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An Evaluation of Norovirus Persistence in Estuarine Water and of Methods for its Detection in Treated Sewage Effluent

Margaret H. Fagan
College of William and Mary - Virginia Institute of Marine Science

An evaluation of norovirus persistence in estuarine water and of methods for its detection in treated sewage effluent

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This thesis is submitted in partial fulfillment of

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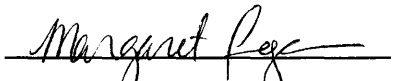

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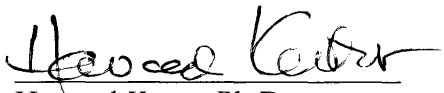
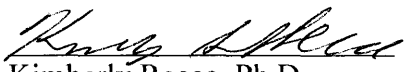
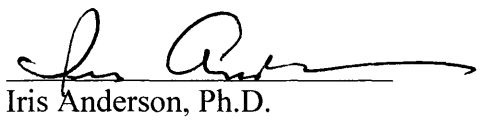


Howard Kator, Ph.D.
Advisor



Kimberly Reece, Ph.D.
Advisor

Corinne Audemard, Ph.D.



Iris Anderson, Ph.D.

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ABSTRACT

Noroviruses are causative agents of epidemic gastroenteritis worldwide. They are highly contagious and are primarily spread by direct human contact, or through food or water. Despite the acknowledged public health importance of these viruses, little is known about their occurrence, removal or inactivation during sewage treatment, or their fate in environmental receiving waters. Noroviruses are particularly resistant to chlorination, and limited molecular data suggest they can be abundant in treated sewage effluents released into receiving waters. Norovirus quantification presents a challenge because the viruses are not easily grown in cell culture, but studies have demonstrated the potential of real-time quantitative reverse transcription polymerase chain reaction (qPCR) to quantify noroviruses in sewage effluent, shellfish, and drinking and surface waters. This study evaluated methods of concentration and RNA extraction for the detection of norovirus in sewage effluent samples from the James River Treatment Plant in Newport News, VA, USA. Sewage samples were concentrated by ultracentrifugation and viral RNA was extracted using the Qiagen RNeasy Mini Kit. Spiking experiments demonstrated a detection limit of 1.5×10^4 norovirus genome copies 20 ml^{-1} sewage effluent. Norovirus was not detected in the collected sewage effluent samples. This study also included *in situ* and *in vitro* experiments designed to investigate the influence of insolation, water temperature, and salinity on the inactivation of norovirus in estuarine water. Additionally, the inactivation of FRNA coliphage, a proposed indicator for enteric viruses, was analyzed to determine if norovirus and FRNA coliphage persistence was similar. Inactivation rates were calculated for the two viruses under experimental conditions, and 95% confidence intervals were estimated to determine if inactivation rates varied significantly between the two viruses in response to environmental factors. *In situ* and *in vitro* experimental data suggested that FRNA coliphage was inactivated significantly faster than norovirus. Although light and water temperature had a significant effect on FRNA coliphage inactivation, norovirus inactivation rates varied little in the *in situ* experiments. *In vitro* experimental data suggested that increased water temperature and salinity do not consistently lead to higher virus inactivation rates; however, taken together, the *in situ* and *in vitro* experimental data suggested that high insolation and salinity have the potential to increase norovirus inactivation rates. The findings of this study suggest that FRNA coliphage is not a suitable surrogate for norovirus in estuarine water.

An evaluation of norovirus persistence in estuarine water and of methods for its detection in treated sewage effluent

INTRODUCTION

This project involved the evaluation of methods for norovirus concentration and detection in sewage effluent samples and the characterization of norovirus and FRNA coliphage inactivation in estuarine water. Both of these viruses are known to occur in treated sewage effluent discharged worldwide (Metcalf et al. 1995; Leclerc 2000) and have been considered as potential candidates for indicators or surrogates of enteric virus pollution (Kator, verbal communication 2006; Leclerc 2000). While several genogroups of noroviruses are human pathogens and have the capability of causing outbreaks of acute nonbacterial gastroenteritis, FRNA coliphages are not pathogenic to humans. Quantitative information on norovirus contamination in environmental waters is limited in the United States and lacking in the Chesapeake Bay region.

Various methods for detecting and quantifying norovirus in sewage effluent samples have been reported in the literature. However, these have been used by few investigators and questions concerning factors such as ease of use, reliability, and reproducibility exist. Therefore, one focus of this project was to evaluate norovirus concentration and detection methods for sewage samples.

In addition, *in situ* and *in vitro* laboratory experiments were performed to examine how factors of sunlight, water temperature and salinity affected virus inactivation. Three *in situ* experiments were conducted, one each in the spring, summer and winter, to examine the

inactivation of norovirus and FRNA coliphage in response to natural variations in environmental factors such as water temperature and sunlight during the different seasons. This information was used to determine inactivation coefficients for each virus under varying temperature and light conditions, or their persistence in the estuarine environment. *In vitro* experiments were also performed at three temperatures (10°, 20° and 30°C) and four salinities (0, 10, 20 and 30 practical salinity units, psu) to supplement and compare with results from the *in situ* experiments.

Norovirus Background

Noroviruses are enteric viruses belonging to the family *Caliciviridae*. Noroviruses are 26-35 nm in diameter, have a positive-stranded, polyadenylated RNA genome of 7.7 kilobases and are protected by a protein capsid (Hutson et al. 2004; Mohamed et al. 2006). Noroviruses are classified into five distinct genogroups, designated GI through GV (Blanton et al. 2006). Genogroups I and II contain the majority of human noroviruses, though GIV strains also infect humans. Noroviruses infecting pigs fall into GII; bovine noroviruses cluster in GIII; and murine noroviruses falls into GV (Hutson et al. 2004). Within genogroup GII, a genetic cluster designated as GII.4 has been reported as the dominant epidemic strain over the last decade in the United States, Ireland, England, the Netherlands, Germany, Japan, New Zealand and Australia (Blanton, et al. 2006).

Noroviruses belong to the most infectious group of causative agents of epidemic gastroenteritis; over the three-year period from 1997 to 2000, noroviruses accounted for 93% of the outbreaks of nonbacterial gastroenteritis in the United States (Lodder and de Roda Husman 2005; Fankhauser et al. 2002). They are believed to cause 23 million cases of nonbacterial gastroenteritis annually in the United States (Mead et al. 1999) and anecdotal evidence suggests

a higher incidence of norovirus-induced gastroenteritis over the last five years. Noroviruses infect and replicate in the gastrointestinal tract of the host where they appear to recognize histo-blood group antigens of the intestinal epithelium (Tan et al. 2006). Transmission is primarily spread by direct human contact, or foodborne and waterborne outbreaks; infection risk is highest following consumption of raw or improperly cooked seafood, or after exposure to contaminated recreational waters (Jothikumar et al. 2005; Laverick et al. 2004). Contaminated shellfish continue to be an important vector for transmission of acute gastroenteritis (CDC 2001).

Enteric viruses, including noroviruses, are commonly introduced to the environment through leaking sewage and malfunctioning septic systems, and, in the case of estuarine and marine waters, sewage outfalls and vessel wastewater discharge (Fong and Lipp 2005). Detected in both raw and treated sewage waters, noroviruses frequently cause waterborne outbreaks of gastroenteritis due to inadequate water treatment (van den Berg et al. 2005). Humans suffering from viral gastroenteritis can excrete between 10^5 and 10^{11} virus particles per gram of stool, resulting in high norovirus concentrations in sewage treatment plants (van den Berg et al. 2005; Ueki et al. 2005; Fong and Lipp 2005), particularly during winter months. Reasons behind the commonly observed peak in norovirus concentrations during the winter are not completely understood, but is likely due to a combination of climatic conditions favoring virus survival and social behaviors that increase the likelihood of person-to-person and foodborne transmission (Lopman et al. 2004; Mounts et al. 2000). Noroviruses are heat stable, well-adapted to survive transit between hosts and particularly resistant to chlorination (Keswick et al. 1985; Griffin et al. 2003).

Studies on human volunteers have shown that the minimum infectious dose for norovirus

may be as low as 6-10 polymerase chain reaction (PCR)-detectable units (Meschke and Sobsey 2001; Schaub and Oshiro 2000). A PCR-detectable unit is determined by serially diluting a PCR-positive sample. The highest dilution that results in a PCR-positive result is designated as 1 PCR-detectable unit and the starting concentration is back-calculated. Therefore, in addition to causing acute disease, they are of public health concern due to their low infectious dose (Fong and Lipp 2005). Furthermore, noroviruses induce a relatively poor and short-lived immune response, causing humans to be susceptible to repeated episodes of norovirus-induced gastroenteritis throughout life (Laverick et al. 2004; Parrino et al. 1977).

Caliciviruses have been reported in wastewater at 10^7 RNA-containing particles per liter, and it has been suggested that they are much more prevalent than previously thought (Griffin et al. 2003). Data specific to noroviruses are mostly limited to Europe and Japan, but numerous studies using varied quantitative methods indicate noroviruses may be abundant (approximate range of 10^5-10^6 copies l^{-1}) in final treated effluents released into receiving waters (da Silva et al. 2007; Katayama et al. 2008; Laverick et al. 2004; Lodder and de Roda Husman 2005; van den Berg et al. 2005). These studies generally found a 1-2 log reduction of norovirus concentrations between influent and effluent samples.

Despite the acknowledged importance of noroviruses as etiologic agents of viral illness, research pertaining to norovirus removal or inactivation during sewage treatment has been limited in the United States. Unlike many waterborne bacterial pathogens, which have been controlled largely by water and wastewater treatment practices, the incidence of water-related viral diseases has remained virtually unchanged over the past several decades (Metcalf et al. 1995). Over two decades ago, the United States Environmental Protection Agency (USEPA)

described the enteric virus group (including norovirus, rotavirus, hepatitis A virus, adenovirus, and enterovirus, etc.) as the most meaningful, reliable and effective virus index for environmental monitoring (Karaganis et al. 1983). Currently, the degree of fecal pollution in receiving waters is monitored using bacterial indicator organisms, i.e., fecal coliforms, *Escherichia coli* (*E. coli*) or the enterococci (USEPA 2000) despite the knowledge that the relationship between virus levels and levels of enteric indicator bacteria is known to vary and it is generally accepted that bacterial indicators inadequately reflect the behavior and survival of enteric viruses (NRC 1993; Gerba et al. 1979; Keswick et al. 1984; Bosch 1998). Therefore, measurement of bacterial levels may be of little relevance to the virological quality of the water and the risk presented by shellfish consumption, swimming or other uses (Laverick et al. 2004; Henshilwood 2002).

Information concerning the occurrence and relative densities of norovirus in treated sewage effluent is lacking for Virginia sewage treatment plants that discharge into the Chesapeake Bay. Norovirus quantification has presented a challenge in the past because until 2007 no cell culture or animal model was available for infection. A three-dimensional small intestinal epithelium model has become available in the course of this study (Straub et al. 2007); however, to develop and maintain the culture is costly, time-consuming and labor-intense. Prior to this model, studies demonstrated (and continue to demonstrate) the potential of real-time quantitative reverse transcription polymerase chain reaction (hereafter referred to as qPCR) assays to quantify noroviruses in sewage effluent, shellfish, drinking and surface water samples (Hot et al. 2003; Haramoto et al. 2004; Loisy et al. 2005; Vinje et al. 2004; Schmid 2004; Pang et al. 2004). The primers and TaqMan probe published by Kageyama et al. (2003) for qPCR

were used in this thesis to evaluate methods for the concentration, detection and quantification of norovirus in sewage effluent samples from the James River Treatment Plant in Newport News, Virginia.

When using PCR techniques for virus detection in environmental samples, sensitivity of virus detection is dependent on the efficiency of virus recovery from the sample and the degree of final purity of the recovered virus (Metcalf et al. 1995), as well as the concentration of compounds that might inhibit the reaction. Viruses (e.g. noroviruses) may be present in low densities, requiring concentration from high sample volumes (Griffin et al. 1999). Concentration of large sample volumes can lead also to concentration of inhibitors like humic compounds within the sample that may lead to PCR interference (Metcalf et al. 1995). In addition, subsequent sample purification to remove inhibitors can diminish virus recovery, a concern when trying to quantify the virus within a sample. So in using PCR to detect norovirus in environmental samples, one must concentrate a large volume and purify it sufficiently without sacrificing virus recoverability (Wyn-Jones and Sellwood 2001). For this project, various published methodologies were evaluated for norovirus recovery and concentration from sewage effluent samples.

FRNA Coliphage Background

Over 140 enteric viruses, including norovirus, have been identified in human feces. To monitor for each of these viruses is impractical from the standpoint of cost, time and specialization of workforce and laboratory equipment. Therefore, to assess sanitary water quality, surrogates or indicators are monitored rather than specific pathogens. An *ideal* indicator has the following characteristics:

- Occurs where pathogens do
- Cannot grow in the environment
- More resistant to disinfection than are pathogens
- Easy to isolate and count
- Can be isolated from all types of water
- Not subject to antibiosis
- Only found in sewage
- Found in higher numbers than pathogens
- Density of indicator should relate to degree of contamination
- Density of indicator should relate to a health hazard or type of pollution (Griffin et al. 2001).

FRNA coliphages are bacteriophages that have been proposed as indicators of pathogenic enteric viruses (Havelaar and Hogeboom 1984; IAWPRC 1991). They are single-stranded RNA viruses with simple cubic capsids that are 24-27 nm in diameter; their genomic size and physical properties are similar to the properties of noroviruses and they share a strong resistance to chlorination (Keswick et al. 1985; IAWPRC 1991; Furuse 1987). FRNA coliphages utilize the sex pilus of F+ *E. coli* for attachment and subsequent reproduction (Griffin et al. 2001; Leclerc 2000). FRNA coliphages have been found in raw sewage at concentrations of 1000 pfu ml^{-1} (Furuse 1987; Havelaar et al. 1990, 1993; IAWPRC 1991); however, they are not always found in human feces (Leclerc et al. 2000).

FRNA coliphages can be easily enumerated using cultural methods in contrast to the molecular methods required to detect and quantify norovirus. Data comparing the persistence of FRNA coliphage and noroviruses in the environment are necessary to decide whether FRNA coliphage can be a suitable indicator for norovirus in the environment.

Norovirus and FRNA coliphage persistence in the environment

Little is known about norovirus fate in environmental receiving waters following discharge from sewage treatment plants. Some studies have indicated that enteric viruses are

able to persist for extended periods in the marine environment, which increases the probability of human exposure by recreational contact and accumulation in shellfish that are consumed uncooked (Fong and Lipp 2005). However, specific information describing norovirus persistence in the estuarine environment, and how factors such as sunlight and temperature affect its inactivation, are limited. Sunlight is known to be a critical inactivating factor to FRNA coliphage and indicator bacteria. FRNA coliphages are inactivated more slowly than indicator bacteria and manifest greater resistance to sunlight than bacteria (Sinton et al. 1999, 2002). A 1995 study (Muller 1995) examined FRNA coliphage inactivation in summer and winter months in the York River. Inactivation rates were determined for FRNA coliphage suspensions at 0.5 and 1 meter depths in the water column and no significant differences were found between inactivation rates at these two depths. Inactivation rates were as follows:

Table 1. FRNA coliphage inactivation rate coefficients.

Season	Treatment	Inactivation rate coefficient ($k\ d^{-1}$)
Summer	Light	8.57
	Dark	5.83
Winter	Light	1.51
	Dark	1.42

The only study found describing norovirus persistence in seawater was a laboratory study using an artificial solar light source (Henshilwood 2002). The study reaffirmed that sunlight exposure decreases viral survival and that reductions in viral levels are less significant than for bacterial indicators such as *E. coli*. These data reiterate that the use of bacterial indicator species for monitoring water quality can underestimate the potential risk from viral contamination (Henshilwood 2002).

Quantitative information describing norovirus persistence is needed to validate the size of buffer zones surrounding sewage discharges, to understand if and when shellfish in restricted

waters can be relayed, to assess the potential effect of a treated effluent on the boundaries of approved shellfish harvesting waters, and to assess risks associated with transient contamination of recreational waters. Until comparative data on the inactivation of norovirus and FRNA coliphage are obtained, it is very difficult to judge if the latter can be an adequate viral indicator (Leclerc et al. 2000).

Study Objectives

Objective 1. To evaluate methods for the concentration, detection and quantification of norovirus in sewage effluent samples.

Sewage effluent samples were collected from the James River Treatment Plant in Newport News, VA. Adsorption/elution, precipitation and ultracentrifugation methods were evaluated for effluent sample concentration. Three RNA extraction methods (Boom et al. (1990), Trizol and Qiagen RNeasy kit) were evaluated as well. Using qPCR, the efficacy of these methods was assessed based on norovirus recovery from effluent samples spiked with genogroup II norovirus-positive stool samples.

Objective 2. To examine the persistence of norovirus and FRNA coliphage in the estuarine environment.

Null hypotheses

- *Light intensity will not have a significant effect on the inactivation of norovirus and FRNA coliphage.*
- *Water temperature will not have a significant effect on the inactivation of norovirus and FRNA coliphage.*
- *Water depth will not have a significant effect on the inactivation of norovirus and FRNA coliphage.*
- *Salinity will not have a significant effect on the inactivation of norovirus and FRNA coliphage.*
- *Comparable inactivation rates in the estuarine environment will be found for FRNA coliphage and norovirus.*

Three *in situ* experiments were conducted to measure the comparative persistence of the viruses as a function of seasonal temperature and light intensity during the spring, summer and winter. The *in situ* experiments were complemented with *in vitro* studies measuring persistence as a function of controlled temperature and salinity.

The *in situ* experiments were conducted adjacent to VIMS (off the Davis Hall pier) in the York River in late spring 2007, summer 2007 and fall 2007. Cell culture bags filled with filtered York River water were spiked with genogroup II norovirus and FRNA coliphage (MS2) and exposed to seasonal conditions. Water within the bags was sampled over a one-week period and norovirus and FRNA coliphage were quantified (using qPCR and plaque assays, respectively) to determine the effect of seasonal environmental conditions on viral persistence.

A limitation of qPCR is that it may not necessarily differentiate between infective and inactivated viruses. To try to rule out the issue of false positives, the extent to which presumed noninfective (capsid-damaged) norovirus RNA was detected as a positive signal by qPCR was evaluated using a treatment method of Nuanualsuwan and Cliver (2002). The treatment premise is that virions possessing a damaged capsid due to light or other damage will be removed through use of a combination of enzymes (proteinase K and RNase) that destroy viral proteins and RNA. Intact, assumedly infectious, virions are not affected; the difference between treated and untreated samples thus reflects the extent of inactive, but positive-by-reverse transcription-PCR, virus present. For each sampling day of the *in situ* experiments, norovirus samples were split to allow half to be treated in this manner and half untreated to determine the extent of intact viruses versus 'free' RNA within the sample.

In vitro experiments were conducted to examine the effects of water temperature and salinity on virus inactivation in dark conditions. Salinities (0, 10, 20 and 30 psu) were chosen to reflect the range of salinity in Virginia marine receiving waters. These experiments consisted of spiking genogroup II norovirus and FRNA coliphages into volumes of filtered marine water and holding the samples at 10°, 20° or 30°C. Water within the tubes was analyzed over a one-week period and noroviruses and FRNA coliphages were quantified (using qPCR and plaque assays, respectively) to determine the effect of water temperature and salinity on virus inactivation. All norovirus samples from the *in vitro* experiments were also treated with proteinase K and RNase as previously described.

METHODS

Detection and quantitation of norovirus in sewage samples.

One liter grab samples of secondary chlorinated effluent were collected from the James River Treatment Plant (design flow of 20 MGD) located in Newport News, Virginia over the 16-month period of method evaluation from August 2006 - December 2007. This plant discharges secondary chlorinated effluents into estuarine waters where shellfish are grown. Samples were collected in sterile 2 L polycarbonate bottles and transported to the laboratory in an insulated container. Samples were kept at 4°C or frozen at -80°C prior to processing.

Methods for the recovery of norovirus from sewage effluents were evaluated. Three different concentration procedures were assessed: adsorption/elution; precipitation; and ultracentrifugation. Figure 1 depicts a flowchart showing how methods were evaluated. Methods were evaluated on water or sewage samples and run in duplicate. In instances where water samples were used for method evaluation, filtered (0.1 □m) deonized (DI) water or artificial seawater (ASW) samples were spiked with a norovirus-positive stool sample (approximately 10^6 -10^7 genome copies ml^{-1}). When the methods were evaluated for sewage effluent samples, sewage effluent samples were spiked with norovirus-positive stool sample. Norovirus-positive stool sample was made available by the Virginia Division of Consolidated Laboratory Services. The stool sample was clarified by centrifuging at 400 x *g* for 10 minutes at 4°C to sediment solids. The supernatant was used for all spiking described in this project. The virus density of the stool sample spiked into samples (reagent-grade water, artificial seawater or sewage effluent)

was estimated based on the qPCR crossing points of the stool sample extractions. A comparable virus density was maintained throughout the evaluation of all methods. Additionally, unspiked sewage effluent samples were processed to determine the baseline level of norovirus, if detectable.

RNA Extraction Methods Tested

Three different methods of RNA extraction were evaluated in this project; however, they share in common the use of guanidinium thiocyanate as a major component for extraction. Guanidinium thiocyanate is a nuclease-inactivating chaotropic denaturing agent, meaning it is capable of disrupting the three-dimensional structure of proteins and RNA.

The Boom et al. (1990) method of nucleic acid purification entails the construction of silica reaction vessels (microcentrifuge tubes containing a silica suspension). A sample is added to a reaction vessel along with a lysis buffer containing guanidinium thiocyanate. The nucleic acids adsorb to the silica and then go through a series of washes before resuspension in RNase-free water.

Trizol Reagent (Invitrogen Corporation, Carlsbad, CA) is a mono-phasic solution of phenol and guanidine isothiocyanate that is used in combination with chloroform to isolate RNA in an aqueous phase. The aqueous phase is precipitated with isopropyl alcohol, washed with ethanol and resuspended in RNase-free water.

Qiagen's RNeasy kit (Qiagen Inc., Valencia, CA) combines the chaotropic properties of a guanidine thiocyanate containing buffer with a silica column that binds RNA. The RNA is purified on the column by additional buffers. The kit is limited in that it can only bind RNA at

least 200 base pairs in length. The RNA extraction methods that were coupled to each of the concentration methods are described below.

Virus concentration and RNA extraction

Adsorption/elution methods for concentrating virus particles

The adsorption/elution methods for concentrating virus particles involve the sample being brought into contact with a matrix that the virus will adsorb to under specific conditions of pH and ionic strength. Negatively-charged nitrocellulose filters known to work well when pre-conditioning the sample with salt or acid or when working with marine samples were used. Using vacuum filtration, viruses are bound to the filter by opposing electrostatic forces. The virus is eluted from the matrix into a smaller volume, generally using a solution of beef extract, skimmed milk or glycine/NaOH of a specific pH. The eluent is then flocculated with strong acid and centrifuged, and RNA is extracted from the pellet.

Wyn-Jones et al. (2000) methodology

Based on Wyn-Jones et al. (2000), 1 L ASW was spiked with 300 □l of norovirus-positive stool sample. The sample was then preconditioned to a pH of 3.5 with hydrochloric acid (HCl) (Mallinckrodt Baker, Phillipsburg, NJ). A 500 ml volume was passed through a 47 mm 0.45 □m nitrocellulose filter (Millipore, Billerica, MA or Whatman, Florham Park, New Jersey) using vacuum filtration. Virions were eluted off the filter with 20 ml 0.1% (w/v) skimmed milk (EMD, Gibbstown, NJ) in 0.05 M glycine buffer pH 9.5 (Bio-Rad, Hercules, CA). The eluent was flocculated by adjusting the pH to 3.5 with HCl. The eluent was centrifuged at 7,000 x *g* for 30 minutes at 4°C, the supernatant was discarded, and the pellet was dissolved in 1 ml 0.15 M

Na_2HPO_4, pH 7.0 (Fisher Scientific, Pittsburgh, PA). RNA was extracted using the Qiagen RNeasy Mini Kit (Qiagen Inc.) following manufacturer protocols.

Fuhrman et al. (2005) methodology

The method described by Fuhrman et al. (2005) involves sample adsorption to nitrocellulose filters with direct lysis from the filter using Qiagen RNeasy Mini Kit RLT lysis buffer. The protocol used was modified slightly from Fuhrman et al. (2005) and began by spiking 1 L ASW with 300 µl norovirus-positive stool sample. A 500 ml volume was passed through a 47 mm 0.45 µm nitrocellulose filter (Millipore or Whatman) using vacuum filtration. The filter was placed in a sterile disposable petri dish (47 mm) and 1 ml of the Qiagen RNeasy Mini Kit RLT lysis buffer was applied to the filter for a 10-minute incubation period. The extraction kit was then used following manufacturer protocols. Norovirus recovery from ASW was promising in comparison to qPCR results for stool sample extractions; therefore, this method was also evaluated for use with filtered estuarine water (1 µm filtered-York River water and 0.22 µm filtered York River water), and sewage effluent samples. To test recovery in these samples, a 1 L sample volume was spiked with 300 µl norovirus-positive stool sample; the filtration and extraction procedures modified from Fuhrman et al. (2005) and described above were followed.

Precipitation methods

Jiang et al. (2001), Schwab, and Affymetrix (2004) methodologies

Based on Jiang et al. (2001) and a personal communication with K. Schwab, precipitation of virus with polyethylene glycol 8000 (PEG) (Promega Corporation, Madison, WI) was evaluated using deionized water and sewage effluent samples.

A 175-200 ml deionized water or effluent sample was spiked with 500 µl of a norovirus-positive stool sample. The sample was mixed with sodium chloride (NaCl) (Fisher Scientific) to a final concentration of 0.2 M and 10% (w/v) PEG 8000 and shaken at 150 rpm overnight at 4°C. The sample was centrifuged at 6,000 x *g* at 4°C for 30 minutes and the supernatant removed by pipetting. RNA was extracted from the pelleted material using 1 ml of Trizol or 3 ml Trizol LS (Invitrogen Corporation) following the manufacturer's protocol. The Trizol extraction was further evaluated, specifically in terms of using multiple Trizol extractions, to increase removal of PCR inhibitors from the sample.

The Trizol extraction method was also combined with the Qiagen RNeasy Mini Kit. Based on guidance from Affymetrix (2004), once the sample had been incubated with Trizol LS and chloroform and centrifuged, the resulting aqueous phase was applied to the spin column and the Qiagen protocol followed.

A DNase digestion (as described by Jiang et al. 2001) was evaluated for removing inhibitors from effluent samples. A DNase treatment has been suggested for treating RNA preparations to degrade trace to moderate amounts of genomic DNA (Ambion 2001). The DNase digestion was applied during the Qiagen RNeasy extraction as indicated in the manufacturer's protocol.

Metcalf and Estes (1994) methodology

The Metcalf and Estes (1994) methodology for virus recovery from oysters was evaluated using PEG-precipitated sewage effluent concentrates. This method entails additional precipitations that further purify the sample, specifically polysaccharides and protein. Deionized water and sewage effluent samples (175 ml) were each spiked with 200 µl of a norovirus-positive

stool sample. Samples were precipitated with PEG and NaCl overnight as previously described and centrifuged at 6,000 x *g* at 4°C for 30 minutes. The supernatant was removed; 3 ml sterile water was used to suspend the pellet. The resuspended pellet was mixed with 1 ml 4X proteinase K (PK) buffer (0.04 M Tris-HCl, 0.02 M Na_2-EDTA; 2% SDS (Sigma-Aldrich, Inc., St. Louis, MO, pH 7.5) and 40 µl PK (20 mg ml^{-1}, Amresco, Solon, OH) and incubated for 30 minutes in a 56°C water bath. Four milliliters of phenol-chloroform (Fisher Scientific) was added to each sample. The sample was vortexed and centrifuged for 7 minutes at 6000 x *g* at 4°C. The aqueous phase was then added to a new tube containing 4 ml phenol-chloroform. The sample was vortexed and centrifuged. The aqueous phase was added to a new tube containing 400 µl 3 M sodium acetate (NaOAc), pH 5.2 and 10 ml 100% ethanol, mixed by inverting and incubated for 30 minutes at -80°C. The sample was then centrifuged for 30 minutes at 15,000 x *g* at 4°C. The supernatant was poured off, and 2.25 ml of 56°C sterile water was added to the tube to suspend the pellet. Each sample was then mixed with 0.9 ml CTAB/NaCl (5% cetyltrimethylammonium bromide (Sigma-Aldrich, Inc., 0.4 M NaCl) and incubated at room temperature for 15 minutes. The sample was then centrifuged for 30 minutes at 15,000 x *g* at 25°C. The supernatant was removed and the pellet was suspended in 0.150 ml of 1 M NaCl by gentle pipetting and the tube washed with 300 µl sterile water. The suspended pellet solution and wash water were added to a new tube containing 30 µl NaOAc and 900 µl 100% ethanol and incubated for 30 minutes at -80°C. Precipitated nucleic acids were pelleted by centrifuging for 30 minutes at 13,000 x *g* at 4°C. The supernatant was discarded and 380 µl 70% ethanol (at -20°C) was added to the pellet. The sample was centrifuged for 10 minutes at 13,000 x *g* at 4°C. The supernatant was discarded and the sample was dried for 15-20 minutes at 45°C, resuspended

with 100 µl DEPC water (diethylpyrocarbonate) (Sigma-Aldrich, Inc.) (at 70°C) and stored at -80°C.

Ultracentrifugation method

Myrmel et al. (2006) methodology

A modified ultracentrifugation method as adapted by Myrmel et al. (2006) from Puig et al. (1994) was evaluated. Sample concentration by ultracentrifugation and adsorption to and elution from silica was tested for recovery of norovirus from sewage effluent. A 13 ml volume of sewage effluent was spiked with 150 µl of a norovirus-positive stool sample. The sample was centrifuged at 100,000 x *g* for 2 hours at 4°C and the resulting pellet dissolved in 5 ml of glycine buffer (0.25 M glycine, 0.15 M NaCl, pH 9.5) by gentle shaking overnight (16 hours) at 4°C. The following morning the solution was diluted to 13 ml with phosphate buffered saline (0.14 M NaCl, 25 µM KH_2PO_4) and centrifuged at 4,300 x *g* for 15 minutes at 4°C to remove particulate material. The supernatant was transferred to another tube and centrifuged at 100,000 x *g* for 2 hours at 4°C. The pellet (viral concentrate) was dissolved in 200 µl phosphate buffered saline and stored at -80°C until RNA extraction.

A variation of the Myrmel et al. (2006) method was also evaluated in which the pellet resulting from the initial 100,000 x *g* centrifugation was dissolved in 200 µl phosphate buffered saline and the RNA was extracted from that pellet.

Two methods were evaluated for RNA extraction from the viral pellets. The first was based on the Boom et al. (1990) method of nucleic acid purification. This method uses reaction vessels with 900 µl lysis buffer (details in following paragraph) mixed with 40 µl silica suspension (details in following paragraph) in a 1.5 ml microcentrifuge tube. A 100 µl viral

concentrate was added to the reaction vessel, vortexed, and incubated at room temperature for 10 minutes. The content was homogenized and centrifuged at 12,000 x *g* and the supernatant decanted as waste. All washing steps entailed fully suspending the silica in the washing solution followed by a 15 second 12,000 x *g* centrifugation. The sample was washed twice with washing buffer (details in following paragraph) and 70% ethanol, followed by a single wash once with acetone. The silica was dried for 10 minutes at 56°C. To elute RNA, 80 µl DEPC water was added to the silica, vortexed and incubated for 10 minutes at 56°C. The sample was vortexed again and centrifuged for 2 minutes at 12,000 x *g*. The supernatant was stored at -80°C prior to use in reverse transcription.

Lysis buffer was made by dissolving 9.3 g guanidinium thiocyanate (GuSCN) (Sigma-Aldrich, Inc.) into 10 ml of the following: 0.1 M Tris hydrochloride (Sigma-Aldrich, Inc.), pH 6.4 with 22 ml of a 0.2 M EDTA (Sigma-Aldrich, Inc.) solution adjusted with NaOH (Acros, Morris Plains, NJ) to pH 8.0 and 2.6 g of Triton X-100 (Sigma-Aldrich, Inc.). Washing buffer was made by dissolving 12 g GuSCN into 10 ml of 0.1 M Tris-hydrochloride, pH 6.4. The silica suspension was made by suspending 12 g silicon dioxide (~99%, 0.5-10 µm) (Sigma-Aldrich, Inc.) in 100 ml demineralized water in a glass cylinder and sedimenting the solution at unit gravity for 24 hours at room temperature. An 86 ml portion of the supernatant was removed, and demineralized water was added to bring the total volume to 100 ml. The settled material was suspended by shaking. After a second sedimentation step for 5 hours at room temperature, 85 ml of the supernatant was removed. The pH of the suspension was adjusted to 2.0 with 120 µl of HCl (32%, w/v).

The second method evaluated for RNA extraction following ultracentrifugation was direct extraction of the viral concentrate using the Qiagen RNeasy Mini Kit. A 600 µl volume of RLT lysis buffer was added to the 200 µl viral concentrate and then the manufacturer's protocols were followed.

Reverse transcription

Extracted RNA was reverse transcribed to cDNA using Qiagen's Omniscript RT kit (Qiagen Inc.). The reverse primer was the same reverse primer used in the qPCR and was designed by Kageyama et al. (2003). The following reagent concentrations were used:

Reverse primer:	2.5 □M
dNTP Mix:	0.5 mM
Buffer RT:	1X
RNase inhibitor:	0.5 units □l^{-1}
Omniscript Reverse Transcriptase:	0.2 units □l^{-1}
RNase-free water:	5 □l 20 □l $reaction^{-1}$
RNA:	1 □l $reaction^{-1}$.

The reaction was incubated at 37°C for 1 hour and cDNA was subsequently stored at -20°C. Different reaction volumes were evaluated to minimize inhibition in qPCR while maintaining the efficiency of the reaction. Using 1 µl of RNA in each reaction, 20 µl, 10 µl, and 5 µl reverse transcription reactions were evaluated. Additionally, a reverse transcription reaction was evaluated using the same reagent concentrations as above with 10 µl of RNA for a final reaction volume of 25 µl.

An additional PCR clean-up step (Qiaquick PCR Purification Kit, Qiagen Inc.) was evaluated for use on the cDNA prior to analysis by qPCR. This purification kit is designed to remove primers, nucleotides, enzymes, salts, and other impurities from DNA samples.

Real-time quantitative PCR

Real-time quantitative PCR (qPCR) was the final step used to determine if norovirus was detectable in a sample, and if so, how many genome equivalents relative to a plasmid standard. A TaqMan assay with primers and probe designed by Kageyama et al. (2003) was used to amplify the highly conserved ORF1-ORF2 junction. The ORF1 region is reported to code for the RNA-dependent RNA polymerase gene, while the ORF2 region is reported to code for the capsid protein gene (Kageyama et al. 2003). Primers were purchased from Invitrogen Corporation and the probe and TaqMan Fast Universal PCR Master Mix were purchased from Applied Biosystems (Foster City, CA).

The TaqMan assay is a fluorogenic 5' nuclease assay that involves a fluorogenic probe designed to anneal specifically to the targeted cDNA fragment in between the forward and the reverse primers. The probe is an oligonucleotide with a reporter fluorescent dye and a quencher dye. While the probe is intact, emission of fluorescence is limited by the proximity of the quencher to the dye. During amplification, the Taq DNA polymerase cleaves the annealed probe by its 5'-3' exonuclease activity, and separates the dye from the quencher, resulting in an increase in fluorescence. Fluorescence emission by the cleaved probe is directly proportional to the amount of target template in the sample, and is measured on a cycle-by-cycle basis during the reaction. The threshold cycle value at which exponential amplification commences is proportional to the amount of initial template target, with higher initial template amounts giving a lower threshold cycle value (Henshilwood 2002).

qPCR amplifications were performed on the Applied Biosystems Real-Time PCR system. For all sample analyses, negative (reagent-grade water) and positive (cDNA generated from a

norovirus-positive stool extraction) controls were run simultaneously for quality control. Per 10 □l reaction, reagent final concentrations were as follows:

Forward primer:	1 pmol $□l^{-1}$
Reverse primer:	1 pmol $□l^{-1}$
Probe:	0.25□M
TaqMan Fast Universal PCR Master Mix:	5 $□l$ reaction^{-1}
Sterile water:	1.3 $□l$ reaction^{-1}
cDNA:	1 $□l$ reaction^{-1}.

Reaction conditions were as follows:

Initial denaturation:	95°C, 10 minutes
45 cycles:	
Denaturation:	95°C, 15 seconds
Annealing:	60°C, 60 seconds.

Plasmid preparation

Relative estimation of norovirus detectable units in sewage effluent necessitated the use of a standard curve. A plasmid was generated containing the PCR product amplified during qPCR. A plasmid is extra-chromosomal DNA into which, in this case, the qPCR sequence was inserted. The plasmid was introduced into *E. coli* and copied millions of times. The plasmid was purified from the *E. coli*, linearized, quantified and a serial dilution was run to estimate norovirus copy number in sewage effluent and water samples.

A standard PCR was run to generate the PCR product. Reagents were from Applied Biosystems. Per 20 □l reaction, reagents concentrations were as follows:

Bovine serum albumin (BSA):	0.4 mg ml^{-1}
$MgCl_2$:	3 mM
Taq polymerase:	0.05 units
Forward primer:	1 □M
Reverse primer:	1 □M
Sterile water:	6.1 $□l$ reaction^{-1}

cDNA:	0.5 □l reaction^{-1}.

Reaction cycling conditions were as follows:

Initial denaturation:	94°C, 10 minutes
40 cycles:	
Denaturation:	95°C, 15 seconds
Annealing:	56°C, 30 seconds
Extension:	72°C, 45 seconds
Final extension:	72°C, 5 minutes.

The PCR product size was confirmed by gel electrophoresis on a 2.5% agarose gel. The PCR product was purified using the Qiaquick PCR Purification Kit (Qiagen Inc.) and dilutions of the purified PCR product were cloned into a plasmid vector using the TOPO TA Cloning Kit (4□l PCR product, 1 □l salt solution, 1□l vector) (Invitrogen Corporation). The vector was transformed into One Shot Competent *E. coli* cells (Invitrogen Corporation) through heat shock (42°C, 30 seconds). Following incubation (37°C, 1 hour), the cells were plated on pre-warmed Luria broth ampicillin (LB amp) plates and incubated overnight at 37°C. A boil preparation (95°C, 4 minutes) was performed on picked colonies in 10 □l sterile water. The boil preparation was used in an M13 PCR to confirm that colonies carried the vector with the cloned PCR product insert. The M13 primers were provided in the cloning kit. Reaction reagent concentrations were as follows for a 20 □l reaction:

BSA:	0.20 mg ml^{-1}
10X Qiagen buffer:	1X
$MgCl_2$:	1.5 mM
dNTPs:	0.2 mM
Forward primer:	1 □M
Reverse primer:	1 □M
Taq polymerase:	0.03 units
Water:	10.9 □l reaction^{-1}
Boil preparation DNA:	1 □l reaction^{-1}.

Reaction conditions were as follows:

Initial denaturation:	94°C, 4 minutes
30 cycles:	
Denaturation:	94°C, 1 minute
Annealing:	54°C, 45 seconds
Extension:	72°C, 30 seconds
Final extension:	72°C, 10 minutes.

The presence of the insert was confirmed using gel electrophoresis on a 2.5% agarose gel. Products of the M13 PCR were sequenced to confirm the product identity. PCR products were treated with a shrimp alkaline phosphatase (SAP) and exonuclease I (*Exo* I) (Amersham Biosciences, Piscataway, NJ) to degrade nucleotides and primers remaining after PCR. The SAP/*Exo* I clean up reagents were as follows:

SAP:	0.5 units
Exo I:	5 units
Dilution buffer:	1 □l
M13 Amp PCR product:	5 □l

This reaction was incubated at 37 °C for 30 min, 80°C for 15 min and 15 °C for 5 s. To verify that the vector had the correct PCR product insert, the M13 PCR products were sequenced bi-directionally using the Big Dye Terminator kit (Applied Biosystems) with M13 sequencing primers. Reaction reagents were as follows:

Big Dye:	0.75 µl
Forward OR Reverse primer:	0.32 µl
5X buffer:	0.875µl
Sterile water:	3.205 µl

Reaction conditions were as follows:

Initial denaturation:	96°C, 1 minute
25 cycles:	
Denaturation:	96°C, 10 seconds
Annealing:	50°C, 5 seconds

Extension:	60°C, 4 minutes.

Sequenced products were precipitated using ethanol/sodium acetate. The following was added to 20 µl of the sequencing reaction:

3M sodium acetate (pH 4.6):	3 µl
Nondenatured 95% ethanol:	62.5 µl
Sterile water:	14.5 µl

Reactions were vortexed and incubated at room temperature for 15 minutes. Tubes were centrifuged at 2000 x *g* for 45 minutes and 3000 x *g* for 30 minutes. With caps removed, tubes were inverted and centrifuged at 50 x *g* for 1 minute. Tubes were again placed upright, washed with 150 µl 70% EtOH, and vortexed for 15 seconds. Tubes were again centrifuged at 2000 x *g* for 10 minutes and 3000 x *g* for 10 minutes. Samples were resuspended in 20 µl Hi-Dye Formamide (Applied Biosystems). Ten microliters of the reaction were loaded onto the ABI Genetic Analyzer (Model #3130*xl*, Applied Biosystems) for sequencing.

After confirming the correct product through sequencing, 3 ml 2YT media (Becton, Dickinson and Company, Franklin Lakes, NJ) was inoculated with transformed bacterial colonies and incubated overnight at 37°C while shaking at 200 rpm. Plasmid DNA was purified from bacterial cultures using a Qiaprep Spin Miniprep Kit (Qiagen Inc.). A *Not* I restriction digestion was performed to linearize the plasmid in 100 µl reactions containing 66 µl purified plasmid DNA, 20.5 µl of sterile water, 10 µl 10X buffer, 1 µl 100X BSA and 2.5 µl *Not* I restriction endonuclease. Plasmid DNA was digested at 37°C for 12 hours followed by 65°C for 20 minutes. Purified and digested plasmid DNA was electrophoresed on a 0.9% agarose gel, stained with ethidium bromide and visualized under UV light.

Digested plasmid DNA was purified using the Qiaquick PCR Purification Kit and

resulting DNA was quantified (Genequant *pro*, Amersham Biosciences). The linearized plasmid was serially diluted to generate a standard curve for use in the qPCR. This serial dilution was run during all qPCR runs to estimate norovirus genome copy number in sewage effluent samples.

Sewage sample collection

Sewage samples were collected from the James River Treatment Plant on the following dates:

- February 13, 2007
- March 1, 2007
- March 8, 2007
- April 18, 2007
- June 28, 2007
- December 6, 2007
- January 25, 2008
- February 22, 2008
- March 18, 2008
- April 17, 2008
- May 13, 2008

Two replicates of approximately 20 ml of sewage effluent (13 ml for the 12/6/07 samples) were concentrated using the Myrmel et al. ultracentrifugation method. For each sample, two replicates of sewage effluent were spiked with approximately 100 µl norovirus-positive stool sample, and similarly concentrated. The viral concentrate was extracted using the RNeasy Mini Kit. A 10 µl RT volume was used for these samples and qPCR was conducted as previously described.

Persistence of norovirus and FRNA coliphage in the estuarine environment.

***In situ* experiments**

Three experiments were conducted over the spring, summer and winter seasons. Transparent (96% solar transmission) Permalife Cell Culture Bags (OriGen Biomedical, Austin,

TX) containing approximately 30 ml filtered (0.22 μm) estuarine water (21 psu) were spiked with either a norovirus-positive stool sample or FRNA coliphage (MS2) suspension yielding a final concentration of approximately10^6 virion genome equivalents ml^{-1} and approximately10^6 plaque forming units (PFU) ml^{-1}, respectively. Two sets of 3 aluminum foil-covered dark control bags and 3 uncovered bags were placed at the water surface and at a 1 m depth in the York River using a tethered float (design shown in Figure 2). The experimental setup was exposed to *in situ* spring, summer and winter conditions. The bags were fitted with a closeable sampling port allowing for aseptic sampling of microbial suspensions and redeployment in water. Samples were collected from each bag at 0, 1, 2, 4 and 8 days. Four 1.5 ml replicates were collected for norovirus. Two 1 ml replicates were collected for FRNA coliphage.

Light extinction from the surface to 1 meter was measured using a LICOR quantum light sensor (Lincoln, Nebraska) on each sampling day. Water temperature data for each sampling day was acquired from the VIMS Virginia Estuarine and Coastal Observing dataset.

FRNA coliphage sample processing

Two 1 ml analytical replicates were taken from each of the three replicate bags on the sampling days. FRNA coliphage was enumerated using a double agar overlay technique (Rhodes and Kator 1991). The sample (or a dilution of the sample in phosphate buffer (0.24 M, pH 7.2)) was combined with 0.2 ml *Salmonella typhimurium* host WG49 (Havelaar and Hogeboom 1984) and 4 ml molten agar medium held at 47°C in a water bath. The resulting suspension was mixed and poured over a base agar layer in a petri dish and allowed to cool to room temperature. Inverted plates were incubated overnight at 37°C and plaques were counted at appropriate dilutions the following morning to determine the PFU ml^{-1}.

Norovirus sample processing

Four 1.5 ml samples were collected at each time point for norovirus sample processing. Two of the samples were treated with RNase and PK (details below) and two were not treated. Samples were concentrated on a 47 mm 0.45 □m nitrocellulose filter. Viruses were lysed directly from the filter based on Fuhrman et al. (2005) using 1 ml RLT lysis buffer. RNA was extracted using the Qiagen RNeasy Mini Kit following the manufacturer's protocol.

RNase and Proteinase K (PK) Treatment

Two analytical replicates were treated with PK (20 units) and RNase (100 ng) and incubated for 30 minutes at 37°C. PK was dissolved in phosphate buffered saline (0.01 M phosphate buffer, 0.0027 M potassium chloride and 0.1378 M sodium chloride, pH 7.4) and RNase was dissolved in a Tris-EDTA buffer (1.0 M Tris–HCl, 0.1 M EDTA, pH 8.0). Forty units of RNase inhibitor (Applied Biosystems) were added to the samples following treatment to protect the RNA during the subsequent extraction from intact virions. Samples were concentrated and extracted as previously described.

RNA was reverse transcribed as previously described using a 5 □l reaction volume. qPCR was conducted as previously described on all norovirus samples.

***In vitro* experiments**

Seawater was collected from the Eastern Shore Laboratory, filtered (0.22 □m) and diluted with reagent-grade water to yield water with 10, 20 and 30 psu values. Norovirus and FRNA coliphage were spiked into the water samples to yield final concentrations of 10^6 virion genome equivalents ml^{-1} and 10^5 plaque forming units (PFU) ml^{-1}, respectively. Three replicate tubes for each microorganism were sampled over the experimental period (days 0, 1, 2, 4 and 8). For each

salinity, experiments were conducted at 10°, 20° and 30°C. Samples were collected and processed following the methods described for the *in situ* experiments; however, only 2 analytical replicates were collected for the norovirus samples. These samples were treated with PK and RNase as previously described to obtain estimates of intact norovirus concentrations.

RNA was reverse transcribed as previously described using a 5 □l reaction volume. qPCR was conducted as previously described on all norovirus samples.

Figure 1. Summary of concentration and extraction method evaluation.

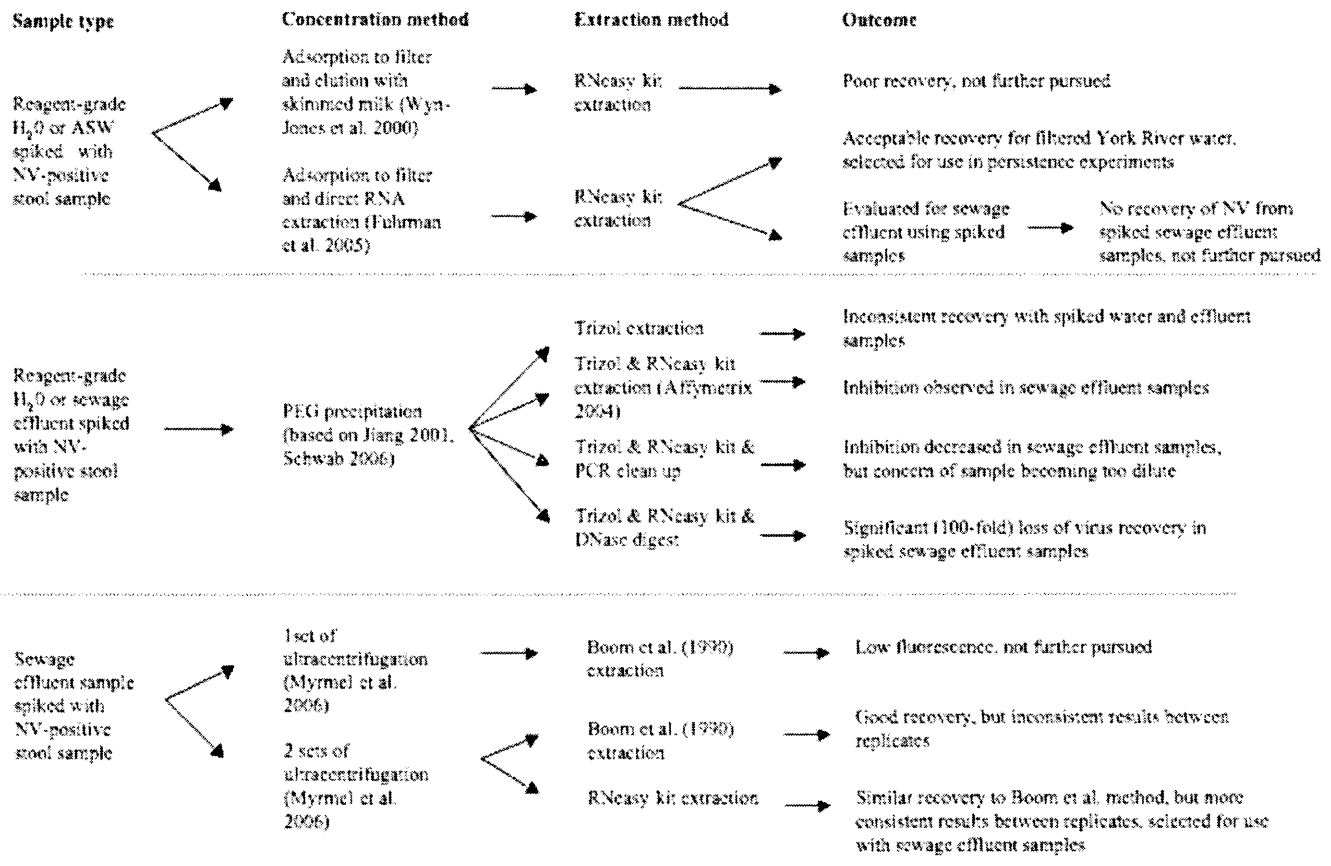
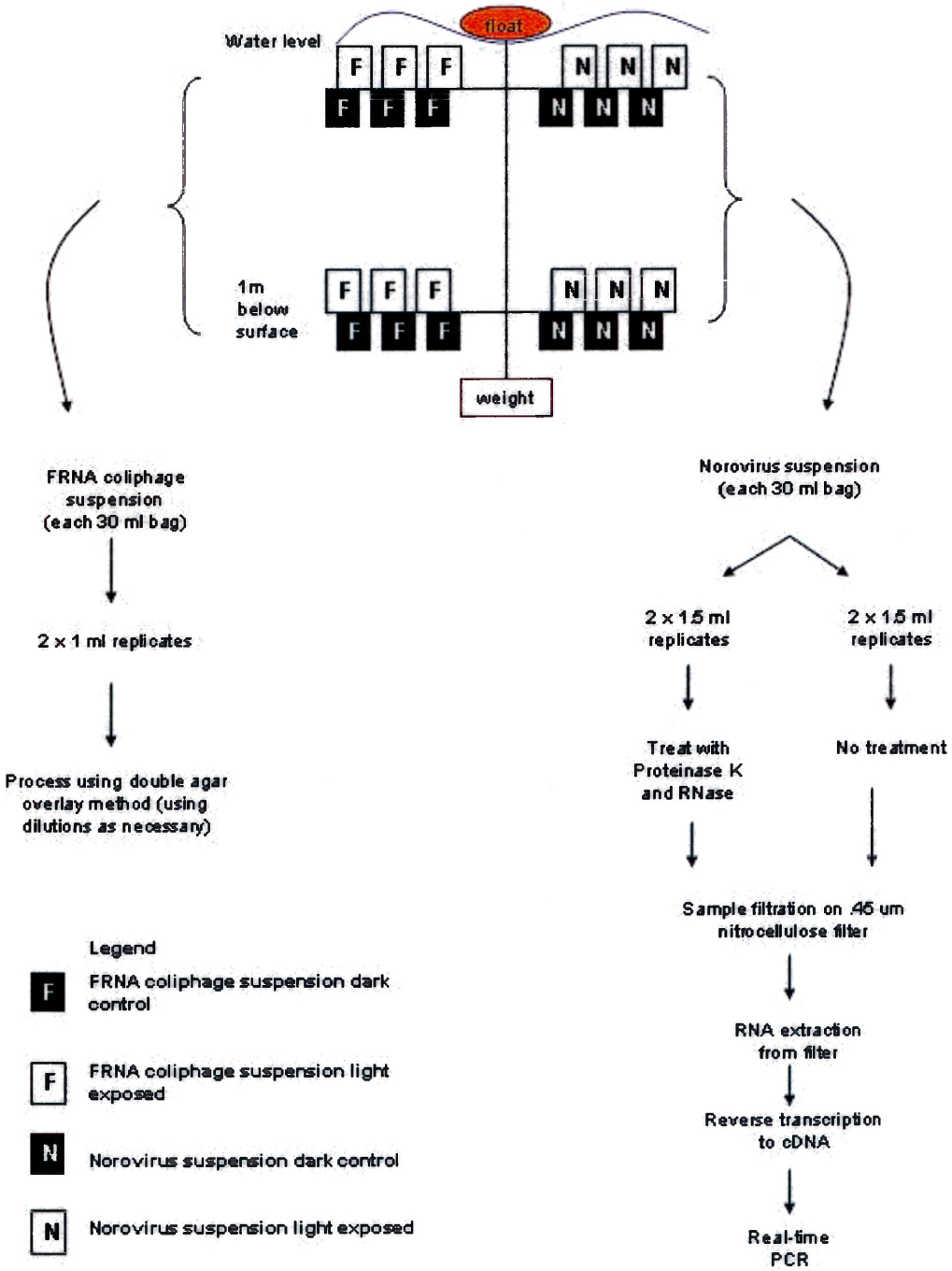


Figure 2. Experimental set up and sample analysis.



RESULTS

Detection and quantitation of norovirus in sewage samples

Virus concentration and RNA extraction

Adsorption/elution methods

Wyn-Jones et al. (2000) methodology

The Wyn-Jones et al. (2000) methodology was evaluated using both DI and ASW samples spiked with norovirus-positive stool sample. Using a 20 µl RT reaction volume and qPCR as previously described, qPCR crossing points were between 7 and 11 threshold cycles later than qPCR results for a sample extracted directly from stool. The higher crossing points suggest a loss of recovery of at least 100- to 1,000-fold when the virus was spiked into the water samples. This method was rejected for use in detecting norovirus in sewage effluent samples or in the *in situ* or *in vitro* experimental samples.

Fuhrman et al. (2005) methodology

The Fuhrman et al. (2005) method was evaluated using ASW, sewage effluent, and filtered (1 µm) York River water. Using a 20 µl RT reaction volume and qPCR as previously described, ASW samples spiked with norovirus-positive stool sample had crossing points comparable to a stool sample extraction; however fluorescence was much lower for spiked ASW than for stool sample extractions, indicating PCR inhibition in the ASW sample. Sample fluorescence is measured relative to the background fluorescence in a qPCR reaction and is

unitless. However, the fluorescence level is useful for inferring the efficiency of the reaction, with higher fluorescence indicating a more efficient reaction. Fluorescence of stool sample cDNA was approximately 6.5 while ASW sample cDNA was approximately 0.5.

In using this method with sewage effluent spiked with norovirus-positive stool, norovirus was not detected by qPCR following a 20 µl RT reaction. Dilution of the sewage effluent sample cDNA did not result in the detection of norovirus.

Using a 20 µl RT reaction volume and qPCR as previously described, this method was evaluated for filtered (1 µm) York River water. Crossing points were found to be comparable to direct stool sample extraction; however, fluorescence continued to be much lower than fluorescence for stool sample extractions (fluorescence of about 0.5 compared to 7, respectively). Sample RNA and sample cDNA was diluted (a serial 10-fold dilution) to determine what the cause of the qPCR inhibition was. Fluorescence remained low in both dilution RNA and cDNA samples (approximately 0.5), indicating that the PCR inhibitors are brought through the initial extraction and reverse transcription to qPCR.

York River water was further filtered (0.45 µm and 0.22 µm) and spiked with norovirus-positive stool sample. Filtering of the water samples resulted in slightly higher crossing points (3 threshold cycles, or about a 10-fold loss of recovery), but fluorescence was either comparable to the stool sample extraction (7) or slightly lower (5).

The Fuhrman et al. (2005) method was selected for use in the *in situ* and *in vitro* experiments.

Precipitation methods

Jiang et al. (2001), Schwab, and Affymetrix (2004) methodologies

As explained in Methods, various iterations of PEG precipitation, Trizol extractions, and the RNeasy Viral Mini Kit were evaluated. A 20 µl RT reaction was used for the samples processed using PEG precipitation methods followed by qPCR as previously described.

PEG precipitation followed by a single Trizol extraction showed that norovirus could be detected by qPCR in effluent spiked with norovirus-positive stool sample. Crossing points for spiked effluent samples were comparable to reagent-grade water samples spiked with norovirus-positive stool sample; however fluorescence for the effluent samples was almost undetected, though crossing point values were calculated.

As previously evaluated, RNA was serially diluted to determine if PCR inhibition could be reduced to improve amplification and fluorescence. Crossing points of the 1:10 dilution resulted consistently in an increase of approximately 3.3 threshold cycles, the interval indicative of a 1:10 dilution; however fluorescence remained very low.

Because recovery of spiked samples was at times inconsistent (appeared to be a result of organics being carried through the Trizol reaction), the RNeasy Mini Kit was evaluated using the aqueous phase of the Trizol extraction. When comparing the crossing points of norovirus-positive stool samples and reagent-grade water samples spiked with norovirus-positive stool sample using this method, the stool sample extractions had crossing points of about 5 threshold cycles lower than the spiked water samples. This would suggest a loss of viral recovery of almost 100-fold when comparing this method for stool sample extraction and spiked water sample extraction.

Despite the potential recovery issues, the Trizol-RNeasy Mini Kit method was subsequently evaluated for use with sewage effluent samples. Spiking of reagent-grade water samples and effluent samples with norovirus-positive stool sample resulted in comparable crossing points for both types of samples; however, fluorescence for effluent samples remained very low.

To try to remove inhibitors from the effluent samples, multiple Trizol extractions were run on stool-spiked samples, using reagent-grade water as a control and evaluating spiked effluent samples. The final aqueous phase of the extractions was processed through the RNeasy Mini Kit column. Performing 4 Trizol reactions until there was not a noticeable interphase, norovirus was detectable in spiked reagent-grade water samples with crossing points 3 to 4 threshold cycles later than when using a single Trizol extraction. Norovirus was not detected in spiked sewage effluent samples.

An additional step evaluated for its ability to remove PCR inhibitors from sewage effluent samples was a DNase digestion during RNA extraction with the RNeasy Mini Kit. This treatment resulted in qPCR crossing points 1 threshold cycle later for spiked reagent-grade water samples in comparison to a PEG precipitation, Trizol-RNeasy extraction method. However crossing points for spiked effluent samples were 6 threshold cycles later in one replicate and undetermined in a second replicate. The higher crossing points of the spiked sewage effluent samples suggested at least a 100-fold loss of norovirus as compared to the spiked reagent-grade water sample.

Metcalf and Estes (1994) methodology

In evaluating this method, norovirus was detected by qPCR (using a 10 □l RT reaction) in sewage effluent samples that had been spiked with norovirus-positive stool sample. A relatively high level of fluorescence was seen in these samples (3-4). When using this method for spiked reagent-grade water samples, no norovirus was detected by qPCR.

Ultracentrifugation method

Myrmel et al. (2006) methodology

The first step in evaluating this method was determining if one or two rounds of ultracentrifugation were necessary. When sewage effluent samples spiked with norovirus-positive stool sample had undergone one round of ultracentrifugation, qPCR crossing points were either similar to or higher than (by about 3 threshold cycles) the crossing points of spiked effluent samples that had undergone 2 rounds of ultracentrifugation. Additionally, spiked effluent samples ultracentrifuged twice had a higher level of fluorescence than spiked effluent samples ultracentrifuged only once, suggesting that two sets of ultracentrifugation improved removal of PCR inhibitors. Therefore, subsequent tests of steps in this method were following two rounds of ultracentrifugation.

To further evaluate this method, a serial dilution of norovirus-positive stool sample was spiked into sewage effluent to determine the sensitivity of the concentration and extraction methodology. Norovirus was not detected in one of the spiked replicates. Norovirus was detected by qPCR in the second replicate, however the crossing points for this sample were much higher (by 5 threshold cycles) than those seen in the previous evaluation of this method. For the replicate that had detectable norovirus, the 1:10 and 1:100 dilutions also had calculated crossing

points by qPCR. However the crossing points did not show the 3.3-cycle increase in 10-fold dilutions customarily seen: the 1:10 dilution had crossing points 2 threshold cycles higher than the stock spike and the 1:100 dilution had crossing points 3 threshold cycles higher than the stock spike.

A norovirus-positive stool sample was serially diluted and extracted using the Boom et al. (1990) method to determine the detection limit of the extraction method. Crossing points for the stock stool extraction indicated that approximately 100-fold more norovirus was recovered from the stool suspension than from the norovirus-positive stool spiked into the sewage effluent. Norovirus was detected in the serial dilution of the stool sample down to a 1:100 dilution; however, crossing points did not exhibit the 3.3 cycle increase indicative of a 10-fold serial dilution.

The viral concentrates resulting from the sewage effluent samples spiked with a serial dilution of norovirus-positive stool sample were also extracted using the RNeasy Mini Kit. The kit extraction appeared to increase viral recovery: the crossing points indicated there was only about a 10-fold loss of virus between the stool sample extraction and the sewage effluent sample spiked with norovirus-positive stool. When using the kit, crossing points were about three threshold cycles lower than for the same samples extracted with the Boom et al. (1990) method. Norovirus was detected down to a 1:100 dilution, but crossing points did not exhibit the 3.3-cycle increase as expected of a 10-fold dilution.

A similar spiking experiment was conducted in which a serially diluted norovirus-positive stool sample was spiked into sewage effluent samples, down to a 1:10,000 dilution. These samples were concentrated by ultracentrifugation, extracted with the RNeasy Mini Kit and

run alongside the plasmid standard (plasmid results detailed below). Similar to previous results, norovirus was detected in spiked sewage samples down to a 1:100 dilution. By running these samples alongside the plasmid standard, it was estimated that single copies of virus were detected in spiked sewage effluent samples for each 10 µl qPCR reaction, or 1.5 x 10^4 genome copies 20 ml^{-1} sewage effluent. These results also indicated that the serially diluted samples had approximately a 3.3-cycle difference between 10-fold dilutions, indicating an improvement in the sensitivity compared with not using the kit.

Reverse transcription

The 20 µl RT reaction volume was initially used as suggested by Qiagen Inc. Work done by William Jones III (personal communication) showed that reducing the master mix volume to 5 µl while using 1 µl of template RNA increased qPCR sensitivity reflected by decreasing crossing points. When comparing the 20 µl RT volume to the 5 µl RT volume, the smaller volume resulted in crossing points between 3 and up to 6 threshold cycles lower. Crossing points were not, however, necessarily reproducible from run to run. This could account for the range of differences seen between 20 µl reactions and 5 µl reactions. Regardless, the decrease in RT volume improved detection between 10- and 100-fold.

Additional evaluation of RT reaction volumes (William Jones III, personal communication) indicated that volumes as small as 5 µl were not ideal for use with samples likely to contain more inhibitors (e.g. sewage effluent). By looking at both crossing points and fluorescence, it was found that a 10 µl reaction was a good compromise between sensitivity and inhibitor dilution. Therefore, a 5 µl volume was use for experimental samples (made up of

filtered water spiked with norovirus-positive stool sample); and a 10 µl volume was used for sewage effluent sample RNA.

When using 10µl template RNA in a 25 µl RT reaction, qPCR crossing points for spiked sewage samples increased by 3 threshold cycles, as compared to using 1 µl template RNA in a 10 µl RT reaction. The higher crossing points suggest that the increased volume of template RNA is causing inhibition in the qPCR.

Use of the Qiaquick PCR Purification Kit to remove PCR inhibitors following RT for spiked DI samples resulted in crossing points 7 to 11 threshold cycles higher than without this additional step. A dilution factor of 10 should be considered however, because 5 □l of cDNA was purified and eluted into 50 □l buffer prior to qPCR. This step resulted in an increase of 4 threshold cycles for spiked sewage effluent samples. Fluorescence for purified samples was markedly higher than that for samples where this step was not used.

Plasmid preparation

A plasmid containing the norovirus GII region amplified during qPCR was generated as described in Methods. The purified linearized plasmid as quantified (Genequant *pro*) had 126 µg ml^{-1} DNA with a 260/280 ratio of 1.909 (indicating high quality of the DNA). To determine the copy number of the linearized plasmid stock solution, the following was calculated:

Mass of 1 plasmid molecule = 4.43×10^{-18} g
Linearized plasmid concentration = 1.26×10^{-7} g μl^{-1}
1.26×10^{-7} g / 4.43×10^{-18} g μl^{-1} = 2.84×10^{10} copies μl^{-1} plasmid stock

By serially diluting the plasmid stock solution and detecting it using qPCR, the detection limit was estimated to be the 10^{-11} dilution (one to one hundred trillion dilution). The standard curve generated from the serial dilution (an example shown in Figure 3) exhibited a high r^2 value

and a slope approximating the optimal crossing point differences between a 10-fold dilution (3.3 threshold cycles).

Sewage samples

Norovirus was not detected in unspiked sewage effluent samples using qPCR. For spiked sewage samples, an average of 1.0x 10^5 genome copies 20 ml^{-1} sewage effluent was detected with a range from 1.0 x 10^3-1.0 x 10^6 genome copies 20 ml^{-1} sewage effluent.

Persistence of norovirus and FRNA coliphage in the estuarine environment.

***In situ* experiments**

The experimental conditions for the spring, summer and winter experiments are outlined in Table 2. Light attenuation (LICOR) data were recorded each of the sampling days for the spring and summer experimental periods. During the winter experiment, light attenuation data were only available for days 4 and 8. For each experimental period, the radiation values were plotted against depth to determine the light attenuation coefficient for the period.

Table 2. *In situ* experimental conditions.

	Experimental period	Water temperature (°C)	Light attenuation coefficient (K_d) (m^{-1})	22-year average monthly insolation (kWh $m^{-2}d^{-1}$)*	PAR from Taskinas Creek, VA (mM m^{-2})†
Spring	May 23-June 1, 2007	19.2-25.7; average = 21.7	1.97	5.96	55,517
Summer	July 31-August 8, 2007	26.5-30.8; average = 28.6	0.995	5.08	48,249
Winter	February 11-19, 2008	6.79-10.2; average = 8.05	0.523	2.97	21,457

Legend:
PAR = photosynthetically active radiation
* Values specifically incident on a horizontal surface (NASA, 2008).
† Taskinas Creek station (37° 24' 50.76 N, 76° 42' 44.53 W), approximately 15 miles northwest of the experimental setup (NERRS CDMO, 2008); PAR value is average over experimental period.

FRNA coliphage counts and norovirus copy number were determined for each condition (light-exposed surface, dark surface, light-exposed 1 meter, and dark 1 meter) for each sampling day. The data were $\log_{10}$ transformed and plotted against time. Figures 4-6 show FRNA coliphage counts and RNase/PK-treated (treated) and untreated norovirus copy number persistence during the spring, summer and winter experimental periods, respectively. Treated norovirus data was collected for the winter experiment.

RNase and Proteinase K Treatment

The RNase and PK treatment was evaluated to determine if there was a difference in viral genomic copy number between treated and untreated norovirus samples. A trial was run in which 10 stool-spiked water suspension samples were treated and 10 were not treated. In comparing the resulting copy numbers from this trial by a t-test, it was determined that there was no effect ($p>0.05$) of this treatment per se on virus copy number.

Further analysis of the spring and summer persistence experiment data is described in the Data Analysis section to follow.

***In vitro* experiments**

FRNA coliphage counts and norovirus copy numbers were determined for each salinity and temperature condition described in Methods for each sampling day. The data were $\log_{10}$ transformed and plotted against time. Figures 7-9 show the FRNA and norovirus persistence for 10°C, 20°C and 30°C, respectively.

Data analysis

Using the persistence data from the *in situ* and *in vitro* experiments, inactivation rates were calculated for each of the experimental conditions. For each experimental treatment (spring

surface light exposed, spring surface dark, etc. and 0 psu, 10°, 0 psu, 20°C, etc), the virus counts (PFU ml^{-1} or genome copies ml^{-1}) of the three replicates for each sampling day were used in a nonlinear regression to calculate a slope value, or inactivation rate k d^{-1}. Using an SAS (SAS Institute, Inc., Cary, NC) program written by M. Newman (1995), virus counts were natural log transformed and a linear model fit was used to estimate the inactivation rate and initial virus density. These estimates were used as initial estimates in an iterative, non-linear regression from which final estimates were generated. The 95% confidence intervals were calculated using the following equation:

$$x \pm t(S/\sqrt{n})$$

where x is the mean of the replicates; t is the critical t-value of the t-distribution; S is the standard deviation of the replicates; and n is the number of replicates. Inactivation rates were considered not to be significantly different if the 95% confidence intervals overlapped.

An analysis of inactivation rates and 95% confidence intervals was used to determine if there was a significant treatment effect of RNase and PK for norovirus samples in the *in situ* experiments. Figure 10 shows the inactivation rates with 95% confidence intervals for treated and untreated norovirus samples from the *in situ* experiments. For all experimental conditions, the confidence intervals overlapped for the inactivation rates of both untreated and treated norovirus samples. Because a treatment effect was not observed and there was more untreated norovirus data available than treated norovirus data, untreated norovirus data was used for the purpose of comparing norovirus inactivation rates to FRNA coliphage inactivation rates. Figure 11 shows the inactivation rates with 95% confidence intervals for the *in situ* experiments. Table 3 summarizes the FRNA coliphage and norovirus inactivation rates, the r^2 values associated with

Table 3. Summary of *in situ* FRNA coliphage and norovirus inactivation rates (-k d^{-1}), 95% confidence intervals, regression r^2 values and T_{90} values.

	Spring				Summer				Winter			
	SD	SL	MD	ML	SD	SL	MD	ML	SD	SL	MD	ML
FRNA coliphage	3.08± 0.13	4.60± 0.21	3.21± 0.12	3.89± 0:27	6.31± 0.04	6.02± 0.03	4.91± 0.07	5.40± 0.07	1.32± 0.25	1.56± 0.24	1.25± 0.21	1.51± 0.25
	r^2=0.95	r^2= 0.95	r^2= 0.98	r^2= 0.97	r^2= 0.99	r^2= 1.00	r^2= 0.92	r^2= 1.00	r^2= 0.93	r^2= 0.97	r^2= 0.93	r^2= 0.50
	T_{90}= 1.50	T_{90}= 1.00	T_{90}= 1.43	T_{90}= 1.18	T_{90}= 0.73	T_{90}= 0.77	T_{90}= 0.94	T_{90}= 0.85	T_{90}= 3.48	T_{90}= 2.96	T_{90}= 3.69	T_{90}= 3.05
Untreated norovirus	0.29± 0.21	0.83± 0.19	0.26± 0.33	0.41± 0.19	0.15± 0.20	0.26± 0.16	0.14± 0.18	0.27± 0.27	0.14± 0.14	0.22± 0.18	0.10± 0.15	0.16± 0.14
	r^2=0.84	r^2=0.93	r^2=0.87	r^2=0.90	r^2=0.73	r^2=0.42	r^2=0.64	r^2=0.90	r^2=0.75	r^2=0.83	r^2=0.35	r^2=0.63
	T_{90}= 16.06	T_{90}= 5.57	T_{90}= 17.71	T_{90}= 11.23	T_{90}= 29.94	T_{90}= 17.64	T_{90}= 33.37	T_{90}= 16.97	T_{90}= 33.54	T_{90}= 21.19	T_{90}= 46.01	T_{90}= 28.57

Legend:
SD = surface dark condition
SL = surface light-exposed condition
MD = 1 meter dark condition
ML = 1 meter light-exposed condition

the regression and T_{90} values (ln (0.01/k)). FNRA coliphage inactivation rates were significantly higher than untreated norovirus inactivation rates for all *in situ* experimental conditions. The spring experiment results showed that FRNA coliphage inactivation rates were about 10 times higher than norovirus inactivation rates, except for the light surface condition where FRNA coliphage rate was only about 5 times higher than the norovirus inactivation rate. The summer experiment results showed that FRNA inactivation rates were between 20-40 times higher than norovirus inactivation rates. Finally, FRNA inactivation rates in the winter experiment were about 10 times higher than norovirus inactivation rates for the same period. FRNA coliphage inactivation rates varied significantly as a function of season and light over the *in situ* experimental conditions.

The rate analysis showed that none of the experimental conditions resulted in a significant difference in norovirus inactivation rates except for the spring surface light-exposed condition. The spring surface light-exposed condition was significantly higher than any other norovirus inactivation rate.

Figure 12 shows the FRNA coliphage and untreated norovirus inactivation rates as a function of temperature. A linear regression line is shown when the r^2 value of the linear regression indicated that temperature was predictive of the inactivation rate (a value of 0.80 was selected, i.e., the r^2 value was $\geq$ 0.80). Table 4 shows the linear regression equations and associated r^2 values for FRNA coliphage. Seasonal water temperature differences were predictive of FRNA coliphage inactivation rates. When linear regressions were applied to the norovirus inactivation rates, the r^2 values did not indicate a predictive relationship of seasonal water temperature on norovirus inactivation rates.

Table 4. Linear regression equations and r^2 values for *in situ* inactivation rates as a function of seasonal temperature for a given treatment.

	SD	SL	MD	ML
FRNA coliphage	y = 0.23x - 0.84 r^2= 0.88	y = 0.22x - 0.18 r^2= 1.00	y = 0.17x - 0.25 r^2= 0.98	y = 0.19x - 0.04 r^2= 1.00

Legend:
SD = surface dark condition
SL = surface light-exposed condition
MD = 1 meter dark condition
ML = 1 meter light-exposed condition

Figure 13 shows an analysis of the dark vs. light-exposed treatments for norovirus and FRNA coliphage. A reference line with a slope equal to 1 is plotted; a light effect is inferred if points plot above the line, but not if they plot along the reference line. All points in the norovirus plot are above the reference line, with the spring points deviating highly from the reference line. FRNA coliphage points all plot above the line, except for one summer point; deviations from the line are also highest for the spring points.

Figure 14 shows the inactivation rates with 95% confidence intervals for the *in vitro* experiments and Table 5 summarizes the FRNA coliphage and norovirus inactivation rates, the r^2 values associated with the regression and the T_{90} values. Analysis of the *in vitro* rate data indicated that FRNA coliphage inactivation rates were higher than norovirus inactivation rates for all conditions except 0 psu and 10 psu at 10°C where the confidence intervals overlapped. In the 10°C experiments, FRNA coliphage inactivation rates were between approximately 3.5-7 times higher than norovirus inactivation rates. During the 20°C experiment, much larger differences were seen between FRNA coliphage inactivation rates and norovirus inactivation rates: at 10 psu and 20 psu, FRNA coliphage rates were over 70 times and 130 times higher than norovirus inactivation rates, respectively. Slightly lower differences in rates were seen in the 30°C experiment, with FRNA coliphage inactivation rates ranging from 14-30 times higher than

Table 5. Summary of *in vitro* FRNA coliphage and norovirus inactivation rates (-k d^{-1}), 95% confidence intervals, regression r^2 values, and T_{90} values.

	10°C				20°C				30°C			
	0 psu	10 psu	20 psu	30 psu	0 psu	10 psu	20 psu	30 psu	0 psu	10 psu	20 psu	30 psu
FRNA coliphage	0.28± 0.16 r^2= 0.90 T_{90}= 16.22	0.75± 0.41 r^2= 0.84 T_{90}= 6.15	0.99± 0.31 r^2= 0.84 T_{90}= 4.63	1.34± 0.26 r^2= 0.83 T_{90}= 3.44	1.79± 0.21 r^2= 0.94 T_{90}= 2.57	5.21± 0.10 r^2= 0.76 T_{90}= 0.88	4.15± 0.35 r^2= 0.73 T_{90}= 1.11	4.11± 0.51 r^2= 0.92 T_{90}= 1.12	3.28± 0.25 r^2= 0.95 T_{90}= 1.40	6.16± 0.55 r^2= 0.68 T_{90}= 0.75	8.95± 0.36 r^2= 0.45 T_{90}= 0.51	8.48± 1.08 r^2= 0.94 T_{90}= 0.54
Norovirus	0.04± 0.05 r^2= 0.21 T_{90}= 111.24	0.15± 0.06 r^2= 0.72 T_{90}= 30.54	0.29± 0.12 r^2= 0.46 T_{90}= 15.78	0.22± 0.08 r^2= 0.89 T_{90}= 20.75	0.15± 0.10 r^2= 0.75 T_{90}= 30.32	0.07± 0.06 r^2= 0.34 T_{90}= 64.23	0.03± 0.07 r^2= 0.04 T_{90}= 147.60	0.31± 0.14 r^2= 0.84 T_{90}= 15.08	0.20± 0.06 r^2= 0.93 T_{90}= 23.42	0.20± 0.08 r^2= 0.84 T_{90}= 23.39	0.35± 0.08 r^2= 0.95 T_{90}= 13.07	0.59± 0.18 r^2= 0.86 T_{90}= 7.77

Legend:
psu = practical salinity unit

norovirus inactivation rates.

Figure 15 shows FRNA coliphage and norovirus inactivation rates as a function of temperature. When temperature was predictive of the inactivation rates ($r^2 \geq 0.80$), the linear regression line is shown on the graph. Linear regression equations and r^2 values are shown in Table 6. Similar to the *in situ* experiments, water temperature was always predictive of FRNA coliphage inactivation rates. For FRNA coliphage, the inactivation rates for 0 psu treatments of each temperature were progressively higher: the 20°C rate was 6.3 times that of the 10°C rate; the 30°C rate 1.8 times that of the 20°C rate. Similarly, the 10, 20 and 30 psu treatment inactivation rates were progressively higher with increasing temperature: the 20°C rates were higher than the 10°C rates by a factor of between 3 and 7; the 30°C rates were higher than the 20°C rates by a factor of between 1.2 and 2.2.

Few significant differences were observed between the norovirus inactivation rates based on temperature in the *in vitro* experiments and water temperature was not consistently predictive of norovirus inactivation.

Table 6. Linear regression equations and r^2 values for *in vitro* inactivation rates as a function of temperature at a given salinity.

	0 psu	10 psu	20 psu	30 psu
FRNA coliphage	$y = 0.15x - 1.21$ $r^2 = 1$	$y = 0.27x - 1.37$ $r^2 = 0.88$	$y = 0.34x - 3.26$ $r^2 = 0.99$	$y = 0.36x - 2.50$ $r^2 = 0.98$
Norovirus	$y = 0.008x - 0.03$ $r^2 = 0.94$	r^2 value < 0.80	r^2 value < 0.80	$y = 0.09x + 0.003$ $r^2 = 0.91$

Figure 16 shows FRNA coliphage and norovirus inactivation rates as a function of salinity. When salinity was predictive of the inactivation rates ($r^2 \geq 0.80$), the linear regression line is shown on the graph. Linear regression equations and r^2 values are shown in Table 7.

Comparing salinity, in the 10°C experiment, there was no significant difference between the FRNA coliphage inactivation rate of the 0 psu and 10 psu samples; but 20 and 30 psu treatments had inactivation rates higher than 0 psu samples by a factor of 3.5 and 4.7, respectively. At 20°C, inactivation rates of the 10, 20 and 30 psu treatments were higher than the 0 psu treatment by a factor between 2.3 and 2.9. At 30°C, inactivation rates of the 10, 20 and 30 psu treatments exceeded the 0 psu treatment by a factor between 1.9 and 2.7.

In both the 10° and 30°C experiments, a significant difference was observed between the norovirus inactivation rates of the 0 psu and the 20 and 30 psu treatments; however salinity was not consistently predictive of norovirus inactivation rates. More variability in inactivation rates as a function of temperature and salinity was observed in the *in vitro* experiments as compared to the variability of inactivation rates observed as a function of season and sunlight in the *in situ* experiments. Additionally, some of the confidence intervals associated with the *in vitro* inactivation rates were very narrow due to the small standard deviations associated with replicate measurements of a given experimental treatment.

Table 7. Linear regression equations and r^2 values for *in vitro* inactivation rates as a function of salinity at a given temperature.

	10°C	20°C	30°C
FRNA coliphage	$y = 0.03x + 0.33$ $r^2 = 0.99$	r^2 value < 0.80	$y = 0.18x + 3.96$ $r^2 = 0.84$
Norovirus	r^2 value < 0.80	r^2 value < 0.80	$y = 0.01x + 0.13$ $r^2 = 0.86$

Figure 3. Standard curve generated from 10-fold serially diluted plasmid containing norovirus GII PCR product.

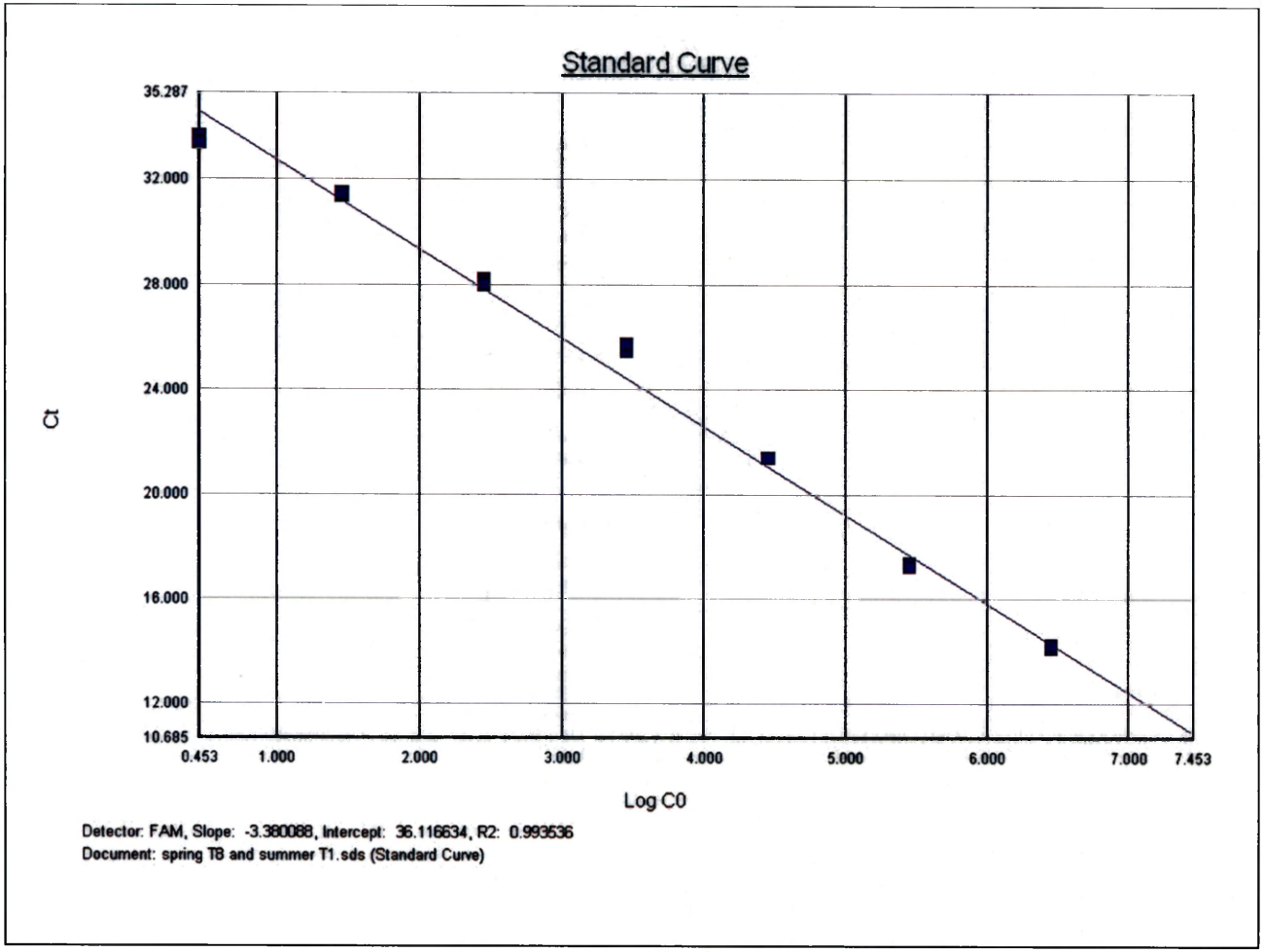


Figure 4. Spring experiment persistence results.

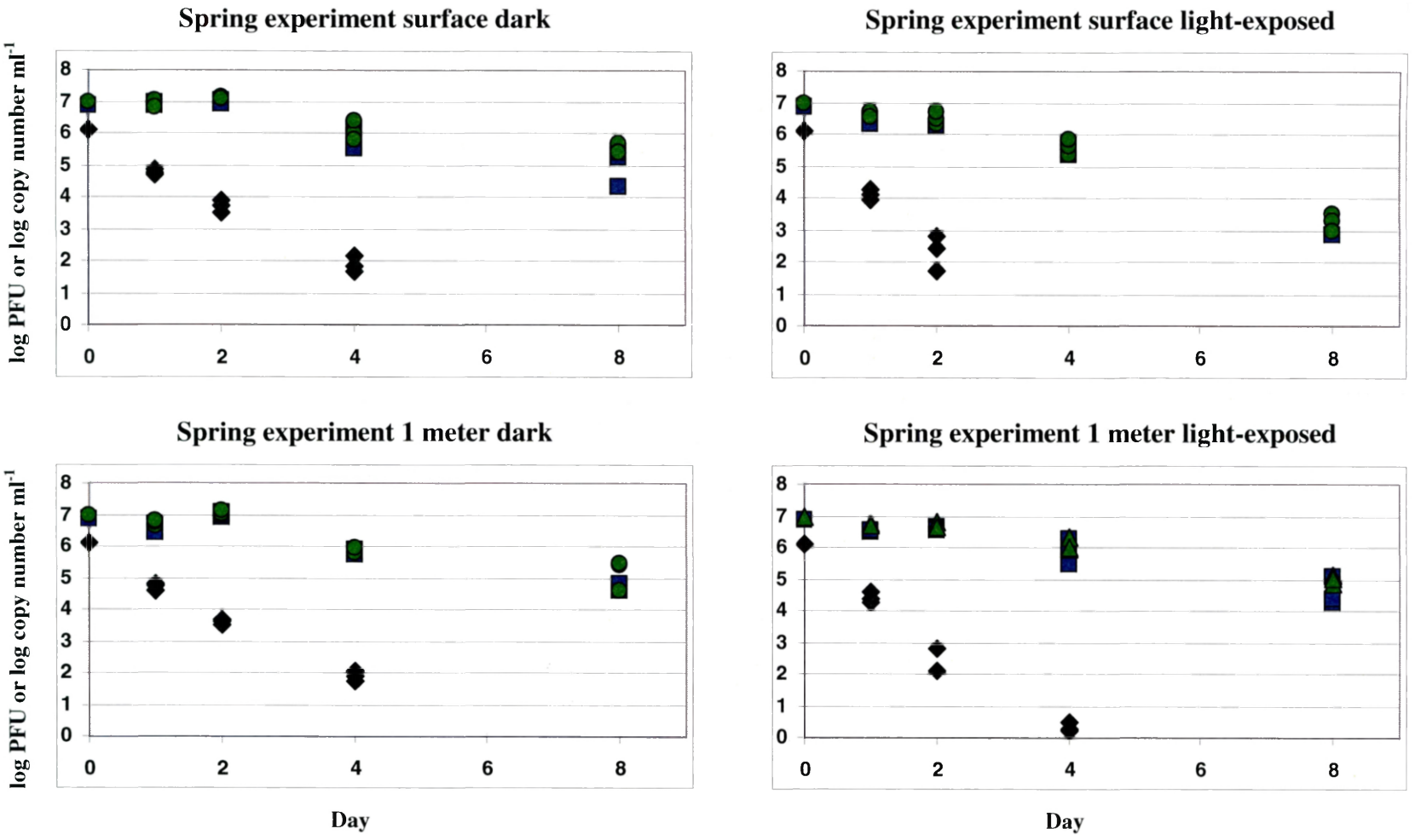


Figure 5. Summer experiment persistence results.

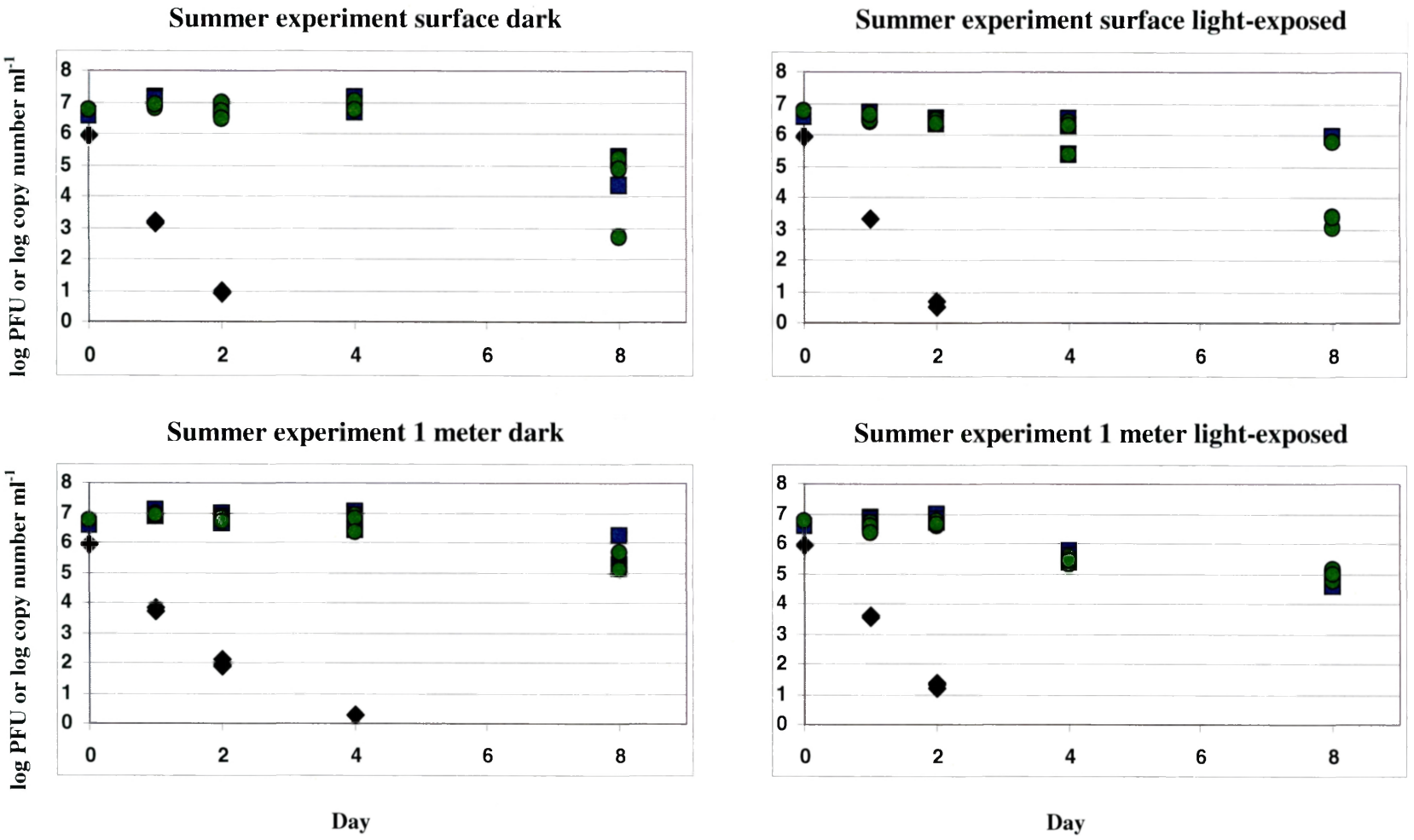
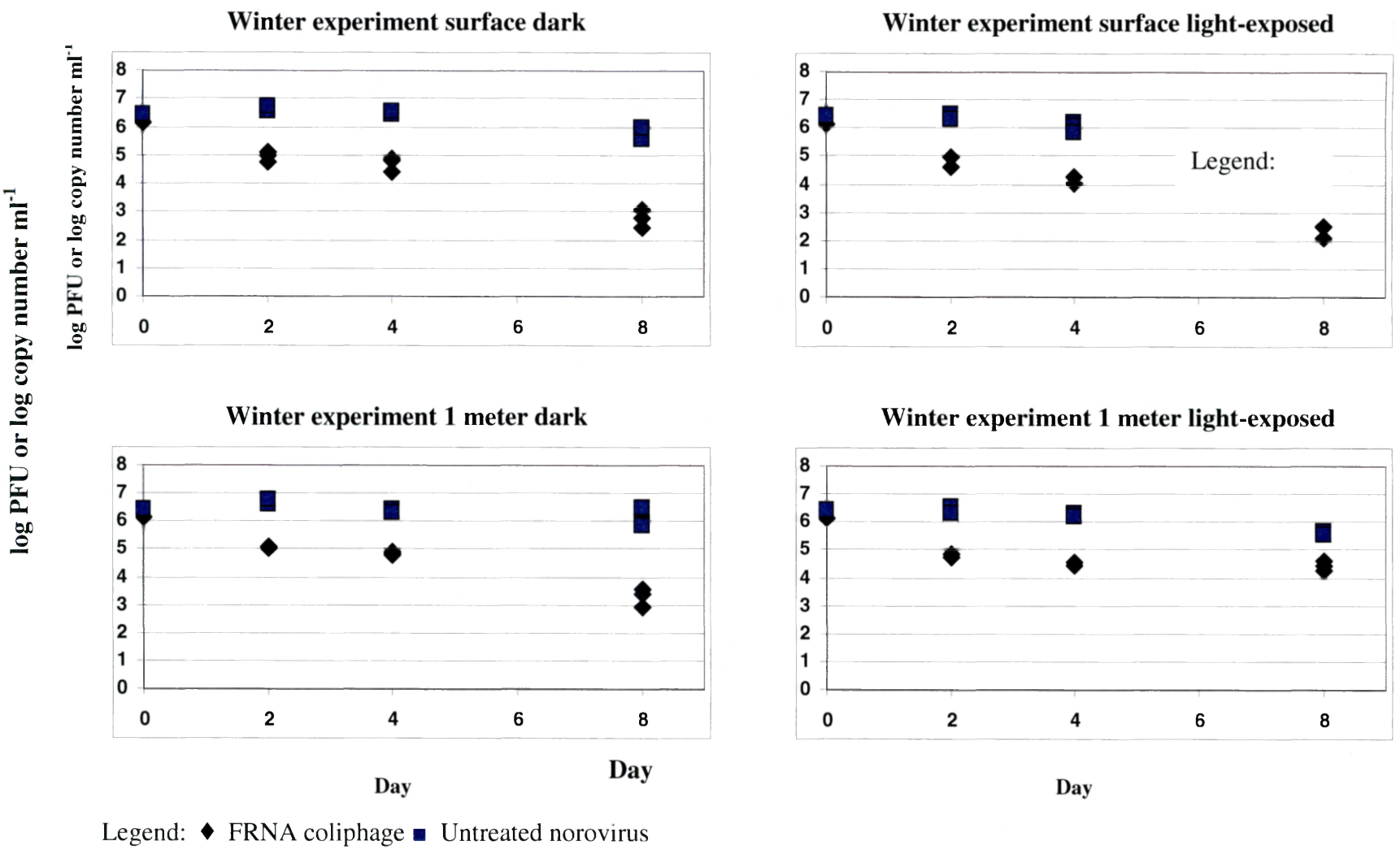


Figure 6. Winter experiment persistence results.



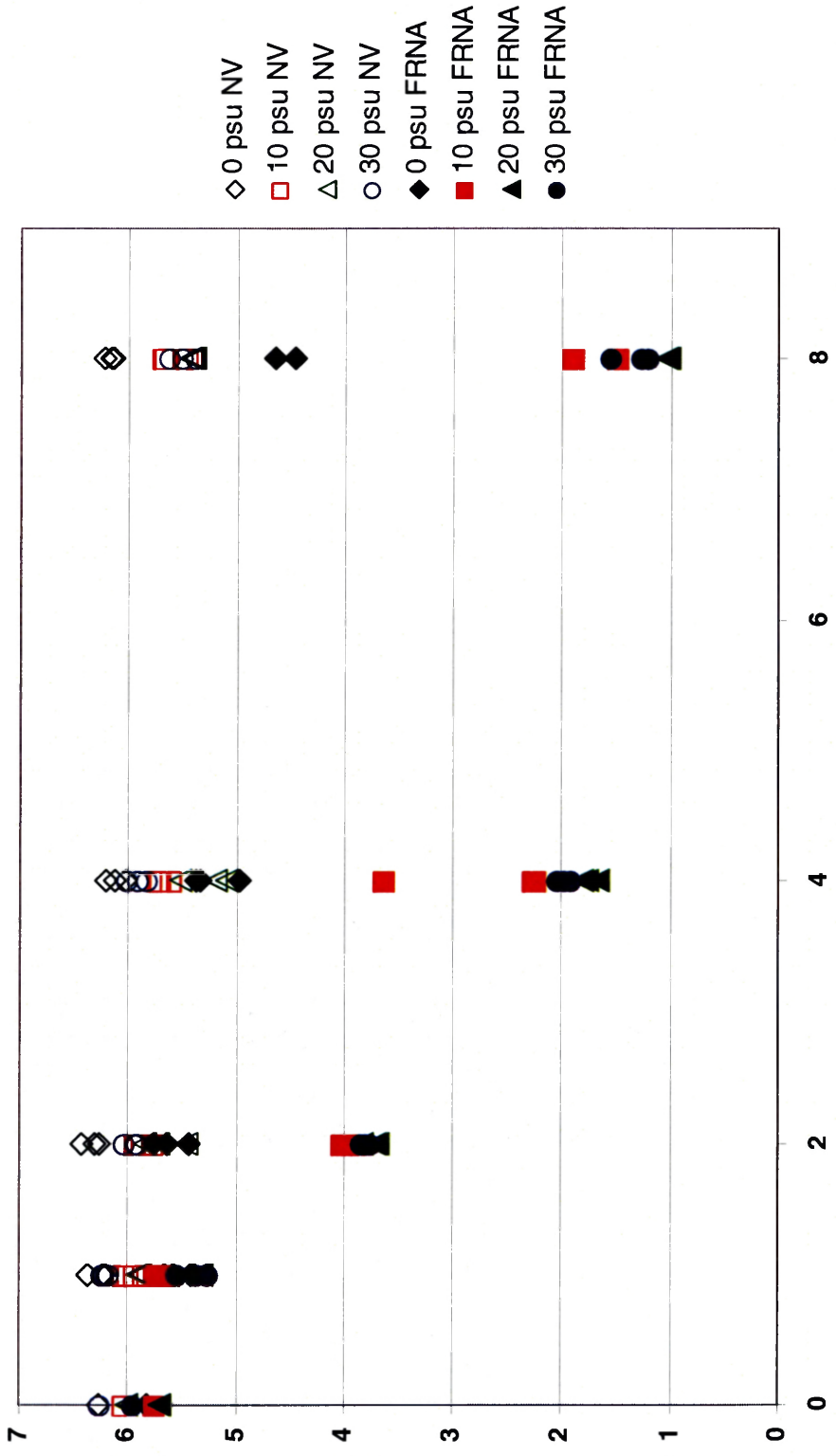

◇ 0 psu NV
□ 10 psu NV
△ 20 psu NV
○ 30 psu NV
◆ 0 psu FRNA
■ 10 psu FRNA
▲ 20 psu FRNA
● 30 psu FRNA
7
6
5
4
3
2
1
0
0
2
4
6
8

Figure 8. 20°C *in vitro* persistence experiment results.

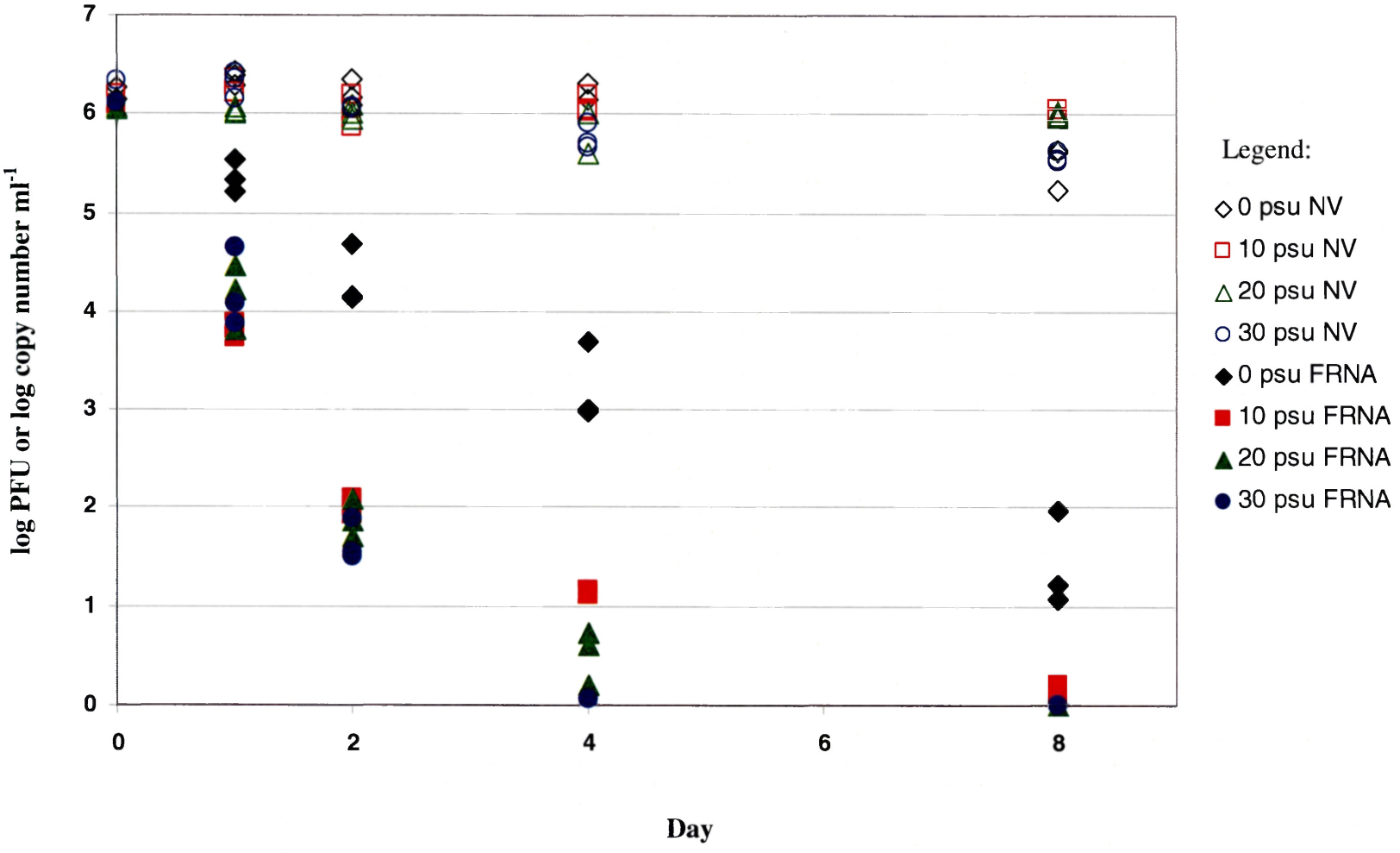


Figure 9. 30°C *in vitro* persistence experiment results.

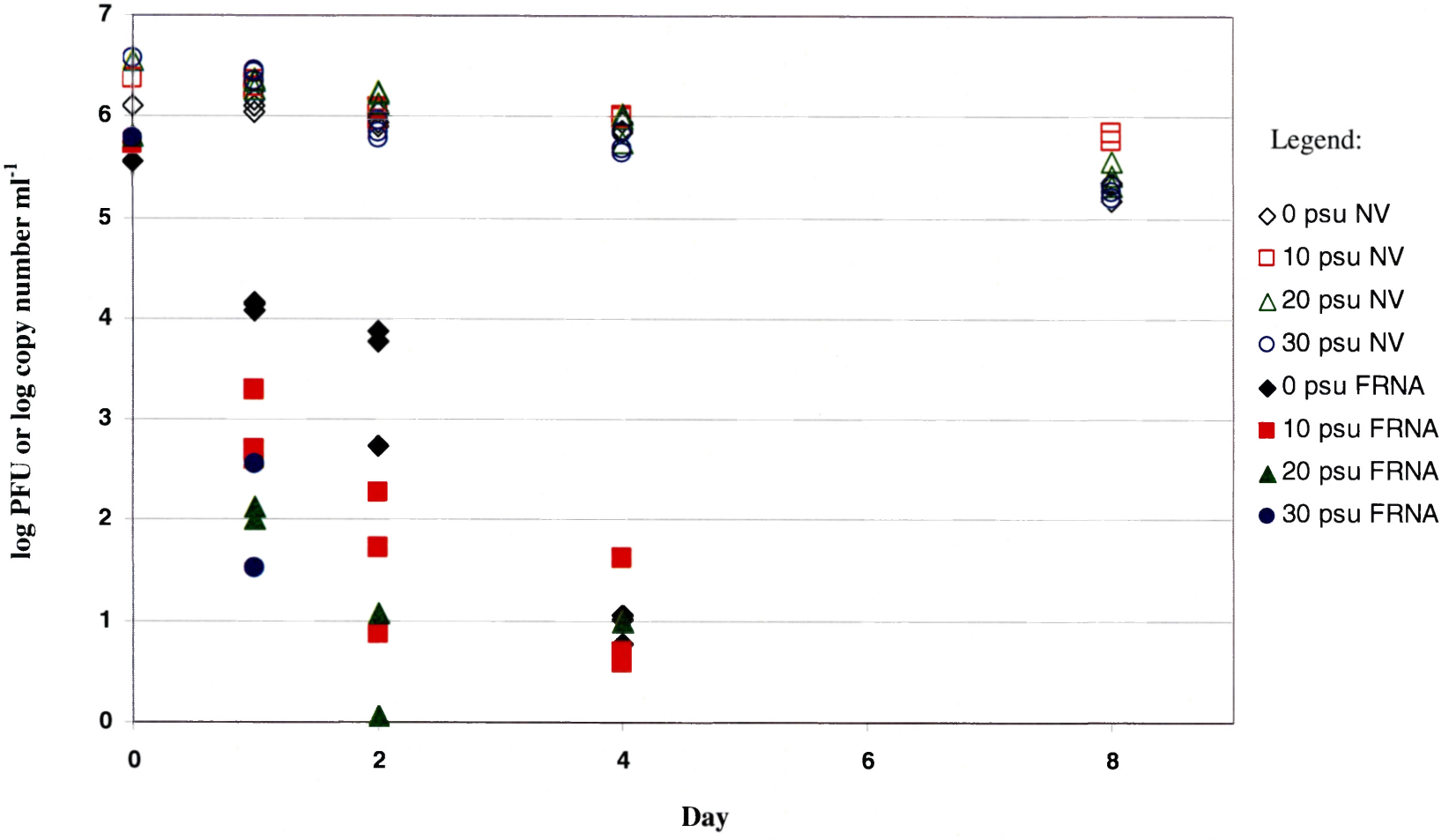
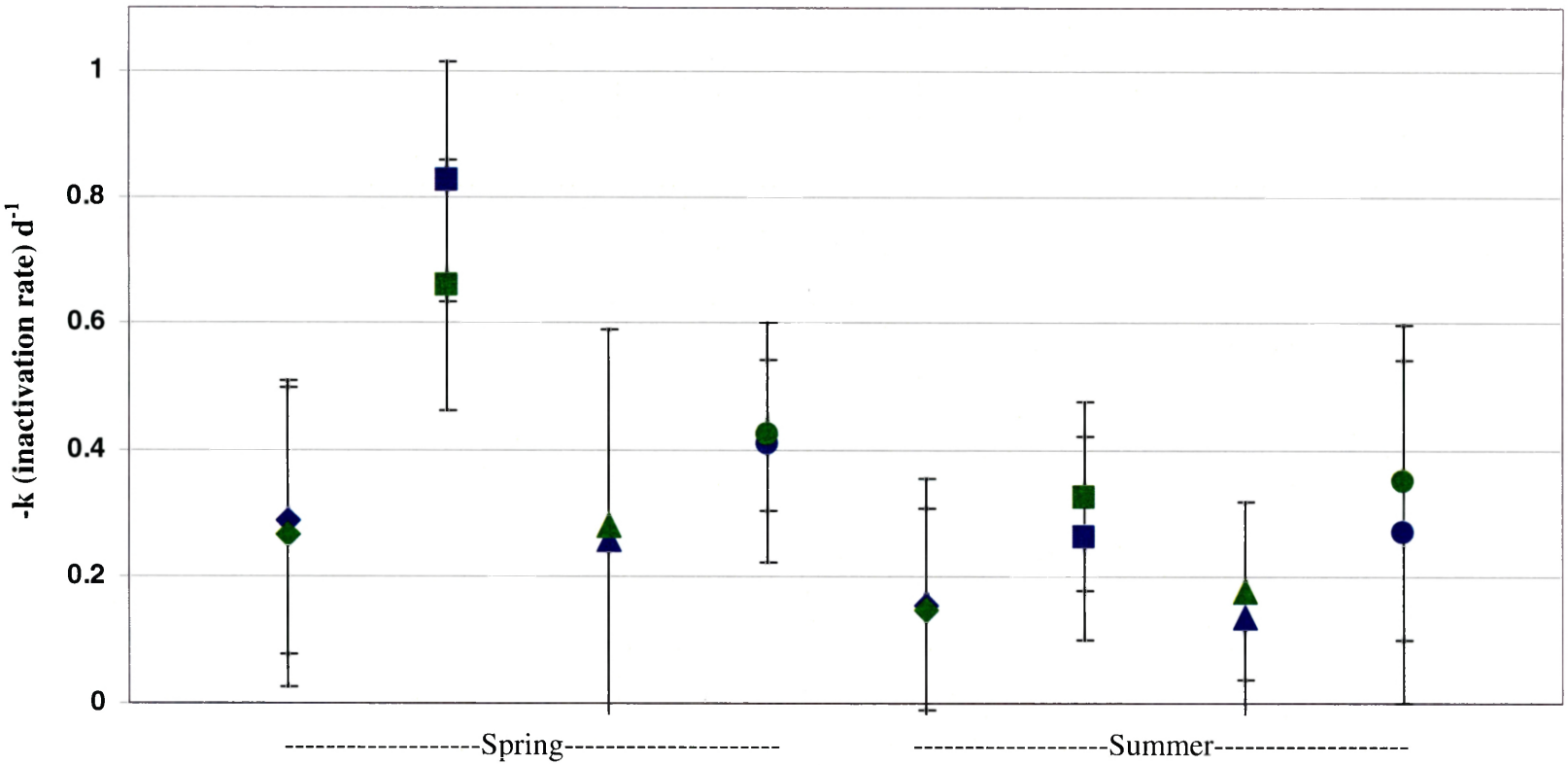


Figure 10. Norovirus sample treatment analysis: inactivation rates showing 95% confidence intervals.

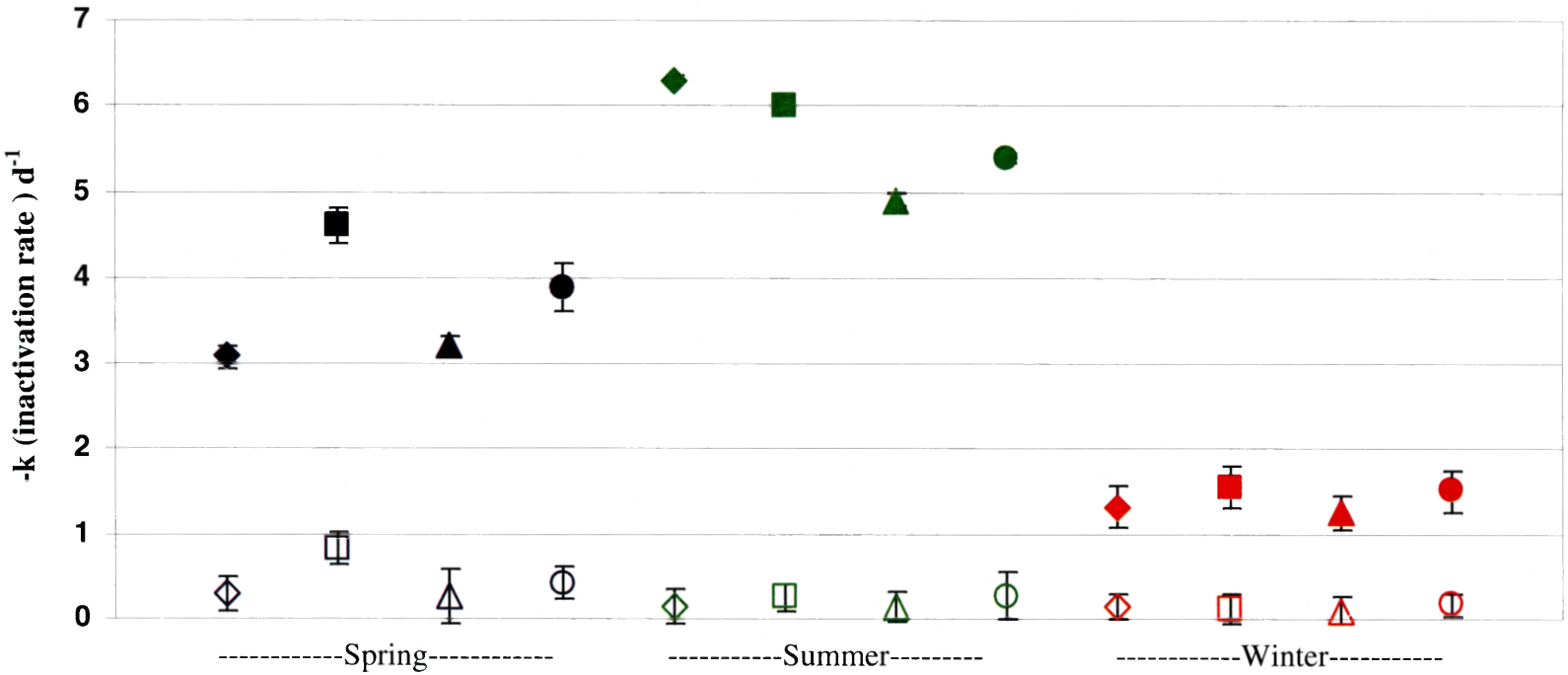


Legend: Untreated norovirus samples in blue; treated norovirus samples in green.

♦ Surface dark condition ■ Surface light condition

▲ 1 meter depth dark condition ● 1 meter depth light condition

Figure 11. ***In situ*** **experiment treatment analysis: inactivation rates showing 95% confidence intervals.**

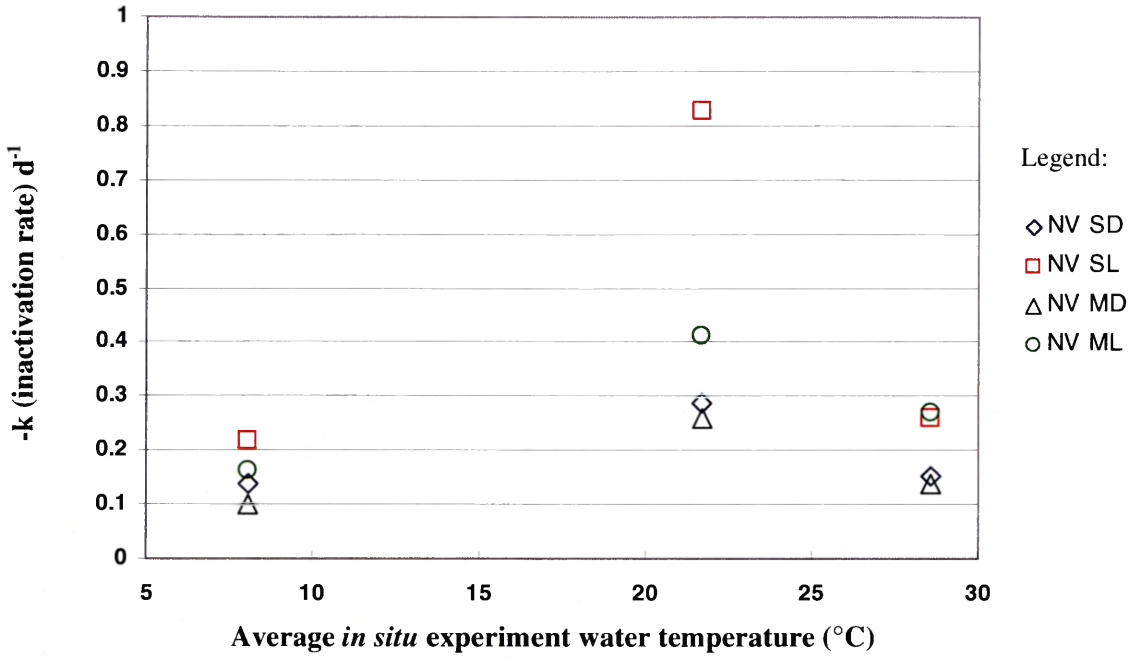


Legend: Closed shapes = FRNA coliphage; open shapes = untreated norovirus.

♦ Surface dark condition ■ Surface light condition

▲ 1 meter depth dark condition ● 1 meter depth light condition

Figure 12. *In situ* experiment inactivation rates as a function of temperature for a given treatment.



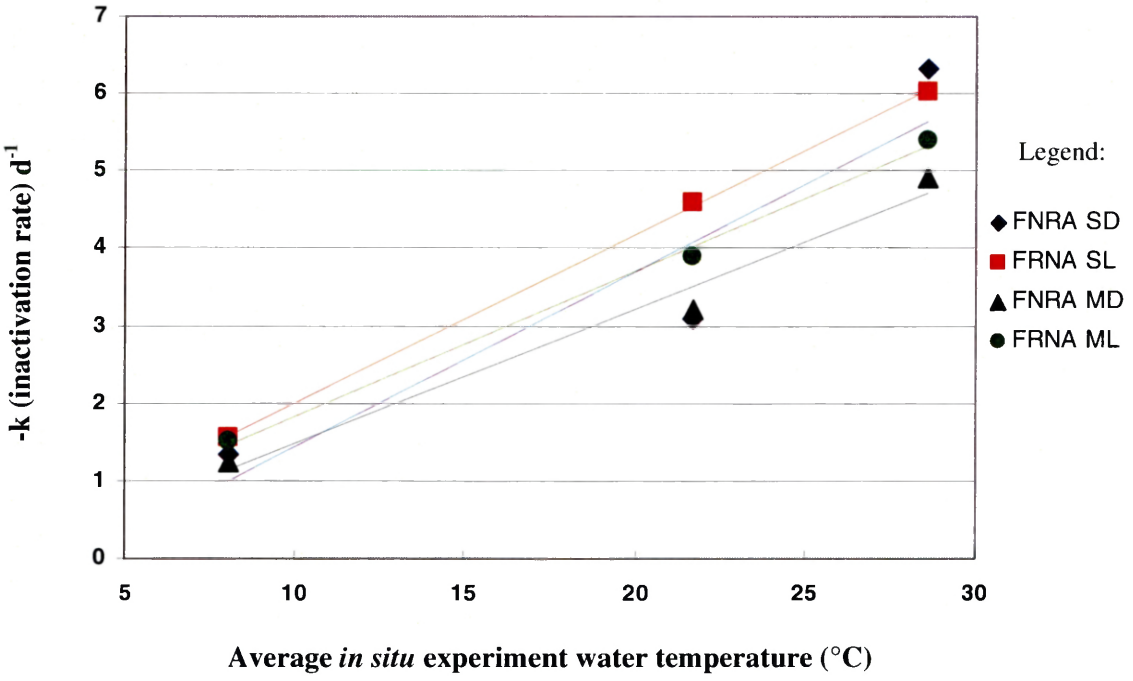
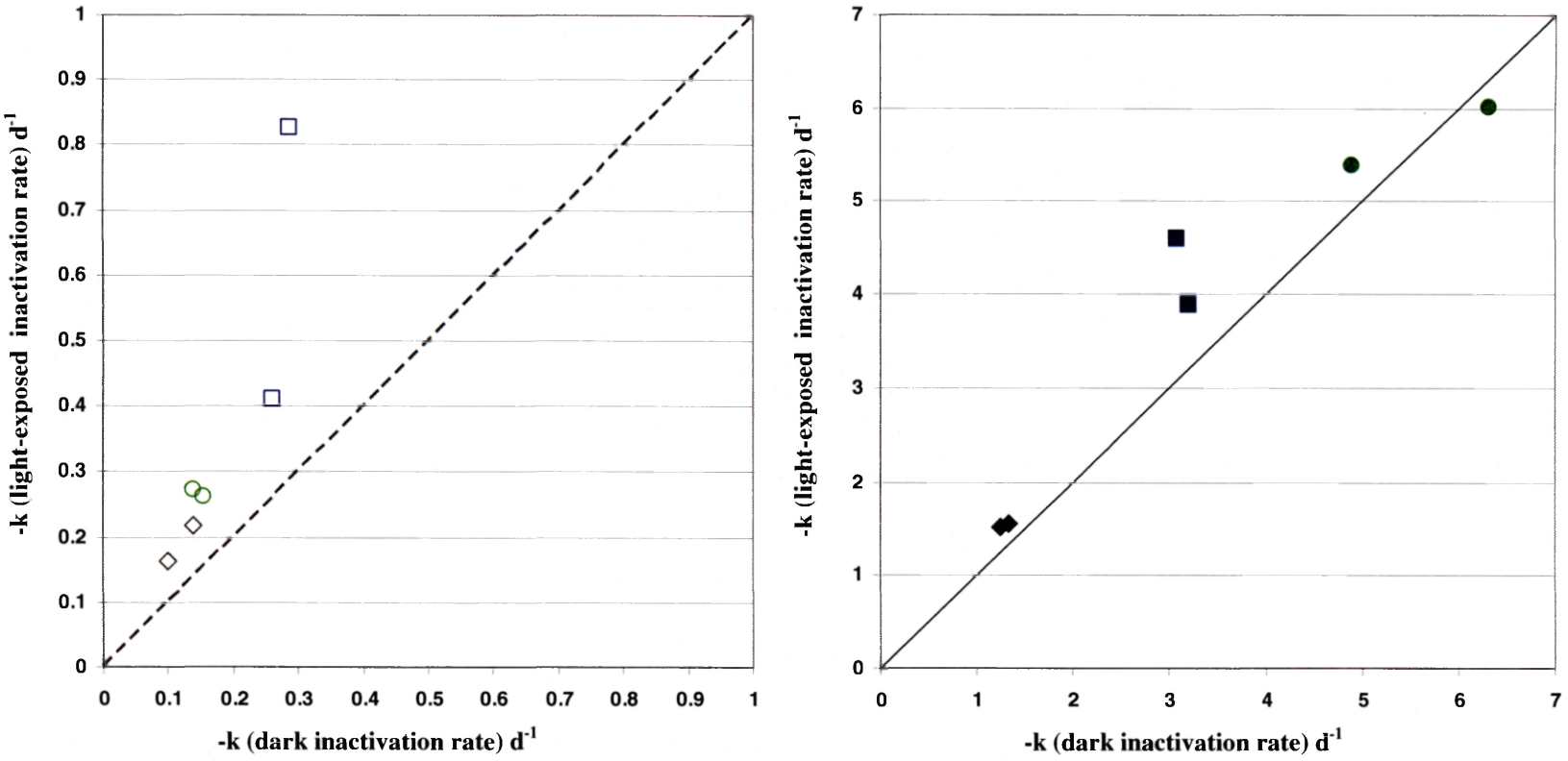


Figure 13. ***In situ*** **experiment dark treatment inactivation rates vs. light-exposed treatment inactivation rates.**

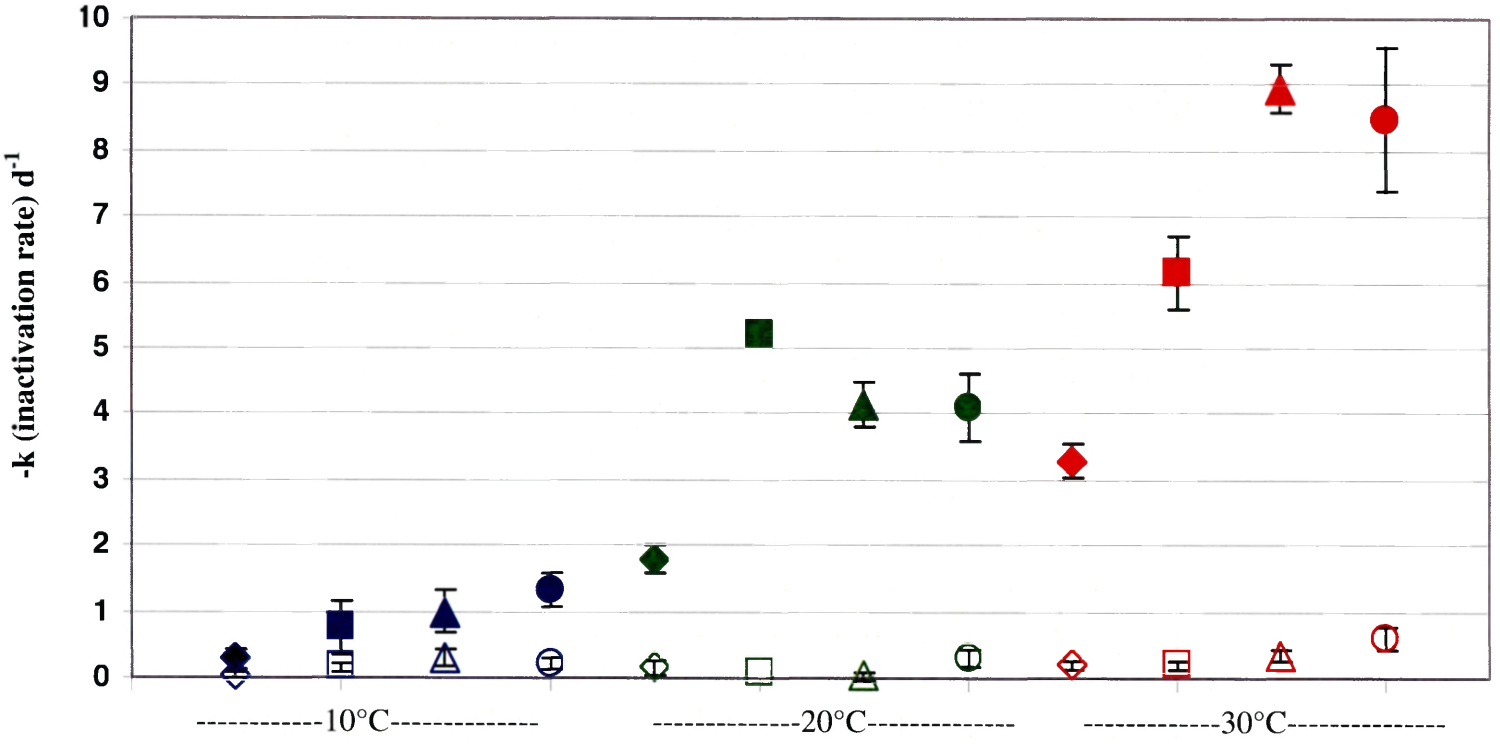


Legend: Open shapes = untreated norovirus; closed shapes = FRNA coliphage

♦ = winter ■ = spring ● = summer

Theoretical reference line has a slope = 1.

Figure 14. ***In vitro*** **experiment treatment analysis: inactivation rates showing 95% confidence intervals.**

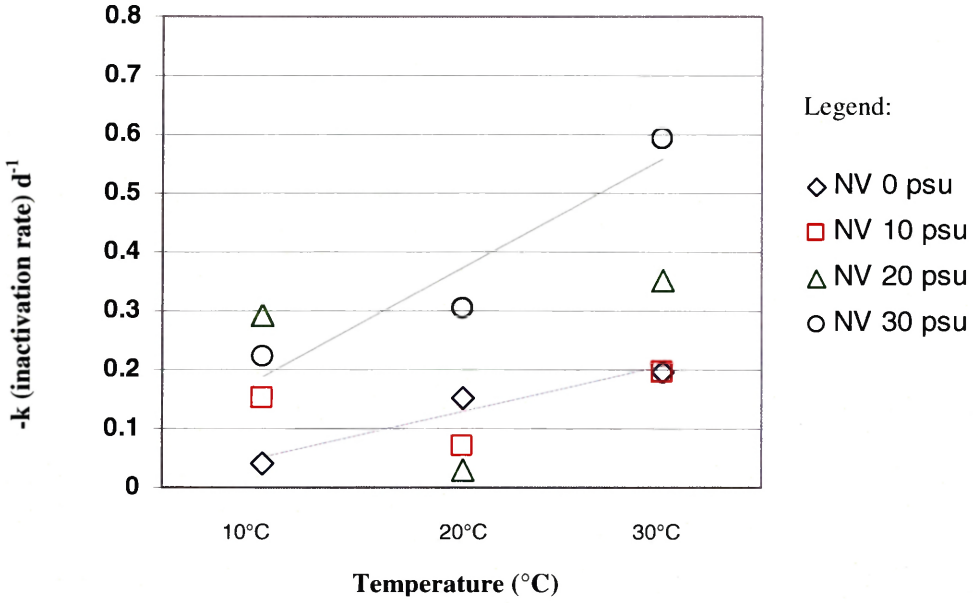


Legend: Closed shapes = FRNA coliphage; open shapes = untreated norovirus.

♦ 0 psu ■ 10 psu

▲ 20 psu ● 30 psu

Figure 15. ***In vitro*** **experiment inactivation rates as a function of temperature at a given salinity.**



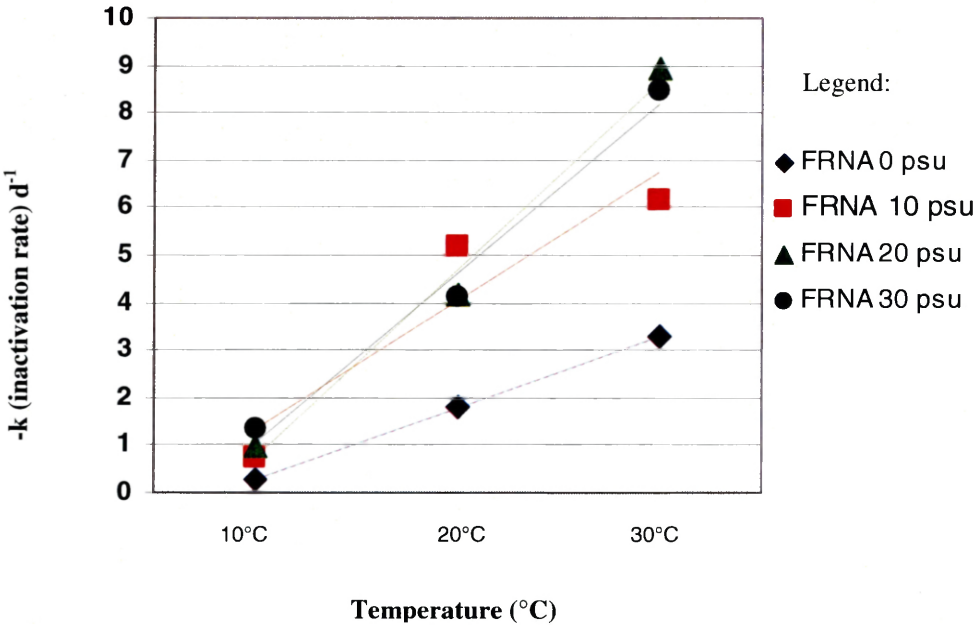
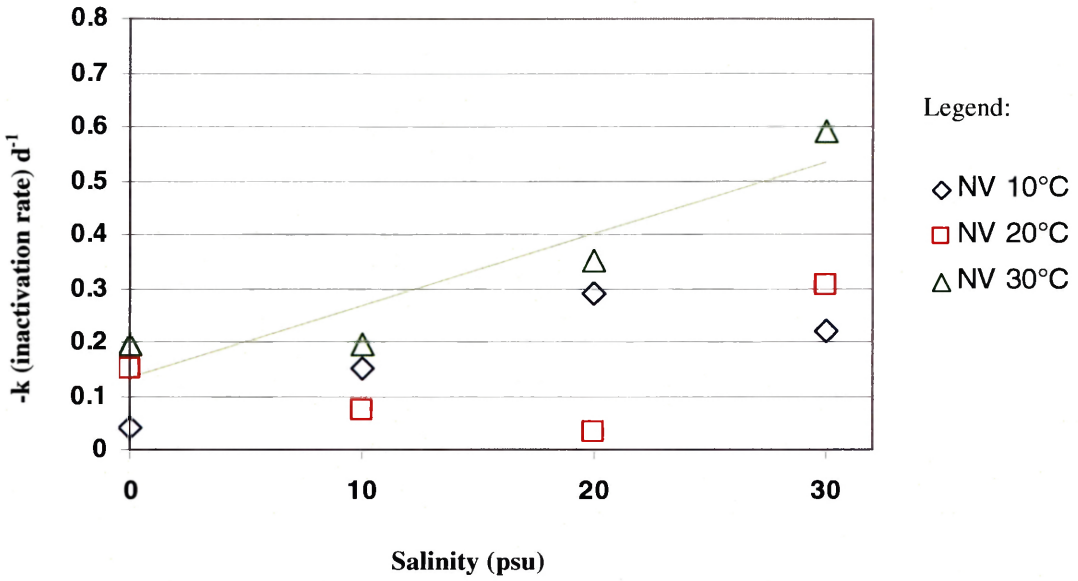
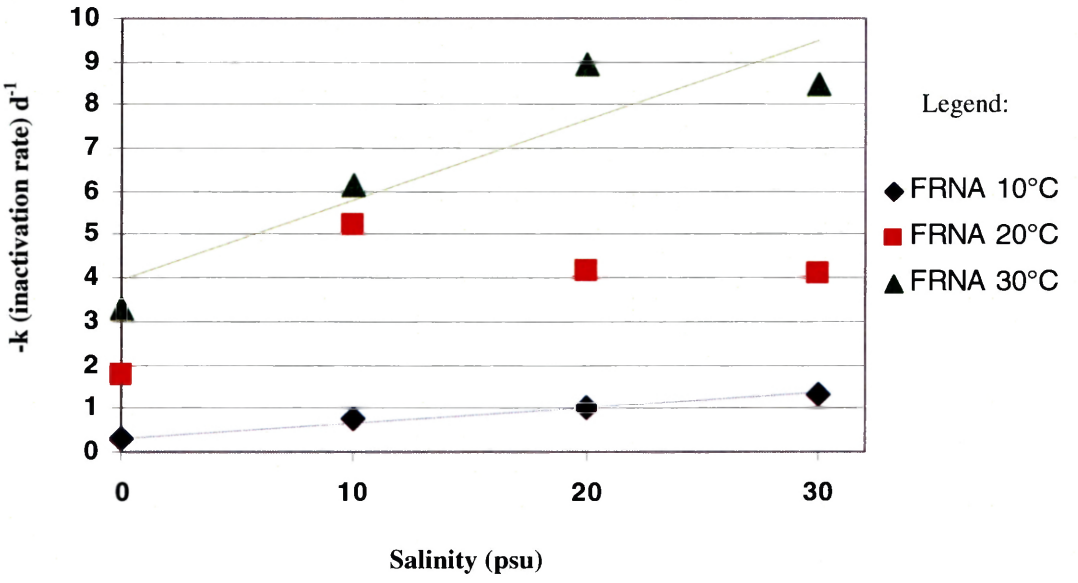


Figure 16. ***In vitro*** **experiment rates as a function of salinity at a given temperature.**





Discussion

Virus Concentration and RNA Extraction

Concentration methods

Several methods for concentrating sewage effluent samples were evaluated. When assessing sample adsorption to negatively charged nitrocellulose filters and elution with skimmed milk-glycine buffer, results indicated poor recovery of norovirus spiked into DI water samples. A Wyn-Jones and Sellwood (2001) review reported this method to have an average recovery efficiency of 50% for enteric viruses in water samples. In this study, norovirus was recovered in the range of 0.1-1% and the method was not pursued further.

A method applied to sewage effluent and seawater samples based on adsorption to negatively charged nitrocellulose filters followed by direct RNA lysis from the filter was evaluated (Fuhrman et al. 2005). When assessing this method for sewage samples, norovirus was not detected by qPCR for sewage samples spiked with norovirus; therefore, the method was not pursued further for sewage effluent samples.

When initially evaluating the Fuhrman et al. method (2005) or environmental water samples, low levels of fluorescence in qPCR were encountered when analyzing 1 μm-filtered York River water samples spiked with norovirus. Inhibitors of enzymatic reactions, including reverse transcription and PCR, are often found in environmental samples. Therefore, to reduce the potential problems with inhibitor presence in the water samples, York River water was further filtered to 0.22 μm, removing the 0.22 -1 μm-sized material potentially inhibiting the

reverse transcription of qPCR (e.g. particulate organic matter, humic material in that size range and microbiota). qPCR fluorescence improved dramatically upon filtering the York River water. Fuhrman et al. (2005) reported a recovery efficiency ranging from 1-25% for poliovirus seeded into seawater; similar results were observed in this study when spiking norovirus in filtered estuarine water. Due to the sufficient recovery of the virus using this method, the lack of sample manipulation, and the fact that it was not time-consuming, this procedure was used for recovery of norovirus from the in situ and in vitro persistence experiments.

Phillipson et al. (1960) reported PEG precipitation methods to be rapid, inexpensive, and nondestructive of viruses. Lewis and Metcalf (1988) reported a 50% and 66% rate of recovery from estuarine water samples for hepatitis A and rotavirus, respectively. In evaluating PEG precipitation methods for sewage sample concentration and norovirus recovery, both low virus recovery (a 10-100 fold loss) and reduced signal fluorescence due to inhibitors carried through to qPCR were observed. A follow-up step of PCR purification improved fluorescence, but further diluted the sample. As virus recovery was already a limiting factor, this method was not pursued any further.

The Metcalf and Estes (1994) methodology yielded positive qPCR results only for norovirus-spiked sewage samples, not norovirus-spiked DI water samples. Without comparison to a 'clean' water sample, it was difficult to gauge the efficiency of the method for sewage effluent. Due to the time-consuming nature of this method, it was not selected for use with sewage effluent samples.

The ultracentrifugation method modified by Myrmel et al. (2006), which was originally described by Puig et al. (1994), was determined to be optimal in terms of virus recovery,

inhibitor removal and minimal manipulation for sewage effluent samples. Puig et al. (1994) found ultracentrifugation to be a fairly simple procedure suitable for samples containing high or low levels of fecal contamination and reported a 50-70% recovery efficiency of poliovirus from sewage samples. Additionally, the ultracentrifugation method does not add any known PCR-inhibitors (for elution or otherwise) and was also reported to remove or inactivate inhibitors of reverse transcription or PCR. When comparing norovirus recovery from spiked sewage samples to norovirus-positive stool extractions, crossing points for the concentrated spiked sewage samples were only slightly higher (1-2 crossing points) than those for norovirus-positive stool extractions, suggesting minimal virus loss using this concentration method. The similar crossing points of the stool extraction and spiked sewage samples suggested a virus recovery efficiency similar to or higher than that of Puig et al. (1994).

Extraction methods

In the evaluation of RNA extraction methods, the RNeasy Mini Kit was determined to be optimal for consistent RNA recovery from replicate samples. Additionally, it was not a time-consuming procedure and required limited use of hazardous components (i.e., no chloroform or phenol were necessary).

In assessing Trizol extractions, high concentrations of RNA were recovered from sewage effluent samples, but not necessarily the targeted norovirus RNA. The extraction of non-target RNA appeared to deleteriously affect the ability to subsequently detect norovirus by qPCR. When conducting a single Trizol extraction, qPCR showed evidence of inhibition, but when conducting multiple Trizol extractions to try to remove PCR inhibitors, there was substantial loss of RNA and norovirus was no longer detectable.

In evaluating the Boom et al. (1990) method for RNA extraction from norovirus-spiked sewage samples, results indicated that the detection limit for norovirus recovery was similar to that for the RNasy Mini Kit. However when comparing the Boom et al. (1990) method to the RNeasy Mini Kit for stool extractions, the kit had a lower detection limit (1 genome copy 10 μl^{-1} qPCR reaction vs. 100 genome copies 10 μl^{-1} qPCR reaction). Based on the detection limit and ease of kit use, RNA was extracted from the viral concentrates using the RNeasy Mini Kit.

Sewage sample processing

Eleven secondary sewage effluent samples were collected and eight were initially processed (February 2007 - February 2008); norovirus was not detected in the sewage samples not spiked with norovirus-positive stool samples. The three remaining samples (March - May 2008) were not processed by qPCR. When processing the spiked sewage samples, an average 1.0 x 10^5 genome copies 20 ml^{-1} sewage effluent was detected with a range from 10^3-10^6 genome copies 20 ml^{-1} sewage effluent. The detection limit of 1.0 x 10^3 -1.0 x 10^4 genome copies 20 ml^{-1} observed is unfortunately close to the norovirus concentrations generally reported in treated sewage effluent (2.0 x 10^3-2.0 x 10^4 copies 20 ml^{-1}). Based on this detection limit, it is difficult to conclude if norovirus was present in the sewage effluents analyzed for this study, as it may be present at concentrations lower than detectable by these methods. Other studies have reported lower detection limits (using 2-200 times the volume of this study): 100 genome copies l^{-1} from 40 milliliters sewage effluent (da Silva et al. 2007), single PCR detectable units l^{-1} from 200-400 liters sewage effluent (Lodder and de Roda Husman 2005), 0.047 PCR detectable units ml^{-1} from 1 liter sewage effluent (Katayama et al. 2008). These studies also include subsequent RNA purification procedures (CTAB extractions, ultrafiltration, gel chromatography). The lack of a

universal standard for quantification makes it difficult to clearly interpret these values, but it is notable that these studies consistently detect norovirus in sewage, often throughout the year. It is unclear whether differences in levels of sewage treatment or inhibitor presence could be responsible for the norovirus densities estimated in the other study countries (France, the Netherlands, Japan). Regardless, the high detection limit for this study is a definite concern for the ability to detect norovirus in sewage effluent in the Chesapeake Bay region.

When evaluating the selected methods for sample concentration and RNA extraction, a stool sample high in titer for norovirus was used to test virus recovery from sewage effluent samples. It was concluded that inhibition of either reverse transcription and/or qPCR was low when recovering high concentrations of norovirus, evidenced by only small differences in qPCR crossing points between norovirus-positive stool sample and that spiked into the sewage sample. However when the norovirus-positive stool sample was more dilute than a 1:100 dilution, the norovirus spike was not detected in sewage effluent samples.

Humic compounds are often cited as a major inhibitory factor in environmental samples (Wilson 1997). In this study, when norovirus was present in a high concentration (10^6 ml^{-1}) in the sewage effluent samples, and the ultracentrifugation method was used, humic substances might not have been as inhibitory as when norovirus was present in lower concentrations because of the overall proportion of humic substances to norovirus. To test if humic substances or similar environmental inhibitors are an inhibitory component, the viral concentrate or viral concentrate RNA could be serially diluted and processed with qPCR to determine if norovirus can be detected at a lower concentration.

A second possibility is inhibition due to nontarget DNA or RNA, especially as the target norovirus RNA becomes more dilute in the sample. Tebbe and Vahjen (1993) reported that high levels of nontarget DNA could inhibit PCR, specifically when working with soil samples. Significant inhibition was not observed when spiking higher titers of norovirus (10^6 ml^{-1}) into sewage effluent samples; however norovirus was not detected at lower concentrations, possibly because nontarget substances within the sewage effluent are inhibiting norovirus extraction, reverse transcription or amplification at lower concentrations. Experiments spiking both norovirus-positive stool sample and FRNA coliphage into reagent grade water and sewage effluent would clarify whether the presence of a second nontarget virus (FRNA coliphage) inhibits the quantification of norovirus in the water or sewage effluent samples.

In the studies cited above where lower detection limits were found, higher volumes of sewage effluent were concentrated. Concentrating higher volumes might aid in overcoming some of the limitations outlined in this work. Additionally, the previous studies concentrated and extracted RNA with additional purification procedures (including gel chromatography, ultrafiltration, and methods similar to those of Metcalf and Estes (1994) originally designed for shellfish samples) that could also prove useful in improving virus recovery at lower concentrations. These steps require additional time, and there is the concern that additional purification steps can result in virus loss. However, it is evident from this study that a more sensitive method is required to confirm the presence or absence of norovirus in sewage effluent samples of this region.

Persistence Experiments

In situ experiments

In situ experiments were conducted to examine the persistence of norovirus and FRNA coliphage in response to environmental factors during three seasons: spring 2007, summer 2007 and winter 2008. To the best of our knowledge, this is the first study in which norovirus suspensions were exposed to *in situ* physical conditions in an estuarine environment. Quantitative real-time PCR (qPCR) was used to quantify norovirus density over each 8-day experimental period. In parallel with norovirus, FRNA coliphage suspensions exposed to identical environmental conditions were evaluated for persistence over the 8-day period, using a plaque assay.

Virus inactivation rates for both norovirus and FRNA coliphage were calculated for the experimental conditions (light-exposed and dark treatments at the water surface and at 1 meter) for each seasonal experiment. Norovirus inactivation rates did not vary significantly as a function of season, light treatment or depth treatment with the exception of the spring surface light-exposed condition that was significantly higher than all other norovirus inactivation rates (Figure 11). The norovirus inactivation rate for the light-exposed surface condition was almost three times higher than the dark control at the surface. Additionally, a plot of dark vs. light-exposed inactivation rates (Figure 13) indicated a light effect on norovirus inactivation. All points plotting above the "reference line", specifically spring rates that deviated the most from the line, indicated the effect of light to increase norovirus inactivation rates. The *in situ* results show that sunlight exposure resulted in a faster inactivation rate for norovirus, similar to what was reported by Sinton et al. (1999) for FRNA coliphages and somatic coliphages. The

inactivation rates for light-exposed norovirus treatments were 1.6 – 2 times higher (though not significantly different) than the dark treatments in the summer experiment and the 1 meter light-exposed condition in the spring. Twenty-two year average insolation flux for the spring experimental month of May was higher (5.57 kWh m^{-2} d^{-1}) than that for the summer experimental month of August (5.08 kWh m^{-2} d^{-1}). Additionally, photosynthetically active radiation (PAR) data (NERRS CDMO 2008) indicated an average PAR for the spring experimental period of 55,517 mM m^{-2} versus 48,249 mM m^{-2} for the summer experiment period. Though PAR does not include the ultraviolet wavelengths known to be most damaging to viruses, PAR does correlate well to total insolation, and therefore can serve as a proxy for the potential for sun damage. The higher insolation and PAR values for the spring period might explain the significantly higher inactivation rate for light-exposed norovirus in the spring.

The 95% confidence interval analysis (Figure 11) indicated that light exposure had a significant effect on FRNA coliphage infectivity resulting in higher inactivation rates for both the surface and 1 meter treatments in the spring. The plot of dark vs. light-exposed inactivation rates (Figure 13) complements the 95% confidence interval analysis, with spring points deviating most from the reference line. The FRNA inactivation rate was higher for the light-exposed condition at 1 meter, but not at the surface in the summer. Phage inactivation in sunlight-exposed seawater occurs when solar radiation results in damage to the capsid and/or nucleic acid genome. Sinton et al. 1999 reported that FRNA coliphage were susceptible to all components of the solar spectrum below 556 nm, though not particularly susceptible to damage by UV-B wavelengths (responsible for direct photobiological damage). At wavelengths above 329 nm, viruses are more affected by photochemical mechanisms that act through photo-sensitizers to

damage virus capsids. In conducting optical filter experiments to examine what portion of the light spectrum was responsible for virus inactivation, Sinton et al. (1999) concluded that the majority of capsid damage was as a result of photochemical mechanisms consistent with longer wavelengths (329 nm-556 nm).

It is unusual to note that the dark surface summer treatment had a higher inactivation rate than the light-exposed surface summer treatment. There is the possibility that the bags were not constantly submerged due to very low tides, and uneven heating of the bags might have occurred if an arm of the float was out of the water for a period of time during daylight. Though there was a significant difference between the light-exposed and dark surface treatments, it was only by a factor of 1.05 that the dark surface inactivation rate exceeded the light-exposed inactivation rate.

A depth effect was observed in the spring and summer for FRNA coliphage inactivation rates (Figure 11). In both seasons, the inactivation rate of the light-exposed FRNA coliphage at the 1 meter depth was lower than that at the surface. This was anticipated as sunlight is attenuated by the water and particulates within the water column. However, the first wavelengths to be attenuated are the shorter wavelengths of the solar spectrum, allowing the longer wavelengths to penetrate deeper. FRNA coliphages are also susceptible to longer wavelengths, possibly explaining why there is only a factor of 1.2 and 1.1 between the surface and 1 meter inactivation rates for the spring and summer, respectively.

Sunlight exposure did not have a significant effect on FRNA coliphage inactivation rates in the winter (Figure 11). Insolation and PAR values for the winter experimental period were 2.97 kWh m^{-2} d^{-1} and 21,456 mM m^{-2}, approximately half that of the insolation and PAR values seen in spring and summer. The light-exposed winter treatments both had higher inactivation

rates than the dark controls as evidenced by Figure 13; however none of these treatments showed a significant difference from one another.

Significant differences seen among FRNA coliphage inactivation rates were related to season (Figure 11). Figure 12 indicates that water temperature was predictive of FRNA coliphage inactivation rates for all treatments. All spring inactivation rates were higher than those for winter (by a factor of 2.3-3.0), and all summer inactivation rates were higher than those for winter and spring (by a factor of 3.5-4.8 and 1.3-2.0, respectively). Exposure of enteric viruses to temperatures ranging from 22-35°C can damage nucleic acids and protein capsids of viruses, negatively affecting virus adsorption to the host cell and preventing replication due to enzyme inactivation (reviewed in Bitton, 1980). Lo et al. (1976) consistently found when studying enteroviruses in seawater that there was a direct relationship between virus stability and water temperature: the higher the water temperature, the greater rate of virus inactivation. Allwood et al. (2005) reported increased inactivation of viruses (FRNA coliphage and feline calicivirus) in chlorinated water as a function of water temperature; and Ngazoa et al. (2008) found significantly higher reduction of norovirus genomic material in river water at 25°C compared to 4°C.

Considering norovirus inactivation rates and persistence did not parallel that of FRNA coliphage, the effect of environmental temperature on these viruses could reflect differences in mechanisms of inactivation and structural stability. FRNA coliphage was assayed by an infectivity assay whereas norovirus was assayed by RNA (genome copy) detection, raising the question whether the norovirus RNA detected is representative of infectious norovirus. An assay of infectivity can assist in the determination of the health risk caused by infectious virions;

however a nucleic acid assay is beneficial in that it can detect viruses recalcitrant to replication in cell culture (Gassilloud et al. 2003, Sobsey et al. 1998, Rose et al. 1997). In comparing infectivity assays to RNA detection of feline calicivirus, poliovirus and FRNA coliphage (MS2), Bae and Schwab (2007) reported that in laboratory positive control waters, minimal viral RNA losses were observed over the 3-5 week sampling time for all viruses tested while infectivity was significantly reduced over time for all tested viruses. Despite their limitations, nucleic acid assays are still useful in that they directly assay norovirus, the known human pathogen, for which a routine infectivity assay is still lacking. Additionally, Duizer et al. (2004) concluded in their study comparing calicivirus infectivity to nucleic acid detection using known means of inactivation (thermal, UV irradiation, ethanol, chlorine, pH) that quantitative real-time assays were a valuable tool in detecting reduction in infectivity, because a higher crossing point was correlated to some degree of viral inactivation.

Duizer et al. (2004) also specifically noted the utility of conducting quantitative qPCR following an enzymatic treatment of PK and RNase (Nuanualsuwan and Cliver 2002), used in the *in situ* experiments in this project. Significant differences were not observed between inactivation rates for the untreated and treated norovirus samples (Figure 10); therefore it was concluded that only intact RNA encapsulated within the norovirus protein capsid was detected, and that any RNA released as a result of capsid damage was destroyed by environmental factors of sunlight or water temperature. Using this method prior to infectivity and nucleic acid assays for feline calicivirus, Nuanualsuwan and Cliver (2002) reported that the enzymatic digestion prevented an RT-PCR positive result on viruses that were no longer infective based on an infectivity assay. Based on the aforementioned results, the results of this thesis suggest that the

norovirus RNA detected by qPCR in the *in situ* and *in vitro* experiments was from intact, and therefore potentially infectious norovirus.

There are a few concerns with concluding that all detected RNA was representative of infectious virus. The enzymatic digestion method was initially used on suspensions with 10^3 PFU ml^{-1}. Experiments were begun with uncertainty regarding virus density because the plasmid standard was not generated. The method was used as published, but on norovirus concentrations of approximately 10^6-10^7 genome copies ml^{-1}. The higher virus density could be a possible reason for why a difference was not seen between treated and untreated norovirus suspensions. However as virus density decreased over the experimental period (in the summer light-exposed surface condition as low as 10^3 genome copies ml^{-1}), an increasing difference between the untreated and treated norovirus samples was not observed. Without a parallel infectivity assay, it can only be concluded that the RNA detected was from intact norovirus.

This thesis study provides new information with respect to norovirus nucleic acid persistence in response to environmental factors and is unique to previous studies of norovirus persistence because norovirus is exposed to *in situ* environmental conditions. Even in the presence of spring- and summer-time insolation and water temperature, norovirus RNA was observed to persist for at least 8 days, underscoring the robustness of norovirus and potential for waterborne spread of gastroenteritis. Additionally, T_{90} values (Table 3) indicate the potential for norovirus RNA to persist in the environment up to 46 days, while 90% of FRNA coliphage is inactivated after no more than 3.5 days. It is important to note that the rates calculated in this study are conservative: aged filtered (0.22 μm) estuarine water was used, reducing its virucidal properties. Regardless, norovirus RNA has the potential to persist for long periods of time.

In vitro experiments

In vitro experiments were conducted to complement the *in situ* experiments, and to determine the effect of temperature and salinity on virus inactivation in a dark temperature-controlled setting.

A distinct temperature effect was observed in the inactivation rates of FRNA coliphage with increased temperatures resulting in higher inactivation rates (Figure 14). Figure 15 corroborates that water temperature is consistently predictive of FRNA coliphage inactivation rates. Additionally, a salinity effect was observed (Figures 14 and 16), most notably in the 10°C and 30°C treatments, with higher inactivation rates accompanying higher salinity. Gerba and Scaiberger (1975) reported that salinity could increase viral aggregation, resulting in a single PFU for an aggregate. In this instance, the salinity effect is likely more an artifact associated with quantifying the virus than actual damage to the infectivity of the virus.

Based on a comparison of inactivation rates, the effect of salinity on FRNA coliphage inactivation appears to have a diminishing effect with increasing temperature, underscoring the importance of temperature on virus infectivity. In studying the stability of human enteroviruses in estuarine and marine water, Lo et al. (1976) reported that the salinity of water had only minor effects on virus survival, and that water temperature was the critical factor in affecting virus survival.

Similar to the *in situ* experimental results, norovirus inactivation rates did not exhibit as broad a range as FRNA coliphage inactivation rates in response to temperature or salinity. Few significant differences were observed between the norovirus inactivation rates based on temperature (Figure 14); however Figure 15 does show that temperature was predictive of

norovirus inactivation rates for the 10°C and 30°C treatments. The 20°C treatment does not follow a similar trend however and some of the inactivation rates calculated for the 20°C samples were among the lowest inactivation rates for all *in vitro* samples.

In both the 10° and 30°C experiments, a significant difference was observed between the inactivation rates of the 0 psu and the 20 and 30 psu treatments (Figure 14). The more saline treatments at 10° and 30°C had higher inactivation rates (by a factor of 5.4 to 7.0 and 1.8-3.0, respectively). However Figure 16 suggests that salinity is only predictive of norovirus inactivation rates at 30°C. It is difficult to conclude from these results whether salinity would play a significant role in norovirus inactivation, and it is unclear by what mechanism increased salinity would affect norovirus inactivation.

Based on the variability of norovirus inactivation rates in the *in vitro* experiments, it is difficult to clearly define a significant effect of temperature or salinity. Though some trends are observed, significant differences in inactivation rates were not observed to state an effect. Additionally, inactivation rates associated with the 20°C experiment do not follow the trends observed in the 10° and 30°C experiments, further confounding a generalization of a temperature or salinity effect.

Examined together, the *in vitro* results complemented the *in situ* results for both viruses. For FRNA coliphage, the *in vitro* results substantiated that water temperature is an important factor in the decrease of virus infectivity. In terms of norovirus persistence, the norovirus inactivation rate for the light-exposed spring condition was significantly higher than all *in situ* rates and all *in vitro* rates except the 30°C, 30 psu rate. This would suggest that conditions of high insolation and high salinity are most detrimental to norovirus persistence. FRNA coliphage

inactivation rates failed to parallel those of norovirus in both sets of experiments. While the inactivation rates are conservative, the differences in T_{90} values (Table 5) are notable: norovirus RNA has the potential to persist over 100 days in dark conditions, while 90% of FRNA coliphage was inactivated after no more than 16 days. The difference in assay endpoint is most likely a contributing factor to the significant differences seen in these studies.

Based on the findings of this study, FRNA coliphage persistence did not parallel norovirus persistence in estuarine water samples, implying that FRNA coliphage is not an acceptable indicator predictive for norovirus in the marine environment. FRNA coliphage inactivation rates were higher and more variable than norovirus inactivation rates in both *in situ* and *in vitro* experiments, with norovirus far outlasting FRNA coliphage. Though qPCR has its limitations, at present it is the most efficient method in use for the detection of norovirus in environmental samples. Considering the low infective dose of norovirus, an overly-conservative detection method like qPCR, especially coupled with an RNase and proteinase K treatment like the one used in this study, might be a better means of screening water samples than monitoring for a potential indicator like FRNA coliphage.

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VITA

Margaret H. Fagan

Born in Roslyn, New York, 8 July 1980. Graduated from Sacred Heart Academy, Hempstead, NY in 1998. Earned a B.S. in Biology from Washington and Lee University, Lexington, VA in 2002. Entered the Master's program at the Virginia Institute of Marine Science, College of William and Mary, Gloucester Point, VA, in 2005.