1 **Strategies to reduce norovirus (NoV) contamination from oysters under**
2 **depuration conditions**

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8 Key words: Pacific oysters, Norovirus, Depuration, Purification

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10 **The published version of this paper can be found at:**
11 https://authors.elsevier.com/sd/article/S0278-6915(20)30399-9

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25 **Abstract**

26 Depuration of oysters can effectively reduce levels of *E. coli,* however, may not be
27 effective in safeguarding against viral contamination (EFSA 2012). These trials
28 assess the removal of Norovirus Genogroups I and II (NoV GI and GII) and F+ RNA
29 bacteriophage genogroup II (FRNAP-II) from oysters under depuration using
30 molecular and viability assay methods. Our results show consistently better removal
31 of NoV GII compared with Nov GI. We found approximately 46% removal of NoV GII
32 at 18°C after 2 days and 60% after 5 days compared with a maximum of 16% NoV GI
33 removal. Twice the rate of NoV GII removal was achieved at 18°C compared with 8°C
34 after 5 days. Results suggest better NoV removal when depuration water salinity is
35 close to that prevailing in the harvesting area. Trials investigating algal feeding,
36 light/dark and disturbance from pump vibration did not show any significant effect.

37 We found that FRNAP-II was more readily removed than NoV. No significant
38 difference was found between the rate of removal (as measured by RT-qPCR) and
39 inactivation (as measured by bioassay) of FRNAP-II. This indicates that reduction in
40 FRNAP-II may be primarily due to physical removal (or destruction) rather than *in situ*
41 inactivation of the virus.

42

43

44 ## 1. Introduction

45 Across most of Europe, Norovirus (NoV) illness associated with the consumption of
46 raw or lightly cooked oysters mainly occurs in the winter months (November to March).

47 In the UK, our experience has been that there is a close association between low
48 seasonal environmental temperatures and NoV presence in oysters. Conversely, NoV
49 levels generally decline markedly in the summer months.
50
51 Purification, also referred to as depuration, is generally the main treatment method
52 employed for shellfish from class B production areas under EU legislation. In England
53 and Wales, the majority of areas are class B and so this treatment step is particularly
54 significant. Depuration is known to rapidly and effectively reduce levels of *E. coli,* the
55 statutory indicator of faecal contamination. However, even if bacterial end product
56 standards are achieved, depuration may not be effective in safeguarding against viral
57 contamination (EFSA 2012). Aside from the immediate health effects, NoV outbreaks
58 are also damaging to the shellfish industry in terms of the general public's confidence
59 in these products.
60

61 NoVs can be classified into at least seven different genogroups, each of which can be
62 further divided into different genotypes. Genogroups I, II and IV are those associated
63 with human illness (Ramirez *et al* 2008). Most NoVs that infect humans belong to
64 genogroups GI and GII (*Vinjé et al 2000).* NoV from Genogroup II, genotype 4
65 (abbreviated as GII.4) currently account for the majority of adult outbreaks of
66 gastroenteritis.

67

68 A comprehensive review of NoV depuration was undertaken by McLeod *et al* in 2017.
69 This review notes that NoV GII strains are more prevalent than NoV GI in NoV
70 outbreaks generally, although there is a greater proportion of GI strains specifically in

71 shellfish-associated outbreaks. One explanation for this might be the suggested
72 binding of NoV GI strains (Le Guyader *et al*, 2006) to the midgut digestive diverticula
73 of oysters (more so than NoV GII strains) making GI removal from oysters more
74 difficult, thus leading to a higher proportion of GI outbreaks associated with shellfish
75 than other sources of infection. It is proposed that the binding, initially at least, of GII
76 strains to the gills and mantle may make them more susceptible to inactivation or
77 account for less efficient bioaccumulation of GII strains (Maalouf *et al*, 2011). McLeod
78 *et al* 2017 conclude that there are strain-specific variations in binding patterns. GII
79 infections from shellfish are nevertheless still significant given their greater prevalence
80 generally in NoV outbreaks in the community.

81

82 Polo *et al* 2015 developed a mathematical model to characterize the kinetics of viral
83 elimination during depuration of Manila clams (*Venerupis philippinarum*) and
84 Mediterranean mussels (*Mytilus galloprovincialis*) previously subjected to
85 bioaccumulation with Hepatitis A Virus or Murine NoV-1 (as a surrogate for human
86 norovirus). Depuration was carried out over 7 days and it was observed that there
87 were effectively two viral loads: one susceptible to depuration and the other non-
88 susceptible to depuration. The latter may be due in part to binding. The two shellfish
89 species used in this study exhibited different depuration behaviour. This highlights
90 that assumptions on depuration efficacy need to be made with care and should
91 recognise that the outcomes for different species may vary.

92

93 McLeod *et al*, also report that surrogate virus indicators such as F+ RNA
94 bacteriophage (FRNAP) tend to be more quickly removed from oysters than NoV.

95 Furthermore, they note that the more rapid reduction values for such surrogate viruses
96 may be, at least in part, due to the quantitation of infectious surrogate virions compared
97 with NoV quantitation by PCR genome detection i.e. the NoV PCR method cannot
98 provide information on infectivity so this method can only indicate actual removal or
99 destruction of the genome segment targeted in the test, whereas the FRNAP bioassay
100 effectively indicates removal and/or destruction as well as loss of infectivity. In this
101 way it is possible that NoV values may be misleadingly high when compared with
102 surrogate infectious virus values as they fail to represent any potential loss of NoV
103 infectivity. Polo *et al*, 2018 propose the use of Tulane virus as a surrogate for NoV as
104 it also appears to exhibit binding in shellfish, recognizing human histo-blood group
105 antigens. It may, therefore, behave more similarly to NoV under depuration conditions
106 and thus may be more suited specifically for future depuration trials. One
107 disadvantage with Tulane virus, however, is that it does not occur naturally in human
108 sewage or typically in the environment in Europe. It is generally isolated from
109 macaque stools (Tian *et al*, 2013), and so would have to be artificially administered to
110 the shellfish prior to any trials.
111
112 Combining RT-qPCR testing with a viability assay for infectious FRNAP has been
113 reported to have the potential to better estimate health risks, and to better predict the
114 presence of infectious norovirus than RT-qPCR testing alone (Lowther, 2019).
115
116 From previous depuration trials (Neish, 2013) investigating total NoV removal, we
117 found relatively slight reduction of total (GI and GII) NoV by RT-qPCR but much better
118 reduction of viable FRNAP. In order to address the question of whether the viability
119 of NoV might be being reduced more than the reduction in PCR signal (assuming

FRNAP viability is a satisfactory indicator of NoV viability), the trials in our study were run with FRNAP genogroup II (FRNAP-II) by both RT-qPCR and viability assay testing alongside NoV testing by RT-qPCR. FRNAP-II was used in this study due to its association with human faecal contamination (Lee et al., 2011; Stewart-Pullaro et al., 2006).

The effect of feeding shellfish during depuration has been inconclusive in studies reported so far (Mcleod *et al*, 2017) and so this study also aims to investigate this further.

We focused on Pacific oysters (*Crassostrea gigas*), as this is the species that dominates the oyster trade in Europe and is implicated in most bivalve-associated illness outbreaks.

2. Material and methods

2.1 Source of contaminated oysters

Experiments were conducted using oysters naturally contaminated from the environment. This was partly because of difficulties encountered in previous studies in reliably contaminating oysters experimentally using either primary sewage effluent or a concentrated clinical source of NoV (the latter, in any case, being difficult to obtain). It was also considered that naturally contaminated oysters would give the most representative illustration of ‘real-life’ NoV depuration behaviour. Given the seasonal occurrence of NoV in the UK, and needing to ensure sufficient levels of NoV, trials had to be limited to a restricted window from December to March when NoV levels in the community are normally at their highest.

143 A large quantity of oysters was placed near a continuous sewage outfall serving a
144 sewage treatment works with secondary treatment and storm overflow facility (dry
145 weather flow 6,800m^3 per day). These oysters were kept *in situ* for approximately 2
146 weeks prior to the start of the trials. After 2 weeks of this contamination, oysters were
147 washed with potable water on arrival at the laboratory to remove any external
148 sand/sediment in line with usual commercial practice and legislative requirements.

149 **2.2 Depuration experiments**

150 All experiments, apart from experiment 3, were undertaken in identical stnadrad
151 design small-scale shallow tank commercial depuration systems (Seafish, 2018)
152 manufactured by TMC (Tropical Marine Centre, UK). These were carried out at the
153 Cefas experimental tank facility using a 12 hour light/dark regime (except in the
154 light/dark experiment 4) approximating to the environment of a typical commercial
155 business premises. Tanks measured 1375 mm (length) by 1120 mm (width) by 750
156 mm (height), with a working volume of 600 litres. Oysters in these tanks were held in
157 Alibert 41042 trays, 2 layers of oysters per tray, maximum 125 oysters (average oyster
158 size 120g = maximum 15kg per tray). Trays in the bottom layer were raised off the
159 base of the tank by 25 mm to avoid recontamination by voided and settling faecal
160 material.

161 The depuration procedures employed followed UK commercial best practice (as
162 described in Seafish, 2018) unless otherwise stated. Seawater was recirculated
163 (except for experiment 3) as standard for these commercial systems at a rate of 25
164 litres/min and disinfected by passing recirculated water via a chamber containing 2 x
165 25 watt UV lamps. The temperature of seawater in the tanks was maintained by TECO
166 Seachill TR60 chiller/heaters. Dissolved oxygen levels were monitored and

167 maintained at around 7.5 mg/L, well above the 50% saturation level (broadly
168 equivalent to 5mg/L at 15°C) generally recognised as being the minimum advisable
169 for the depuration of oysters (FAO, 2009). The working volume of water in each
170 experiment was adjusted to ensure the shellfish to water ratio reflected that of
171 commercial purification practices for this type of system in the UK i.e. minimum 1:6
172 shellfish to water ratio.

173

174 For experiment 3, smaller modified experimental tanks (approx. 1000 mm length, by
175 50 mm width, by 20 mm depth) were used due to the more specific water supply
176 requirements for this study - natural unfiltered seawater on continuous replacement
177 was used. Oysters in these tanks were not placed in trays but were instead placed
178 directly on mesh inserts covering the base of the tanks to raise them approximately
179 3cm above the bottom.

180 All waters discharged from these experiments to the sewerage network from the Cefas
181 experimental facility were sterilised with ozone to prevent any release of pathogens
182 into the wider environment.

183

184 **2.3 Experiment 1 - Time and temperature**

185 120 oysters were placed in each of 2 tanks of natural filtered and UV disinfected
186 seawater at 12°C. The water temperature of one tank was then gradually adjusted to
187 8°C and the other to 18°C over a period of 12hrs to avoid temperature shocking or
188 stressing the oysters. The 120 oysters in each tank were evenly distributed across 4

189 trays stacked in 2 levels in 400L of seawater. After a period of 2.5 days (c. 60 hours)
190 the tanks were carefully drained down to below the top trays of oysters which were
191 then carefully removed, ensuring no disturbance of any settled shellfish faecal material
192 that may cause recontamination of the oysters still submerged below. The remainder
193 of the oysters continued the depuration process for a further 2.5 days. At the end of
194 the cycle, the tanks were drained down and the remaining oysters were removed for
195 analysis and shelf life testing.

196 **2.4 Experiment 2 - Salinity**

197 120 oysters were placed in each of 2 tanks of natural filtered and UV disinfected
198 seawater at 12°C. One tank was maintained at 35 parts per thousand (ppt) seawater
199 (effectively ‘full strength’ seawater around the UK) and the other was diluted with
200 potable quality freshwater to 25ppt, more typical of the estuarine salinity with which
201 the oysters we used would have been more familiar. The 120 oysters in each tank
202 were divided equally between 4 trays stacked in 2 levels in 400L of seawater. The
203 sampling process and arrangements were as described above for experiment 1.

204 **2.5 Experiment 3 - Natural flow-through seawater vs filtered seawater.**

205 240 oysters were divided evenly across 4 smaller purpose-built tanks (approximately
206 80 in each) in the Cefas secure experimental facility with controlled flow-through of
207 natural seawater – this was only available in this facility and not the room used for the
208 other experiments. Two of the tanks were supplied with unfiltered seawater and the
209 remaining two with 2μm filtered seawater. Each source of water was warmed to 15°C
210 and passed through the tanks at a rate of 2L/min to maintain sufficient dissolved
211 oxygen levels. The use of 4 tanks rather than 2 (as was used for the other

212 experiments) was due to the smaller size of the tanks used in this particular experiment
213 – twice the number of tanks had to be used to ensure a sufficient number of oysters
214 for testing under the 2 different treatments. The sampling process was essentially as
215 described above for experiment 1.

216 **2.6 Experiment 4 - Light vs Dark**

217 120 oysters were placed in each of 2 tanks with one being kept in 24hr darkness and
218 the other maintained in 24hr light (normal fluorescent strip lights). Seawater salinity
219 was maintained at 35ppt, with it being UV disinfected and maintained at 15°C. The
220 sampling process was as described above for experiment 1.

221 **2.7 Experiment 5 - Feeding**

222 360 oysters were divided evenly across 3 tanks containing filtered natural seawater
223 maintained at 14°C. One tank of oysters was given a daily feeding regime of live algae
224 grown on-site; a second tank of oysters was given a daily feeding regime of a
225 commercially available algal feed (details below) and a third tank of oysters was not
226 fed at all.

227 The commercial algal feed used was Shellfish Diet 1800® (Reed Mariculture). This is
228 a concentrated blend of 4-20 µm algae used for oyster hatcheries and broodstock
229 containing *Isochrysis, Pavlova, Tetraselmis*, *Chaetoceros calcitrans*, *Thalassiosira*
230 *weissflogii* and *Thalassiosira pseudonana*.

231 The Shellfish Diet was administered to the tanks after first passing through a 50 µm
232 sieve to disrupt any aggregations of cells. The feed was added to the tanks at the

233 manufacturer's recommended dose of 0.3 – 1.2 ml of formulation per adult animal per
234 day.

235 The live algae were grown at Cefas and comprised 3 species: *Diacronema* (previously
236 known as *Pavlova*), *Tetraselmis* and *Chaetoceros.* A suspension of these was added
237 to the oyster tanks twice a day to obtain a similar cell concentration in the experimental
238 tanks to that of the Shellfish Diet at approximately 2 x 10^6 cells per ml.

239 Pouring feed into the tanks was undertaken carefully so as to avoid causing any
240 resuspension of settled shellfish faecal material in the tanks which have might
241 recontaminated the oysters.

242 The sampling process and arrangements were as described above for experiment 1.

243

244 **2.8 Experiment 6 - Disturbance**

245 120 oysters were placed in each of 2 tanks of natural filtered seawater of salinity 35ppt
246 and maintained at 12°C. One tank was recirculated as per commercial practice with
247 the pump attached to the side of the tank running continuously. It has been suggested
248 that vibration from the pump might cause some disturbance of the shellfish so this was
249 the 'disturbance' treatment. A second tank was modified with an overflow to allow
250 continuous flow-through of filtered seawater from a remotely pumped source without
251 the tank's own pump running, thus no vibration or 'disturbance' of the shellfish. The
252 sampling process and arrangements were as described above for experiment 1.

253 **2.9 Sampling**

254 The studies reported here were all run over a period of 4 to 5 days. For all treatments, 255 three oyster samples were taken and tested at each of three time points: before 256 depuration, mid-way through the cycle and at the end of the cycle. Each sample 257 comprised a minimum of 10 individual oysters to allow for differences in NoV levels 258 between them.

259 For the commercial scale tanks used in all experiments except experiment 3, trays 260 were arranged in such a way that one sampling occasion equated to one full layer of 261 trays. At each sampling time point, the tanks were carefully drained down to below 262 the level of the oyster trays. Oysters to be sampled (no longer submerged) were then 263 carefully removed, this was to ensure that there was no resuspension of any settled 264 shellfish faecal material which might contaminate any remaining shellfish submerged 265 in the trays below. The remainder of the oysters continued the depuration process 266 until the end of the study cycle (day 4 or 5). At the end of the cycle, tanks were drained 267 down and the remaining oysters were removed for analysis and shelf-life testing.

268

269 Each study treatment was run in a single separate tank e.g. for the temperature studies 270 one tank was run at 8°C and the other at 18°C. Resource constraints prevented 271 running tanks in duplicate at each treatment.

272

273 **2.10 Shellfish testing**

274 **Viral RNA extraction and RT-qPCR**

275 For quantification of NoV and F+ bacteriophage genogroup II (FRNAP-II), a sub-
276 sample of oysters were shucked and the digestive glands dissected. These glands
277 were further divided into sub-samples for RNA isolation and FRNAP-II culture. RNA
278 isolation and RT-qPCR analysis were carried out for NoV genogroups I and II
279 according to ISO-15216-1. Briefly, two grams of digestive gland were finely chopped
280 and digested in a solution of 100 µg/ml proteinase K. The homogenate was
281 centrifuged and the supernatant was retained. RNA was isolated from 500 µl of
282 supernatant using the NucliSENS magnetic bead system (BioMerieux, France) and
283 eluted into 100 µl of elution buffer. RT-qPCR for NoV was carried out using the QNIF4
284 and NV1LCR primers, and TM9 probe for GI (da Silva et al. 2007; Hoehne and
285 Schreier 2006; Svraka et al. 2007) and the QNIF2 and COG2R primers, and QNIFS
286 probe for GII (Kageyama et al. 2003; Loisy et al. 2005). Triplicate 25 µl total volume
287 RT-qPCR reactions were carried out using the RNA UltraSense® one-step RT-qPCR
288 system (Invitrogen) (final concentrations of 1× Reaction Mix, 500 nM forward and 900
289 nM reverse primers, and 250 nM probe, plus 0.5 µl Rox and 1.25 µl Enzyme Mix per
290 reaction). The isolated RNA was further analysed by RT-qPCR to enumerate FRNAP-
291 II in the same way as NoV but using the FRNAP Genogroup II primers and probes
292 (Wolf et al. 2008).

293 **FRNAP-II bioassay**

294 Infectious FRNAP-II was quantified in the remaining digestive glands using a 5x3
295 most-probable number (MPN) method followed by confirmatory RT-qPCR as follows.
296 A host culture was prepared by growing *Escherichia coli* HS(pFamp)R) (ATCC
297 700891) at 37°C for 16 hours in tryptone yeast glucose broth (TYGB) consisting of 1%
298 tryptone (Oxoid, UK), 0.1% yeast extract (Oxoid, UK) and 0.8% sodium chloride in

299 deionised water. Digestive gland tissue was homogenised in a 1:1 mass ratio of 0.1%
300 peptone water (Oxoid, UK) using a Waring blender before being centrifuged at 3000 x
301 g for 10 minutes and the supernatant retained. Two tenfold serial dilutions of the
302 supernatant were made in 0.1% peptone water for use in the MPN assay. A phage
303 culture broth was created containing 0.38‰ calcium chloride dihydrate, 1.25‰
304 glucose, 0.06‰ streptomycin sulfate, 0.06‰ ampicillin, 12.69‰ tryptone, 1.27‰ yeast
305 extract 10.15‰ sodium chloride and 12.5% host culture. MPN culture was carried out
306 in 2 ml wells of a 96-well plate using 800 µl of phage culture broth and 200 µl of
307 supernatant or diluted supernatant. For each sample, five replicates were used for
308 each supernatant dilution. Negative and positive growth controls were included on
309 each plate. For the negative growth control, 200 µl 0.1% peptone water was used in
310 place of the supernatant. For the positive growth control, 200 µl of the FRNAP-II
311 reference strain GA was used at 1000 PFU/ml. Plates were covered with a breathable
312 adhesive cover (Breathe-EASIER, Sigma-Aldrich, UK) and incubated at 37°C for 16
313 hours. Following incubation, 100 µl of chloroform was added to ach culture well and
314 mixed by repeat pipetting. The plates were covered and centrifuged at 3000 xg for 10
315 minutes and the aqueous layer was retained for further analysis. Supernatants were
316 diluted 100-fold in Tris-EDTA buffer, mixed by vortex and heated to 60°C for 5 minutes
317 and allowed to cool to room temperature. This was then used as an RNA template in
318 RT-qPCR. RT-qPCR was carried out in the same way for FRNAP-II as described
319 previously, but using a total reaction volume of 6 µl and using 2 µl of RNA template.
320 Additionally, in each RT-qPCR reaction, mengovirus RNA (10,000 gc/µl) and primers
321 and probes were used as an internal amplification control. A negative amplification
322 control (Tris-EDTA buffer) and a positive amplification control (5000 gc/µl GA RNA)
323 were used in each RT-qPCR run. RT-qPCR reactions negative for both FRNAP-II and

324 mengovirus were considered to be inhibited and were repeated. The MPN for FRNAP-
325 II was calculated using the combination of positive and negative RT-qPCR reactions
326 according to Jarvis et al. (2010).

327

328 **2.11 Shelf life test**

329 At the end of each trial, 12 similar-sized oysters from each treatment regime were
330 placed and held in open plastic bags in a refrigerator at 4°C. Oysters were then
331 checked periodically (every 3 to 4 days) and assessed for mortality. Death was
332 confirmed by oysters not responding to a percussion test (i.e. not closing upon
333 tapping) or were obviously gaping.

334

335 **2.12 Data analysis**

336 All statistical analyses were performed using R statistical software R (R Core Team,
337 2019) and the graphics package *ggplot* (Wickham, 2016). A linear regression was
338 performed for each trial for estimating the treatment effect on NoV and bacteriophage
339 counts over time. Data was log-transformed for satisfying the normality of the
340 residuals. As a unique set of samples was collected before treatment; pre-depuration
341 data was treated as the initial datapoint for each treatment level.

342

343 **3. Results**

344 The study focused on concentration changes in replicated samples taken from the
345 same experimental unit. The variability between the samples was taken into account
346 when fitting the linear model to the data and is reflected by the significance of the
347 estimates.

348 As shown in Tables 1 and 1a, very few treatments had a statistically significant effect
349 at the 5% level (bold p-value, Table 1) on NoV and phage counts over time. In Table
350 1, the sign of the treatment effect is indicated after each p-value ("+" = increase, "-" =
351 decrease). These results demonstrated that the temperature and salinity trials
352 returned the most promising results in terms of NoV removal. Table 1a represents
353 quantitation values for the different viruses in terms of change in concentration per
354 hour and these are directly associated with the p-values from Table 1.

355

356 The results of the temperature trial and salinity trials are plotted using a simple line
357 graph (figures 1 and 2) – the average result of three samples is plotted at each data
358 point. The variability between the samples was taken into account when fitting the
359 linear model to the data and is reflected by the significance of the estimates (rather
360 than indicating the standard deviation). Comparing these graphs it can be seen that
361 temperature appears to show the most consistent and convincing pattern of removal.
362 Datapoints for the salinity trials appear to be higher at the end of the cycle than mid-
363 cycle for NoV GI at 35ppt and FRNAP II MPN at 35 ppt, suggesting an increase.
364 However, this may be a reflection of the variability in results within the triplicate testing
365 and the fact that some of these results were close to the limit of quantification for the
366 relevant test.

367

368 Figure 3 shows the individual results plotted with regression lines added for the
369 temperature trial. This shows a higher degree of NoV GII removal than NoV GI, with
370 FRNAP-II being better removed than both (pattern of FRNAP-II by PCR removal being
371 similar in terms of gradient to FRNAP-II by MPN), which is confirmed by lower p-values
372 for FRNAP-II than NoV GII and GI (Table 1).

Treatment	Factor	GI	GII	FRNAP-II
Temperature	*8*	0.78 -	0.10 -	**0.02 -**
	18	0.66 -	**0.01 -**	**0.006 -**
Salinity	*25 ppt*	**0.002 -**	**0.003 -**	**0.0002 -**
	35 ppt	0.29 -	**0.014 -**	**0.006 -**
Light	*Light*	0.42 +	**0.02 -**	0.77 +
	Dark	0.85 +	0.09 -	0.17 -
Filter	*Filtered*	0.39 +	**0.04 -**	0.73 -
	Unfiltered	0.79 +	0.09 -	0.80 -
Feeding	*Algae*	0.29 -	0.048 -	0.50 -
	Diet	0.21 -	**0.017 -**	0.08 -
	No Food	0.052 -	**0.003 -**	0.13 -
Disturbance	*Disturbance*	0.21 +	0.51 -	0.07 +
	No Disturbance	0.86 +	0.59 -	0.42 +

373

374 *Table 1. Numbers in GI, GII and FRNAP columns represent P-values associated with*
375 *the effect of the treatment estimated with linear regressions by treatment levels over*
376 *time (null hypothesis H0: the treatment has no effect on NoV or phage counts over*
377 *time). Significance level is chosen at 5% (values in bold represent p-values under the*
378 *5% level).* "+" represents increase, "-": represents decrease.

379

Treatment	Factor	GI	GII	FRNAP-II
Temperature	*8*	-1.525	-2.610	**-11.480**
	18	-2.305	**-5.916**	**-14.030**
Salinity	*25 ppt*	**-11.370**	**-10.045**	**-5.295**

	35 ppt	-5.988	**-9.215**	**-4.833**
Light	*Light*	1.739	**-2.467**	0.343
	Dark	0.670	-2.792	-1.698
Filter	*Filtered*	2.493	**-2.406**	-0.413
	Unfiltered	0.563	-2.507	-0.351
Feeding	*Algae*	-9.358	-13.594	-1.448
	Diet	-12.920	**-18.743**	-3.750
	No Food	-13.587	**-20.469**	-2.937
Disturbance	*Disturbance*	5.280	-2.724	1.252
	No Disturbance	1.358	-3.684	1.434

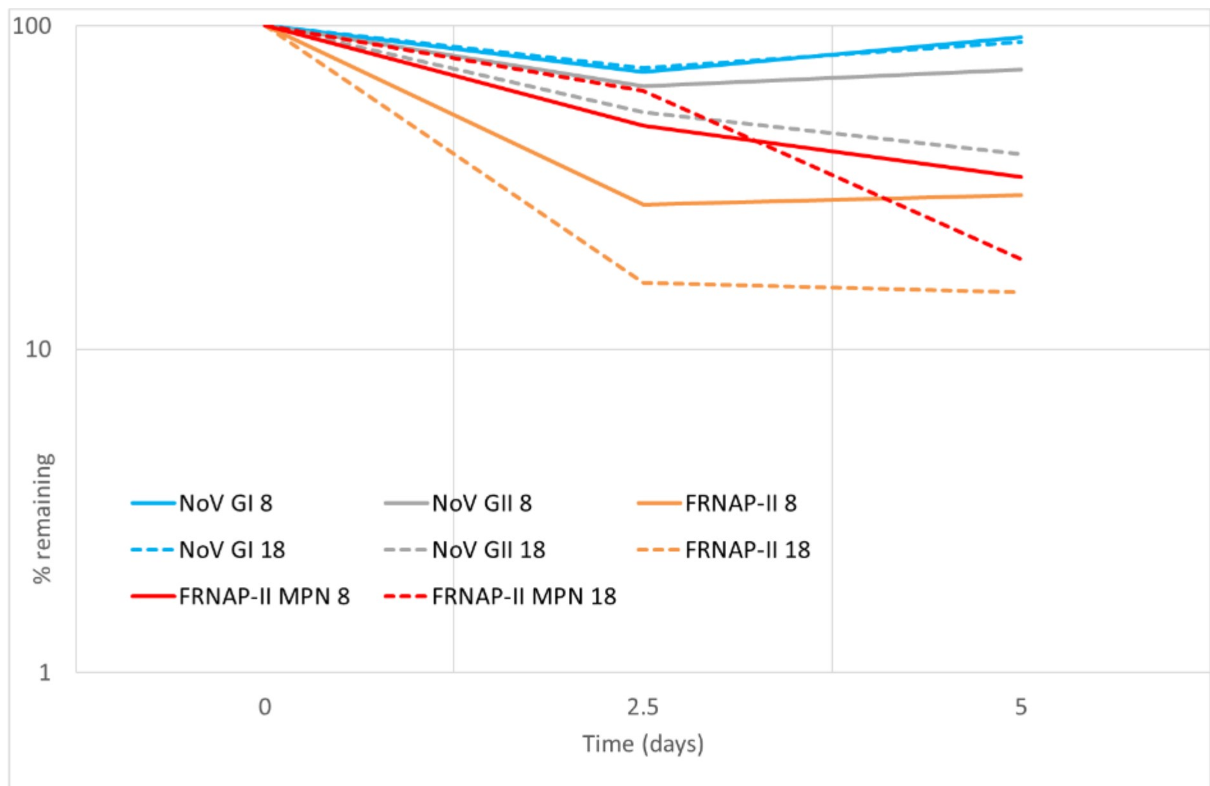
380

381 *Table 1 a. Table summarises the estimates for the different viruses and treatments.*

382 *The values correspond to the change in concentration per hour and are directly*

383 *associated with the p-values from Table 1.*

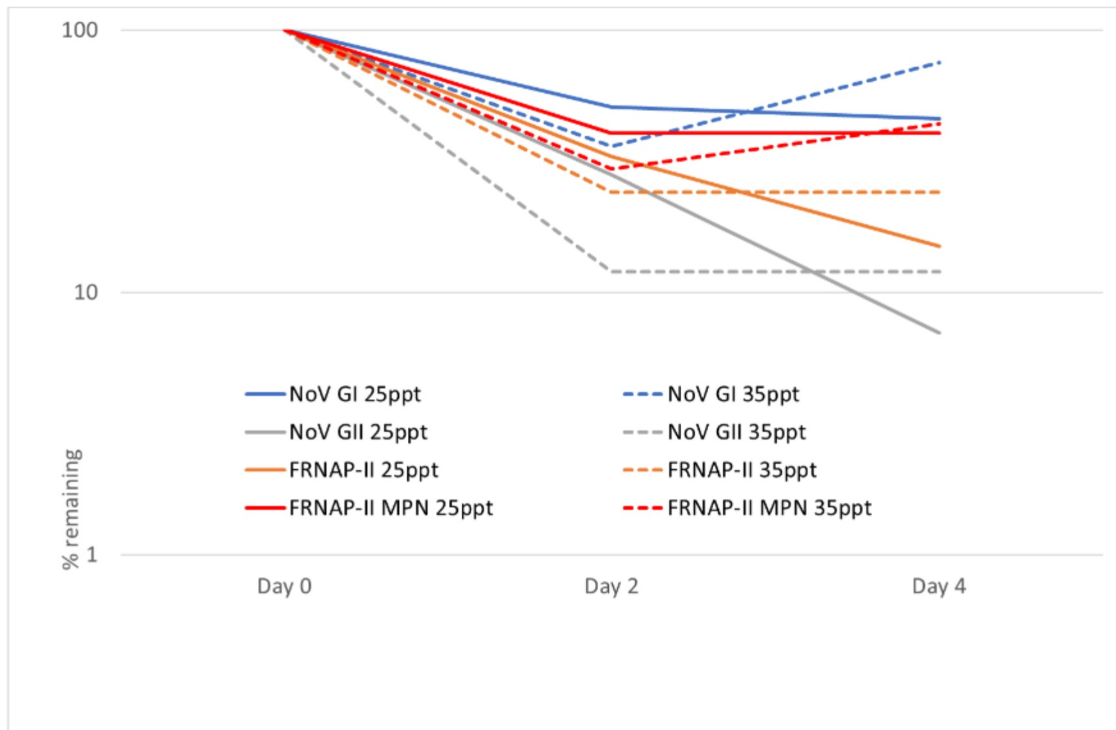
384



385

386 *Figure 1. Removal of NoV with time at temperatures of 8 and 18°C*

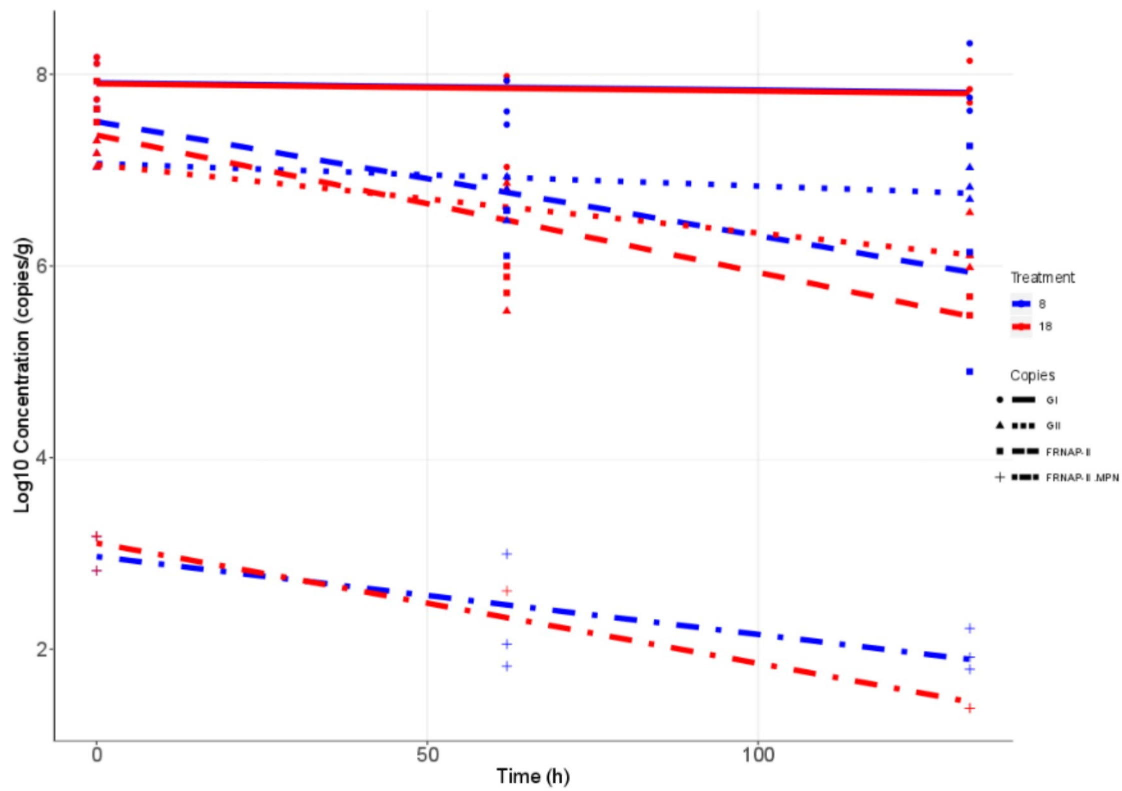
387



388

389 *Figure 2. Removal of NoV with time at salinity 25 and 35ppt.*

390



391

392 *Figure 3. Comparison of NoV GI, NoV GII, FRNAP-II by PCR and MPN bioassay removal. Observed*
393 *individual counts (copies/g - points) over time (h) with linear regression (line) by treatment (blue: 8ºC;*
394 *red: 18ºC). GI: solid line and circle point; GII: dotted line and triangle point; FRNAP-II by PCR: dashed*
395 *line and squared point; FRNAP-II by MPN boassay: alternate dotted and dashed line with + symbol.*

396

397 In summary, we found approximately 46% removal of NoV GII at 18°C after 2 days
398 and 60% after 5 days compared with a maximum of 16% NoV GI removal. Twice the
399 rate of NoV GII removal was achieved at 18°C compared with 8°C after 5 days. Trials
400 show better NoV removal at salinity closer to that prevailing in the harvesting area (25-
401 30ppt). Trials investigating the effect of algal feeding, light/dark and disturbance from
402 pump vibration vs no vibration did not produce any significant effects in terms of NoV
403 removal.

404

405 **4. Discussion**

406 The analytical costs and practicalities of running the trials limited the number of
407 samples and replicates collected by treatment. Although the statistical analysis may
408 lose power with small sample sizes, the study provides an initial indication of the
409 treatment trends and effects. Another limitation of this work was that NoV levels in the
410 environment were falling as trials progressed to the extent that, in the final validation
411 trial, pre-depuration levels were close to the limit of quantification for the test.
412 Consequently, the results of this final trial in particular are considered to be of little use
413 and are not included.

414 Figure 3 highlights that FRNAP-II would not appear to be a satisfactory indicator of
415 NoV under the depuration conditions employed in these trials as it is more readily
416 removed. The key feature of an indicator is that it should be present in higher numbers
417 than the target pathogens and, in a depuration context, should show a similar or lesser
418 tendency for removal. Furthermore the removal rates of FRNAP-II as shown by PCR
419 (i.e. presence of genome) and bioassay (presence of viable phage) appear to be
420 similar, although starting values were different, suggesting that the decreasing values
421 for both as the depuration cycle progresses may be due mainly to actual removal of
422 bacteriophage from the shellfish rather than any inactivation of virus remaining *in situ*.
423 In other words, the reduction in bacteriophage values appears to be due largely to
424 removal rather than *in situ* inactivation.
425

426 In terms of NoV removal, these trials provide evidence that elevated temperature
427 during depuration can achieve statistically significant removal of NoV, however, the
428 extent of removal appears to depend on the strains of NoV present. Our trials showed
429 consistently better removal of NoV GII compared with NoV GI. We found
430 approximately 46% removal of NoV GII at 18°C after 2 days and 60% after 5 days
431 compared with a maximum of 16% NoV GI removal. Twice the rate of NoV GII removal
432 was achieved at 18°C compared with 8°C after 5 days. The difference in removal may
433 be due to differences between NoV strains in their propensity for binding to the oyster
434 gut as reported by Maalouf *et al*, 2011. It should be noted, however, that non-
435 infectious NoV particles may also be more easily removed as damaged capsids could
436 have reduced binding ability (Manuel *et al,* 2018) and this effect may be significant if
437 the proportion of damaged capsids differed between the two genogroups found in
438 these trials.

439

440 From a practical point of view, our findings suggest that depuration can be effective in
441 reducing NoV levels and thus the potential for illness in oysters consumers, but the
442 level of effectiveness will depend on the strains circulating in the environment which
443 changes over time as new strains emerge.

444

445 Our trials show that salinity levels had a statistically significant effect on NoV removal
446 (better removal at 25ppt than 35ppt) and this is likely to relate to the range of salinities
447 that our oysters had experienced in the production area from which they had been
448 harvested. These originated from an upper estuarine source where salinities typically
449 range from 25-30ppt.

450

451 It is well known that both temperature and salinity have a fundamental effect on bivalve
452 physiology and can influence their level of activity so our findings with respect to these
453 two factors are not unexpected. The filtering activity of Pacific oysters in the UK has
454 been found to decline to negligible levels below 8°C, hence this value being
455 recommended for this species in the UK as a minimum in commercial depuration
456 facilities (Seafish, 1994) and this being the lower temperature value chosen for our
457 study. Similarly, the maximum recommended depuration temperature for Pacific
458 oysters in the UK to avoid unduly stressing them is 18°C and so this value was chosen
459 as the upper temperature in this study.

460 Lower salinity values have been found to be limiting for Pacific oysters in depuration
461 with a minimum value of 20.5ppt being recommended in the UK for commercial
462 depuration facilities (Seafish, 2018). The salinity values chosen in our trials were

463 25ppt, to be similar to that experienced in the harvesting area from which the trial
464 oyster were obtained and 35ppt, the maximum encountered in UK waters.

465 Shelf life experiments for the most successful temperature and salinity regimes trialled
466 showed no difference up to 12 days post-depuration, suggesting these options might
467 be commercially viable from the subsequent shellfish saleability point of view.

468 Trial results suggested that NoV removal was not significantly influenced by feeding
469 the oysters, according to the algae preparations we used. In fact there was a
470 suggestion that NoV removal might have been very slightly better with no feeding at
471 all. Table 1 indicates a possible effect of NoV GII removal with both Shellfish Diet but
472 the highest level of significance was with no feeding.

473 There was no obvious difference found in NoV removal between depurating shellfish
474 in a light or dark environment.

475 Similarly, no benefit was observed between using either filtered or unfiltered (i.e.
476 potential food particles removed) seawater i.e. No difference between natural
477 seawater flowing through the system on a constant renewal basis and recirculated
478 seawater in a closed system.

479 Finally, we found no difference in NoV removal between 'disturbance' from pump
480 vibration and no disturbance i.e. vibration from the pump attached to the tank did not
481 appear to adversely affect NoV removal.

482 In terms of removal rates observed in our study compared with previous studies using
483 Pacific oysters, findings in the literature vary. The general observation from all these
484 studies can be made that the combination of increased time and temperature do help
485 increase removal of NoV, but the extent varies. One possible explanation for this may
486 be due to differences in binding between NoV strains previously described. Rupnik *et*

487 *al*, 2018 reported that depuration for 9 days at an elevated temperature of 13.3°C was
488 able to achieve 90% of oysters <100 copies per gram (only 55% had <100 copies per
489 gram pre-depuration) and these oysters were found to be commercially viable for sale
490 at the end of the depuration cycle. Results from a study by Greening *et al* (2003)
491 suggested 10.6 days were required to achieve a 1 log reduction in NoV GII at 12-18°C.
492 Dore *et al*, 2010 report 23.1 days to achieve the same level of reduction of NoV GII at
493 15-17°C. Previous studies at our laboratory reported a 0.5 log reduction of GII NoV at
494 16°C, in a UV system (Neish 2013). Total Norovirus reduction (no distinction was
495 made in these trials between GI and GII) was more apparent at 16°C than 8°C,
496 although even after 14 days depuration, 59% of the initial norovirus load was still
497 remaining at this temperature. Our current study showing 60% (approximately 0.5 log)
498 removal of GII after 5 days at 18°C represents a marked improvement on the Neish
499 study results at 16°C and is perhaps more similar in terms of trajectory to the findings
500 of the Greening study.
501
502 Polo *et al* 2014 found a significant difference in the viral uptake and removal rates
503 between Manila clams (*Venerupis philippinarum*) and Mediterranean mussels (*Mytilus*
504 *galloprovincialis*) using murine norovirus as a surrogate for human norovirus. This
505 suggests that differences between species in terms of their NoV removal are possible
506 and that the results from our trials may therefore not be extrapolated to other species
507 without specific confirmatory studies.
508
509

5. Conclusions

- These trials provide evidence that elevated temperature during depuration can achieve significant removal of NoV, however, the extent of removal appears to depend on the strains of NoV present. Our trials showed consistently better removal of NoV Genogroup II (GII) compared with NoV Genogroup I (GI). We found approximately 46% removal of NoV GII at 18°C after 2 days and 60% after 5 days compared with a maximum of 16% NoV GI removal. We found no difference in shelf-life between oysters from trials held at 8 or 18°C for up to 12 days post depuration.
- Twice the rate of NoV GII removal was achieved at 18°C compared with 8°C after 5 days.
- Slightly improved NoV removal was found at 25ppt compared with 35ppt. This difference may relate to the range of salinities that the oysters have experienced in the production area from which they have been harvested and suggests the importance of ensuring that salinities in depuration tanks are matched as closely as possible to the typical salinity prevailing in the harvesting area from which the shellfish have originated.
- No effect of feeding was observed, in fact our trials suggest that NoV removal may be slightly better with no feeding at all.
- No obvious difference was found between depurating shellfish in a light or dark environment.
- No benefit of filtered vs unfiltered water was observed i.e. No difference between natural seawater flowing through the system on a constant renewal basis and recirculated seawater in a closed system.

534 • Vibration from the pump attached to the tank did not appear to make any
535 difference to NoV removal compared with the no vibration control.

536

537 ***Acknowledgements***

538 *This project has received funding from the European Union's Horizon 2020 research and*
539 *innovation programme under Grant Agreement no. 773400 (SEAFOODTOMORROW). This output*
540 *reflects the views only of the author(s), and the European Union cannot be held responsible*
541 *for any use which may be made of the information contained therein.*

542

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668

669 Tables and Figures:

670 Table 1

Treatment	Factor	GI	GII	FRNAP-II
Temperature	*8*	0.78 -	0.10 -	**0.02 -**
	18	0.66 -	**0.01 -**	**0.006 -**
Salinity	*25 ppt*	**0.002 -**	**0.003 -**	**0.0002 -**
	35 ppt	0.29 -	**0.014 -**	**0.006 -**
Light	*Light*	0.42 +	**0.02 -**	0.77 +
	Dark	0.85 +	0.09 -	0.17 -
Filter	*Filtered*	0.39 +	**0.04 -**	0.73 -
	Unfiltered	0.79 +	0.09 -	0.80 -
Feeding	*Algae*	0.29 -	0.048 -	0.50 -
	Diet	0.21 -	**0.017 -**	0.08 -

	Factor			
	No Food	0.052 -	**0.003 -**	0.13 -
Disturbance	*Disturbance*	0.21 +	0.51 -	0.07 +
	No Disturbance	0.86 +	0.59 -	0.42 +

671 Table 1a

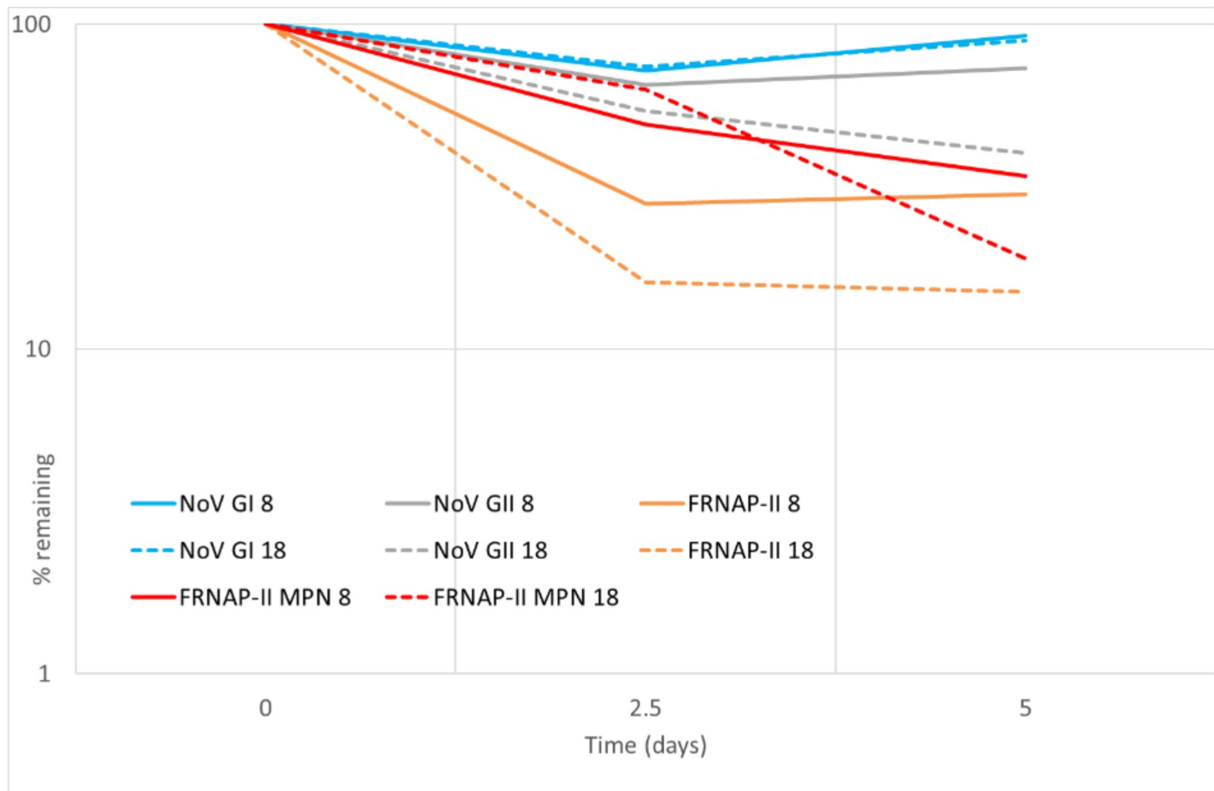
672

Treatment	Factor	GI	GII	FRNAP-II
Temperature	*8*	-1.525	-2.610	**-11.480**
	18	-2.305	**-5.916**	**-14.030**
Salinity	*25 ppt*	**-11.370**	**-10.045**	**-5.295**
	35 ppt	-5.988	**-9.215**	**-4.833**
Light	*Light*	1.739	**-2.467**	0.343
	Dark	0.670	-2.792	-1.698
Filter	*Filtered*	2.493	**-2.406**	-0.413
	Unfiltered	0.563	-2.507	-0.351
Feeding	*Algae*	-9.358	-13.594	-1.448
	Diet	-12.920	**-18.743**	-3.750
	No Food	-13.587	**-20.469**	-2.937
Disturbance	*Disturbance*	5.280	-2.724	1.252
	No Disturbance	1.358	-3.684	1.434

673

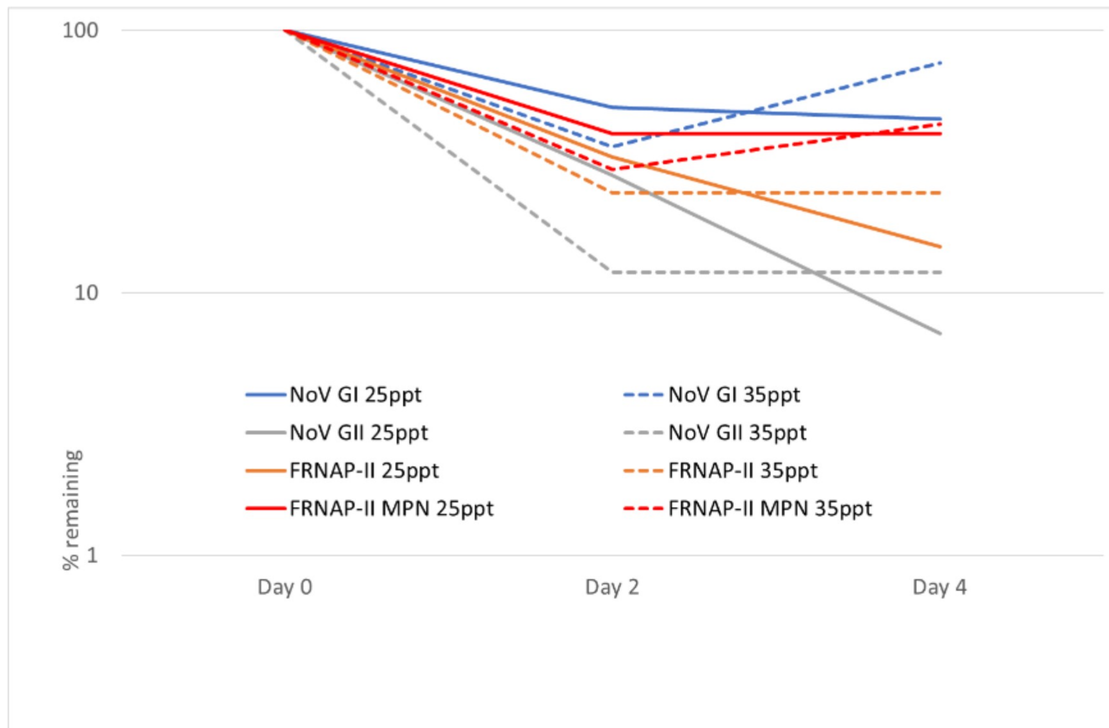
674

675



676 Figure 1

677



678 Figure 2

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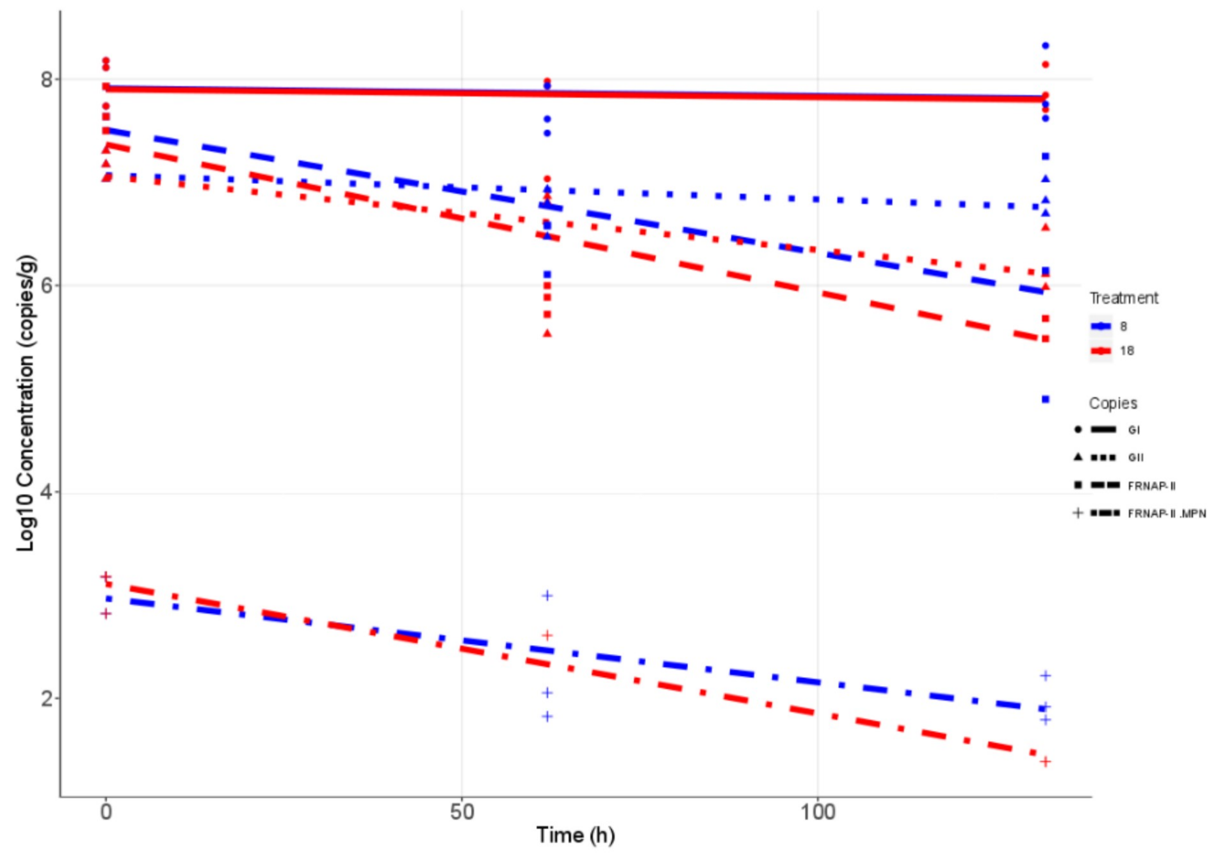

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681 Figure 3

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