1 Investigation of F-RNA bacteriophage as a tool in re-opening Australian oyster growing areas
2 following sewage spills.

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9   **Abstract**

10   Oysters contaminated with human enteric viruses from sewage are implicated in foodborne
11   outbreaks globally. Bacteriophages have been identified as potential indicators for these
12   viruses, but have not been used in shellfish management outside of the USA. This study
13   aimed to determine the background levels of F-RNA phage in five Australian oyster growing
14   areas with a history of sewage spills and closures, over an 18 month period. In addition,
15   oysters from five growing areas impacted by adverse sewage events were investigated for F-
16   RNA phage, *Escherichia coli*, norovirus (NoV) and hepatitis A virus (HAV). F-RNA phage
17   ≤ 60 pfu/100 gm shellfish flesh were found to represent a conservative background level in
18   the surveyed areas. Following two of the five sewage spills, elevated phage levels were
19   observed in most sample sites less than 4 days post spill. By 7 days, most sites from all events
20   had phage < 30 pfu/100 gm. NoV was detected in day 1 and day 6 samples from one event
21   when all phage were ≤ 30 pfu/100 gm. NoV was also detected in a day 3 sample from another
22   event with < 30 phage pfu/100 gm, however, multiple replicate samples had elevated phage
23   levels. The results of this study add evidence on the potential use of F-RNA phage as a tool in
24   early re-opening of oyster harvest areas post sewage spills. However, it also highlights the
25   need to better understand situations where phage testing may be ineffectual, and the
26   importance of sampling at multiple sites and over multiple time points, to effectively capture
27   evidence of contamination.

28   **Key words:** shellfish management; food safety; foodborne viruses; norovirus; hepatitis A
29   virus; *Escherichia coli* bacteriophage MS2.

30   **Introduction**

31   The human enteric viruses norovirus (NoV) and hepatitis A virus (HAV) are the most
32   commonly reported foodborne viral pathogens associated with shellfish harvested from

sewage contaminated waters (Bellou et al. 2013). Indicator bacteria, coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish and are internationally used in shellfish management programs (ASQAAC 2016; EC 2004; FDA 2015). Numerous studies have established that bacteria are inadequate as stand-alone indicators of the risk of human enteric viruses and do not reflect the presence or absence of enteric viruses (Lowther et al. 2018, Flannery et al. 2009; Dore and Lees 1995). Viruses are morphologically different to bacterial cells and exhibit significantly different resistance and susceptibility responses to environmental conditions including desiccation, UV irradiation and water and sewage treatment processes (Blatchley et al. 2007; Stewart et al. 2008).

Bacteriophage, or “phage”, have been identified as potential indicators or surrogates for human enteric viruses due to their similarities in morphology, behaviour in water environments and resistance to disinfectant treatments (Havelaar et al. 1986). Phages are viruses which specifically only infect bacteria. The somatic coliphage, male specific RNA coliphage (F-RNA phage or F-specific phage) and the phage of *Bacteroides* are of most interest for water quality testing and as indicators of viral pollution in shellfish (WHO 2001). In particular, F-RNA phage (*Lentiviridae*) are similar in size and morphology to many of the human enteric viruses, and also contain a single stranded RNA genome. Furthermore, F-RNA phage replicate in the gastrointestinal tract of animals and are unlikely to multiply in the aquatic environment (Havelaar et al. 1986).

In 2009, the US Interstate Shellfish Sanitation Conference officially included the application of F-RNA phage as an indicator of viral contamination of bivalve shellfish from sewage (FDA 2015). The US National Shellfish Sanitation Program (NSSP) permits re-opening of a growing area contaminated with raw untreated sewage earlier than the obligatory three week closure if shellfish samples collected at least 7 days after the contamination event have F-RNA phage levels that do not exceed the critical limit of 50 pfu/100 gm shellfish, or are

below predetermined background F-RNA phage levels. Similar regulations were introduced in the Australian Shellfish Quality Assurance Program (ASQAP) Operations Manual in 2016 (ASQAAC 2016), however, no prior studies into background levels of F-RNA phage in Australian growing areas or shellfish had been undertaken. The aim of this study was to establish the baseline levels of F-RNA phage in five Australian growing areas which have a history of being impacted by sewage spills and closures and to evaluate the potential to implement F-RNA phage testing as a management tool following sewage spills in shellfish growing areas in Australia.

Material and Methods

Background FRNA phage study

Five “at risk” Australian oyster growing areas as determined by a history of previous sewage spills, were selected to undertake an 18 month background study for F-RNA phage. Three growing areas in NSW, Camden Haven (Port Macquarie-Hastings Council; population 84,441), Brisbane Water (Central Coast Council; population 339,394) and ShoalhavenGoodnight Island and Berrys Bay (Shoalhaven Council; population 103,061), and two in Tasmania, Pittwater and Island Inlet (Sorell Council; population 14,774), were selected for the study (Table 1) (ABS, 2019). In Australia, growing area microbial risk management occurs through the use of environmental indicators and/or end-product testing. The environmental and bacterial triggers for harvest closure of these growing areas are outlined in Table 2. To determine background phage levels, sampling from these five growing areas was undertaken monthly for 18 months, from July 2016 to December 2017 inclusive, unless a potential sewage contamination event occurred. All background phage study shellfish samples were sent chilled on ice bricks, and received at SARDI Food Sciences Laboratories within 5 days (average 2.4 days and median 2.0 days) of collection. Arrival

temperatures ranged from 6.6°C to 28.6°C (average 16.3°C and median 16.7°C). Samples each consisted a minimum of 12 live oysters. Sydney Rock (*Saccostrea glomerata*) and Pacific oysters (*Crassostrea gigas*) were received from NSW and Tasmania, respectively. F-RNA phage enumeration was done within 24 hours of arrival; when necessary, samples were stored at $4 \pm 2^\circ\text{C}$ prior to processing.

Adverse sewage case studies

Five adverse sewage events were investigated. The aim was to examine a combination of large (>1000 kL/day) and small (<1000 kL/day) sewage events. The first event investigated was in Moulting Bay (Break O'Day Council; population 6,186) Tasmania in January 2017, a growing area not included in the background study (see Table 2 for closure triggers) (ABS, 2019). The remaining sewage event case studies all occurred in growing areas that were part of the background study. The location and dates of sewage events were as follows: Camden Haven, NSW (March 2017); Brisbane Water, NSW (March 2017); Camden Haven, NSW (December 2017); and Pittwater and Island Inlet, Tasmania (December 2017). Each case study aimed to collect samples from up to five sites potentially impacted by the sewage event based on criteria such as the water flow, geography and tides.

Oyster samples were collected as soon as possible following cessation of the sewage event, ideally one day following the event, 7 days post the event and 21 days post the event. The samples, each consisting of a minimum of 24 live oysters, were collected and transported on chilled ice bricks to SARDI Food Science Laboratories within 5 days (average 2.1 days and median 1.5 days) of collection. Arrival temperatures ranged from 5.4°C to 24.3°C (average 14.9°C and median 15.6°C). One dozen oysters were processed on arrival, or stored at $4 \pm 2^\circ\text{C}$ then processed within 24 hrs of receipt, for *E. coli* and F-RNA phage enumeration. The

105 remaining oysters (n=12) were stored at -80°C for NoV and HAV detection by quantitative
106 reverse transcriptase polymerase chain reaction (RT-qPCR).

107 **F-RNA phage enumeration**

108 The method used was as described in the Centre for Environment, Fisheries and Aquaculture
109 Science (Cefas) standard operating procedure 'Enumeration of male-specific RNA
110 bacteriophages in bivalve molluscan shellfish' Issue 7, 2007 (CEFAS 2007). In brief,
111 damaged or dead shellfish were discarded. Each oyster shell was washed and shucked
112 (n=12/sample). The shellfish flesh and liquor were collected, weighed then homogenised in
113 0.1% peptone water (PW; 1:2 w/v). Homogenates were assayed in batches and could be
114 stored for up to 48 hr at $4 \pm 2^\circ\text{C}$ prior to further analysis. The homogenate, 30-50 mL, was
115 centrifuged at $2,000 \times g$ at room temperature for 5 minutes and the supernatant was decanted
116 into a clean tube for analysis. Molten ($48-50^\circ\text{C}$) overlay tryptone yeast extract glucose agar
117 1% (TYGA1) containing 0.1% calcium glucose solution (3% $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ and 10% glucose)
118 and 0.01% nalidixic acid solution (2.5% nalidixic acid in 0.2M NaOH) was inoculated with
119 one mL of the *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F' 42 lac:Tn5);
120 NCTC 12484 host bacteria in early log phase and one mL of the sample supernatant
121 (n=10/sample). Overlays were gently mixed, poured onto the surface of the base complete
122 tryptone yeast extract glucose agar 2% (TYGA2) plate and incubated at $37 \pm 1^\circ\text{C}$ for 18 ± 4
123 hrs. Following incubation, the number of plaques were counted. The diameter of the plaques
124 is variable and dependent on the host cell density and thickness of the overlay. Typically, F-
125 RNA phage produce small to medium (0.2-1.0 mm) opaque, circular plaques. Plaques
126 exhibiting typical DNA phage morphology (clear lysis centres and approximately 6 mm
127 diameter) were noted but not counted in the total.

128 Results are expressed as plaque forming units (pfu) per 100 gm shellfish flesh as calculated
129 by the following formula:

130
$$C_{\text{pfu}} = [(N - N_{\text{RNase}}) / n \times F] \times 300$$

131 C_{pfu} confirmed number of F-RNA phage

132 N total number of plaques counted

133 N_{RNase} number of plaques counted on RNase plates (RNase plates were not inoculated for the
134 background study)

135 n number of replicates

136 F dilution factor

137 Each batch of samples tested included various controls including a blank of 0.1% PW and
138 two positive *E. coli* bacteriophage MS2 (ATCC 15597) controls, one at the start of the assay
139 and another at the end, to confirm host bacteria WG49 susceptibility to F-RNA phage
140 (CEFAS 2007). If no phage were detected, the result was expressed as <30 pfu/100 gm
141 shellfish flesh (the limit of detection for this assay). The adverse sewage event study included
142 the addition of RNase overlay agar plates for each sample. This involved the inoculation of a
143 parallel series of overlay agar plates with the addition of $100 \pm 1 \mu\text{L}$ RNase solution (1
144 mg/mL) to each molten TYGA1 sample overlay as described above. The addition of RNase
145 facilitates confirmation of the results. The plaques on the RNase plates are DNA phage,
146 which are subtracted from the total number of plaques on the standard overlay plates.

147 ***Escherichia coli* Most Probable number (MPN)**

148 The method was as described in the New Zealand Ministry for Primary Industries
149 'Enumeration of *Escherichia coli* in Bivalve Molluscan Shellfish, Version 9, 7 February
150 2013' (MPI 2013) with the modification that the homogenate prepared in 0.1% PW for the F-

151 RNA phage analysis was used. The homogenate had been prepared as a 1/3 dilution (2 mL
152 0.1% PW for each 1 gm shellfish). This was further diluted to 10^{-1} by the addition of 70 mL
153 of Peptone saline solution (PSS) to 30 mL of homogenate followed by preparation of a 10^{-2}
154 dilution in PSS. The MPN test was inoculated as follows: five tubes of double strength
155 minerals modified glutamate broth (MMGB) with 10 ± 0.2 mL of the 10^{-1} homogenate, five
156 tubes of single strength MMGB with 1 ± 0.1 mL of the 10^{-1} homogenate and five tubes of
157 single strength MMGB with 1 ± 0.1 mL of the 10^{-2} homogenate and appropriate controls,
158 specifically the positive control inoculated with *E. coli* ATCC 25922 and MMGB only.
159 Tubes were incubated at $37 \pm 1^\circ\text{C}$ for 24 ± 2 hrs. The presence of *E. coli* in tubes positive for
160 acid was confirmed by subculturing onto tryptone bile X-glucuronide medium (TBX) agar
161 plates and incubation at $44 \pm 1^\circ\text{C}$ for 22 ± 2 hrs. *E. coli* colonies were identified by the
162 characteristic blue or blue-green colour. The MPN of *E. coli* was then determined according
163 to the appropriate probability table (USFDA 2010).

164 **Detection of foodborne viruses in oysters**

165 Oyster samples were analysed for NoV GI, NoV GII and HAV as described in the ISO/TS
166 15216 method “Microbiology of food and animal feed – horizontal method for determination
167 of HAV and NoV in food using real-time RT-PCR” with the exception that murine norovirus
168 (MNV) was used as the process control virus (ISO/CEN 2013; Torok et al. 2018). The oyster
169 samples (n=12) were thawed overnight at $4 \pm 2^\circ\text{C}$, scrubbed under potable running water,
170 shucked and oyster meat collected. The digestive tissue (DT) from each oyster was dissected,
171 pooled per sample and finely chopped. A 2 ± 0.2 gm aliquot of the DT was taken, mixed with
172 10 ± 0.5 μL (3.0×10^4 pfu/mL) of the process control virus (MNV-1) and 2 mL of Proteinase
173 K solution (3U/mL; Astral Scientific, Australia), incubated at 37°C for 1hr followed by 60°C
174 for 15 min. Samples were centrifuged at $3,000 \times g$ for 5 min and the supernatant recovered
175 for further nucleic acid extraction using the bioMerieux NucliSENS® Minimag system

(bioMerieux Pty. Ltd. Baulkham Hills, NSW) according to the manufacturer's protocol.

HAV, NoV GI and NoV GII were analysed by RT-qPCR using primers and probes as specified in the ISO/TS 15216-1:2013. Primers and probes for MNV were those specified by Hewitt et al. (2009). Each sample nucleic acid was tested for HAV, NoV GI and NoV GII in duplicate, both the neat and 10^{-1} dilution. Where viral RNA was detected, the RT-qPCR was repeated for confirmation.

Results

F-RNA phage background study

The background levels of F-RNA phage from the six harvest areas representing five growing areas in New South Wales (NSW) and Tasmania are shown in Figure 1. Over an 18 month period, F-RNA phage were detected in 23 samples with the remaining 77 samples reported as having <30 pfu/100 gm shellfish flesh. Spikes in F-RNA phage levels, $\geq 1,000$ pfu/100 gm of shellfish flesh, were detected in two samples from Pittwater, Tasmania and one sample from Brisbane Water, NSW (Figure 1).

The Pittwater sample collected on the 19th April 2017 was taken from an open lease where no rainfall had been recorded for the previous five days, and only 1.4 mm logged over two days on the 13-14th April with salinity at 36.8 Practical Salinity Unit (PSU), a unit of measure equivalent to salinity in parts per thousand. Similarly, the sample collected on the 20th June 2017 was from an open lease, no rain recorded for the previous 15 days and salinity at 35.8 PSU. The environmental triggers for closure in Pittwater are rainfall ≥ 70 mm in seven days at Hobart airport or salinity ≤ 29 PSU. Hence, neither rainfall nor salinity were approaching the closure limits (Table 2).

The Brisbane Water sample collected on the 16th July 2017 was taken from sub-tidal floating baskets in an open lease. This lease is conditionally restricted with closure triggered when

200 rainfall is >40 mm within 24 hrs or salinity at mid-ebb tide is <21 PSU (Table 2). No rainfall
201 had been recorded since 2.6 mm on the 13th July and salinity was 30.7 PSU.

202 The remaining samples with elevated F-RNA phage levels (>60 pfu/gm) were also taken
203 from open leases that did not approach environmental trigger levels of rainfall or salinity. The
204 phage titres in pfu/100 gm shellfish flesh were 120 (19th June 2017, Brisbane Water, salinity
205 level not reported and a total of 2.6 mm rain over the preceding 7 days), 180 (19th June 2017,
206 Island Inlet, 36.2 PSU and no rain the preceding 13 days), 240 (17th July 2017, Island Inlet,
207 36.0 PSU and only 0.2 mm rain on 16th July and none the preceding 11 days), 360 (16th
208 October 2016, Goodnight Island, 31 PSU and only 1.8 mm rain on the 13th October) and 600
209 (18th July 2017, Pittwater, 35.5 PSU and 1.1 mm rain in the preceding 7 days).

210 **Adverse sewage event study**

211 **Moultling Bay, Tasmania (Event 1)**

212 On 20th January 2017, localised heavy rain (89 mm) resulted in a pump station sewage
213 discharge (>100 kL), consisting of untreated sewage and stormwater, into Moultling Bay in
214 Tasmania. The event started and ended on the same day with both incoming and outgoing
215 tides recorded during the event. Stormwater infiltration of the sewerage network resulted in
216 the overflow of two sewage pump stations (SPS) located approximately 2 km from the
217 growing area over a 4 hr period (SPS 1) and 6.75 hrs (SPS 2) period (Figure 2).

218 Thermotolerant coliform levels in three water samples in the vicinity of SPS 1 ranged from
219 280-350 cfu/100 mL, while those in the vicinity of SPS 2 ranged from 8,000-12,000 cfu/100
220 mL on the 20th January. The environmental and bacterial contamination triggers for closure
221 of this growing area are detailed in Table 2. Five sites located approximately 2-6 km from the
222 sewage source were selected for shellfish sampling (Table 3, Figure 2). Pacific oyster
223 samples were collected three (3), ten (10) and twenty-four (24) days post cessation of the

224 sewage event. The results of F-RNA phage, *E. coli* and foodborne virus testing are
225 summarised in Table 3. NoV or HAV were not detected in any samples. Levels of F-RNA
226 phage were 30 pfu/100 gm shellfish flesh or below. *E. coli* levels were high at all sites
227 initially, and persisted at high levels in at least one site across all sampling times.

228 **Camden Haven, NSW (Event 2)**

229 On the 16th March 2017, a SPS spill in heavy rainfall impacted the Camden Haven growing
230 area in NSW. An unknown volume surcharged from the reticulated sewerage network into
231 the Camden Haven River “Discharge Point” via a stormwater drain (Figure 3). The Bureau of
232 Meteorology station at Laurieton recorded 203.0 mm rain accumulated over the preceding
233 three days. This resulted in a 21 day closure for all three harvest areas in the estuary; Gogleys
234 Lagoon, Hanleys Point and Stingray Creek. Samples of Sydney Rock oysters were taken
235 from four sites 0.2 to 1.7 km from the discharge point at three (3), eleven (11) and twenty-
236 five (25) days post cessation of the sewage event (Table 3, Figure 3). In addition, water
237 samples were taken from Camden Haven River at three sites (the sewage discharge point, 50
238 m upstream and 50 m downstream) on the day of discharge and subsequent day for
239 thermotolerant coliform testing. On the day of the discharge, presumptive thermotolerant
240 coliforms cfu/100 mL ranged from 540 (downstream) to 3,900 (upstream), while on the
241 following day they ranged from 3,300 (downstream) to 5,200 (upstream). F-RNA phage in
242 shellfish samples on day three (3) and eleven (11) post sewage event contained <30 to 60
243 pfu/100 gm, while those sampled at day 25 all contained <30 pfu/100 gm. *E. coli* results from
244 day 3 and 11 samples ranged from 110 to 750 MPN/100 gm, while those from day 25
245 samples ranged from <20 to 310 MPN/100 gm. No NoV or HAV were detected in any of the
246 oyster samples (Table 3).

247 **Brisbane Water, NSW (Event 3)**

On the 22nd March 2017, there was an adverse sewage event resulting in 1.4 ML of untreated raw sewage and 1 ML of stormwater discharging over an estimated 4 hr period into Murphys Bay in the Brisbane Water growing area with an outgoing tide. The adverse event was caused by an intense rainfall event of 38 mm over a 40 min period combined with a power outage resulting in the shutdown of the sewer pump stations. Samples of Sydney Rock oysters were taken from five sites 3.4 to 4.2 km from the discharge point: two (sites 2 and 5) with subtidal floating baskets, two (sites 1 and 3) with intertidal cultivation trays and one (site 4) of wild caught oysters (Figure 4). Samples were collected on days two (2), seven (7) and twenty-one (21) post cessation of the adverse event. A trace level of NoV GII was detected in one sample on day two (Table 3). F-RNA phage levels were elevated on day two at 4 of the 5 sites, but returned to background levels by day 7 (Table 3). The *E. coli* levels were raised at all sites on day two and persisted at high levels in one site (site 2) on all occasions (Table 3).

Camden Haven, NSW (Event 4)

On the 6th December 2017, a raw sewage leak from a newly laid line in the Camden Haven growing area was identified. The volume of the spill was estimated to be 1.5 ML, split over 24 hrs, covering both incoming and outgoing tides. Samples of Sydney Rock oysters were collected from four sites one (1), six (6) and twenty-three (23) days post the sewage event. Thermotolerant coliform testing of water at the discharge site and 50 m up and downstream on the day of the event ranged from 6,100 to 10,000 cfu/100 mL. By the second and third day following the event, thermotolerant coliform levels had dropped to 18 to 90 cfu/100 mL and 5 to 14 cfu/100 mL, respectively. The day 23 samples were further impacted by heavy rainfall of 177 mm recorded at the Laurieton weather station over the previous 24 hrs. The distance from the surcharge (Spill Site) to the oyster sample sites was approximately 0.3 to 3.3 km (Figure 3). The results of F-RNA phage, *E. coli* and enteric virus testing are summarised in Table 3. NoV was detected in oysters from all four sites on day one (1), but only at site 16 on

day six (6). There were no elevated F-RNA phage levels from any of the samples. *E. coli* levels were raised in the day one samples, had dropped by day 6, but had increased even higher in the day 23 samples, as compared to day one samples.

Pittwater and Island Inlet, Tasmania (Event 5)

On the 4th December 2017, there was a sewage overflow into the Pittwater and Island Inlet growing areas as a result of heavy rainfall. Rainfall of 66 mm was recorded near the growing area with 86 mm recorded higher in the catchment. Stormwater infiltration of the sewerage system led to an unknown volume of untreated sewage discharging at SPS3, SPS4 and SPS5 and 1.2 ML (discharge point) of partially treated (screened and chlorinated) sewage discharging into the growing areas (Figure 5). In addition, 2-3 kL sewage discharge overflowed from sewerage lines due to a tree root blockage on the 8th December (Figure 5). Pacific oysters were collected from Pittwater leases, 4.1 to 7.6 km, and Island Inlet leases, 2.8 to 5.8 km, from the discharge point on day 3, 7 and 23 post cessation of the sewage event (Figure 5).

The results of F-RNA phage, *E. coli* and enteric virus testing are summarised in Table 3. Trace levels of NoV were found in oysters from one site (Island Inlet 5) on day 3 only. F-RNA phage levels were elevated in two samples on day 3 and one day 23 sample. *E. coli* levels were elevated in all day 3 samples and some of day 7 samples. The day 23 sample with high F-RNA phage levels also had a very high *E. coli* level.

Discussion

Food safety risk management for bivalve shellfish has traditionally relied on enteric bacteria, coliforms and *E. coli*, as indicators of faecal contamination. F-RNA phage have been suggested as an alternative foodborne viral indicator in shellfish management. The NSSP allows closure of shellfish growing areas following a sewage spill to be reduced if samples

collected at least 7 days post contamination with raw untreated sewage discharge have F-RNA phage <50 pfu/100 gm shellfish, or below predetermined background levels (FDA 2015). This critical limit is based on data from a UK study which showed that when mean levels of F-RNA phage were below 50 pfu/100 gm then no NoV was detected (FDA 2009; Dore et al. 2000). A review by Hodgson et al. (2017) supported investigations into the use of F-RNA phage as an indicator in contaminated shellfish sites and post adverse sewage events. However, under normal growing conditions, the data were variable and did not substantiate the use of F-RNA phage data for assessment of risk (Hodgson et al. 2017).

Information on background phage levels and use as a potential tool in shellfish management within Australia was lacking. The current F-RNA phage background study of five Australian growing areas in two states, determined a baseline of 60 pfu/100 gm shellfish flesh in all areas. Titres higher than 60 pfu/100 gm shellfish flesh and very high spikes, although rare, were noted in some samples collected in winter months from Brisbane Water, NSW and Pittwater and Island Inlet, Tasmania, while these growing areas were open for harvest. The testing methodology used in this study did not differentiate among the sources of these phages. Further analysis would have been required to identify specific genogroups, which are a better indicator of whether the contamination source was human or animal (Long et al. 2005).

The aim of the adverse sewage event study was to confirm the validity of using F-RNA phage as an indicator of human enteric viruses in bivalve shellfish. An appropriate indicator should be readily detected and present in larger numbers when the target pathogens are present. A conservative indicator may or may not be present in the absence of the pathogens, however, it should be detected if the pathogen is detected. The viral pathogens of interest, NoV and HAV, were not detected in two of the five adverse events studied; Moulting Bay, Tasmania in January 2017 and Camden Haven, NSW in March 2017. It is worth noting that these two

322 events were similar, both due to pump station spills in heavy rainfall, leading to elevated *E.*
323 *coli* levels and low phage titres of <30 to 60 pfu/100 gm shellfish flesh in samples taken less
324 than 11 days post cessation of the event. Moulting Bay was not included in the background
325 study but all of the day 24 samples had titres of <30 pfu/100 gm, indicating the background
326 level in this area is likely to be very low. Based on the low level of phage and lack of viral
327 detection, the oyster leases in these two adverse events do not appear to have been heavily
328 impacted by the sewage spills, possibly due to the dilution effect of the rainfall run-off, the
329 local water movement, or a combination of both.

330 The Brisbane Water spill in March 2017 was larger in volume leading to contamination by
331 the F-RNA phage and *E. coli* in 4 of the 5 samples tested at day 2. These four contaminated
332 samples were closer to the spill site than the uncontaminated sample, with highest F-RNA
333 phage levels detected in the two leases closest to the spill. In this case, F-RNA phage levels
334 and NoV co-occurred, albeit the NoV was in trace amounts.

335 The Camden Haven spill in December 2017 resulted in undetectable F-RNA phage in all
336 samples which tested positive for NoV, and some for multiple genogroups of NoV, at days 1
337 and 6. This result is concerning, suggesting F-RNA phage is not always a good indicator for
338 human pathogenic enteric viruses.

339 The Pittwater and Island Inlet adverse event in December 2017 resulted in both elevated F-
340 RNA phage and NoV detection in day 3 samples, although at different sites. Generally risk
341 managers will take a conservative approach, regarding elevated F-RNA phage at any site as
342 indicative of foodborne viral contamination. It then follows that both the geographic and
343 environmental complexity of harvest area, as well as sampling numbers need to be carefully
344 considered when planning F-RNA phage sampling.

345 Furthermore, the Pittwater and Island Inlet adverse event highlighted the fact that the leases
346 closest to the discharge site may not always be those which are most contaminated. The most
347 contaminated samples were in the north and centre of Pittwater, well away from the discharge
348 points, whilst sites closer to the discharges showed less contamination. This is likely due to
349 the nature of the harvest areas and complexity of water movements combined with incoming
350 and outgoing tides. A previous investigation by Brake et al. (2018) of the spatial and
351 temporal distribution of NoV, HAV and *E. coli* in oysters following a contamination event in
352 NSW found similar results, with NoV detected in the sample furthest (8.2 km) from the spill
353 location and no NoV detected in a sample 6.8 km from the spill, although it was noted that
354 the source may have been outfall from other sewage treatment plants (Brake et al. 2018). For
355 spills involving multiple harvest areas and complex water movements it is recommended that
356 multiple sites are sampled. Our results indicated that the number of sites contaminated varies
357 with multiple factors impacting on the level of contamination in the shellfish, including the
358 spill volume, level of treatment of the sewage, dilution in receiving waters, distance of leases
359 from the source and incoming or outgoing tides. At a minimum, for less complex growing
360 areas and situations, no fewer than 5 samples are recommended for F-RNA phage testing.

361 In the collective adverse sewage event studies, F-RNA phages were below the level of
362 detection (30 pfu/100 gm shellfish flesh) on 53 of the total 76 samples. F-RNA phages were
363 detected in 16 samples in the absence of human enteric viruses and F-RNA phages were only
364 detected with NoV on one occasion. Low levels of human enteric virus RNA were detected in
365 samples below the level of detection for F-RNA phage in 6 samples, associated with two of
366 the sewage events, although F-RNA phage was detected in a concurrent sample during one of
367 these events. Unlike the current study, a good correlation between PCR detectable F-RNA
368 genogroup II and NoV was found in a UK survey of oysters at retail (Lowther et al. 2019).
369 However, a recent EU survey of NoV in market ready oysters harvested from Class A

370 production areas, equivalent to the Australian open for harvest classification, also showed a
371 prevalence of 24.7% (EFSA 2019), which is far greater than that of <2% reported nationally
372 in Australian oysters at production (Torok et al. 2018).

373 The adverse sewage event data from this study should be considered collectively for each
374 sewage event, where any one site or sample occasion with an elevated F-RNA phage level is
375 indicative of sewage contamination and foodborne viral risk for that entire harvest area.

376 Under these considerations, two of the three events where NoV was detected had elevated
377 levels of F-RNA phage, and one did not. Although, in the latter case, *E. coli* was elevated and
378 would have resulted in closure of the harvest area. This highlights the need to also consider
379 other environmental and microbiological triggers for closure specified for that harvest area.

380 We have compared F-RNA phage levels to NoV as measured by RT-qPCR, however, we do
381 not know how these estimates exactly relate to the risk of infection to the consumer. The
382 molecular diagnostic method for human enteric viruses, is based on amplification of the viral
383 RNA genome which may not necessarily originate from an infectious virion. Therefore, no
384 comment can be made regarding the infectivity of the NoV detected in these shellfish.

385 Determination of infectivity ratios via comparison of total PCR detectable F-RNA genogroup
386 II with that of cultivable F-RNA genogroup II revealed that <10% of PCR detectable phage
387 were infectious (Lowther et al. 2019). However, evidence has shown that there is a risk of
388 infection at any level of NoV contamination. For example, an investigation into the NoV
389 outbreak in 2013 involving Tasmanian oysters, which led to 525 identified cases, showed that
390 unquantifiable trace levels of NoV GII RNA were detected in two un-shucked oyster samples
391 (Lodo et al. 2014).

392 In conclusion, level of F-RNA phage in five at risk growing areas were low with 60 pfu/100
393 gm representing background levels for these areas. The limited adverse sewage event

investigations were inconclusive as to the value of using F-RNA phage as an indicator of human enteric viruses in bivalve shellfish management in the Australian context. The results do not confirm the presence of F-RNA phage on all occasions with detectable levels of NoV viral genomes, although it is not known if these detections represented infective virus particles. This study investigated only five sewage events of which three were small to medium in size, <1000 kL and involved rainfall events. Hodgson et al., (2017) noted that there is more evidence supporting the use of F-RNA phage as a virus indicator in shellfish from contaminated sites, but less for sewage spills associated with rainfall events (Scholes et al. 2009). We recommend further analysis of samples for F-RNA phage, NoV and HAV after adverse sewage events to provide more information on the effectiveness of this tool. In addition, epidemiological data must be included to verify, if possible, the safety of shellfish from growing areas where the reopening has been brought forward following an adverse event. Under current conservative management practices in Australia, the risk of illness due to enteric viruses from shellfish is low (Torok et al. 2018).

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1 **Table 1:** Summary of the potential pollution sources in the growing and harvest areas investigated in FRNA phage background study

Growing area, Harvest area			
Classification	Depuration required prior to sale	Potential pollution sources	Sewage treatment
Camden Haven, Hanleys Point, NSW			
Conditionally restricted ¹	Yes	Sewage pipes, previous outbreak, small settlement upstream, mixed farming and forested land	Level of treatment: tertiary, membrane bioreactor, hypo and UV disinfection. Type of disinfection: chlorine. Volume of discharge: up to 10,800 kL. Volume of receiving waters/exchange rate of receiving waters: total exchange approximately 5 days. Distance of effluent discharge (under normal conditions) from the growing area: >10 km via ocean. Type of receiving waters: complex system including estuarine.
Brisbane Water, Murphys Bay, NSW			
Conditionally restricted	Yes	Heavy population, large number of boats, high recreational use, high risk pump stations, sewage pipes, storm water, birds	Level of treatment: secondary, sedimentation/aeration/clarification and sludge removal. Type of disinfection: none. Volume of discharge: 18 to 20 GL (2015-2017). Volume of receiving waters/exchange rate of receiving waters: very complex estuary, variable rates. Distance of effluent discharge (under normal conditions) from the growing area: 25 km/ Type of receiving waters: open marine however growing area is a complex system including estuarine.
Shoalhaven, Goodnight Island, NSW			
Conditionally approved ²	Following high rainfall only	Multiple pump stations, storm water, recreational use, boats	Serviced by 5 WWTPs to tertiary level. No discharge into the Shoalhaven region – closest discharge into ocean > 9 km from Goodnight Island.
Shoalhaven, Berrys Bay, NSW			
Conditionally restricted	Yes	Underperforming on-site sewerage management system from 24 houses, large drain with high <i>E. coli</i> counts from unknown source (agricultural/domestic)	As for Goodnight Island.
Pittwater, Zone 3, Tasmania			
Conditionally approved	No	Industrial & agricultural catchment, high risk SPS drains, outfall further away, birds	Level of treatment: tertiary, membrane bioreactor, membrane filtration. Type of disinfection: chlorination and UV. Volume of discharge: 800 kL/day. Volume of receiving waters/exchange rate of receiving waters: exchange rate 1-3 days. Distance from the growing area: 4.1 km. Type of receiving waters: complex system including estuarine.
Island Inlet, Zone 4, Tasmania			
Conditionally approved	No	Sewage outfall, some small boating, stormwater, very good flushing	Level of treatment: tertiary, membrane bioreactor, membrane filtration. Type of disinfection: chlorination & UV. Volume of discharge: 800 kL/day. Volume of receiving waters/exchange rate of receiving waters: very shallow receiving waters.

		exchange rate approx. 1 day. Distance from the growing area: 2.8 km. Type of receiving waters: complex system including estuarine.

2

3 ¹ Conditionally restricted growing areas are defined in Section 3.6.1 of the ASQAP Manual of Operations as “A comprehensive sanitary survey
4 finds that the area will be open for the purposes of harvesting shellfish for relaying or depuration for a reasonable period of time and the factors
5 determining this period are known, predictable and are not so complex as to preclude a reasonable management approach” (2). Conditionally
6 restricted growing areas are closed for harvest for any purpose when *E. coli* in shellfish exceed 10.0 MPN/gm, or when thermo-tolerant
7 coliforms in water exceed 70 cfu per 100 mL.

8 ² Conditionally approved growing areas are defined in Section 3.4.1 of the ASQAP Manual of Operations as “ comprehensive sanitary survey
9 finds that the area will be open for the purposes of harvesting shellfish for a reasonable period of time and the factors determining this period are
10 known, predictable and are not so complex as to preclude a reasonable management approach” (2). Conditionally approved growing areas are
11 closed for harvest for direct sale to market when *E. coli* in shellfish exceed 2.3 MPN/gm, or if thermo-tolerant coliforms exceed 14 cfu per 100
12 mL.

Table 2: Environmental and bacterial triggers for harvest areas involved in background study and adverse sewage events.

Growing area, Harvest area	Environmental triggers for harvest closure	Bacterial contamination trigger
Shoalhaven, Goodnight Island, NSW (Background and adverse event study)	Approved conditions when: <40 mm of rainfall within 48 hrs, <60 mm of rainfall within 7 days, salinity measured and averaged at all sites at mid-ebb tide >26 PSU Restricted conditions when: <50 mm of rainfall within 48 hrs, salinity measured and averaged at all sites at mid-ebb tide >22 PSU. Closed when: ≥50 mm of rainfall within 48 hours, salinity measured and averaged between all sites at mid-ebb tide <22 PSU	Open for direct harvest for market under normal conditions. Downgraded to restricted operations (relay or depuration only) when: exceeds 14 cfu per 100 mL OR <i>E. coli</i> in any shellfish sample exceeds 2.3 MPN per gm Closed for all harvest activities when: level of faecal coliforms at any sample site exceeds 70 cfu per 100 mL OR <i>E. coli</i> in any shellfish sample exceeds 10.0 MPN per gm
Shoalhaven, Berrys Bay, NSW (Background and adverse event study)	Closed when ≥40 mm of rainfall within 48 hrs or salinity <18 PSU	Open for restricted operations (relaying and depuration only) under normal conditions. Closed all harvest activities when: level of faecal coliforms at any sample site exceeds 70 cfu per 100 mL OR <i>E. coli</i> in any shellfish sample exceeds 10.0 MPN per gm
Camden Haven, Hanleys Point, NSW (Background and adverse event study)	Closed when >40 mm of rainfall within 48 hrs, >100 mm of rainfall within 7 days, salinity measured at sites 11 or 12 at mid-ebb tide was <18 PSU	As for Shoalhaven
Camden Haven, Stingray Creek, NSW (Adverse event study)	Closed when >40 mm of rainfall within 48 hrs, >100 mm of rainfall within 7 days, salinity measured at any water site at mid-ebb tide was <18 PSU	As for Shoalhaven
Camden Haven, Gogleys Lagoon, NSW (Adverse event study)	Approved conditions when:	As for Shoalhaven
Conditionally Approved dual-management		

	<20 mm of rainfall within 24 hrs, <50 mm of rainfall within 7 days, salinity measured at any site at mid-ebb tide was >20 PSU Restricted conditions when: <50 mm of rainfall within 48 hrs, <100 mm of rainfall within 7 days, salinity measured at any site at mid-ebb tide was >20 PSU Closed when: >50 mm of rainfall within 48 hrs, >100 mm of rainfall within 7 days, salinity measured at any site at mid-ebb tide was <20 PSU	
Brisbane Water, Murphys Bay, NSW (Background and adverse event study)		As for Shoalhaven
Conditionally Restricted	Closed when >40 mm of rainfall within 48 hrs, salinity <21 PSU.	
Pittwater, Zone 3, Tas (Background and adverse event study)		
Conditionally Approved	Closed when ≥ 70 mm rain in 7 days at Hobart Airport, salinity ≤ 29 PSU	Open for direct harvest to market under normal conditions. Closed when: TC¹ confirmed in water >14 cfu/100 mL, TC presumptive >21 cfu/100 mL OR <i>E. coli</i> in a shellfish sample of one dozen animals > 2.3 MPN/gm, 2 of 5 samples > 2.3 MPN/gm or 1 of 5 samples > 7 MPN/gm
Island Inlet, Zone 4, Tas (Background and adverse event study)		
Conditionally Approved	Closed when ≥ 20 mm rain in 3 days at Hobart Airport if no salinity data, salinity ≤ 32.8 PSU	As for Pittwater
Moulting Bay, Zone 1, 2, 4 and 5, Tas (Adverse event study)		
Conditionally Approved	Closed when: Zone 1 and 2 ≥ 40 mm rain in 3 days at Pyengana or St Helens, salinity ≤ 30 PSU, Georges River ≥ 12 cumecs² for zone 1 or ≥ 8 cumecs for zone 2 Zone 4 and 5 ≥ 35 mm rain in 3 days @ Pyengana or St Helens, salinity ≤ 30 PSU, Georges River ≥ 8 cumecs for zone 4 or ≥ 12 cumecs for zone 5	As for Pittwater

¹ TC thermotolerant coliforms

² cumecs = cubic metre per second

1 **Table 3:** Adverse sewage event studies

Samples	Distance from spill (km)	F-RNA phage pfu/100 g	<i>E. coli</i> MPN/100 g	NoV GI	NoV GII	HAV
Moultling Bay, Tas 20th January 2017 (Event 1)						
Day 3						
Zone 1 sample 1	2.9	<30	70	ND ¹	ND	ND
Zone 1 sample 2	2.0	<30	430	ND	ND	ND
Zone 2 sample 3	5.9	30	700	ND	ND	ND
Zone 4 sample 4	5.9	<30	700	ND	ND	ND
Zone 5 sample 5	6.0	<30	310	ND	ND	ND
Day 10						
Zone 1 sample 1	2.9	<30	160	ND	ND	ND
Zone 1 sample 2	2.0	<30	750	ND	ND	ND
Zone 2 sample 3	5.9	<30	20	ND	ND	ND
Zone 4 sample 4	5.9	<30	20	ND	ND	ND
Zone 5 sample 5	6.0	<30	40	ND	ND	ND
Day 24						
Zone 1 sample 1	2.9	<30	90	ND	ND	ND
Zone 1 sample 2	2.0	<30	700	ND	ND	ND
Zone 2 sample 3	5.9	<30	40	ND	ND	ND
Zone 4 sample 4	5.9	<30	<20	ND	ND	ND
Zone 5 sample 5	6.0	<30	<20	ND	ND	ND
Camden Haven, NSW 16th March 2017 (Event 2)						
Day 3						
Hanleys Point site 17	1.7	<30	750	ND	ND	ND
Stingray Creek site 15	1.2	60	110	ND	ND	ND
Hanleys Point site 16	0.2	<30	750	ND	ND	ND
Stingray Creek site 20	1.7	30	430	ND	ND	ND
Day 11						
Hanleys Point site 6	1.7	<30	700	ND	ND	ND
Stingray Creek site 15	1.2	<30	220	ND	ND	ND
Hanleys Point site 16	0.2	60	700	ND	ND	ND
Stingray Creek site 20	1.7	<30	500	ND	ND	ND
Day 25						
Hanleys Point site 6	1.7	<30	<20	ND	ND	ND
Stingray Creek site 15	1.2	<30	20	ND	ND	ND
Hanleys Point site 16	0.2	<30	20	ND	ND	ND
Stingray Creek site 20	1.7	<30	310	ND	ND	ND
Brisbane Water, NSW 22nd March 2017 (Event 3)						
Day 2						
Site 1	4.2	180	310	ND	ND	ND
Site 2	3.7	150	>18000	ND	(44.4, ND) ² (38.6, ND)	ND
Site 3	3.5	480	1,300	ND	ND	ND
Site 4	3.4	420	3,500	ND	ND	ND
Site 5	3.9	<30	750	ND	ND	ND
Day 7						
Site 1	4.2	<30	220	ND	ND	ND
Site 2	3.7	<30	>18000	ND	ND	ND
Site 3	3.5	<30	110	ND	ND	ND
Site 4	3.4	<30	110	ND	ND	ND
Site 5	3.9	<30	160	ND	ND	ND
Day 21						
Site 1	4.2	<30	220	ND	ND	ND
Site 2	3.7	<30	>18000	ND	ND	ND
Site 3	3.5	<30	90	ND	ND	ND
Site 4	3.4	<30	110	ND	ND	ND
Site 5	3.9	<30	500	ND	ND	ND
Camden Haven, NSW 6th Dec 2017 (Event 4)						

Day 1							
Gogleys site 13	3.0	<30	160	(37.9, 36.9) (36.5, 35.7)	ND		ND
Gogleys site 14	3.3	<30	40	(37.0, 38.3) (35.5, 36.2)	ND		ND
Stingray Creek site 15	1.1	<30	1700	(36.5, 38.3) (35.9, 37.7)	(41.7, 41.7) (36.5, 37.3)		ND
Hanleys Point site 16	0.3	<30	1300	(36.6, 39.2) (35.0, ND)	(40.8, 41.9) (37.2, ND)		ND
Day 6							
Gogleys site 13	3.0	30	<20	ND	ND		ND
Gogleys site 14	3.3	<30	20	ND	ND		ND
Stingray Creek site 15	1.1	<30	20	ND	ND		ND
Hanleys Point site 16	0.3	<30	90	(40.2, 41.1) (36.1, 36.8)	ND		ND
Day 23							
Gogleys site 13	3.0	<30	4800	ND	ND		ND
Gogleys site 14	3.3	<30	720	ND	ND		ND
Stingray Creek site 15	1.1	<30	330	ND	ND		ND
Hanleys Point site 16	0.3	<30	390	ND	ND		ND
Pittwater and Island Inlet, Dec 2017 (Event 5)							
Day 3							
Pittwater A	7.6	120	2,400	ND	ND		ND
Pittwater B	6.7	120	500	ND	ND		ND
Pittwater D	4.6	30	1,300	ND	ND		ND
Pittwater E	4.1	30	160	ND	ND		ND
Island Inlet 2	3.5	<30	500	ND	ND		ND
Island Inlet 3	3.9	30	500	ND	ND		ND
Island Inlet 5	5.8	<30	500	ND	(38.7, 37.9) (42.8, 35.6)		ND
Day 7							
Pittwater A	7.6	30	220	ND	ND		ND
Pittwater B	6.7	<30	<20	ND	ND		ND
Pittwater D	4.6	30	<20	ND	ND		ND
Pittwater E	4.1	<30	220	ND	ND		ND
Island Inlet 1	2.8	<30	40	ND	ND		ND
Island Inlet 2	3.5	<30	40	ND	ND		ND
Island Inlet 3	3.9	<30	310	ND	ND		ND
Island Inlet 5	5.8	<30	<20	ND	ND		ND
Day 23							
Pittwater A	7.6	<30	<20	ND	ND		ND
Pittwater B	6.7	<30	36	ND	ND		ND
Pittwater E	4.1	<30	<20	ND	ND		ND
Island Inlet 1	2.8	<30	<20	ND	ND		ND
Island Inlet 2	3.5	<30	<20	ND	ND		ND
Island Inlet 3	3.9	1,260	3,150	ND	ND		ND
Island Inlet 5	5.8	<30	36	ND	ND		ND

2 ¹ Not detected

3 ² Ct values are for neat samples in duplicate. Each PCR was repeated to confirm the result.

Figure 1: FRNA phage background levels over 18 months in three NSW and two Tasmanian oyster growing areas. The solid horizontal line corresponds to the conservative background level of 60 pfu/100 gm shellfish flesh. The dashed horizontal line corresponds to the limit of detection of 30 pfu/100 gm shellfish flesh. Only samples with ≥ 30 pfu/100 gm are shown.

Figure 2: Adverse sewage Event 1 (Moulting Bay, Tasmania). Red dots indicate the source of the sewage discharge. Blue dots indicate oyster sampling sites.

Figure 3: Adverse sewage events for Camden Haven, NSW. Red dots indicate the source of the sewage discharge; “Discharge Point” for Event 2 and “Spill Site” for Event 4. Blue dots indicate oyster sampling sites.

Figure 4: Adverse sewage Event 3 (Brisbane Water, NSW). Red dots indicate the source of the sewage discharge. Blue dots indicate oyster sampling sites.

Figure 5: Adverse sewage Event 5 (Pittwater and Island Inlet, Tasmania). Red dots indicate the source of the sewage discharge. Blue dots indicate oyster sampling sites.