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ORIGINAL PAPER

Evaluation of a Male-Specific DNA Coliphage Persistence Within Eastern Oysters (*Crassostrea virginica*)

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Abstract

Male-specific coliphages (MSCs) are currently used to assess the virologic quality of shellfish-growing waters and to assess the impact of sewage release or adverse weather events on bivalve shellfish. Since MSC can have either DNA or RNA genomes, and most research has been performed exclusively on RNA MSCs, persistence of M13, a DNA MSC, was evaluated for its persistence as a function of time and temperature within Eastern oysters (*Crassostrea virginica*). Oysters were individually exposed to seawater containing a total of 10^{10} to 10^{12} pfu of M13 for 24 h at 15 °C followed by maintenance in tanks with as many as 21 oysters in continuously UV-sterilized water for up to 6 weeks at either 7, 15, or 22 °C. Two trials for each temperature were performed combining three shucked oysters per time point which were assayed by tenfold serial dilution in triplicate. Initial contamination levels averaged $10^{6.9}$ and ranged from $10^{6.0}$ to $10^{7.0}$ of M13. For oysters held for 3 weeks, $\log_{10}$ reductions were 1.7, 3.8, and 4.2 $\log_{10}$ at 7, 15, and 22 °C, respectively. Oysters held at 7 and 15 °C for 6 weeks showed average reductions of 3.6 and 5.1 $\log_{10}$, respectively, but still retained infectious M13. In total, this work shows that DNA MSC may decline within shellfish in a manner analogous to RNA MSCs.

Keywords Bacteriophage · Shellfish · Virus · Oysters

Bivalve shellfish, being filter feeders, can efficiently bioconcentrate pathogens within their digestive tracts and tissues (Schwab et al. 1998; Wang et al. 2008). Worldwide, regulatory standards for shellfish are principally based on levels of fecal bacteria observed in harvest waters and/or shellfish meats (Richards 1985). While *Escherichia coli* and fecal coliform standards can provide key information about the potential hygienic quality of shellfish, and have been effective at reducing enteric bacterial illness, such as typhoid fever (Rippey 1994), it is well understood that bacterial standards have shortcomings when it comes to preventing viral illnesses associated with shellfish, such as human norovirus (HuNoV) and hepatitis A virus ([HAV]; Doré and Lees 1995; Richards 1988). The main challenge is that fecal bacteria do not persist as efficiently as enteric viruses within the environment, and specifically within oyster tissues (Richards et al. 1988). Therefore, while low bacteria counts may suggest limited recent fecal impact, viruses, which are hard to detect and infectious at low doses, may be present (Allwood et al. 2003; Kingsley and Richards 2003; Love et al. 2010; Provost et al. 2011).

As a result, male-specific coliphages (MSCs) have gained attention as a presumptive norovirus and fecal virus surrogate, as well as a general indicator of sewage-contamination for shellfish (Hartard et al. 2017). Currently, MSCs are commonly utilized for sanitary surveys when assessing the impact of sewage treatment plants (Hartard et al. 2016). Both MSCs and fecal viruses are not substantially inactivated by traditional chlorine-based sewage treatment (Luther and Fujioka 2004; Kingsley et al. 2017; Purnell et al. 2016). While bacteriophage and fecal viruses numbers do decline

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somewhat due to sludge settlement, substantial amounts of viable MSCs and fecal viruses are released in treated sewage effluent (Kitajma et al. 2014; Simmons and Xagoraraki 2011; Pouillot et al. 2015). For the US National Shellfish Sanitation Program (NSSP), MSCs are currently used as an impact indicator after floods and sewage releases which trigger automatic 3-week harvest closures (NSSP 2017). Under these regulations, if the male-specific coliphage levels within shellfish tissues are low enough, shellfish beds can be re-opened, and the mandatory closure can be lifted prior to the end of the prescribed 3-week closure.

Male-specific coliphages have a number of appealing characteristics but as with any indicator, also come with some caveats. The appeal of MSCs is that they may persist in the environment and within shellfish meats in a manner analogous to human enteric viruses, or at least be more persistent than fecal bacteria (Burkhardt et al. 1992a; Dore et al. 2000; Flannery et al. 2009; Kingsley et al. 2018). Furthermore, host bacteria for MSCs require temperatures above 30 °C to make the male pili which are the attachment targets for MSCs (Burkhardt et al. 1992a; Primrose et al. 1982) and *E. coli* strains that are genetically able to express F pili are not prevalent in the environment (Shaw and Cabelli 1981). Thus, when MSCs are found in the environment, their original source was probably from the infection of bacteria derived from the gut of warm-blooded animals (Hata et al. 2016; Hartand et al. 2015, 2017; Primrose et al. 1982). Generally speaking, their presence is not considered to be as a result of replication in environmental or aquatic settings, although exogenous phage amplification probably does occur as nascently shed fecal bacteria that express pili encounter MSCs within raw sewage (Havelaar and Pot-Hogeboom 1988). One drawback for MSCs is that < 5% of humans are actively shedding MSCs at any given time (Havelaar et al. 1990; LeClerc et al. 2000). For this reason, they are not considered good indicators of fecal contamination derived from a single individual or a few persons (Havelaar et al. 1990; LeClerc et al. 2000; Cornax et al. 1994). Therefore, overboard waste discharge or perhaps malfunctioning septic systems, which could potentially contain pathogenic enteric viruses, may not contain MSCs. It is noteworthy that while the presence of elevated MSCs certainly suggests the potential presence of fecal viruses, elevated MSCs are not always found within shellfish implicated in outbreaks (Croci et al. 2000; Flannery et al. 2009).

Academic and regulatory interest in male-specific coliphage is principally because male-specific coliphage strains are nonenveloped, and many MSCs contain single-stranded RNA genomes of the same approximate size as fecal RNA viruses such as HAV and norovirus (Burkhardt et al. 1992a). What is less widely recognized is the fact that not all male-specific coliphages have RNA genomes. Indeed, filamentous DNA male-specific coliphages are also prevalent in raw and treated sewage (Cole et al. 2003; Long et al. 2005), especially in surface waters during warmer months (Cole et al. 2003) suggesting that they are environmentally stable. Therefore, it is conceivable that regulatory assumptions and decisions that may be in part based on research performed with RNA male-specific phage, may not be entirely valid, since DNA male-specific coliphage may not persist within shellfish in a manner analogous to RNA male-specific bacteriophage. Previously, the effect of water temperature was evaluated on the persistence of MS-2, a common male-specific RNA phage (Kingsley et al. 2018) within Eastern oysters. Since a sewage release also will likely contain DNA male-specific coliphage as well as RNA male-specific coliphage, we have characterized the persistence of a representative DNA male-specific coliphage, M13, in oysters (*Crassostrea virginica*) as a function of time and water temperature.

Materials and Methods

Bacteriophages and Oysters

The M13 bacteriophage strain was obtained from the American Type Culture Collection (15669-B1 ATCC, Manasas VA, USA) and propagated by inoculation of *E. coli* strain Hfr D (ATCC 15669) in growth media (10 g tryptone, 1.0 g dextrose, 5.0 g NaCl in 1 L deionized water) at 37 °C for 24 h. Lysed bacterial cells were pelleted at 3000 × g , and the supernatant was filtered with a 0.2-µm filter (ThermoFisher, Fair Lawn NJ) and stored at 4 °C prior to use. Medium-to-large Chesapeake Bay oysters (*C. virginica*) were obtained from a local dealer in Grasonville, MD, USA who harvested from approved areas. Shucked weights were approximately 8–15 g each. Oysters were placed in laboratory aquaria within 24 h, and acclimated to 28–30 ppt natural seawater for 1 week at 15 °C with daily seawater changes. Seawater was obtained from the University of Delaware Marine Laboratory, Lewes, DE.

For each trial, 21 acclimated oysters were individually placed in 21 1-L plastic beakers containing 500 mL of 28–30 ppt seawater and 10 mL of M13 phage (4×10^9 to 2×10^{11} pfu/mL) at 15 °C overnight (approximately 16–20 h). Oysters were then subsequently rinsed with fresh seawater and placed collectively within 40 L tanks containing approximately 30 liters of seawater with air stone aeration and a recirculating UV sterilizing unit as previously described by Kingsley and Richards (2003). Seawater was changed daily. Three oysters were removed, and M13 levels were evaluated after 0, 1, 2, 3, 4, 5, and 6 weeks after contamination. Each experiment was independently performed in duplicate with seawater held at 7, 15 or 22 °C. Oysters were not fed although some phyto- and zooplankon was present within the natural seawater. Testing

of experimental oysters did not reveal any evidence of prior bacteriophage contamination.

M13 Bacteriophage Assay

Extraction and testing of bacteriophage was as previously described (Kingsley et al. 2018) and was adapted from Debartolomeis and Cabelli (1991) and the US NSSP (Anon 2009). In brief, three oysters were shucked, pooled, and weighed. BD Bacto-tryptone broth (Becton Dickson Co., Sparks, MD) equal to twice the oyster volume was added. The oysters were homogenized in a waring blender and clarified by centrifugation. Serial dilutions of extracted bacteriophages were added to log phase *E. coli* (HfrD strain) followed by addition to soft 52 °C top agar and plating on agar plates containing streptomycin and ampicillin (50 g/mL) followed by incubation overnight at 37 °C and enumeration of observed plaques. The limit of detection was < 20 PFU for each group of three oysters tested. Log reduction calculations were based on bacteriophages observed immediately after contamination in comparison with oysters up to 6 weeks post contamination.

Modeling of Survival Curves

The linear model assumes that all the bacteriophages in a population have identical resistance to lethal treatments and their inactivation is governed by first-order kinetics (Schaffner and Labuza 1997).

$$\log \frac{N}{N_0} = -\frac{t}{D}, \tag{1}$$

where N_0 is the initial number of bacteriophage (PFU/g); N is the number of survivors after an exposure time t (PFU/g); D (decimal reduction time) is the time (weeks) required to destroy 90% of the bacteriophages and is a measure of the resistance of a bacteriophage to lethal treatments; and t is the treatment time (weeks).

The Weibull model assumes that bacteriophages in a population have different resistances, and a survival curve is just the cumulative form of a distribution of lethal agents.

$$\log \frac{N}{N_0} = -bt^n, \tag{2}$$

where b and n are the scale and shape factors, respectively (Peleg and Cole 1998). The Weibull distribution corresponds to a concave upward survival curve if $n < 1$, concave downward if $n > 1$, and linear if $n = 1$.

The mean squared error (*MSE*) values were used to compare the two models. The smaller the *MSE* values, the better the model to fit the data (Neter et al. 1996).

$$MSE = \frac{\sum (predicted - observed)^2}{n - p}, \tag{3}$$

where n is the number of observations and p is the number of parameters to be estimated.

Survival curves were fitted using the PROC REG procedure of SAS (Release 9.4, SAS Institute Inc., Cary, NC) for the linear models and PROG NLIN procedure of SAS for the nonlinear models.

Results

Individual oysters were contaminated with M13 bacteriophage upon exposure to 10^{10}–10^{12} pfu in seawater for 24 h, followed by holding within aquaria for up to 6 weeks. Initial testing after contamination indicated that all oysters were contaminated with $> 10^6$ pfu of M13. Overall, the rate of M13 reduction within shellfish increased as water temperatures increased and with longer holding times. Oysters held at 7 and 15 °C still retained M13 at six weeks after exposure (Table 1) with average reductions of 3.6 and 5.1 $\log_{10}$, respectively. For one trial at 22 °C, unexpected shellfish mortality resulted in termination of one trial study at 5 weeks, but after 5 weeks at 22 °C, M13 PFU reduction averaged 5.06 $\log_{10}$ for both trials (Table 1). For the US NSSP-prescribed shellfish closure period of 3 weeks, the declines in mean M13 PFU for 7, 15, and 22 °C were 1.7, 3.8, and 4.2 $\log_{10}$, respectively.

The survival of bacteriophage in oysters as a function of time and seawater temperature is shown graphically in Fig. 1. For seawater temperature of 7 °C, visual inspection of the data indicated that the linear model fits the data almost as well as the nonlinear Weibull model. This observation was confirmed by examining the *MSE* values for the linear and Weibull models, which were quite similar (Table 2). However, the shapes of both 15 °C and 22 °C survival curves were characterized by a rapid initial drop in bacteriophage counts followed by tailing caused by a diminishing inactivation rate. Visual inspection of these survival curves indicated

Table 1 Reductions of M13 bacteriophages within live oysters held for 6 weeks

Weeks post M13 contamination	$\log_{10}$ PFU reduction observed		
	7 °C	15 °C	22 °C
1	0.79 ± 0.55	1.72 ± 0.37	2.49 ± 0.12
2	1.59 ± 0.04	2.51 ± 0.12	2.93 ± 0.77
3	1.70 ± 0.64	3.77 ± 0.18	4.21 ± 0.98
4	2.64 ± 0.64	3.02 ± 0.49	5.09 ± 0.06
5	2.62 ± 0.55	4.47 ± 1.16	5.06 ± 0.48
6	3.58 ± 0.96	5.13 ± 0.57	4.97[a]

Data represent mean of two replicates ± standard error. Three oysters per time point were combined and assayed in triplicate

[a]Indicates one trial only

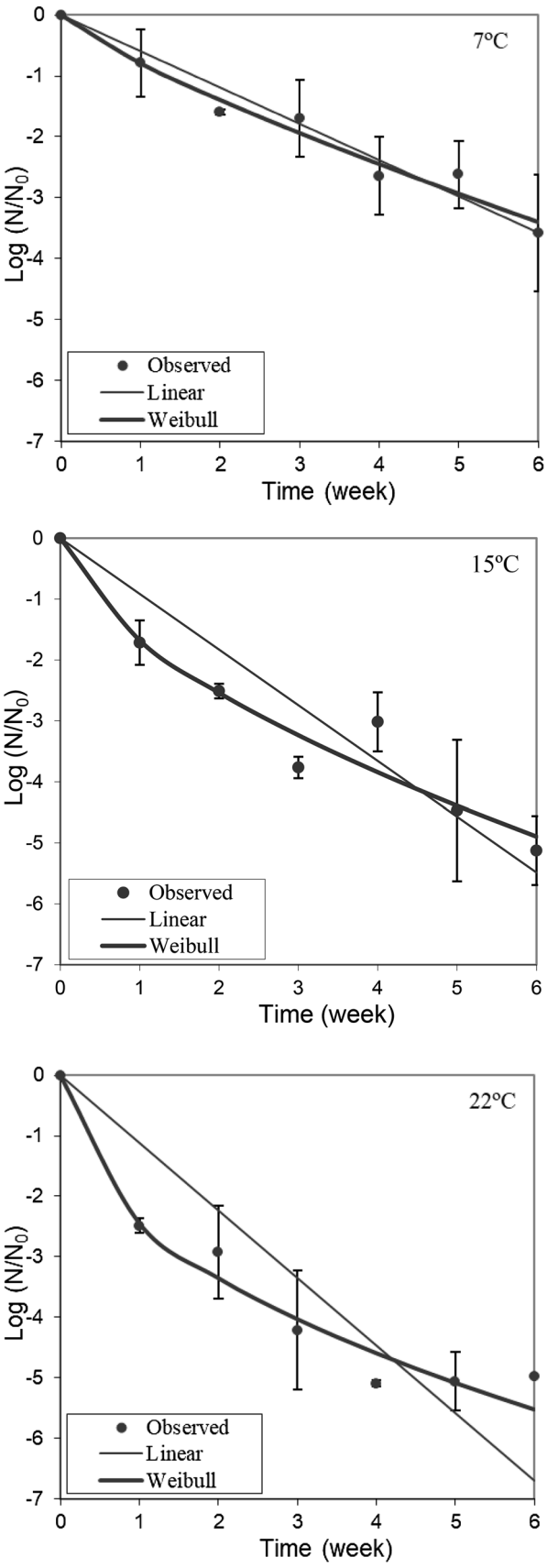

Fig. 1 Survival curves of M13 bacteriophages at three seawater temperatures. Data are the mean of two replicates and were fit with linear and Weibull functions. Error bars represent ± 1 standard error

that a nonlinear model, rather than a linear model, would better describe the data. Fitting the survival data with the linear and nonlinear models, and comparing the goodness-of-fit scores of the models, confirmed this observation since the *MSE* values at 15 °C and 22 °C for the Weibull model were better than those for the linear model (Table 2).

Discussion

To our knowledge, this is the first report to specifically evaluate DNA MSC persistence within oysters. As expected, M13 bacteriophage persistence within shellfish was inversely influenced by water temperature with greater persistence observed at lower temperatures, in general agreement with other bacteriophages and viruses (Choi and Kingsley 2016; Kingsley et al. 2018; Burkhardt et al. 1992b; Love et al. 2010; Dore and; Lees 1995). Comparison of log reductions indicates the persistence rates within oysters of the MS-2 RNA phage (Kingsley et al. 2018) and the M13 DNA phage are somewhat similar. For example, here log reductions 3 weeks post contamination were 1.7, 3.8, and 4.2 $\log_{10}$ at 7, 15, and 22 °C, respectively (Table 1). While for MS-2, 3 week post contamination $\log_{10}$ reductions were 2.3, 2.9, and 4.6 $\log_{10}$ at 7, 15, and 24 °C, respectively (Kingsley et al. 2018). For 6 weeks post contamination at 7 °C, MS-2 had a 3.5 $\log_{10}$ decline (Kingsley et al. 2018), while M13 had a 3.6 $\log_{10}$ decline (Table 1). In contrast, at 15 °C, MS-2 had a 4.0 $\log_{10}$ decline (Kingsley et al. 2018) versus a 5.1 $\log_{10}$ decline for M13 reported here. Given that these two phage types have similar behavior within Eastern oysters, results here suggest that genetic distinctions between DNA and RNA MSCs may not be of functional importance for shellfish management. The concept that water temperature needs to be a consideration regarding the decision of when to re-open human waste-impacted shellfish beds is also reiterated by this study. Presumably, bacteriophage persistence is a function of optimal temperatures for shellfish pumping and metabolism, which is generally thought to be nonexistent below 2 °C and limited below 10 °C, but shellfish pumping and metabolism may vary to some degree for shellfish of different species, different geographic locations, and different seasons (NSSP 2017).

The ideal indicator for virally contaminated shellfish should fulfill several criteria. It should be relatively absent, or at least present at low levels in clean water and ubiquitously present at elevated concentrations in unsanitary water, it should be easy to identify and quantitate, and it should persist within the environment and within shellfish in a way that resembles enteric

Table 2 Comparison of goodness-of-fit scores of the linear and Weibull models

Treatment	*MSE*	
	Linear	Weibull
7 °C	0.42	0.43
15 °C	0.74	0.52
22 °C	1.17	0.44

viruses. MSCs are certainly easily quantified, and acknowledging its limitations as a fecal indicator, it is ubiquitous in sewage and not ordinarily found within the environment at high levels. It is the last criteria, shellfish persistence, where the value of MSCs is currently debated. Ideally MSCs should bioaccumulate and persist within shellfish in a manner analogous to pathogenic human viruses such as HAV and HuNoV. Despite recent reports that HuNoV has been successfully cultured (Ettayebi et al. 2016; Jones et al. 2015), evaluating extracted norovirus viability from shellfish remains problematic, precluding direct comparison of temporal viability with MSCs. Previously it was tentatively concluded that decline of viable MS-2, an RNA phage, was slower than the RNA decline rate observed by RT-qPCR for HuNoV GI.1 (Choi and Kingsley 2016; Kingsley et al. 2018). Based on the similarities between M13 and MS-2 within oysters, that conclusion would also appear to hold for M13. However, recent work by Hartard et al. (2018) working with FRNAPH-II phage and human norovirus compared viability and RNA genome decay rates from sewage-impacted oysters (*Crassostrea gigas*) held at 12 °C. This work suggests that temporal viability reductions may be substantially greater than temporal genomic RNA reductions for the FRNA subgroup II bacteriophage. Put another way, infectious bacteriophage may be inactivated faster than the inactivated phage is cleared from shellfish, as measured by phage RNA. Furthermore, Hartand et al. (2018) noted that the observed genomic declines of the FRNA phage are in fact substantially slower than observed declines for genomic RNA for GII norovirus, inferring that norovirus may be more rapidly inactivated within shellfish than bacteriophage.

Caveats that should be considered when interpreting the results of this study include the concept that overnight accumulation of M13 bacteriophages was performed at 15 °C because researchers have reported that oysters do not uptake well the bacteriophages at temperatures above 20 °C (Burkhardt and Calci 2000; Nappier et al. 2008). Bacteriophage used in this study was not derived from sewage samples. How constant lower levels of bacteriophage sequestered within, or in association with, fecal particles rather than as a one-time overnight bolus of laboratory-generated bacteriophage, as performed here, may influence persistence could be an important consideration. Also raw and treated sewage released into estuaries likely contain multiple strain types of both RNA and DNA MSCs, and thus, the assumptions based on M13 results here, and MS2 results previously (Kingsley et al. 2018), should be considered as tentative. Whether there is substantial persistence variability among other MSC phage types with respect to shellfish remains to be determined.

In conclusion, results reported here indicate that temperature can substantially affect persistence of filamentous DNA male-specific coliphage M13 in a manner analogous to the RNA male-specific coliphage MS2 within shellfish. Overall, results reported here suggest that high levels of MSC—observed to persist after shellfish beds have been impacted by sewage—will, on a temporal basis, reflect the presence of both RNA and DNA MSCs.

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