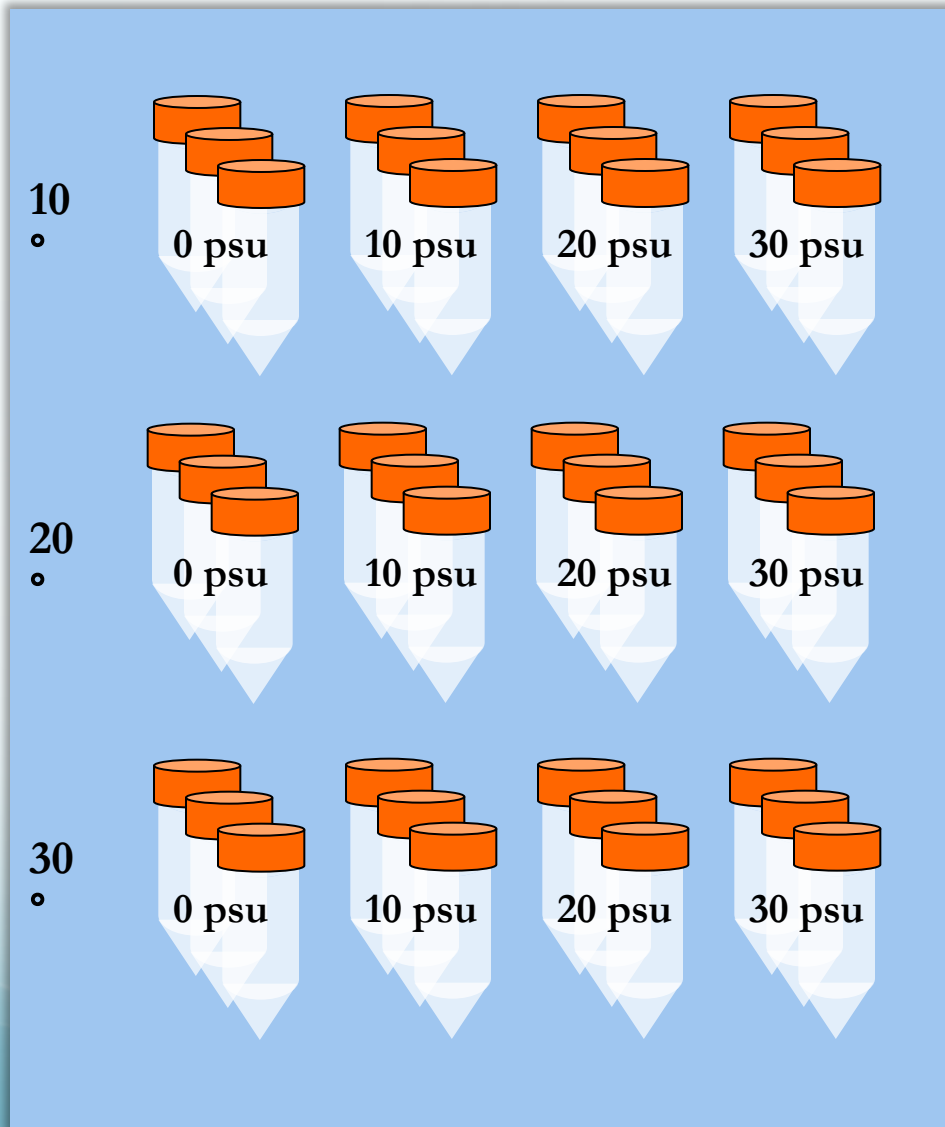


- Are MSC's suitable to assess stability (temperature/sunlight) of enteric viruses in the marine environment?

# *In vitro* setup and sample processing



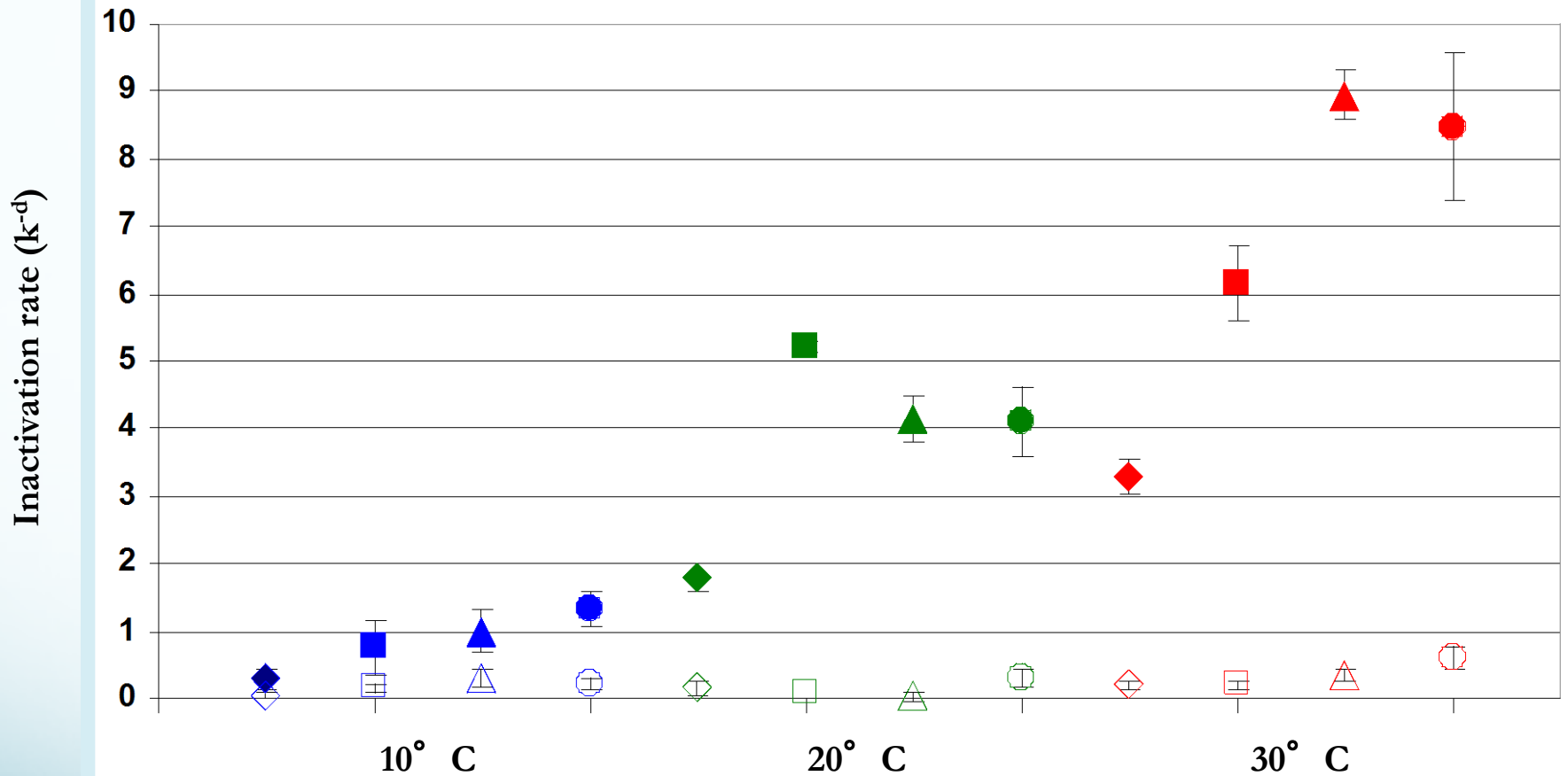
Filtered water was spiked with norovirus and FRNA coliphage

Suspensions incubated at specified temperatures

Water sampled at 0, 1, 2, 4 and 8 days

Samples processed for norovirus and FRNA coliphage

# Inactivation rates— *In vitro* experiments



Closed shapes = FRNA coliphage; open shapes = norovirus

Bars indicate 95% confidence intervals

◆ 0 psu

■ 10 psu

▲ 20 psu

● 30 psu

Note: norovirus enzymatic pre-treatment (Nuanualsuwan and Cliver, 2002, 2003) was done to decrease chances of detecting non-infective particles

# In situ setup and norovirus sample processing

- F** FRNA coliphage suspension dark control
- F** FRNA coliphage suspension light exposed
- N** Norovirus suspension dark control
- N** Norovirus suspension light exposed

FRNA coliphage sample processing at each time point

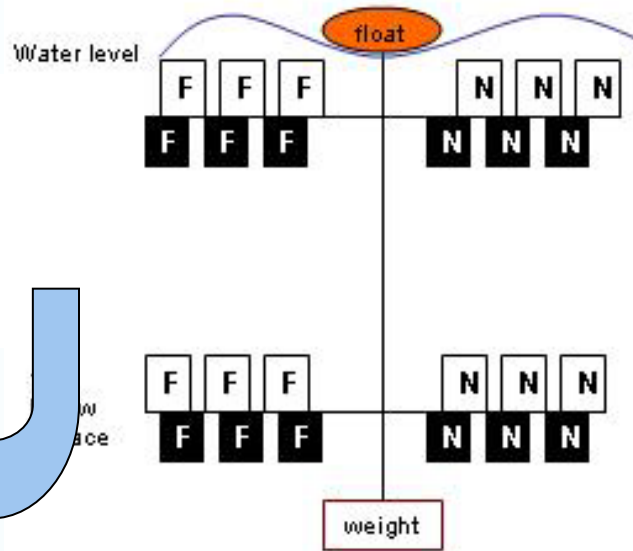
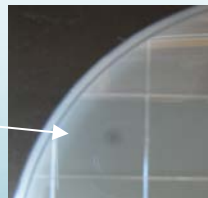
2 x 1 ml replicate samples



+

*S. typhimurium* WG49 host and overlay agar

Count plaque forming units



Norovirus sample processing at each time point

2 x 1.5 ml replicate samples



No treatment

2 x 1.5 ml replicate samples



RNase and proteinase K treatment

Concentrate on 0.45 μm nitrocellulose filter

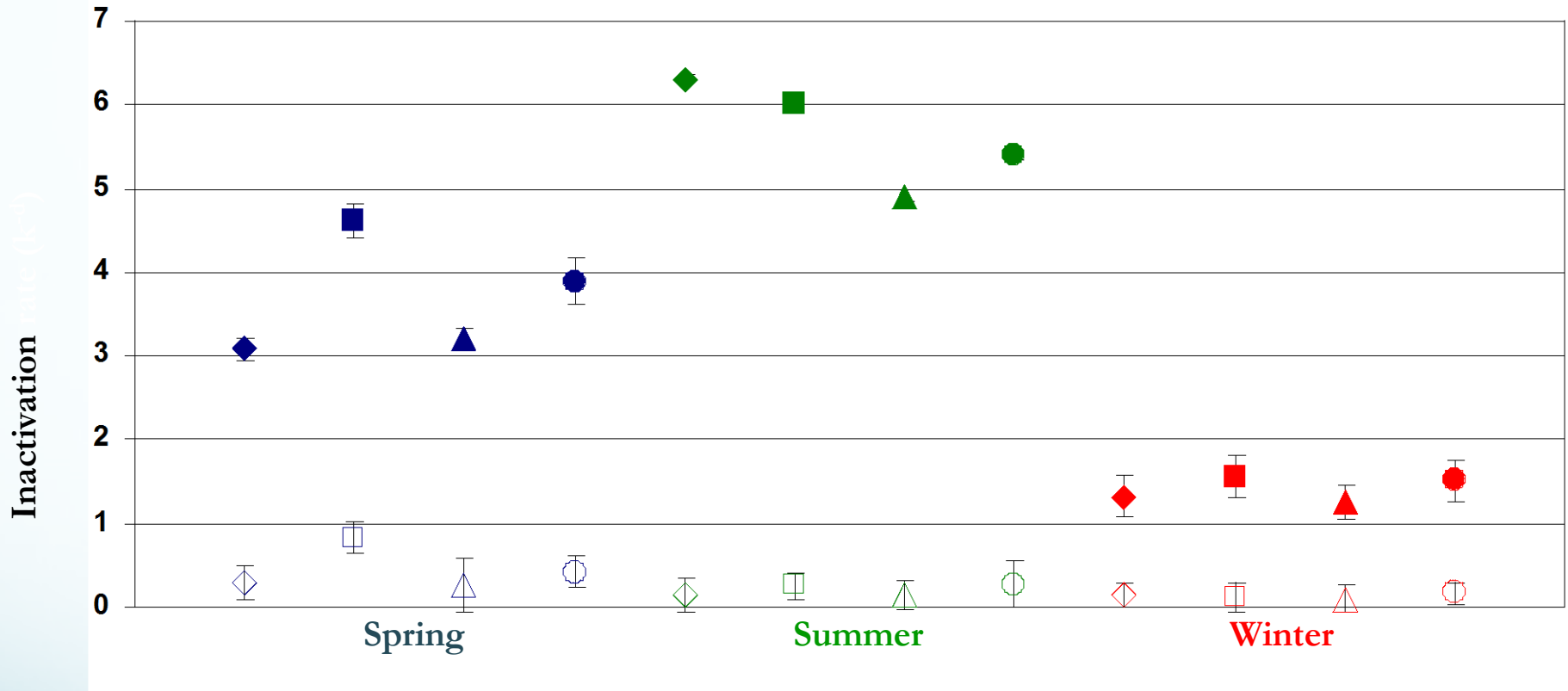


Extract and reverse transcribe RNA



Determine virus copy number using qPCR

# Inactivation rates— *In situ* experiments



Closed shapes = FRNA coliphage; open shapes = untreated norovirus

Bars indicate 95% confidence intervals

◆ Surface dark condition

■ Surface light condition

▲ 1 meter depth dark condition

● 1 meter depth light condition

MSC testing detects infectious agents while current RT-qPCR assays likely detect infectious and non-infectious NoV. Does this level of potential overestimation by RTqPCR err on the side of public health safety; is this overestimation acceptable? If not, why?

The following protocol is most efficient in our hands:

- Release norovirus from shellfish tissues using proteinase K as described by Jothikumar et al. 2005.
- Supernatant containing released virus is collected for RNA extraction.
- The MagMAX AI/ND RNA isolation kit from Ambion (MagMAX) is the most effective.
- Viruses belonging to GI and GII are detected using two separate qPCR assays targeting each genogroup (Loisy et al. 2005).
- Subset of positive samples are confirmed by DNA sequencing

# MSC and norovirus in two oyster species

Table 5. Relationship between initial mean FRNA coliphage densities and norovirus occurrence.

Survey Date	Initial mean FRNA concentration <i>C. ariakensis</i>	Norovirus	Initial mean FRNA concentration <i>C. virginica</i>	Norovirus
11/13/06	25475	2/4	11051	2/4
11/29/06	28900	0/4	9176	0/4
4/7/2007	776	4/4	114	4/4
4/24/07	2020	0/4	80	1/4
10/29/07	230	4/4	210	2/4
4/30/08	22063	2/4	14988	2/4
5/27/08	7061	0/0	5970	1/4

- Is there a general association between MSC and NoV levels in naturally occurring shellfish? Is there an association between these levels and rates of illness? Is this association related to season/temperature?
- Several studies have shown that instead of being passively bioaccumulated in shellfish, noroviruses appear to bind specifically to antigenic structures expressed by bivalve gastrointestinal epithelial cells (e.g. Le Guyader et al. 2006, Tian et al. 2006, 2007), which are similar to human gut histo-blood group antigens.



# MSC and norovirus in two oyster species (natural contamination at a WWTP outfall)

Table 5. Relationship between initial mean FRNA coliphage densities and norovirus occurrence.

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4/30/08	22063	2/4	14988	2/4
5/27/08	7061	0/0	5970	1/4

Norovirus detected using a nested qPCR assay (Myrmel et al. 2004)

# MSC and norovirus in clams, *M. mercenaria*

- Clams collected from one aquaculture site were positive for FRNA coliphage with mean levels of 65 pfu/100 g meat suggesting non-point/animal sources. No norovirus detected.

Table 1. VIMS microbiological results for clams exposed for ca. 2 weeks at the WWTP outfall and Condemnation Line 1 ("Cond 1") in Hampton Roads. Samples retrieved and analyzed Nov. 28, 2007.\* Clams were sourced from a commercial dealer. For some samples that were positive for NoV the PCR amplification products were sequenced to determine whether genogroup I, II or both were detected.

Site	Replicate	Density 100 g <sup>-1</sup> shellfish			NoV	Genogroup
		Fecal coliforms	<i>E. coli</i>	FRNA phage		
"Cond 1"	A	20	20	17	0/2	
	B			90	0/2	
	A	20	20	330	0/2	
	B			226	0/2	
	A	18	18	106	0/2	
	B			92	1/2	GI
	A	<18	20	18	2/2	GI and GII
	B			<18	2/2	GI
	<b>Means</b>		<b>&lt;19</b>	<b>20</b>	<b>&lt;159</b>	
	WWTP	A	20	<18	140	2/2
B				198	0/2	
A		45	45	334	0/2	
B				398	1/2	
A		100	18	488	1/2	
B				388	0/2	
A		<18	<18	570	0/2	
B				424	0/2	
<b>Means</b>			<b>&lt;46</b>	<b>&lt;25</b>	<b>368</b>	

\*Fecal coliforms and *E. coli* densities determined using the APHA 5-tube MPN with EC-MUG as the medium. FRNA coliphage measured following a proposed FDA method but using *Salmonella typhimurium* WG49 as the assay host. NoV occurrence indicated as ratio of analytical replicates that were positive by real-time PCR. Water samples collected at the time of shellfish retrieval:

"Cond 1" - <1.8 fecal coliforms and *E. coli* per 100 ml; <1 FRNA phage per 100 ml; 22.8 psu, 12.1°C

WWTP outfall - 2.0 fecal coliforms and *E. coli* per 100 ml; <1 FRNA phage per 100 ml; 22.6 psu, 11.8°C.

Table 2. VIMS microbiological results for clams exposed for ca. 3 weeks at the WWTP outfall and Condemnation Line 1 (Cond 1) in Hampton Roads. Samples retrieved and analyzed Dec. 4, 2007.\* Clams were sourced from a commercial dealer.

Site	Replicate	Density 100g <sup>-1</sup> shellfish			NoV	Genogroup	
		Fecal coliforms	<i>E. coli</i>	FRNA phage			
“Cond 1”	A	<18	<18	<18	2/2		
	B			18	2/2		
	A	20	20	36	2/2		
	B			54	2/2		
	A	<18	<18	36	1/2		
	B			54	2/2		
	A	20	20	268	2/2		
	B			214	2/2		
	<b>Means</b>		<b>&lt;19</b>	<b>&lt;19</b>	<b>&lt;87</b>		
			7				
	WWTP	A	8	78	54	2/2	GI
		B				2/2	GII
A		45	45	72	2/2		
B				72	2/2		
A		78	78	320	2/2	GI and GII	
B				410	2/2		
A		230	230	178	2/2		
B				90	2/2		
<b>Mean</b>			<b>108</b>	<b>108</b>	<b>159</b>		

\*Fecal coliforms and *E. coli* densities determined using the APHA 5-tube MPN with EC-MUG as the medium. FRNA coliphage measured following a proposed FDA method but using *Salmonella typhimurium* WG49 as the assay host. NoV occurrence indicated as ratio of analytical replicates that were positive by nested PCR. Water samples collected at the time of shellfish retrieval:

Cond 1 – 2.0 fecal coliforms and *E. coli* per 100 ml; <1 FRNA phage per 100 ml; 23.1 psu, 10.1° C

WWTP outfall - <1.8 fecal coliforms and *E. coli* per 100 ml; <1 FRNA phage per 100 ml; 22.5 psu, 9.9° C

Table 3. VIMS microbiological results<sup>a</sup> for clams exposed for periods indicated at various locations in Hampton Roads during the spring of 2008. Values are means of four replicate clam homogenates.

Date	Sample	Means of replicates			NoV	
		FC 100ml <sup>-1</sup> or g <sup>-1</sup>	EC 100 ml <sup>-1</sup> or g <sup>-1</sup>	FRNA 100 ml <sup>-1</sup> or g <sup>-1</sup>	GI	GII
3/27/08	0 day clams	<1.8E+01	<1.8E+01	65	0/4	0/4
4/14/08	Craney Island, 19 day clams	<1.9E+01	<1.9E+01	9	1/4	2/4
	Nansemond River, 19 day clams	<2.5E+01	<1.9E+01	78	0/4	0/4
	Ragged Island, 19 day clams	<1.8E+01	<1.8E+01	47	4/4	0/4
4/21/08	Hampton Creek, 26 day clams	2.1E+02	1.7E+02	8	3/4	0/4
	Broad Bay, 26 day clams	<1.9E+01	<1.9E+01	6	4/4	0/4
5/13/08	Broad Bay, 48 day clams	1.3E+03	1.3E+03	60	4/4	0/4
	Hampton Creek, 48 day clams	4.1E+02	3.1E+02	95	4/4	0/4

Date	Sample	Means of replicates			NoV	
		FC 100ml <sup>-1</sup> or g <sup>-1</sup>	EC 100 ml <sup>-1</sup> or g <sup>-1</sup>	FRNA 100 ml <sup>-1</sup> or g <sup>-1</sup>	GI	GII
5/19/08	Craney Island, 54 day clams	<1.8E+01	<1.8E+01	7	4/4	0/4
	Ragged Island, 54 day clams	2.0E+03	1.2E+03	8	4/4	0/4
	Nansemond River, 54 day clams	<1.9E+01	<1.8E+01	16	3/4	0/4