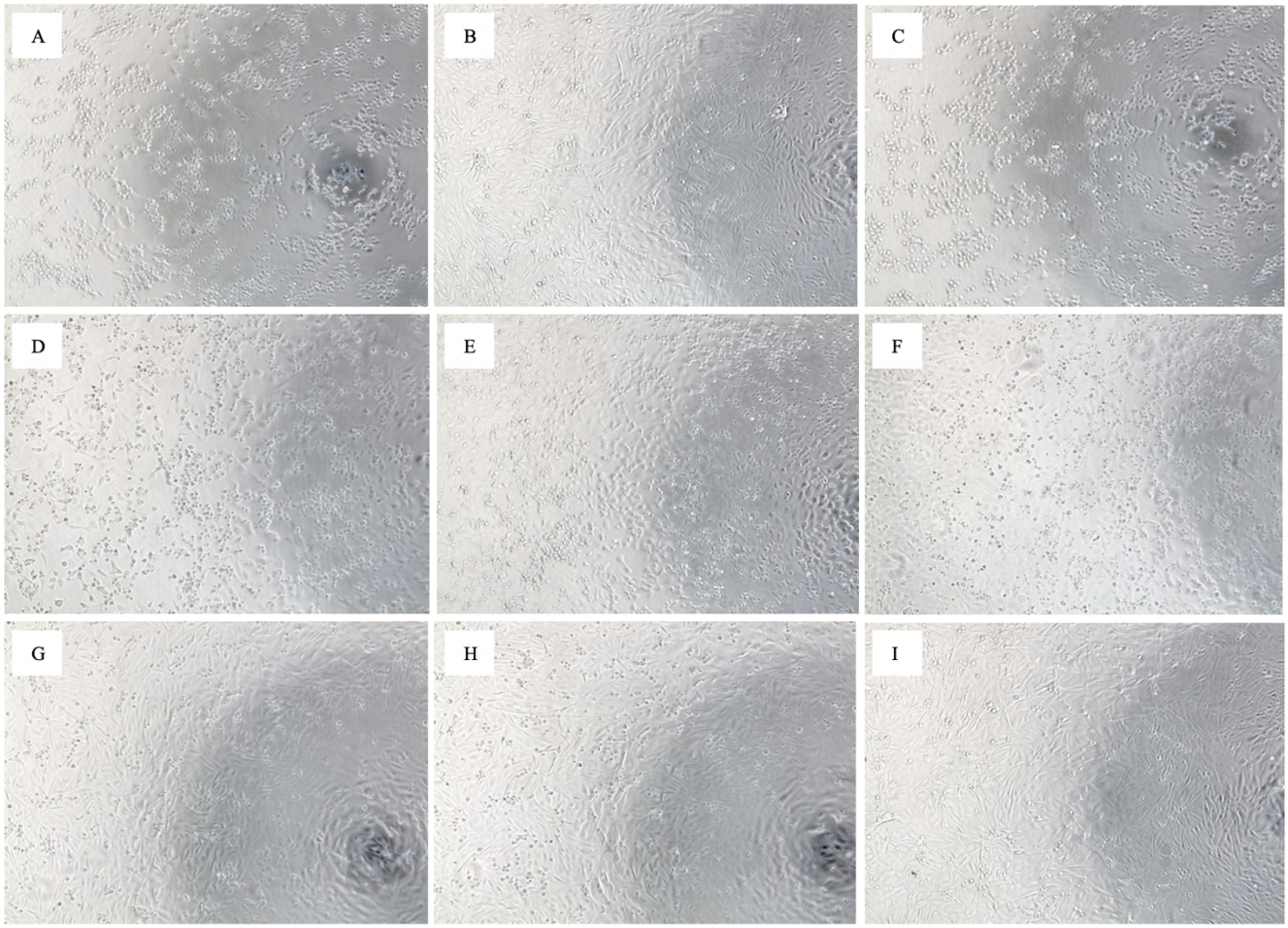

Figure 3.11: The ASK-cells infected with the samples with ISAV isolated using the lentivirus solution on samples of different dilutions of virus spiked in seawater and PBS and SW samples were analyzed for CPE in a microscope. Panel A shows the cells infected by the positive control, panel B the negative control, panel C virus diluted 10^{-1}, panel D virus diluted 10^{-2}, panel E virus diluted 10^{-3}, panel F virus diluted 10^{-4}, panel G SW, panel H PBS and panel I the virus diluted 10^{-1} and boiled. All samples are shown at 4x magnification. The pictures are from day 8 after infection.

The results from the plate reading in microscope of the ISAV infected cells show cytopathic effect in the positive control and in the cells infected by all the different dilutions of isolated virus. In the wells with negative control, boiled virus sample, PBS and SW, the cells look normal and not infected by virus.

CPE results (plate reading) IPNV

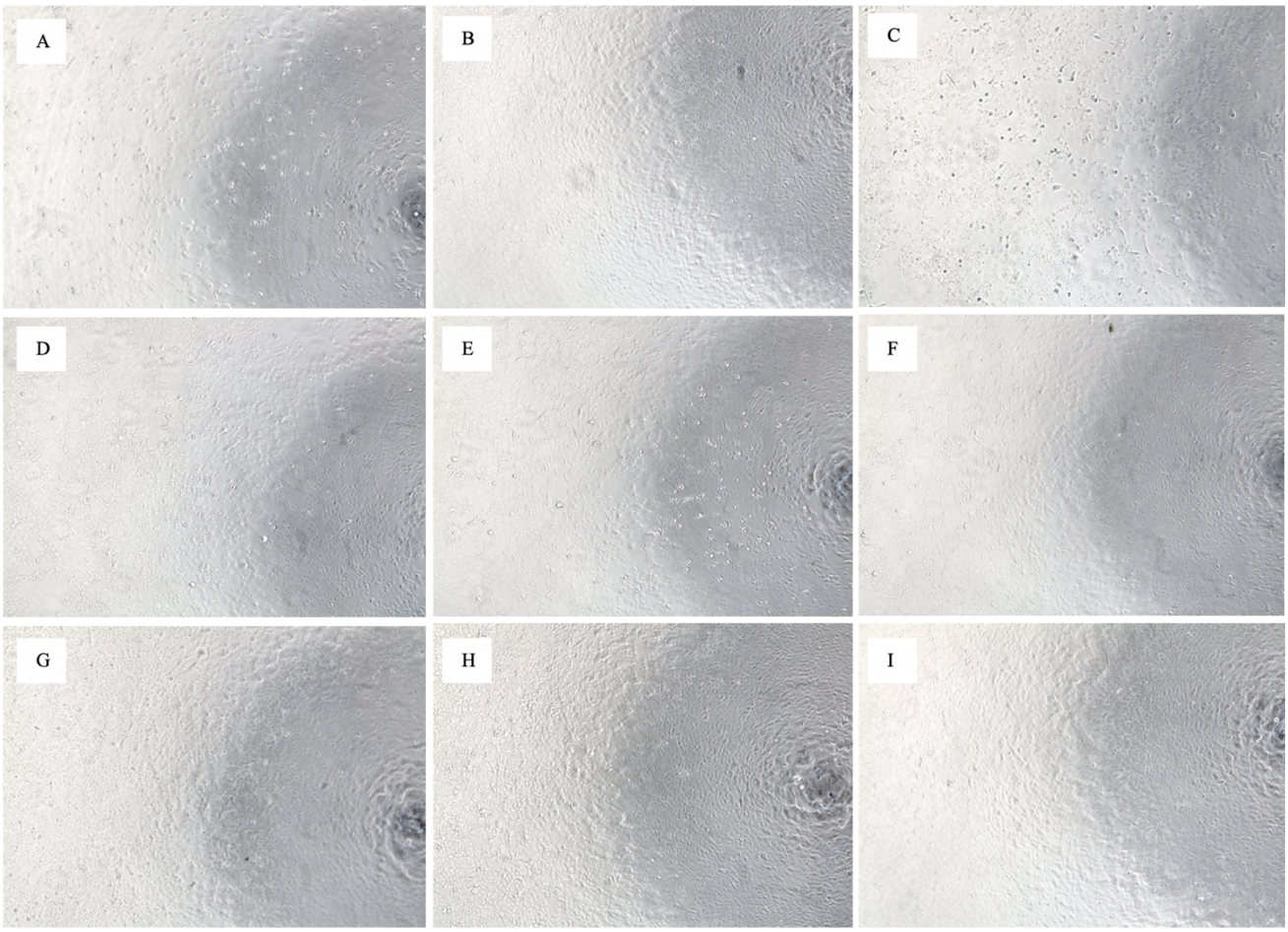

Figure 3.12: The CHSE-cells infected with the samples with IPNV isolated using the lentivirus solution on samples of different dilutions of virus spiked in seawater and PBS and SW samples were analyzed for CPE in a microscope. Panel A shows the cells infected by the positive control, panel B the negative control, panel C the virus diluted 10^{-1}, panel D the virus diluted 10^{-2}, panel E the virus diluted 10^{-3}, panel F the virus diluted 10^{-4}, panel G the SW, panel H the PBS and panel I the virus diluted 10^{-1}. All samples are shown at 4x magnification. The pictures are taken 8 days after infection

The plate reading for the cells with the IPNV samples show that it is hard to see cytopathic effect or cell lysis for the CHSE cells infected by isolated IPNV.

3.5.2 Manual Nanotrap® Wastewater Protocol

The virus isolation from a small volume of seawater by using the Nanotrap protocol was done by adding 20 µL of the magnetic beads to each sample and incubate them for 10 minutes so the beads could bind to the viral particles. The samples were then put in a magnetic rack to isolate the beads. The supernatant was removed, and the beads washed. The beads in the A samples were then resuspended in 150 µL of PBS and 560 µL lysis buffer and incubated again, before the beads were removed by transferring the supernatant into new tubes. The A samples were then analyzed by RNA isolation and RT-qPCR. For the B samples, the samples with the beads still in them were resolved in 500 µL medium. All B samples were split in two tubes, and half of them were boiled prior to being put on cells.

The Ct results for the A samples of both ISAV and IPNV before and after the virus isolation was preformed are given in Figure 3.13. The IFAT results from the ISAV infected cells are given in Figure 3.14, the ISAV CPE results in Figure 3.15 and the IPNV CPE results in Figure 3.16.

Ct results

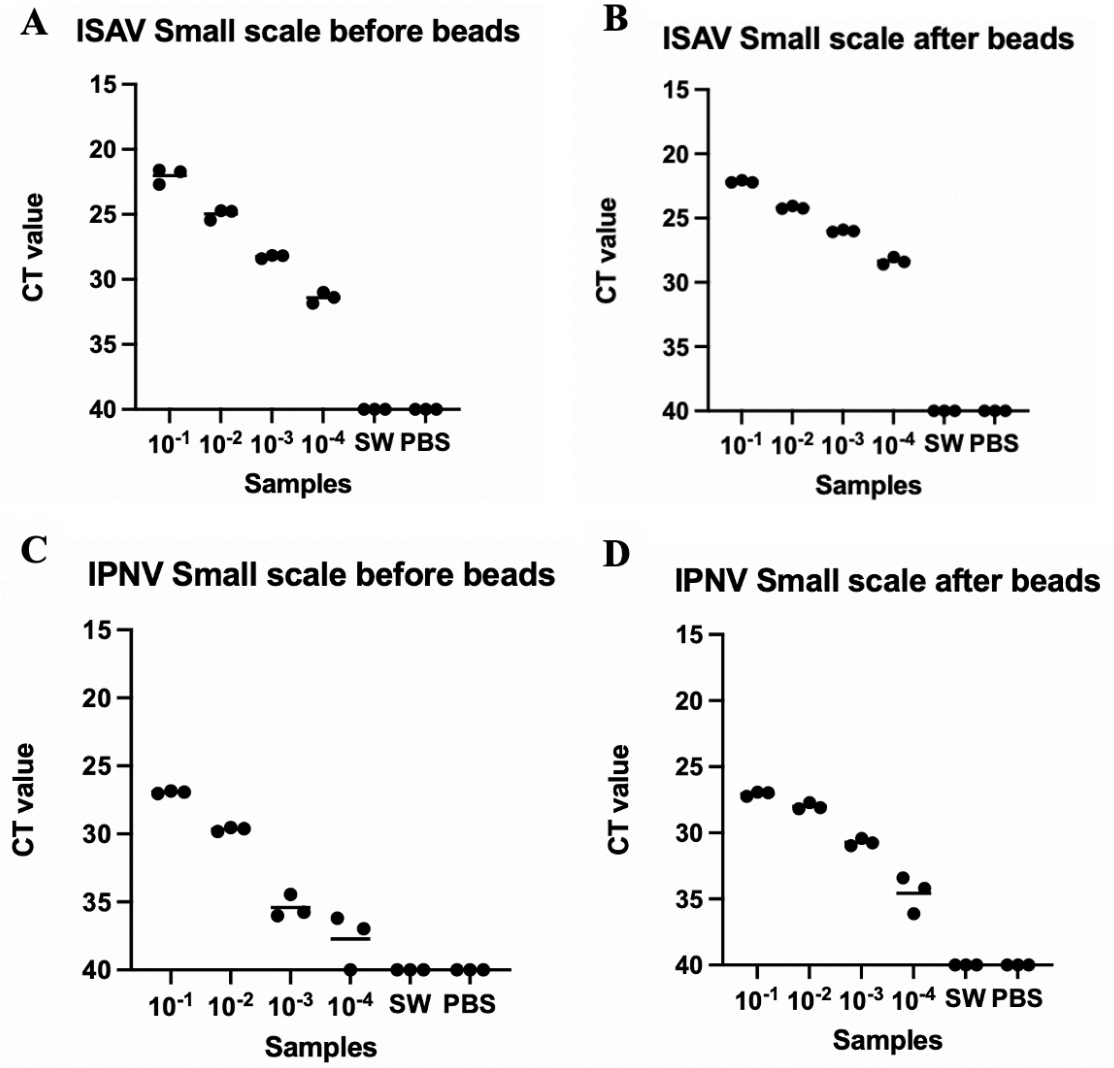

Figure 3.13: The Nanotrap method was used to isolate virus from samples with seawater spiked with virus (ISAV and IPNV) in the dilutions 10^{-1}, 10^{-2}, 10^{-3} and 10^{-4}, as well as SW samples and PBS samples. All samples were tested in duplicates (A and B). The A samples were analyzed by RT-qPCR, and the B samples were put on cells. 100 µL was taken out of each of the A samples for both viruses before the PEG solution was added and analyzed by RT-qPCR. For ISAV, the Ct results from the samples before the beads were added are given in panel A, and the results after the virus isolation in panel B. For IPNV, the before beads samples are given in panel C and the results after virus isolation in panel D. All samples were tested in triplicates.

The results from the RT-qPCR show that viral RNA is detected from all virus dilutions, both before and after virus isolation. The 10^{-1} sample has the lowest Ct value and the 10^{-4} samples the highest Ct value for both viruses. The Ct value is generally a little lower after isolation than before for both viruses. All samples with SW and PBS are negative.

IFAT ISAV

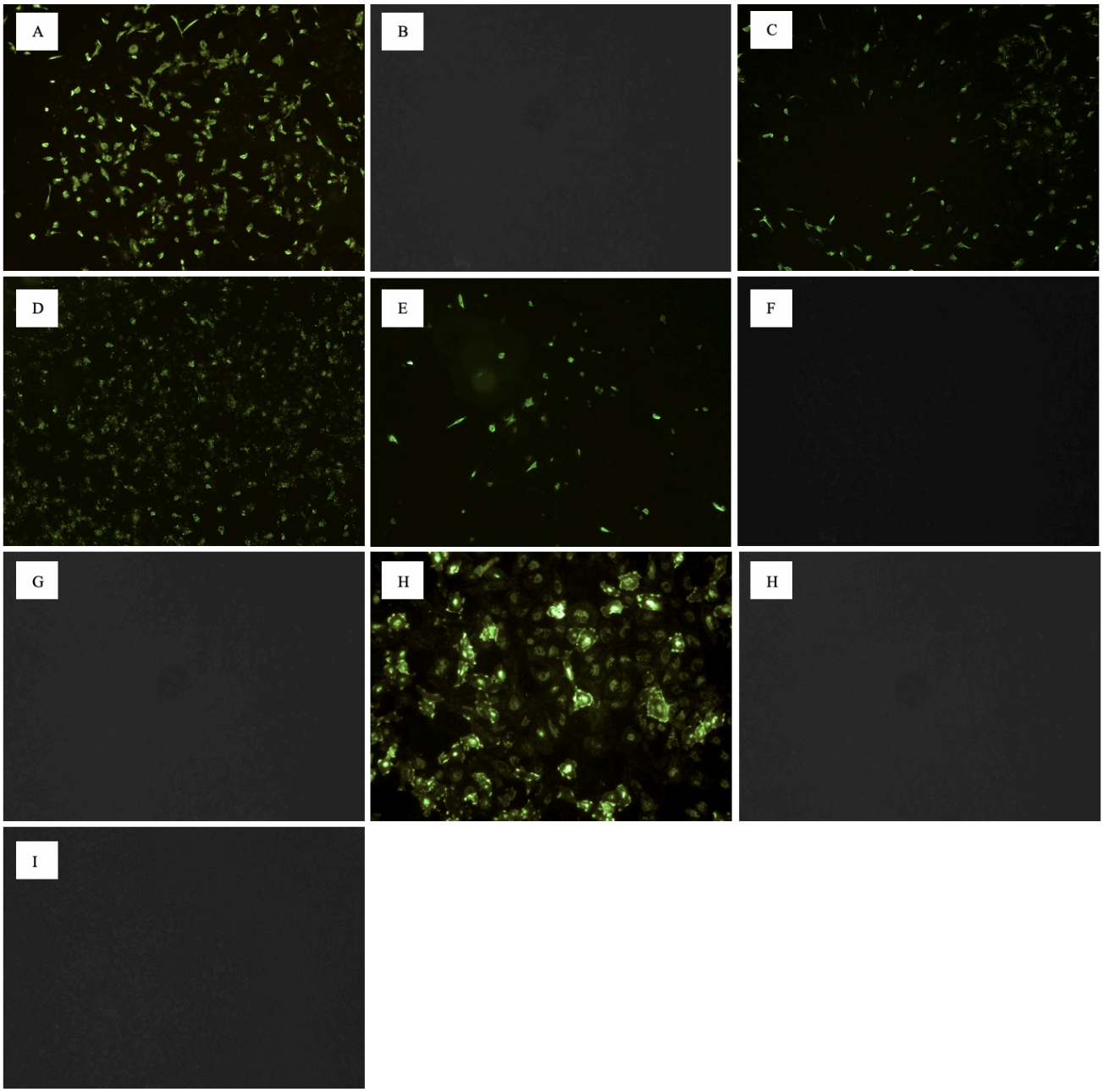

Figure 3.14: IFAT at day 8 after infection was used to analyze the ASK-cells infected with samples of ISAV isolated using the Nanotrap magnetic beads on small scale samples of different dilutions of virus spiked in seawater. Panel A shows the positive control, panel B negative control, panel C 1:10 dilution, panel D 1:100 dilution, panel E 1:10000 dilution, panel F 1:10 000 dilution, panel G 1:10 dilution boiled, panel H 1:100 dilution in 10x magnification, panel I SW and panel J PBS. All samples, except for H, are shown at 4x magnification

The IFAT results from the ISAV samples show that the cells in the wells with the positive control and the virus dilutions 10^{-1}, 10^{-2} and 10^{-3} were infected. The wells with the lowest concentration of virus, 10^{-4}, were not infected. No cells were infected by the seawater or the PBS samples either. The boiled samples of virus were also all negative.

Plate reading (CPE) ISAV

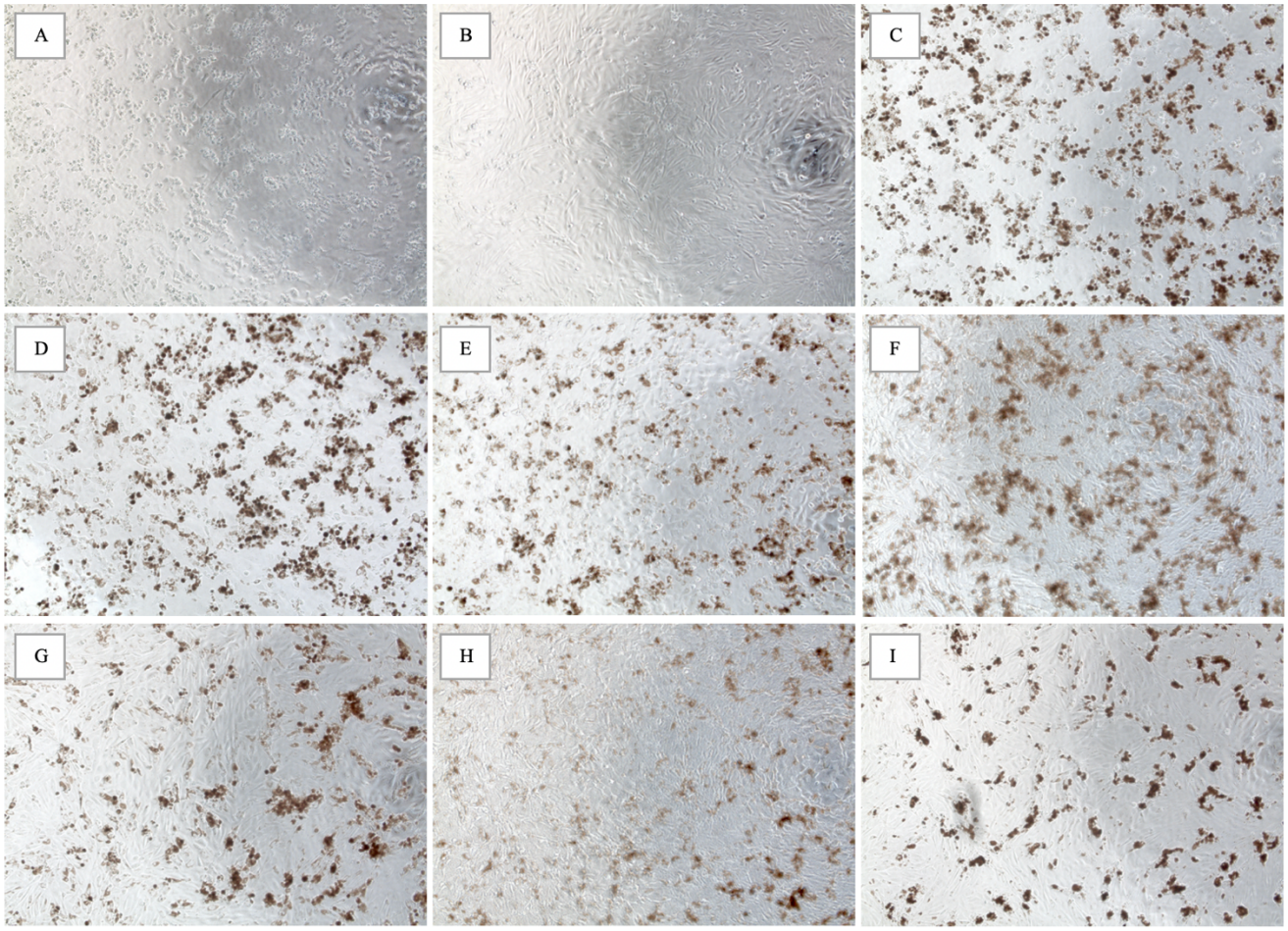

Figure 3.15: The ASK-cells infected with the samples of ISAV isolated using the Nanotrap method on samples of virus dilutions, PBS and SW were analyzed for cytopathic effect in a microscope at day 8 after infection. Panel A shows the cells infected by the positive control, panel B the negative control, panel C the virus diluted 10^{-1}, panel D the virus diluted 10^{-2}, panel E the virus diluted 10^{-3}, panel F the virus diluted 10^{-4}, panel G the SW, panel H the PBS and panel I the virus diluted 10^{-1}. All samples are shown at 4x magnification.

The results from the plate reading in microscope of the ISAV infected cell plates, show cytopathic effect in the positive control and in the wells with the dilutions 10^{-1} – 10^{-3}. The cells in the negative control, the dilution 10^{-4}, the PBS and SW looks more normal, but the beads covering the cells makes it difficult to conclude if there is cytopathic effect or not.

Plate reading (CPE) IPNV

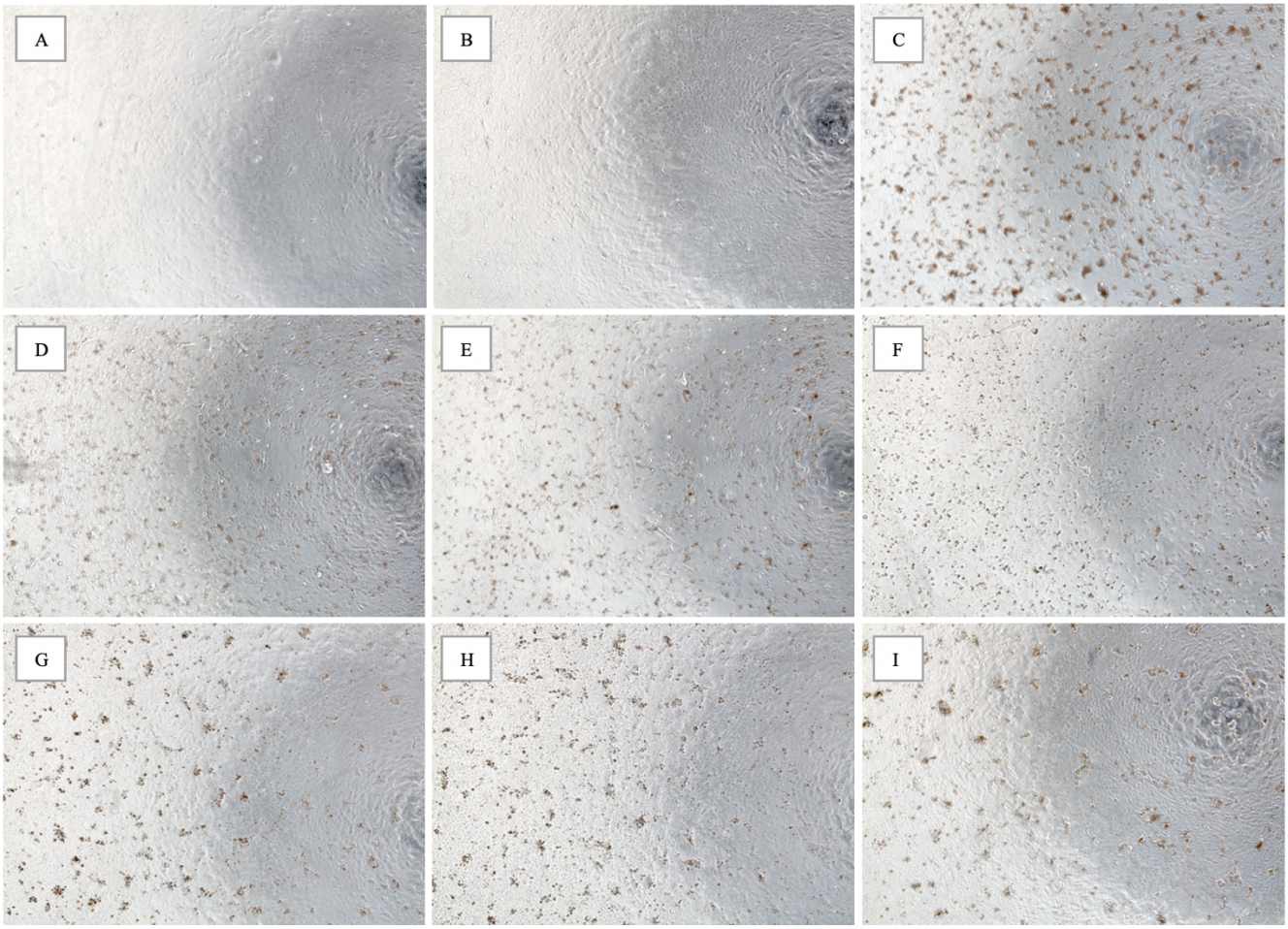

Figure 3.16: The CHSE-cells infected with the samples of IPNV isolated using the Nanotrap method on samples of virus dilutions, PBS and SW were analyzed for cytopathic effect in a microscope at day 8 after infection. Panel A shows the cells infected by the positive control, panel B the negative control, panel C the virus diluted 10^{-1}, panel D the virus diluted 10^{-2}, panel E the virus diluted 10^{-3}, panel F the virus diluted 10^{-4}, panel G the SW, panel H the PBS and panel I the virus diluted 10^{-1}. All samples are shown at 4x magnification.

The results from the plate reading in microscope of the IPNV infected cell plates, show that it is difficult to see cytopathic effect in the cells because of the magnetic beads that was in the samples when they were put on cells.

3.6 Virus isolation from a larger volume of seawater results

The Lentivirus concentrator solution method and the Manual Nanotrap Wastewater protocol were also tested to isolate ISAV and IPNV spiked in larger volumes of seawater (30 ml). Both methods were tested for virus samples with 100 µL of virus spiked in 30 ml of seawater, seawater samples (30 ml) and PBS samples (30 ml). As for the small volume samples, all samples were in duplicates (A and B). The A samples were analyzed by RNA isolation and RT-qPCR, and the B samples were put onto cells and incubated for 6 hours, before the virus was removed and replaced with medium. The cell plates were analyzed after seven days by IFAT or CPE.

100 µL was taken out of each of the A samples for both viruses and methods before the isolation methods were performed and analyzed by RNA isolation and RT-qPCR.

3.6.1 Lentivirus concentrator solution

For testing the lentivirus concentrator solution, 10 ml of the lentivirus concentrator solution was added to each of the samples (virus, SW, and PBS sample for each virus in duplicates) and mixed by shaking (Figure 2.4). The tubes were incubated on a rotary mixer in the cold room at 4°C overnight. The samples were then spun down and the supernatant removed. The pellets in the A samples were resuspended in 1 ml PBS. 100 µL was taken out and analyzed by RT-qPCR. The pellets in the B samples were resuspended in 500 µL of medium and split into two tubes each. Half of the samples were boiled prior to them being put on cells. When the virus samples were added to the plates, the plates were incubated for 6 hours, before the virus was removed and replaced with 200 µL of medium (L15 with 2% FBS and 2% antibiotic) before they went back in the incubator for seven days. At day seven, the ISAV plates were analyzed by using the indirect fluorescent antibody technique (IFAT) and plate reading, while the IPNV plates were analyzed by plate reading in the microscope.

The Ct results for the A samples of both ISAV and IPNV before and after the virus isolation was preformed are given in Figure 3.17. The IFAT results from the ISAV infected cells are given in Figure 3.18, the ISAV CPE results in Figure 3.19 and the IPNV CPE results in Figure 3.20.

CT results

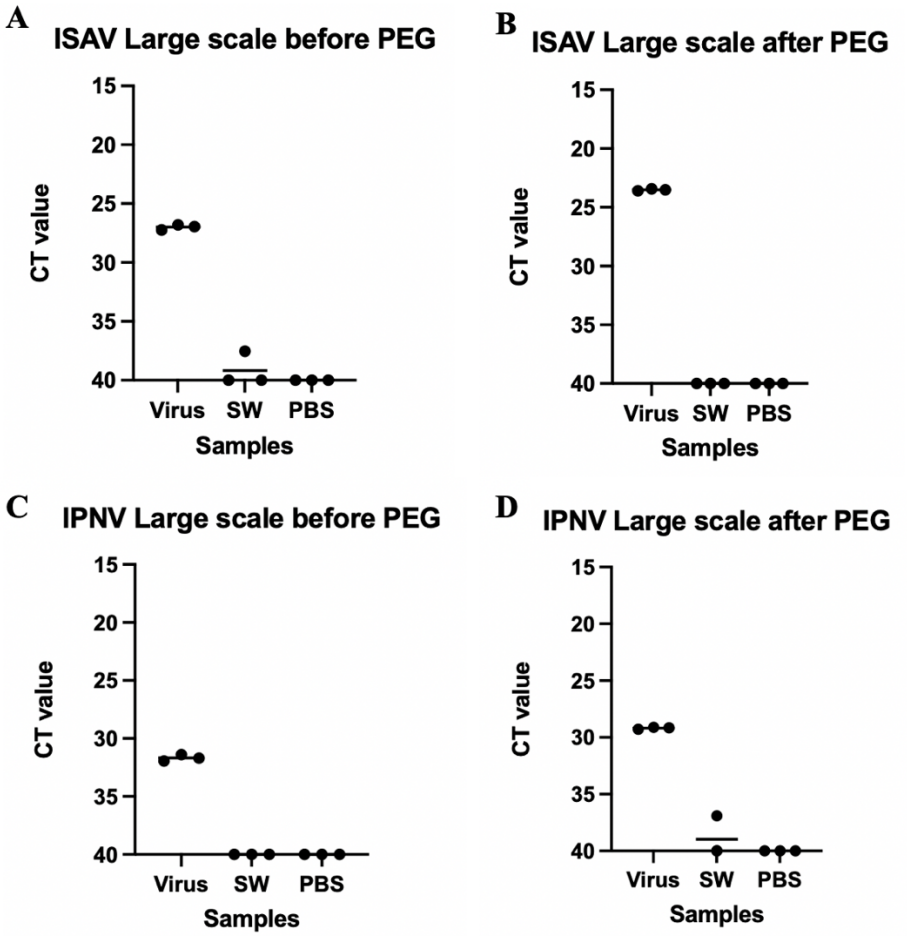

Figure 3.17: The lentivirus concentrator solution was used to isolate virus from samples with a larger volume of seawater (30 ml) spiked with 100µL virus (ISAV, IPNV) and from SW and PBS samples (30 ml). All samples were tested in duplicates (A and B). The A samples were analyzed by RT-qPCR, and the B cells were put on cells. 100 µL was taken out of each of the A samples for both viruses before the PEG solution was added and analyzed by RT-qPCR. For ISAV, the Ct results from the samples before the beads were added are given in panel A, and the results after the virus isolation in panel B. For IPNV, the before beads samples are given in panel C and the results after virus isolation in panel D. All samples were tested in triplicates.

The Ct results from virus isolation from large volume of seawater using the Lentivirus method show that viral RNA was detected from all sample spiked with virus, both before and after virus isolation. One of the triplicates was positive for both ISAV in SW before isolation and for IPNV after isolation.

IFAT ISAV

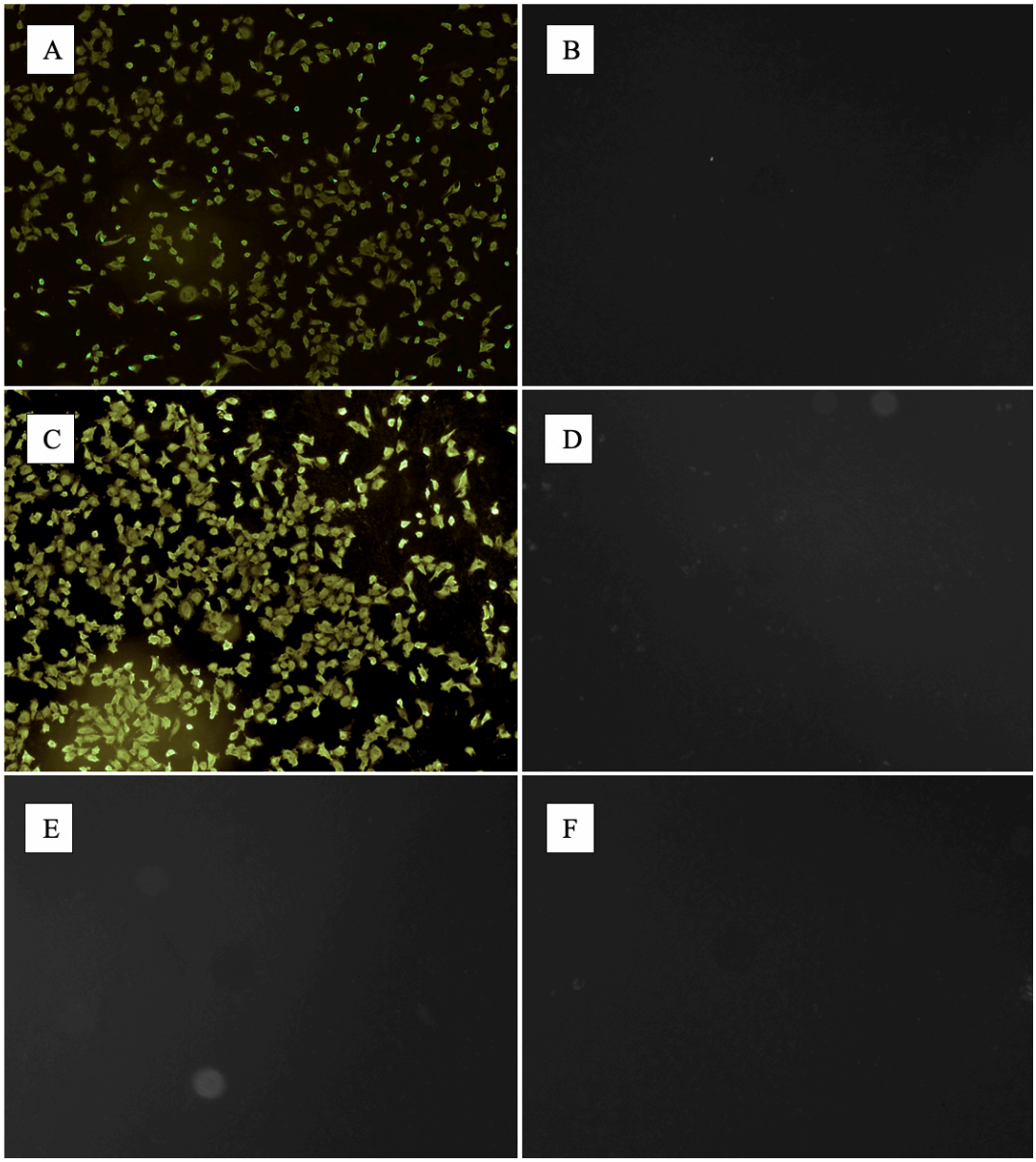

Figure 3.18: IFAT at day 7 after infection was used to analyze the ASK-cells infected with the samples with ISAV isolated using the Lentivirus solution on the large-scale samples of virus spiked in seawater, samples with just seawater and samples with just PBS. Panel A shows the positive control, panel B the negative control, panel C the precipitated virus sample, panel D the boiled virus sample, panel E the SW and panel F the PBS. All samples are shown at 4x magnification.

The IFAT results show that cells were infected in the wells with positive control and the virus sample that was not boiled. All boiled samples, the PBS sample, SW samples and negative controls were not infected.

CPE (plate reading) ISAV

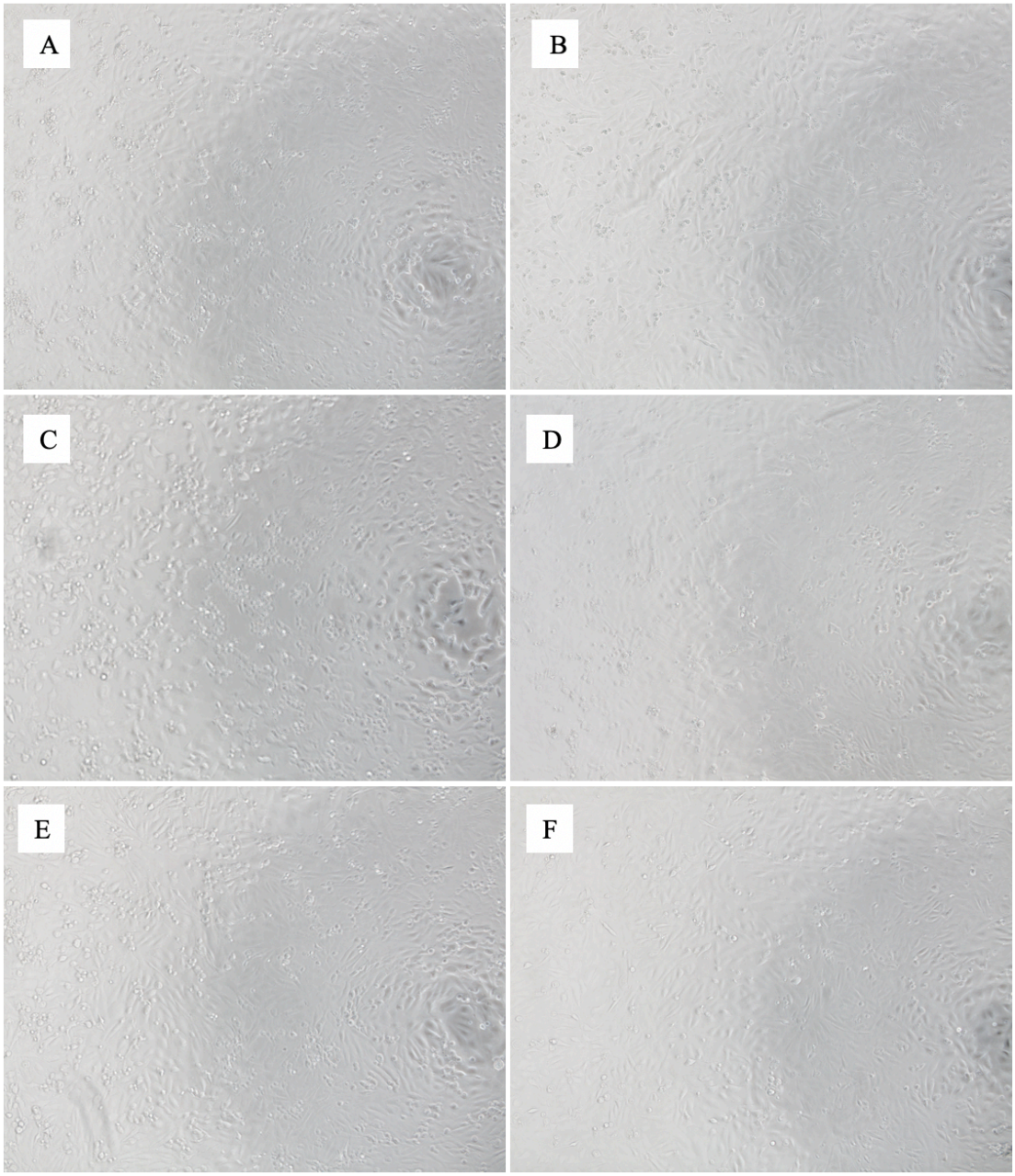

Figure 3.19: The ASK-cells infected with the samples of ISAV isolated using the Lentivirus concentrator solution on samples of virus dilutions, PBS, and SW in larger volumes of seawater were analyzed for cytopathic effect in a microscope at 7 days after infection. Panel A show the cells infected by the positive control, panel B the negative control, panel C the sample containing virus, panel D the boiled virus sample, panel E the SW samples and panel F the PBS samples. All samples are shown at 4x magnification.

The results from the plate reading of the ISAV cell plate shows cytopathic effect in the wells with infected by the positive control and the samples spiked with virus. The other wells have more normal looking cells, but it is hard to determine if there is cell lysis or not.

CPE (plate reading) IPN

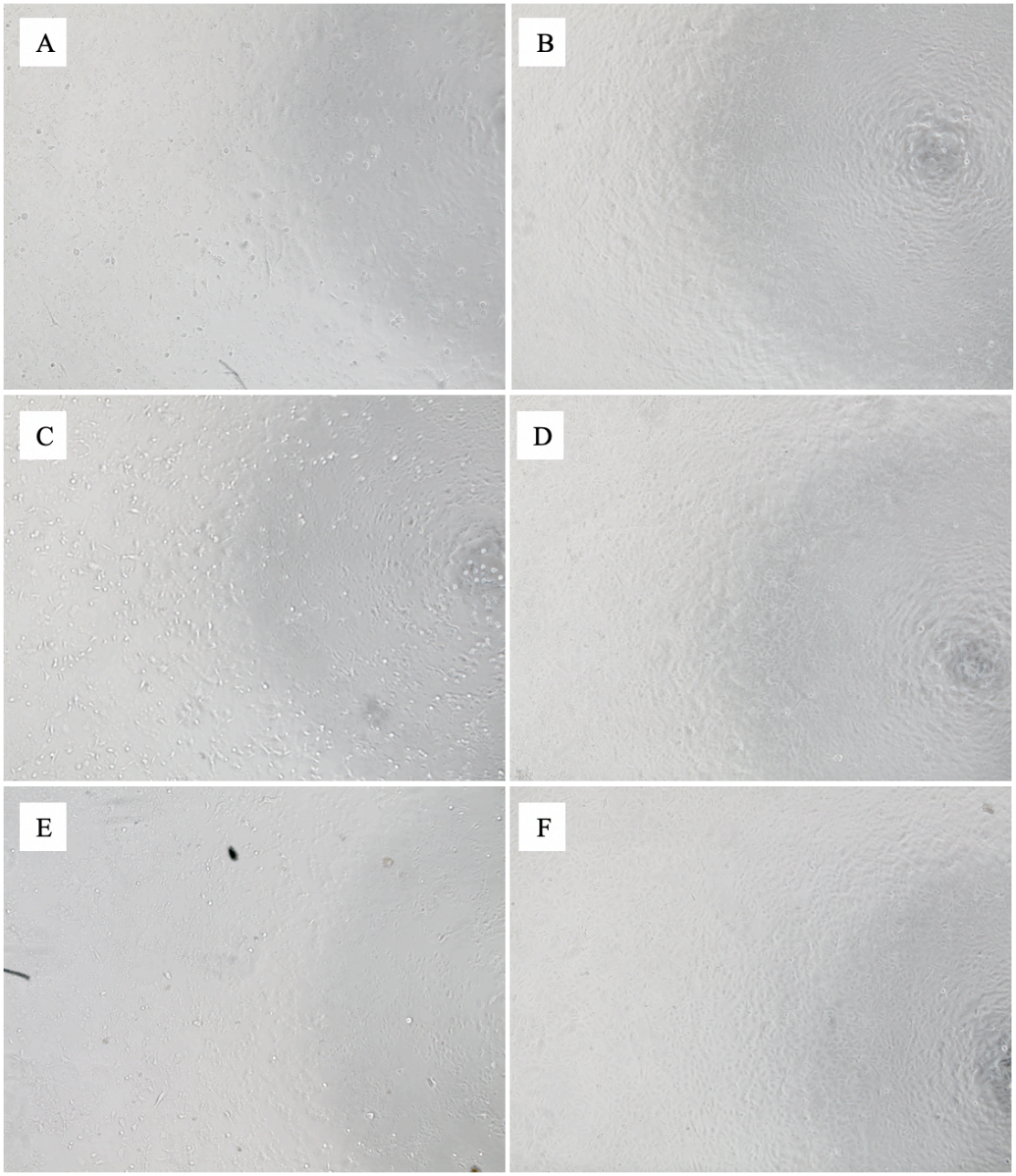

Figure 3.20: The CHSE-cells infected with the samples of IPNV isolated using the Lentivirus concentrator solution on samples of virus dilutions, PBS, and SW in larger volumes of seawater were analyzed for cytopathic effect in a microscope at 7 days after infection. Panel A show the cells infected by the positive control, panel B the negative control, panel C the sample containing virus, panel D the boiled virus sample, panel E the SW samples and panel F the PBS samples. All samples are shown at 4x magnification.

In the plate reading results for IPN, cytopathic effect is seen in the positive control and in the samples containing isolated virus. The negative controls, boiled samples and PBS samples were all negative. In panel E, with the SW sample, some cytotoxicity was observed.

3.6.2 Nanotrap

The Nanotrap wastewater protocol was also tested for isolating ISAV and IPNV from 30 ml samples. 150 µL of magnetic beads was added to each tube and mixed by inversion. The tubes were then incubated for 2 hours, before the tubes were put in a magnet to isolate the beads. The supernatant was removed and then the beads were washed two times in molecular grade water. The water was removed and the pellet in the A tube was resuspended in 150 µL PBS for RT-qPCR and 560 µL virus lysis buffer (buffer AVL). The samples were incubated for 10 minutes, before they were put back in the magnet. The lysate solution was then separated from the beads. The A samples were then RNA isolated and analyzed by RT-qPCR.

The B tubes were resuspended in 500 µL medium (L15 + 2% FBS + 2% antibiotics), and then split into two tubes per sample. Half of the samples were boiled. Then, all samples were put on cells on plates, ISAV on ASK cells and IPNV on CHSE cells, and incubated for 6 hours before the virus was removed and replaced with 200 µL. The plates were incubated again for 7 days, before the cells were analyzed with IFAT or plate reading in microscope.

The Ct results for the A samples of both ISAV and IPNV before and after the virus isolation was preformed are given in Figure 3.21. The IFAT results from the ISAV infected cells are given in Figure 3.22, the ISAV CPE results in Figure 3.23 and the IPNV CPE results in Figure 3.24.

CT result

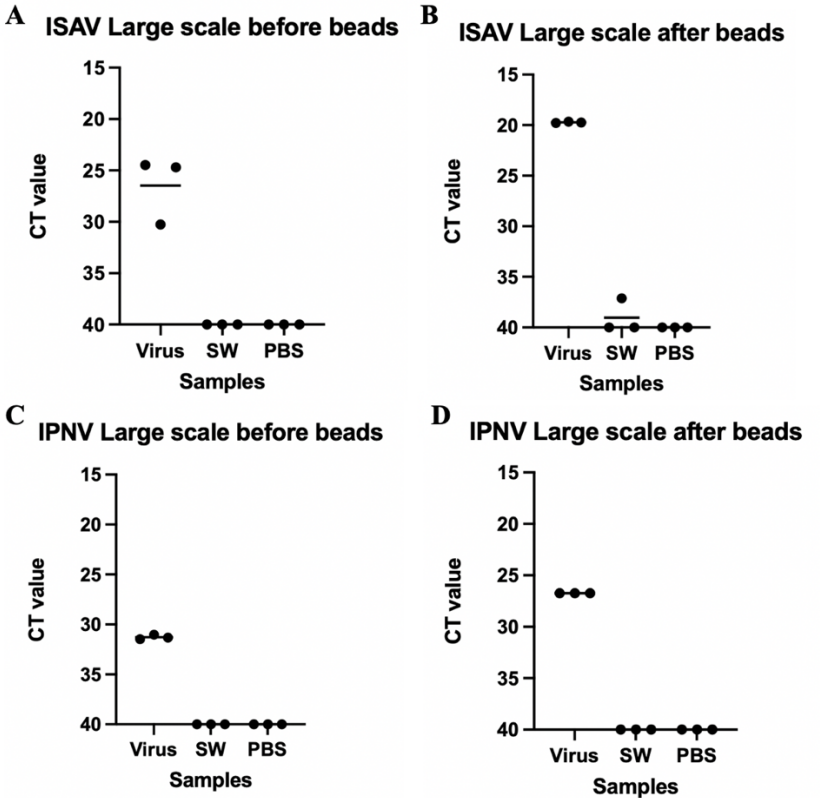

Figure 3.21: The Nanotrap method was used to isolate virus from samples with a larger volume of seawater (30 ml) spiked with 100µL virus (ISAV, IPNV) and from SW and PBS samples (30 ml). All samples were tested in duplicates (A and B). The A samples were analyzed by RT-qPCR, and the B cells were put on cells. 100 µL was taken out of each of the A samples for both viruses before the PEG solution was added and analyzed by RT-qPCR. For ISAV, the Ct results from the samples before the beads were added are given in panel A, and the results after the virus isolation in panel B. For IPNV, the before beads samples are given in panel C and the results after virus isolation in panel D. All samples were tested in triplicates.

The Ct results from the large volume samples isolated by the Nanotrap method show that viral RNA was detected from all samples spiked with virus, both before and after virus isolation. One triplicate from the ISAV seawater sample after isolation was also positive. All the other PBS and SW samples are negative both before and after virus isolation. The Ct value is lower in the samples after isolation than before.

IFAT ISAV

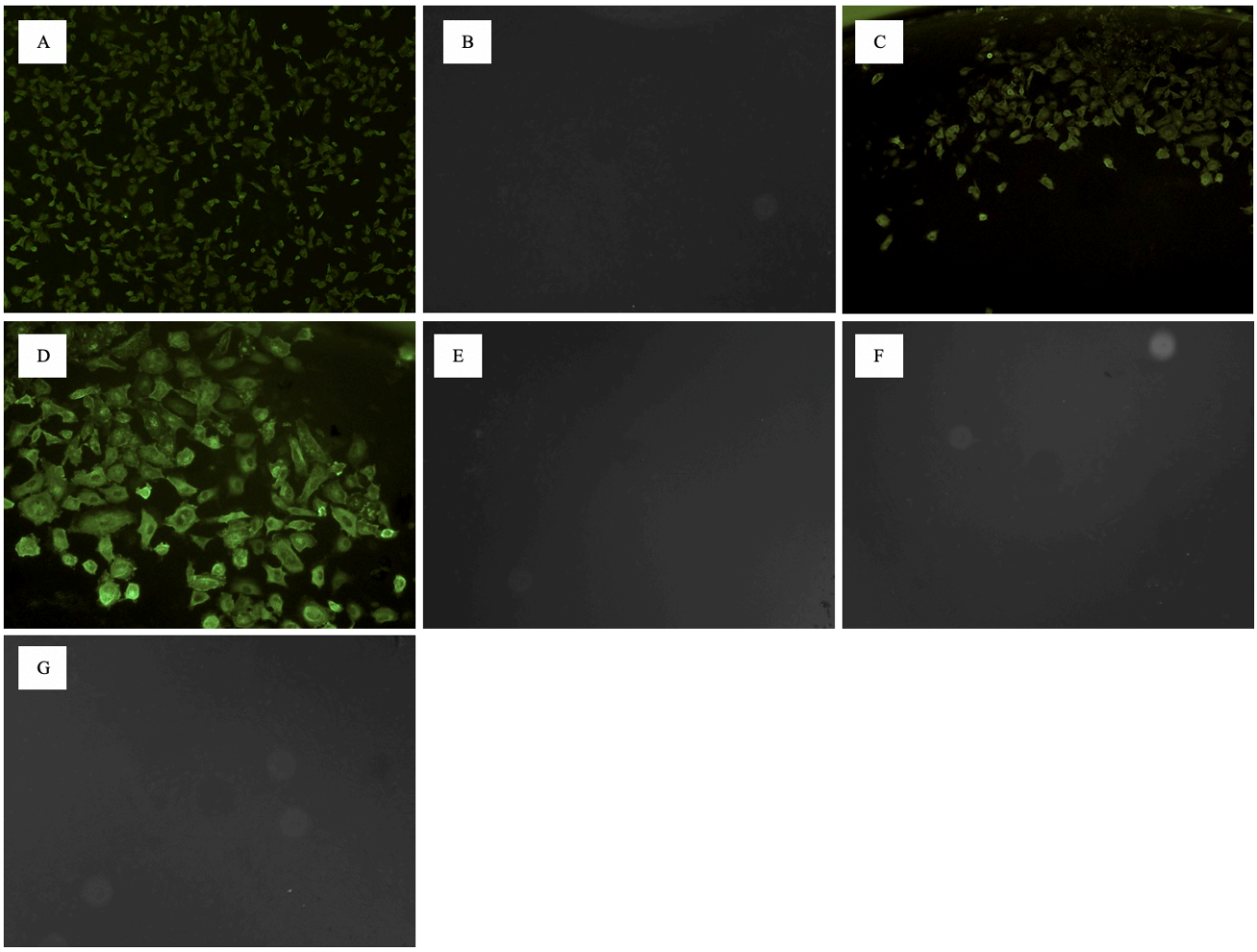

Figure 3.22: IFAT at day 7 after infection was used to analyze the ASK-cells infected with the samples with ISAV isolated using the Nanotrap magnetic beads on the large-scale samples of virus spiked in seawater, samples with just seawater and samples with just PBS. Panel A shows the positive control, panel B the negative control, panel C the virus, panel D the virus at 10x magnification, panel E the boiled virus sample, panel F the SW and the panel G the PBS. All pictures, except for D are shown at 4x magnification.

The IFAT results of the ASK cells infected with the large volume samples isolated by using the Nanotrap method show that cells were infected in the wells with the positive controls and in the samples with spiked virus. All negative controls, boiled samples and SW and PBS samples were negative.

CPE (Plate reading) ISAV

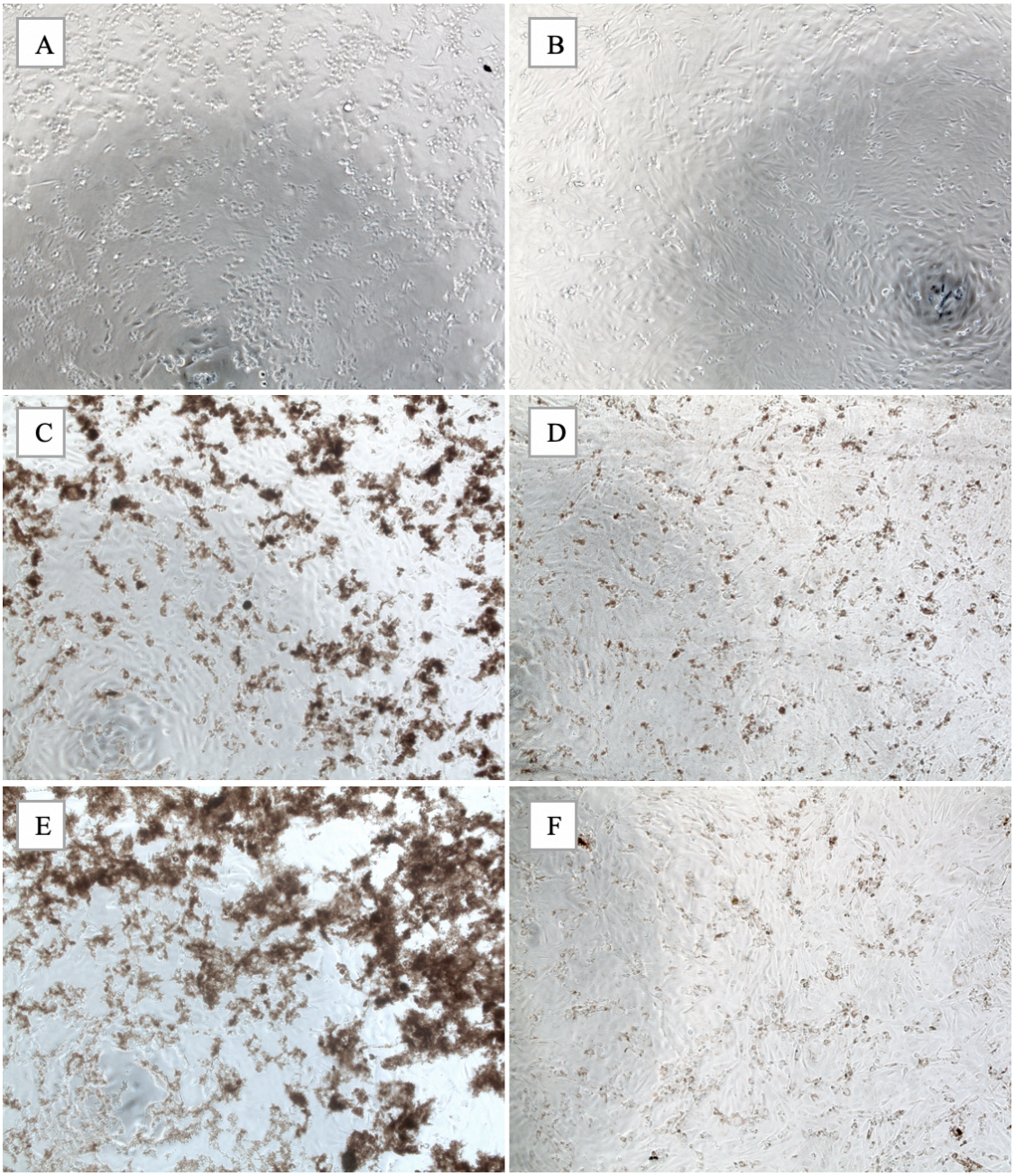

Figure 3.23: The ASK-cells infected with the samples of ISAV isolated using the Nanotrap method of samples of virus dilutions, PBS, and SW in larger volumes of seawater were analyzed for cytopathic effect in a microscope at 7 days after infection. Panel A show the cells infected by the positive control, panel B the negative control, panel C the sample containing virus, panel D the boiled virus sample, panel E the SW samples and panel F the PBS samples. All samples are shown at 4x magnification.

The plate reading results of the plate with ASK cells infected by the samples isolated using the Nanotrap method show that there is cytopathic effect in the positive control and in the sample spiked with virus. The boiled virus samples and the PBS sample looks negative. In the SW samples, there are too many beads to decide if there is any cell lysis.

CPE (Plate reading) IPNV

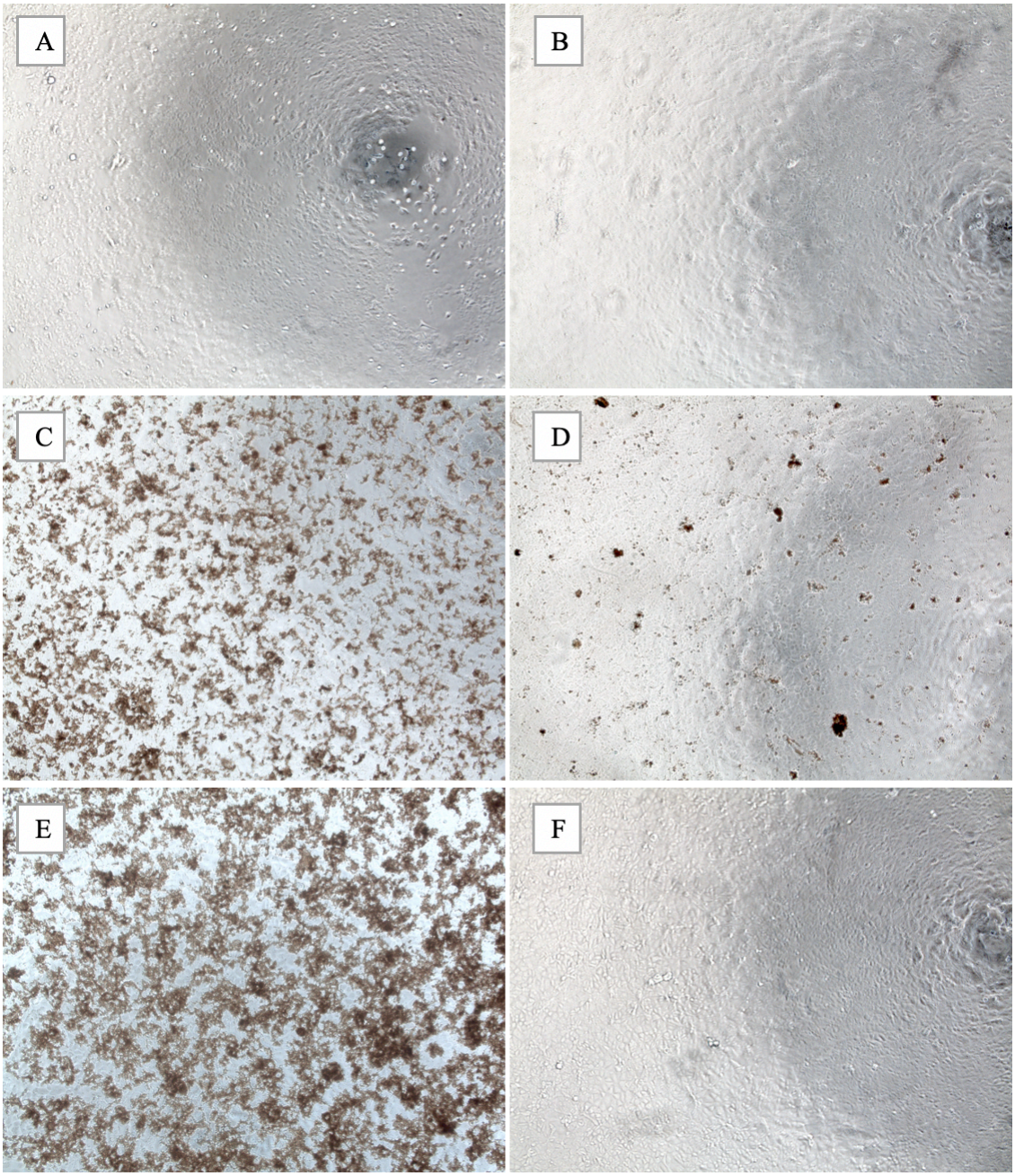

Figure 3.24: The CHSE-cells infected with the samples of IPNV isolated using the Nanotrap method of samples of virus dilutions, PBS, and SW in larger volumes of seawater were analyzed for cytopathic effect in a microscope at 7 days after infection. Panel A show the cells infected by the positive control, panel B the negative control, panel C the sample containing virus, panel D the boiled virus sample, panel E the SW samples and panel F the PBS samples. All samples are shown at 4x magnification.

The plate reading of the IPNV isolated using the Nanotrap show that it is hard to determine if there is cytopathic effect in the wells or not because of the high density of beads in the samples.

4 Discussion

Easier isolation and detection of virus from seawater could reduce the need for sampling of fish, and it would be more cost-efficient and resource saving than existing methods. It could also lead to earlier detection of disease, and thereby earlier implementation of biosecurity measures (Bernhardt, *et al*., 2021). Previously described isolation methods of aquatic viruses from seawater include the virus-absorption-elution technique (Polaczyk, Roberts and Hill, 2007; Andersen, Hodneland and Nylund, 2010a; Jarungsriapisit et al., 2016b), chemical flocculation (John *et al.*, 2011) and ultrafiltration membrane methods (Bernhardt *et al.*, 2021; Weli, *et al.*, 2021a; Weli, *et al.*, 2021b; Weli, *et al.*, 2021c; Mota *et al*., 2022). Some of these methods are time consuming and demanding of equipment to perform, so a simpler method for virus isolation from seawater would be beneficial in research and potentially also in the field (Bernhardt, 2021). It has also been difficult to determine if the virus isolated using these methods has been live infectious or not, because the virus often is detected only by RT-qPCR.

Due to the SARS-CoV2 pandemic, there has been an increased interest in developing methods for isolation of virus from wastewater. Methods based on magnetic particles has been developed, and PEG based methods have also been adapted to isolation of SARS-CoV2 from wastewater. Some of these methods might be used as more efficient and less resource demanding methods for isolation of aquatic viruses from seawater as well.

The aim of this study was to test and evaluate the PEG-based methods Intact Virus Precipitation Reagent, and Lentivirus Concentrator solution, and the magnetic beads method Nanotrap Magnetic Virus Particles to isolate virus (ISAV, SAV, IPNV and VHSV) from medium and seawater. After the initial testing, the Nanotrap method and the Lentivirus concentrator solution was tested again to isolate ISAV and IPNV from both small and large volumes of seawater. The isolated virus was then analyzed by RT-qPCR as before, but also put on cells and incubated for seven days before it was analyzed by IFAT or CPE to see if it was live-infectious. This study shows that all three methods look promising in their ability to isolate virus. The study also indicates that the Lentivirus concentrator method and the Nanotrap method can isolate live infectious ISAV and IPNV.

After the initial testing of all three methods, the rest of the project was done by testing the Lentivirus concentrator solution and the Nanotrap method to isolate only ISAV and IPNV due to time limitations. ISAV and IPNV was chosen because while ISAV and SAV are the most interesting viruses in research, they are difficult to analyze in regular microscope because they don't lyse cells as clearly as IPNV and VHSV. IPNV and VHSV are easier to evaluate in a light microscope, so we chose to use IPNV instead of SAV for the next part of the project. Only antibodies compatible with ISAV was available for this project, so the cells infected with ISAV could be analyzed by IFAT, but not the other viruses.

The Invitrogen method and to the Lentivirus method are both PEG based and quite similar and had relatively similar results in the initial testing, so we chose to only use one of them moving forward.

4.1 Dilution series

A dilution series of each virus was made in medium and seawater and tested by qPCR to get an overview of how much virus is detected from the different dilutions. The results from the dilution series (Figure 3.1 for medium and Figure 3.2 for SW samples) show that ISAV is detected in lower concentrations than the other viruses in both medium and seawater. This might indicate that the RT-qPCR assay for ISAV is more sensitive than those for the other viruses.

A challenge with making a dilution series is that when making dilutions, the samples are not totally homogenous, which makes it possible that less virus than desired for the correct concentration gets transferred to the next tube in the dilution series. This can lead to inaccurate concentration of virus in each sample, but it still gives an indication.

4.2 Invitrogen Intact Precipitation reagent

The Invitrogen intact precipitation reagent protocol is easy to follow and has few steps. Also, the only equipment needed is a centrifuge. However, this procedure takes some time, because the samples are incubated overnight and then centrifuged for 30 minutes. According to the Invitrogen protocol, the incubation time could be shortened to reduce the time needed for this method, but that would have to be tested. A limitation to this method is that it can process up to 100 mL of cell culture media. The reagent used comes premade from the manufacturer in

50 mL bottles, and the ratio between precipitation reagent and the virus-containing media is supposed to be 1:2. This makes this method not suited for precipitation of virus from larger volume samples.

The RT-qPCR results show that the Invitrogen method capable of precipitating all four viruses from both medium and seawater (Figure 3.3 and 3.4), but in seawater it is only the samples with the highest virus concentration that was detected, except for ISAV, where virus was detected in all dilutions.

4.3 Lentivirus concentrator solution, MD Anderson

The lentivirus concentrator solution is a method with few steps that are easy to perform, and not much equipment needed. As for the Invitrogen method, this method does take some time because of overnight incubation followed by centrifugation for 60 minutes, but there might be possible to shorten the incubation time without compromising the efficiency (Torii *et al.*, 2022). The method has some limitations in how many samples that can be analyzed at the same time due to limited space in the centrifuge. The lentivirus method was also easy to use to isolate virus from larger volume samples (30 ml). The PEG solution is cheap and easy to make, and it can be stored in the fridge until use, so it can be made in large amounts at the same time.

When the method was first tried in the initial running of the method, a pellet was formed in all samples from both medium and seawater. In the trials with the small and large volume samples that were put on cells, no pellet was observed in any of the samples, but virus was still precipitated. In these samples, as much of the supernatant as possible was removed, and then the virus yield was resuspended by pipetting up and down in the area where the pellet would form, as described in the MD Anderson protocol.

In the initial testing by RT-qPCR, it was shown that this method is able to isolate all four viruses from both medium and seawater. All four viruses were detected from both medium and seawater dilutions (Figure 3.5 and 3.6). From seawater, virus was only detected from the highest concentration samples for all viruses. When the method was tested a second time to isolate ISAV and IPNV from small volumes of seawater, the RT-qPCR results also show that the method is able to isolate virus (Figure 3.9). Both viruses were detected both before and after the isolation protocol was performed. The Ct values were a little lower after isolation

than before for both viruses. Virus was also isolated using the same method to put on cells to see if the isolated virus was infectious. The IFAT results from the cells infected with ISAV (Figure 3.10), show that the isolated ISAV from all dilutions was able to infect the cells, and thereby was live infectious. For the IPNV infected CHSE cells (Figure 3.12), it is difficult to determine if the cells are infected or not, because it is hard to see the difference between normal cells and lysed cells. These plates should also be analyzed by IFAT, but that was not done in this project due to the lack of anti-IPNV antibodies.

The Lentivirus concentrator solution was also tested to isolate virus from larger volumes of seawater (30 ml). The RT-qPCR results (Figure 3.17) detected for both viruses before and after isolation which shows that virus was isolated. The Ct is lower after than before isolation in both viruses, it has to be pointed out that for the before sample, 100 µl was taken out directly from the sample spiked with virus, while in the after sample, the whole volume of 30 ml seawater spiked with virus was isolated and then the precipitated pellets were analyzed. The isolated virus was also put on cells, and the IFAT results from the plate infected with ISAV (Figure 3.18) show that the isolated virus did infect the cells. The cells incubated with boiled samples, seawater or PBS were not infected. The plate reading of the ISAV plate (Figure 3.19) shows cytopathic effect in the cells infected by the virus-containing sample. The cells in the wells with SW, PBS, boiled samples, and negative controls looks normal, but hard to decide. In the plate reding of the IPNV infected cells (Figure 3.20), it is hard to see cytotoxicity, but it looks like there is lysis in the cells infected by virus containing samples. It also looks like there is some cytotoxicity in the SW sample, but this may be due to something that is cytotoxic to the cells in the seawater.

By plate reading of both ASK cells infected by ISAV and CHSE cells infected by IPNV, it does not seem like the residual PEG in the samples caused any toxicity to the cells. It did not look like the seawater samples and PBS samples caused toxicity to the cells either.

4.4 Manual Nanotrap Wastewater protocol

The Nanotrap method is a fast method for virus isolation and the only equipment needed is a magnetic rack, so it is a method that can easily be performed. The protocol does consist of more steps than the PEG based methods, with several steps of washing, use of the magnetic rack to separate the beds and then removing supernatants to isolate the virus from the rest of

the sample and then separate it from the beads. This requires many tubes for each sample and can get unmanageable with a lot of samples. It also takes a lot of time with many samples.

This method was also a bit difficult to use to isolate virus from larger volume samples because there were so many beads added to each sample. When the samples were put in the magnetic rack, they needed several minutes for all the nanoparticles to aggregate and settle on the magnetic area, so this is time consuming when analyzing several samples. It was also noted that the magnetic area on the racks used was too small for all the beads to attach, so a lot of beads were removed with the supernatant. The method could probably be improved by using different types of magnetic racks with stronger and larger magnets.

There is no published protocol for getting the virus off the beads yet, so the beads were left in the samples when put on cells, as suggested by the manufacturer of the protocol. This made it difficult to evaluate the cells by plate reading using only light microscopy and to look for cytotoxic effect because it was hard to see the cells covered in the beads. Fortunately, it didn't look like the beads were toxic to the cells.

The RT-qPCR results (Figure 3.7 and 3.8) from the initial testing shows that the method can isolate virus from ISAV from medium and seawater, but in the medium samples, SAV, IPNV and VHSV were not detected. In the seawater samples, all the viruses were detected in the samples with the highest concentration, and ISAV and IPNV were detected from all dilutions. When the Nanotrap method was tested again to isolate virus from small volume samples the RT-qPCR results (Figure 3.13) shows again that the method is able to isolate virus. Both ISAV and IPNV were detected from all dilutions, both before and after virus isolation. The Ct value was a little lower after isolation for both viruses. The IFAT of the ISAV infected cells (Figure 3.14) show that the virus isolated from the dilutions 10^{-1}, 10^{-2} and 10^{-3} were infectious to the cells. The cells with the 10^{-4} dilution were not infected. No cells were infected by the boiled samples, or seawater or the PBS samples either. The plate readings (Figure 3.15 and 3.16) from the NT method are hard to evaluate due to all the beads put on the cell with the isolated virus. It does look like there is some cell lysis in the wells infected by virus samples, but it can't be determined for sure. It looks like the nanobeads have no toxic effect on the cells, but this can't be confirmed either.

Testing of the method on larger volume samples (30 ml) were also performed. The RT-qPCR results (Figure 3.21) confirms that both viruses were detected, both before and after isolation. By using the IFAT on ISAV infected cells (Figure 3.22), it was shown that only the cells infected by the sample with virus were infected. This also confirmed that the isolated virus was infectious. The plate readings of the cells infected with ISAV (3.23) and with IPN (3.24) isolated from large volume samples, shows that there was high density of beads in the samples that were not boiled, which made it hard to decide if there were cell lysis or not. There were not as many beads in the PBS samples or in the boiled samples because a lot of the beads attached to the tubes during boiling, but the beads that were left were more clumped together.

When the samples from both the Lentivirus and the Nanotrap methods were put on cells, samples of just PBS and seawater were also added to see if they had any toxic effect on the cells. However, no cytotoxic effect was seen. All the samples were also boiled as a negative control. No cytotoxic effect was seen from those samples either.

Based on the results in this study, it looks like all three methods are able to isolate virus from seawater. The virus isolated by both the Lentivirus concentrator solution and the Nanotrap magnetic beads methods are proven to be live infectious. The PEG based methods requires more time that the bead-based method, but the protocols are easier to follow, and less work is needed. The PEG based methods are also less expensive and more easily available than the Nanotrap particles.

For further research, it would be interesting to test the methods on samples with lower concentrations of virus, on even larger sample volumes and on several samples for each dilution. However, for virus isolation from larger volumes, the virus would likely need to be concentrated out of the seawater on a filter before these methods could be used.
In this study, all testing of the methods were only done on one sample for each dilution due to a limited quantity of viral stocks. It would also be good to test the methods on more natural samples, not only on samples spiked directly with virus. It would also be interesting to infect cells with the isolated SAV and VHSV. It was also more difficult than expected to analyze

IPNV infected CHSE cells for the presence of CPE by light microscopy, so it would be better to analyze these cells with IFAT or a different method to be able to measure infection. Analysis by IFAT worked very well for ISAV.

Natural seawater contains salts and other RT-qPCR inhibitors that might affect the virus quantification. Bernhardt et al (2021) used RT-droplet digital PCR in their study because it is more sensitive and has a higher intolerance to inhibitors, so this could be done in this project as well, but this method is more expensive and not so available as regular RT-qPCR.
The seawater used could also be pre-filtrated to remove unicellular algae and other particulate material that could affect the results, but in this experiment, it was decided to keep the seawater as natural as possible.

These methods could possibly be useful for analyzing seawater samples from laboratory research up to probably 50-100 ml. For even larger volumes, and for analyzing environmental seawater samples, a filtration step would be needed prior to using these methods due to the volumes of seawater needed to get enough virus for testing. The methods tested in this project could however still be useful for these samples after the filtration step, because when the water is filtrated by a filter, buffer would be needed to wash the virus off the filter. The buffer would dilute the virus again, but then the PEG or Nanotrap method could be used to concentrate the virus further from the buffer solution before it could be analyzed by PCR or IFAT.

With some further testing, these methods show great potential to be used to isolate and detect aquatic viruses from laboratory

5 Conclusion and future perspective

This study show that both the PEG based methods (Invitrogens Intact Virus Precipitation Reagent and MD Andersons Lentivirus concentrator solution) and the magnetic particle method (Ceres Nano Manual Nanotrap Wastewater protocol) tested in this project are able to isolate ISAV, SAV, IPNV and VHSV from seawater. Furthermore, the Lentivirus and the Nanotrap method showed ability to isolate live infectious virus from seawater, from both small (1 ml) and larger (30 ml) volumes. While all methods worked to isolate virus, the Lentivirus solution method was the easiest to perform, so this method would be recommended for further testing.

It is likely that all methods tested could be used to isolate and detect infectious virus for laboratory use or from field samples, but further testing of the methods would be beneficial. Moving forward, it would have been interesting to test the methods on samples with lower concentration of virus and on samples collected from laboratory studies or from field, not only on seawater samples spiked directly with virus.

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7 Appendix

7.1 List of kits and chemicals used

Product name	Brand name	Catalogue number
AgPath-ID™ one-step qRT-PCR	Applied biosystems®	
Phosphate Buffered Saline	EuroClone	ECB5004L
Leibovitz´s L-15 Medium + GlutaMAX™	gibco	
Intact Precipitation Reagent	Invitrogen by Thermo Fisher Scientific	10720D
Dulbecco's Phosphate Buffered Saline	SIGMA-ALDRICH	Lot No. RNBJ1059
Hanks´ Balanced Salt Solution	SIGMA-ALDRICH	
Biotechnology grade water	VWR LIFE SCIENCE	
Buffer RLT Lysis buffer	QIAGEN	Lot No. 169013677
RNeasy® Mini Kit (50)	QIAGEN	74104
QIAamp® Viral RNA Mini Kit (50)	QIAGEN	52904
RT-PCR buffer	Applied biosystems by Thermo Fisher Scientific	Lot 20051328
RNAse-free water	Sigma-Aldrich	

7.2 Protocol for the Intact Virus Precipitation Reagent (Invitrogen)

- Spike virus in medium or seawater in desired dilutions in 2 mL tubes
- Add 0.5 volumes of Intact Precipitation Reagent (500 µL to 1 mL samples)
- Mix by pipetting
- Incubate the samples in rotory mixer overnight in cold room (4°C)
- Centrifuge at 10,000 x g for 30 minutes
- Remove supernatant without disturbing the pellet
- Resuspend the pellet in 100 µL PBS (or medium)
- Store at -80°C until use

7.3 Protocol for the Lentivirus Concentrator Solution (MD Anderson)

The PEG solution (concentrator):

- Dissolve 80 g PEG-8000 and 14.0 g NaCl in 80 mL 1x PBS
- Adjust pH to 7.0 ~ 7.2
- Put on magnetic stirrer until everything is resolved
- Store in fridge until use

Virus concentration protocol:

- Spike virus in medium or seawater in desired dilutions in 2 ml tubes
- Add 1 volume of PEG solution to 3 volumes of virus dilution (333 μL to 1 mL samples)
- Mix by shaking
- Incubate the samples in rotory mixer overnight in cold room (4°C)
- Spin down at 1600 x g for 60 minutes at 4°C
- Remove supernatant without disturbing the pellet
- Resuspend the pellet in 100 μL PBS (or medium)
- Store at -80°C until use

7.4 Protocol for the Manual Nanotrap Wastewater protocol (CERES Nano)

- Spike virus in medium or seawater in desired dilutions in 2 ml tubes
- Add 150 µL of magnetic virus particles to each sample
- Mix by inverting
- Incubate the samples at RT for 10 minutes, invert after 5 minutes
- Put the samples in a magnetic rack
- Discard supernatant
- Add 1 ml molecular grade water to the samples and resuspend the beads by pipetting
- Put the samples back in the magnetic rack and remove supernatant
- Add 150 µL PBS and 560 µL QIAGEN Virus lysis buffer (Buffer AVL) from the QIAGEN QIAamp Viral RNA Mini Kit
- Resuspend the pellets by pipetting and vortex
- Incubate at RT for 10 minutes
- Put the samples back in the magnet to separate the beads from the solution
- Remove the supernatant into a new tube and discard pellet
- Follow the QIAGEN QIAamp Viral RNA Mini Kit protocol for RNA isolation