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2 **THERMAL INACTIVATION OF HUMAN NOROVIRUS SURROGATES IN**
3 **OYSTER HOMOGENATE**

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Abstract

Human norovirus (HNV) is the most frequent causative agent of foodborne diseases in the US. Raw and undercooked oysters are commonly associated with outbreaks caused by HNV. Many guidelines recommend that shucked oysters be boiled for at least three minutes, but it is not clear this thermal treatment can inactivate HNV. The objective of this research was to evaluate whether this recommendation was sufficient to inactivate two HNV surrogates, murine norovirus (MNV-1) and Tulane virus (TV) in oyster homogenate as well as to determine their thermal inactivation kinetics. Inoculated oyster homogenate was heated in boiling water and circulating water bath at 49 to 67°C for different time durations. After 3 minutes of boiling, both MNV-1 and TV titers decreased to below the detection limits. First-order model and Weibull model were used to describe thermal inactivation kinetics. TD=1 values from Weibull mode are used as an analog to D values in first-order model. The D values of MNV-1 and TD=1 values ranged from 28.17 to 0.88 min and 26.64 to 0.78 min at 49 to 67°C, respectively. The D values of TV and TD=1 values ranged from 18.18 to 1.56 min and 19.35 to 1.56 min at 49 to 63°C, respectively. The kinetics demonstrated that at temperatures greater than 58°C, TV was much more heat sensitive than MNV-1. As the temperature increased over 58°C, the inactivation of both viruses occurred at a faster rate. Boiling treatment for 3 minutes as recommended by FDA for cooking shucked oysters, inactivated MNV-1 and TV in oyster homogenate below detection limit. One minute heating of TV at 63 °C or MNV-1 at 67 °C in contaminated oyster homogenate reduced the viral titers below the detection limits. Our research identified effective combinations of time and temperature to inactivate two HNV surrogate viruses, and thus provides insights on thermal

48 processing to reduce the risk of foodborne viral illness outbreaks associated with
49 consumption of oysters.

50 **Keywords:** Thermal processing, MNV, TV, oyster, D-value

51 1. Introduction

52 In the United States, human norovirus (HNV) is the most common cause of
53 acute gastroenteritis, leading to 19-21 million illnesses, 56,000-71,000 hospitalizations
54 and 570-800 deaths each year (Hall et al., 2013). It is considered the most frequent
55 causative agent causing more than half of foodborne diseases in the US cases each
56 year (Scallan et al., 2011). Norovirus causes acute gastrointestinal infection with
57 common symptoms include diarrhea, vomiting, nausea, abdominal cramping, chills,
58 headache, dehydration and a high-grade fever (Li et al., 2013). It is highly contagious
59 because only a few particles have the potential to cause infection (Donaldson et al.,
60 2008). It can be spread by contaminated food or water, through person to person
61 contact and via cross contamination surfaces (Hall et al., 2013). Raw and undercooked
62 oysters are commonly involved in outbreaks caused by HNV; they are filter feeders
63 and concentrate and retain viruses derived from the environment (Lees, 2000). From
64 1998 to 2015, 5362 foodborne outbreaks in the US were caused by norovirus, in 86
65 outbreaks of which oyster is the vehicle (Foodborne Outbreak Online Database
66 (FOOD Tool), 2016). In 2016, approximately 75 people in the outer Cape Cod area in
67 Massachusetts developed norovirus-like illness after eating raw shellfish (NoroCore,
68 2016). In 2002, over 100 people in Italy became ill after consuming norovirus
69 contaminated oyster (Le Guyader et al., 2006).

70 It has been demonstrated that HNV can interact specifically with carbohydrate
71 structures in the bivalve digestive organ (Le Guyader et al., 2000). Viruses cannot

72 multiply in food or in the environment, but they can persist for several days or weeks
73 without loss of infectivity (Seitz et al., 2011). Typical methods used to prevent
74 bacterial growth in food products may not be effective against viruses (Jaykus, 2000).
75 Therefore, the potential presence of HNV in oysters poses a serious health threat to
76 consumers and is an important concern for health authorities (Hewitt and Greening,
77 2004). It is then essential to understand whether current seafood handling and
78 processing can mitigate HNV survival and persistence in oysters.

79 Recently, Dr. Mary Estes and her research team have successfully grown
80 human norovirus in enterocytes in stem cell–derived, nontransformed human intestinal
81 enteroid monolayer cultures with bile (Ettayebi et al., 2016). However, before the
82 research, the main difficulty that hampers research of HNV is there is no in vitro cell
83 culture system or small animal model. As a result, most of research relies on viral
84 surrogates, including feline calicivirus (FCV) and murine norovirus (MNV-1). FCV is
85 structurally different from HNV and it is a respiratory virus and very sensitive to low
86 pH (2.0 to 4.0) (Cannon et al., 2006; Li et al., 2012). MNV-1 has been shown to be
87 more similar to HNV immunologically, biochemically, genetically, and molecularly.
88 And it belongs to genus norovirus and is also resistant to acid and heat, and highly
89 stable and persistent in the environment (Cannon et al., 2006; Li et al., 2012).
90 However, clinical symptoms of gastroenteritis caused by MNV-1, which present as
91 hepatitis, pneumonia, and inflammation of nervous systems, are quite different from
92 that caused by HNV (Karst et al., 2003). More importantly, MNV-1 uses sialic acid as
93 a functional receptor whereas HNV uses HBGA as receptors (Wobus et al., 2006; Tan
94 and Jiang, 2010). Previous research on thermal inactivation showed thatFCV and
95 MNV-1 behaved similarly when heated at 63°C (Cannon et al., 2006). It was reported

96 that Tulane virus (TV), a calicivirus isolated from stools of rhesus macaques,
97 represents a new genus, *Recovirus* (Farkas et al., 2008). TV can be cultivated in rhesus
98 monkey kidney cells (LLC-MK2) and is close to HNV based on its genomic sequence
99 (Farkas et al., 2010). More importantly, like HNV, it recognizes the type A and B
100 HBGAs (Farkas et al., 2010). Therefore, it has the potential for use as a surrogate of
101 HNV. In terms of thermal resistance, TV in culture medium is more heat sensitive
102 than MNV-1 at 50 to 60°C (Hirneisen and Kniel, 2013). However, there is no report of
103 the stability of TV when heated in seafood matrix such as oysters.
104 Thermal processing is one of the most effective methods to reduce viruses in
105 any food product. Cooking oysters thoroughly will impact organoleptic characteristics
106 and can toughen oyster meat, which make them undesirable for consumers. Light
107 cooking may be acceptable to some consumers, but might be insufficient to kill all
108 enteric viruses, since most of the viruses are inside the shellfish and would not be
109 subjected to sufficient heat for their total inactivation (Richards et al., 2010). It has
110 been suggested that an internal temperature of 90°C for at least 90 s is a virucidal
111 treatment. FDA suggests that seafood is cooked to an internal temperature of 63°C
112 (145°F) for 15 seconds (FDA, 2009), which ensures that food-borne bacteria is
113 destroyed. Consumers, without thermometers, can rely on shells to open to determine
114 the doneness of shellfish. However, this practice may be insufficient to reach the
115 virucidal treatment. Previous studies on steaming mussel showed that the mean
116 internal temperature was 83°C when all 50 mussels were tested (Hewitt and Greening,
117 2006). It is also recommended by many guidelines that shucked oyster is simmered or
118 boiled for at least 3 minutes (Villalba et al., 2008; Hicks, 2010). Since there is no
119 specific regulation covering the minimum time–temperature combination for

120 inactivating virus in contaminated oysters, establishment of proper thermal processes
121 for inactivating HNV in a high risk food such as oysters would be essential for
122 protecting public health.

123 The specific objectives of this study were to (1) determine thermal inactivation
124 behavior of murine norovirus (MNV-1) and Tulane virus (TV) in oyster homogenate
125 (2) test 3 minutes of boiling water heating efficacy on inactivation of both viral
126 surrogates in oyster homogenate and (3) compare first-order and Weibull models to
127 understand the kinetics of thermal inactivation behavior of two viral surrogates.

128 **2. Material and Methods**

129 **2.1 Viruses and Cell Lines.**

130 MNV-1 and TV were propagated in RAW 264.7 and LLC-MK2 respectively.
131 Raw 264.7 cells were cultured in high-glucose Dulbecco's modified with 10% heat-
132 inactivated fetal bovine serum (FBS) (Life Technologies) at 37°C under a 5% CO_2
133 atmosphere. MK2-LLC cells were cultured in M199 medium (Mediatech, Manassas,
134 VA) with 10% heat-inactivated FBS (Life Technologies) and penicillin G (100 U/ml)
135 and streptomycin (100 μg/ml) at 37°C under a 5% CO_2 atmosphere. To prepare MNV-
136 1 stock, confluent RAW 264.1 cells were infected with MNV-1 at a multiplicity of
137 infection (MOI) of 1. After 1 h of incubation at 37°C under a 5% CO_2 atmosphere, 25
138 ml of DMEM supplemented with 2% FBS was added. MNV-1 was harvested 2 days
139 after post-inoculation by three freeze–thawing cycles and centrifugation. The same
140 procedures were followed to grow TV except that cells were infected with TV at a
141 MOI of 0.1 and 25 ml of M199 supplemented with 10% FBS was used after 1 h
142 incubation period. Virus was stored at −80°C until use.

143 **2.2 Oyster Sample Preparation and Inoculation**

144 Samples of 25-g oyster meats were homogenized with a homogenizer (OMNI
145 international, Kennesaw, GA, 30144) for one minute, then 2 ml of homogenate were
146 added into a 15-ml conical centrifuge tube (Falcon). The homogenate was inoculated
147 with 0.3 ml of virus (5-6 log_{10} PFU/ml), vortexed thoroughly and stored at 4 °C
148 overnight.

149 **2.3 Temperature –Time Profile of Boiling Water Treatment and Thermal**
150 **Treatment**

151 A thermocouple was placed in the center of the uninoculated homogenate in a
152 15-ml centrifuge tube. Then the tube was immersed in the boiling water for 5 minutes.
153 The thermocouples were connected to a portable data recorder (HH506RA,
154 multilogger thermometer, Omega Engineering) to record the temperature. The water
155 temperature was also monitored. As for the different thermal treatments, the samples
156 were heated in a circulating water bath at 49, 54, 58, 63, and 67°C for up to 5 minutes.
157 The center temperature and water temperature were recorded every 2 s. The
158 temperature-time profiles were tested 4 times. At each time two replicates were
159 conducted.

160 **2.4 Boiling Water Treatment of MNV-1 and TV in Oyster Homogenate**

161 The tubes containing the inoculated oyster homogenate were immersed in
162 boiling water. A thermocouple was placed in the boiling water to monitor the
163 temperature. The samples were treated for 2, 3, and 4 minutes. After boiling water
164 treatments, the tubes were cooled immediately in an ice-water bath for ten minutes.
165 Extraction and plaque assay were conducted for enumeration of viral survivors.

166 **2.5 Thermal Treatment of MNV-1 and TV in Oyster Homogenate**

167 The inoculated oyster homogenate samples were added into centrifuge tubes
168 and heated at 49, 54, 58, 63, and 67°C in a circulating water bath for different
169 treatment times (0 to 60 min). Duplicates were tested at each time. A thermocouple
170 was placed in the water to monitor the water temperature. After the thermal treatment,
171 the tubes were cooled immediately in an ice-water bath for ten minutes. Extraction and
172 plaque assay were conducted for enumeration of viral survivors.

173 **2.6 Extraction MNV-1 and TV from Oyster Homogenate**

174 MNV-1 and TV survivors were extracted from oyster homogenate using the
175 procedures described by Ye et al. (2014) with slight modification. A volume of 18 ml
176 glycine buffer (pH 9.5; 0.1M glycine, 0.3MNaCl) was added into 2 ml homogenate
177 and mixed well at room temperature (21°C). The mixture was centrifuged at 10,000 g
178 for 15 min at 4 °C . An equal volume (20 ml) of 16% polyethylene glycol 8000 (PEG)
179 with 0.525M NaCl was added to the supernatant to precipitate viral particles. After a 1
180 hour precipitation on ice, the mixture was centrifuged at 10,000 g for 10 min at 4°C.
181 The pellet was suspended in 2 ml PBS (pH 7.2), and then samples were stored at -80
182 ℃ until quantified by plaque assay.

183 **2.7 Viral Plaque Assay.**

184 MNV-1 and TV were quantified by plaque assay using procedures by (Li et al.,
185 2013) with slight modifications. For MNV-1, RAW 264.7 cells were grown to 80-90%
186 confluence in six-well plates (Becton, Dickinson and Company, Franklin Lakes, NJ).
187 After 24 h of incubation, cell mono-layers were infected with 400 µl of a 10-fold
188 dilution series of the virus and the plates were incubated for 1 h at 37°C and 5% CO_2
189 with gentle agitation every 10 min. After 1 h, cells were overlaid with 2.5 ml of Eagle

190 minimum essential medium (MEM) supplemented with 0.5% agarose, 5% FBS,
191 0.12% sodium bicarbonate, penicillin G (100 U/ml), streptomycin (100 μg/ml), and
192 amphotericin B (0.25 μg/ml), 25 mM HEPES (pH 7.7), and 2 mM L-glutamine (Life
193 Technologies). Plates were then incubated at 37°C and 5% CO_2 for 2 days, and fixed
194 in 3.7% formaldehyde (Fisher Scientific, Pittsburg, PA) and the plaques were
195 visualized by staining with 0.05% (w/v in 10% ethanol) crystal violet. For TV, plaque
196 assay was slightly different from MNV-1. LLC-MK2 cells were grown to 80-90%
197 confluence in six-well plates. Cells were overlaid with 2.5 ml of M199 medium, 10%
198 fetal bovine serum, penicillin G (100 U/ml), streptomycin (100 μg/ml), and
199 amphotericin B (0.25 μg/ml), and 1.5% agarose. Plates were incubated at 37°C and
200 5% CO_2 for 4 days before enumeration of plaques.

201 **2.8 Modeling of Inactivation Kinetics**

202 First-order kinetics: The first-order kinetic model assumes a linear logarithmic
203 reduction of the number of survivors over treatment time:

204 $$\log(N/N_0) = -t/D$$

205 where N_0=the initial population (PFU/ml); N=the number of survivals after an
206 exposure time t (PFU/ml); D (decimal reduction time)=the time required for one log
207 reduction in the population (min); t=time (min).

208 Weibull model: This model assumes that cells in a population have different
209 resistances and a survival curve is just the cumulative form of a distribution of lethal
210 agents.

211 $$N/N_0 = \exp(-(t/\alpha)^{\beta})$$

212 where α and β are the scale and shape parameters, respectively.

213 For the Weibull model, the time required to obtain a specific log reduction of
214 virus can be evaluated by using shape and scale parameters as shown in equation.

215 $$t_D = \alpha(-\ln(10^{-D}))^{1/\beta}$$

216 where D is the number of decimal reductions.

217 **2.9 Data Analysis and Model Evaluation.**

218 The smaller the root mean square error (RMSE) values, the better the model
219 fits the data. The higher the R^2 value, the better is the adequacy of the model to
220 describe data.

221 $$RMSE = (\sum(\text{predicted-observed})^2)^{-1/2}$$

222 where n is the number of observations.

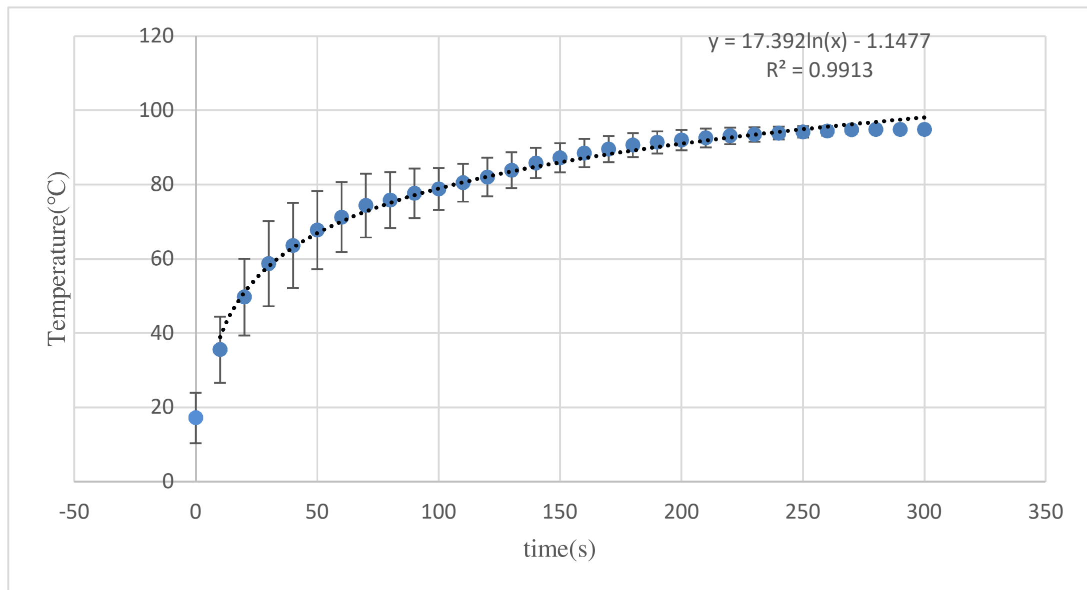
223 The Weibull models were fitted using JMP software (SAS, Cary, NC, USA)
224 Nonlinear model. Tukey's test was used to determine difference between each
225 treatment. Significant difference was reported when $P < 0.05$.

226 **3. Result and Discussion**

227 **3.1 Internal Temperature of Oyster homogenate and Inactivation Effects by**
228 **Boiling Water**

229 The mean temperature of oyster homogenate prior to treatment was 18°C.
230 After 1, 2, and 3 minutes of heating, the internal temperature reached 71, 82, and 91°C
231 respectively. After boiling for 262 s, the mean internal temperature was 95°C and
232 maintained at that level (Figure 1). Compared with previous thermal profile described
233 by Hewitt and Greening (2006), at 1, 2, and 3 minutes, the mean internal temperature
234 reached 60, 80, and 90°C respectively when heating mussels in boiling water, which is
235 similar to our result.

236



237

238 Figure 1 The mean internal temperature- time profile of oyster homogenate under
239 boiling water heating.

240 The initial titers of virus stocks were 6.79 ± 0.17 log_{10} PFU/ml for MNV-1 and
241 4.93 ± 0.10 log_{10} PFU/ml for TV. As shown in Table 1, the recovered titers of
242 inoculated oyster homogenate were 5.77±0.21 log_{10} PFU/ml for MNV-1 and 3.92
243 ±0.19 log_{10} PFU/ml for TV, respectively. Reduction of MNV-1 at 0.73±0.16 and
244 4.35±0.14 log_{10} PFU/ml was obtained by 1 minute and 2 minutes boiling treatments of
245 contaminated oyster homogenate, respectively. However, there was 1.2 ± 0.3 log_{10}
246 PFU/ml reduction of TV when heating the contaminated oyster homogenate in boiling
247 water for 1 minute, but below detection limit when heating for 2 minutes. After 3
248 minutes treatments, neither MNV-1 nor TV was detected in oyster homogenate (< 1

249 $\log_{10}$ PFU/ml). The result indicated that TV was more sensitive to heat than MNV-1 in
250 oyster homogenate when heated by boiling water. In addition, our results suggested
251 that 3 minutes' boiling treatment, which is recommended by FDA for cooking shucked
252 oysters, can inactivate both MNV-1 and TV in oyster homogenate below detection
253 limit. Our findings for the first time compared the inactivation efficacy by boiling
254 water treatments between MNV-1 and TV in oyster homogenate. Different viruses
255 behaved differently in the same food matrix, the oyster homogenate. Oyster matrix
256 plays a protective role against heat inactivation of MNV-1 and TV, which is different
257 from previous findings on heat sensitivity of two viral surrogates in buffer system
258 (Hirneisen and Kniel, 2013). Heating MNV-1 and TV at 70 and 75°C for 2 minutes
259 eliminated both viruses in buffer (Hirneisen and Kniel, 2013), but higher temperature
260 and longer heating time are needed if viral surrogates in oyster homogenate as
261 observed in our research. Additionally, different domestic cooking methods may also
262 have different impacts on virus inactivation in shellfish. Hewitt and Greening (2006)
263 investigated inactivation of HAV in mussels by boiling and steaming. The result
264 indicated that heating by boiling water let the internal point reach higher temperature
265 compared to steaming, which resulted in a higher log reduction in virus titer. It is
266 plausible that boiling instead of steaming for at least 3 minutes could reduce the risk of
267 enteric virus in contaminated shellfish. In our studies, oyster homogenates was spiked
268 with the viruses to obtain more uniform and higher contamination levels, and this
269 contamination might occur during the oyster preparation and handling in the cooking
270 process. However, this contamination could be different with contamination after
271 bioaccumulation of viruses in oyster tissues. In a published research, at 24 h
272 bioaccumulation, TV and MNV-1 accumulated at a significantly higher level in the

273 digestive gland than in the grill and adductor muscles, but no difference was observed
274 at 48 and 72 h bioaccumulation, with virus titer ranged 3-4 log10 PFU/g (Araud et al.,
275 2016). Future research is necessary to determine the thermal inactivation of viruses in
276 oysters contaminated via bioaccumulation.

277 Table 1 Survival and $\log_{10}$ reduction of MNV-1 and TV in oyster homogenate when
278 boiled for 3 minutes. NC means no counts. Data with different letters are
279 significantly from each other (P<0.05)

	Boiling water treatment				Viral reduction ($\log_{10}$PFU/ml)	
virus strain	control	1 minute	2 minutes	3 minutes	1 minute	2 minutes
MNV-1	5.77±0.21	5.04±0.18	1.44±0.26	NC	0.73±0.13a	4.26±0.03c
TV	3.92±0.19	2.70±0.40	NC	NC	1.22±0.26b	>2.92

280

281 **3.2 Thermal Inactivation Kinetics**

282 There is no standard approach available to study thermal stability.
283 Temperatures of 56, 63, 72 and 100°C were recommended by Arthur and Gibson
284 (2014). In addition, First order kinetics and Weibull distribution were used to
285 understand the kinetics of thermal inactivation behavior of two viral surrogates.

286 As shown in Table 2, heating contaminated oyster homogenate at 49°C for 12
287 minutes or at 54°C for 6 minutes only caused mild reduction of MNV-1. However,
288 when the temperature increased to 58 and 67 °C, viral reduction became significant,
289 with ~1.5 $\log_{10}$ reduction at 58°C for 180 s, ~2.5 $\log_{10}$ reduction at 63°C for 120 s. On
290 the other hand, higher temperature had a faster inactivation rate for TV, with
291 approximately 2 $\log_{10}$ reduction after heating at 58°C for 180 s, ~2.5 $\log_{10}$ reduction at
292 63°C for 30 s. One min (60s) heating of TV at 63 °C or MNV-1 at 67 °C in

293 contaminated oyster homogenate both reduced the viral titers below the detection

294 limits. Overall, TV was more sensitive to heat when the temperature > 58°C.

Table 2 Effect of thermal treatment on MNV-1 and TV inactivation in oyster. The unit for recovered titer is $\log_{10}$ PFU/ml

virus strain	Temperature (°C)									
	49		54		58		63		67	
	Treatment	Recovered titer	Treatment	Recovered titer	Treatment	Recovered titer	Treatment	Recovered titer	Treatment	Recovered titer
MNV-1	control	5.16±0.20	control	5.16±0.20	control	5.28±0.29	control	5.36±0.13	control	5.28±0.18
	0 min	4.97±0.35	0 min	4.78±0.41	0 s	4.37±0.43	0 s	4.00±0.32	0 s	2.30±0.12
	3 min	4.83±0.38	1.5 min	4.64±0.43	45 s	4.23±0.53	30 s	3.76±0.37	20 s	1.99±0.15
	6 min	4.78±0.37	3 min	4.53±0.45	90 s	4.10±0.44	60 s	3.39±0.22	40 s	1.44±0.26
	9 min	4.65±0.37	4.5 min	4.42±0.49	135 s	3.89±0.35	90 s	3.15±0.29	60 s	NC
	12 min	4.51±0.36	6 min	4.31±0.53	180 s	3.72±0.41	120 s	2.95±0.32	80 s	NC
TV	control	3.72±0.11	control	3.67±0.14	control	3.71±0.12	control	3.43±0.18	control	3.49±0.09
	0 min	3.26±0.22	0 min	3.23±0.33	0 s	2.62±0.24	0 s	1.50±0.03	0 s	NC
	3 min	3.06±0.25	1.5 min	3.00±0.38	45 s	2.46±0.18	30 s	1.06±0.08	20 s	NC
	6 min	2.90±0.30	3 min	2.84±0.33	90 s	2.11±0.15	60 s	NC	40 s	NC
	9 min	2.68±0.30	4.5 min	2.74±0.31	135 s	1.95±0.16	90 s	NC	60 s	NC
	12 min	2.54±0.40	6 min	2.57±0.31	180 s	1.75±0.04	120 s	NC	80 s	NC

294 The use of high temperatures for food preservation is based on the destructive
295 effects of heat on microorganisms. It has been suggested that the mechanism of
296 thermal inactivation of viruses would be associated with changes in the virus capsid
297 (Bozkurt et al., 2015). The changes in virus capsid during thermal treatment depends
298 on the temperature level (Song et al., 2010). At mild temperatures (<56°C), the
299 inactivation may be caused by the destruction of the viral receptor and structural
300 related changes –the inability to recognize and bind the host cells (Wigginton et al.,
301 2012). Croci et al. (2012) also suggested that the damage to viral receptor binding sites
302 is limited at mild temperature (around 50°C) and the capsid retains the ability to
303 protect the nucleic acid from the environment so the virus still remains infectious
304 (Croci et al., 2012). However, above 60°C, the tertiary protein structure alters,
305 facilitating access of thermal energy to nucleic material. Therefore, the capsid ceases
306 to play a protective role, resulting in the inactivation of nucleic material (Katen et al.,
307 2013). This hypothesis is supported by findings that the inactivation of MNV-1 was
308 rapid when temperature is higher than 60°C, which was likely due to the structural
309 changes of the capsid proteins (Baert et al., 2008). Similar result was also reported by
310 Ausar et al (2006) who observed that norovirus-like virus particles were highly stable
311 up to 55 °C, but, at temperatures above 60 °C, the icosahedral symmetry of the virus-
312 like particle in the capsid of the virus particle was lost and the quaternary structure of
313 the capsid was significantly altered. Thus, our observation that MNV-1 and TV being
314 activated at faster rates at higher temperatures (>58°C) could be due to changes in the
315 tertiary structures of the virus.

316 As shown in Table 3, the D values for MNV-1 from 49 to 67°C were in the
317 range of 28.17 to 0.88 min calculated using the first order model. TD=1 values from

318 Weibull mode which are used as an analog to D values, were in the range of 26.64 to
319 0.78 min (Table 3). For TV, the D values for TV from 49 to 63°C were in the range of
320 18.18 to 1.56 min calculated from first order model and TD=1 calculated from
321 Weibull model were in the range of 19.35 to 1.56 min (Table 3). In previous studies on
322 inactivation of MNV-1 and TV in culture medium, the D values of MNV-1 and TV
323 from 50 to 65°C are in the range of 2.47 min to 0.56 min and in the range of 1.12 min
324 to 0.65 min, respectively (Hirneisen and Kniel, 2013), which disagrees with our
325 results. Food may influence the efficacy of thermal inactivation of virus. Our result
326 indicated that oyster matrix plays a protective role against heat inactivation of MNV-1
327 and TV. Lipid content, as well as protein content, may in fact affect virus inactivation
328 to some extent. It has been suggested that the presence of fat and protein in food
329 matrix protect the cell receptors or cause formation of viral aggregates (Croci et al.,
330 2012). In addition to food matrix, larger D values may also be explained by different
331 come-up time and heating system.

332 Table 3 Coefficients of the first-order and Weibull models for the survival curves of
333 MNV-1 and TV during thermal inactivation. NC means no counts.

Virus strain	T	Weibull distribution					First order model		
		β	α	TD=1(min)	R2	RMSE	D(min)	R2	RMSE
MNV-1	49	1.073	12.25	26.64	0.983	0.029	28.17	0.976	0.031
	54	0.835	6.595	17.91	1	0.004	14.41	0.988	0.022
	58	1.551	2.079	3.56	0.981	0.035	4.60	0.957	0.080
	63	1.245	0.817	1.60	0.996	0.035	1.82	0.973	0.046
	67	1.427	0.433	0.78	1	0.000	0.86	0.974	0.039
TV	49	0.903	7.684	19.35	0.997	0.015	18.18	0.984	0.023
	54	0.927	3.648	8.97	0.997	0.016	8.64	0.991	0.021
	58	1.157	1.393	2.87	0.989	0.035	3.14	0.983	0.044
	63	1	0.678	1.56	1	0.000	1.56	1	0.000
	67	NC	NC	NC	NC		NC	NC	NC

334

335 For Weibull models, the scale factor (α) for MNV-1 ranged from 12.250 to
336 0.433 min at temperature of 49 to 67°C and for TV ranged from 7.684 to 0.678 min at
337 temperature of 49 to 63°C (Table 3). The scale factor is temperature-dependent and
338 reflects the effect of heating environment on the inactivation. The shape factors (β)
339 ranged from 1.073 to 1.427 at temperature of 49 to 67°C and for TV ranged from
340 0.903 to 1.000 at temperature of 49 to 63°C. In contrast to scale factor, the shape
341 factors (β) of the Weibull model will not be influenced by the environment and
342 described the shape of the model. When the $\beta < 1$, the model is an up concave curve
343 and vice versa. When $\beta > 1$, it indicates that remaining survivors become increasingly
344 damaged otherwise indicates that the remaining survivors is able to adapt to the
345 environment and become more resistant (van Boekel, 2002).

346 To compare the goodness of fit of the first-order and Weibull models, the
347 coefficient of determination (R^2), RMSE were calculated (Table 3). For the MNV-1,
348 from 49 to 67°C, the Weibull model had R^2 values of 0.983 to 1.000 and RMSE
349 values of 0.029 to 0.000, whereas the first order model had R^2 values of 0.976 to
350 0.974, and RMSE values of 0.031 to 0.039. For the TV from 49 to 63°C, the Weibull
351 model had R^2 values of 0.997 to 1.000, and RMSE values of 0.015 to 0.000, whereas
352 the first order model had R^2 values of 0.984 to 1 and RMSE values of 0.023 to 0.0006.
353 Overall Weibull model gave a better fit to the survival data of MNV-1 and TV during
354 thermal inactivation because it shows larger R^2 value and smaller RMSE. Other
355 studies also showed that Weibull model was a better fit to express virus inactivation
356 (Araud et al., 2016; Bozkurt et al., 2013). Applying first order model to describe the
357 thermal inactivation of microorganism is based on the mechanistic explanation that

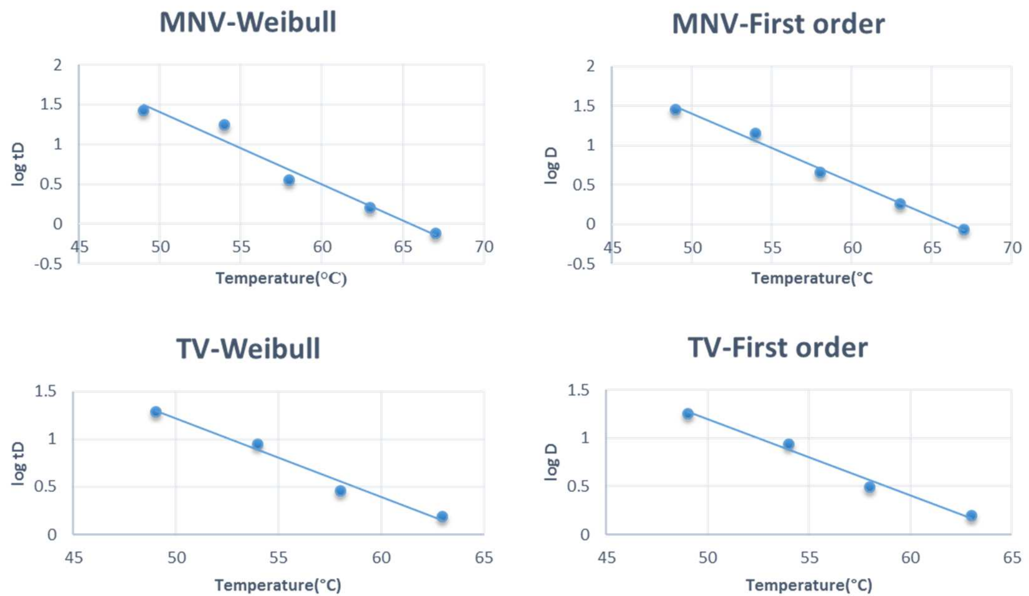
358 death of microorganism is determined by the inactivation of critical enzyme. And
359 enzyme inactivation is governed by first order kinetics. Another view to microbial
360 inactivation considers lethal events as probabilities, rather than as deterministic (van
361 Boekel, 2002). Microbial inactivation is just the cumulative result of a distribution of
362 lethal agents. First order model is a special situation of Weibull distribution when β=1.
363 Accurate model prediction of survival curves would be beneficial to the food industry
364 in selecting the optimum combinations of temperature and time to obtain the desired
365 levels of inactivation. The present results revealed that the Weibull model could be
366 used successfully to describe thermal inactivation of MNV-1 and TV in oysters, which
367 is consistent with finding that Weibull model showed better thermal inactivation
368 prediction of MNV-1 and FCV in mussel (Bozkurt et al., 2014).

369 **3.3 Z Value**

370 The Z value means the change in temperature required for one log reduction in
371 the D value (or t_D for Weibull). It is used for calculating thermal death time (F) under
372 different conditions. Z values were calculated for both models by the thermal death
373 time curve (Figure 2). The Z values for MNV-1 were 10.98°C (R^2, 0.96) using the
374 Weibull model and 11.52°C (R^2, 0.99) for the first-order model. The Z values for TV
375 were 12.25 °C (R^2, 0.98) using the Weibull model and 12.71°C (R^2, 0.99) for the first-
376 order model. Z values of MNV-1 and TV are not comparable because they are
377 calculated from different temperature range (49-67 °C for MNV-1, 49-63°C for TV).
378 The reported Z value of MNV-1 in mussels were 11.62 °C for first order model and
379 9.97°C for Weibull model (Bozkurt et al., 2014), which agreed with our results. There
380 are very limited thermal inactivation data of TV in a seafood matrix. To the best of our
381 knowledge, there are no reported studies on thermal death curve of MNV-1 and TV in

382 oysters. Our result provides some initial insights of MNV-1 and TV thermal

383 inactivation behavior in oysters.



384

385 Figure 2 Thermal death time curves of murine norovirus (MNV-1) for the Weibull
386 model (Z=10.98°C, R^2=0.96) and first-order model (Z=11.52°C,
387 R^2=0.99) and thermal death time curves of Tulane virus (TV) for Weibull
388 model (Z=12.25°C, R^2=0.98) and first order-model (Z=12.71°C,
389 R^2=0.99).

390 **4. Conclusion**

391 Our results suggest that boiling oyster homogenate for 3 minutes may be

392 effective to inactivate HNV to below 1 log_{10} PFU/ml based on the results of two

393 surrogates, MNV-1 and TV. Among the three temperature tested (58 and 63, 67 °C),

394 inactivation at a higher temperature has a faster inactivation rate compared to lower

395 temperatures. The results also indicate MNV-1 was more heat resistant than TV in

396 oyster homogenate, especially >58°C. Lastly, the result of R^2, RMSE and MSE
397 demonstrated that the Weibull model showed better thermal inactivation prediction
398 than the first-order model. Our results give more understanding of the thermal
399 inactivation behavior of norovirus surrogates, which could be beneficial to food
400 industry and consumers by predicting the human norovirus behavior by thermal
401 process so as to prevent foodborne viral outbreaks associated with consumption of
402 oysters.

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