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Oysters Australia IPA: The use of FRNA bacteriophages for rapid re-opening of growing areas after sewage spills

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Foreword

Bivalve shellfish production is an industry well worth supporting. Shellfish are a sustainable, nutritious and resource efficient protein source. Aquaculture is well recognised for its potential as a solution to the long-term challenge of sustainably feeding a rapidly growing global population, however, the growth of the industry must be tempered with consideration for the impact on our finite natural resources. As highlighted in the Blue Frontiers report on the environmental impacts of aquaculture, shellfish production stands out for the benefits to the environment as well as to consumers and the local community[1]. Food safety is the key challenge in the development of a sustainable shellfish industry.

The 19th century discovery of the link between microorganisms and disease led to the 20th century development of effective sanitation systems. Sanitation is attributed to a multidecadal increase in life expectancy in developed countries. Effective shellfish quality assurance programs are a natural extension of sanitation development. Sanitation initially focused on the efficient transport of sewage out of urban areas often discharged directly to the local waterway. Shellfish related illnesses highlighted the need for effective treatment of sewage to ensure that aquatic resources are not degraded. The history of shellfish quality assurance mirrors that of public health systems. The 20th century focus on bacterial pathogens is linked to their ease of detection. Water and shellfish bacterial standards, focused on bacterial indicators, have seen a dramatic decrease in shellfish related outbreaks of bacterial diseases such as typhoid and cholera.

The ability to effectively assess the risk the human pathogenic viruses in shellfish harvest areas will lead to a reduction in food borne illness outbreaks related to shellfish. As deficiencies in current sanitation systems are identified and remediated, the improvements in sanitation benefit all users of the waterway. Increased consumer confidence in the product is linked to increases in demand and improved business confidence. As pathogen detection methods became more sensitive and accessible, reports of shellfish related outbreaks of viral disease have shown a dramatic increase. The bacterial standards which have proven effective at reducing the incidence of bacterial outbreaks have proven inadequate for the management of virus related risks. This study provides knowledge to bridge this gap with an effective and affordable technology that is ready for broadscale implementation.

The development of standardised testing methodology assists the future development of risk management approaches by allowing direct comparison of data from different jurisdictions. The background surveys provide insight into the potential impact that bacteriophage standards could have on industry operation. The difficulty experienced in correlating norovirus risk with bacteriophage levels highlights the technology gap that exists in norovirus testing capability. The outcomes of this study provide industry and regulators with a clear road map for the implementation of bacteriophage testing into the shellfish safety regulatory environment. This step change in the management of shellfish harvest areas will directly contribute to improved safety of harvested shellfish and industry profitability. The potential for bacteriophage to act as an indicator for further pollution source tracking and catchment remediation could yield significant benefits to long term risk reduction efforts.

We thank the project team for their dedication and commitment to complete this work. The significant effort to coordinate sample collection and undertake consultation across such a large geographic area and their responsiveness to short notice requests to process adverse event samples is highly appreciated. This project represents a step forward in the development of the shellfish safety framework which will in turn elucidate areas for the incremental improvement in sanitation systems and broader public health outcomes.

Anthony Zammit
Manager NSW Shellfish Program
NSW Government
Department of Primary Industries Food Authority

[1] Hall, S.J., A. Delaporte, M. J. Phillips, M. Beveridge and M. O'Keefe. 2011. Blue Frontiers: Managing the Environmental Costs of Aquaculture. The WorldFish Center, Penang, Malaysia.

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Acknowledgments

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We also acknowledge the wastewater authority staff for proving information and answering questions.

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Abbreviations

Australian Shellfish Quality Assurance Advisory Committee	ASQAAC
Australian Shellfish Quality Assurance Program	ASQAP
American Type Culture Collection	ATCC
Bureau of Meteorology	BOM
Centre for Environment, Fisheries and Aquaculture Science	Cefas
Colony forming units	cfu
Cysteine lactose electrolyte deficient agar	CLED
Department of Primary Industries, Parks, Water and Environment	DPIPWE
Digestive tissue	DT
Double stranded deoxyribonucleic acid	dsDNA
Dulbecco's modified eagle medium	DMEM
European Committee for Standardization	CEN
European Food Safety Authority	EFSA
European Reference Laboratory Proficiency	EURL
European Union	EU
External control	EC
Fisheries Research and Development Corporation	FRDC

Food Standards Australia New Zealand	FSANZ
Food and Agriculture Organization of the United Nations	FAO
Food and Drug Administration	FDA
Food Safety and Innovation	FSI
F-specific ribonucleic acid	FRNA
Hepatitis A virus	HAV
Hours	hrs
Human norovirus	NoV
International Organisation of Standardisation Technical Specification	ISO/TS
Interstate Shellfish Sanitation Conference	ISSC
Kilometres	km
Male specific coliphage	MSC
Most probable number	MPN
Minerals modified glutamate broth	MMGB
Murine norovirus	MNV
New South Wales	NSW
New South Wales Food Authority	NSWFA
Optical density	OD
Onsite Sewerage Management System	OSMS
Peptone saline solution	PSS
Peptone water	PW
Plaque forming units	pfu
Potential pollution sources	PPS
Practical Salinity Unit	PSU
Primary Industries and Regions South Australia	PIRSA
Quantitative Reverse Transcription Polymerase Chain Reaction	qRT-PCR
Ribonucleic acid	RNA
Ribonuclease	RNase
Revolutions per minute	rpm
Sewage pump station	SPS

Sewage treatment plant	STP
South Australia	SA
South Australian Research and Development Institute	SARDI
Standard deviation	SD
Supervisory control and data acquisition	SCADA
Tasmania	Tas
Thermotolerant coliforms	TC
Threshold cycle	Ct
Tryptone bile X-glucuronide medium	TBX
Tryptone yeast extract glucose agar 1%	TYGA1
Tryptone yeast extract glucose agar 2%	TYGA2
Tryptone yeast extract glucose broth	TYGB
United Kingdom	UK
United States of America	US
Waste Water Treatment Plant	WWTP
World Health Organization	WHO

Executive Summary

What the report is about

The South Australian Research and Development Institute (SARDI) Food Safety and Innovation (FSI) group with the support of the Fisheries Research and Development Corporation (FRDC), TasWater, Central Coast Council, Port Macquarie-Hastings Council, Shoalhaven Council, New South Wales Food Authority (NSWFA), New South Wales Shellfish Program, Oysters Australia and Tasmanian Shellfish Quality Assurance Program (TSQAP) undertook an investigation of the use of FRNA bacteriophages (phages) as indicators of viral contamination in shellfish after adverse sewage events. The human enteric viruses Norovirus (NoV) and Hepatitis A virus (HAV) are the most commonly reported foodborne viral pathogens associated with shellfish. The viruses are bioaccumulated by shellfish when sewage enters water in the growing areas. Oysters were sampled from 'at-risk' growing areas to establish background baseline phage levels from July 2016 to December 2017. Five adverse sewage events were also investigated during the same period. In addition, training in the laboratory techniques for phage enumeration was undertaken at the SARDI FSI laboratory in Adelaide for staff from laboratories in NSW and Tasmania.

Background

Internationally, bivalve shellfish contaminated with human enteric viruses from sewage are implicated in foodborne viral disease outbreaks. Traditionally indicator bacteria, the coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish. Numerous studies have established that bacteria are inadequate as indicators of the risk of human enteric viruses and do not reflect the presence or absence of enteric viruses. Bacteriophages are bacterial viruses and 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.

In 2009, the US Interstate Shellfish Sanitation Conference (ISSC) officially included the application of phages as indicators of viral contamination of bivalve shellfish from sewage. The US National Shellfish Sanitation Program (NSSP) permits re-opening of a growing area earlier than the obligatory three week closure following a sewage spill if shellfish samples collected at least 7 days after the contamination with raw untreated sewage discharge have FRNA phage levels that do not exceed the critical limit of 50 pfu/100 g shellfish, or are below predetermined background FRNA phage levels. Similar regulations were introduced in the Australian Shellfish Quality Assurance Program (ASQAP) Operations Manual in 2016, however, no studies into levels of phages in Australian growing areas or shellfish had been undertaken.

Aims/objectives

- Establish baseline levels of FRNA bacteriophages in "at risk" Australian growing areas.
- Determine appropriate sampling plans for FRNA bacteriophages in shellfish following sewage incidents.
- Enable implementation of FRNA phage levels as a management tool for use following adverse sewage incidents in bivalve shellfish growing waters.
- Train laboratories to be competent in using appropriate testing methodologies for FRNA phages in shellfish.

Methodology

The background study was conducted over an 18 month period from July 2016 to December 2017. Five "at risk" growing areas were sampled on a monthly schedule. Three areas in NSW and two in Tasmania were selected on the basis of a history of previous sewage spills and closures. Samples were sent to the SARDI Food Safety Laboratory and analysed for FRNA phages using the method as described in the Centre for Environment, Fisheries and Aquaculture Science (Cefas) standard operating procedure. Five adverse sewage events that occurred in 2017 were also investigated, two in Tasmania and three in NSW. Samples were collected as soon as possible after the event ceased, then 7 and 21 days post the event. In addition to analysis for FRNA phages using the Cefas method, these samples were also analysed for *E. coli* by Most Probable Number (MPN) as described in the New Zealand Food Safety Authority Guidelines 'Enumeration of *Escherichia coli* in Bivalve Molluscan Shellfish, Version 6, 17 November 2006' and NoV and HAV according to the ISO/TS 15216 method 'Microbiology of food and animal feed – horizontal method for determination of HAV and NoV in food using real-time RT-PCR'.

A guidance document has been prepared for regulators and growers to assist in the application of FRNA phage levels as a management tool following adverse sewage incidents in bivalve shellfish growing waters.

State based regulators and laboratories were invited to nominate appropriate staff to participate in training in the procedures for FRNA phage enumeration in bivalve shellfish.

Results/key findings

Background FRNA phage levels have been established for the five 'at-risk' growing areas. A baseline of 60 pfu/100 g shellfish flesh is appropriate for all areas. Titres higher than 60 pfu/100 g shellfish flesh and very high spikes were noted in some samples collected in winter months from Brisbane Water NSW, Island Inlet and Pittwater Tas. The sources of these have not been elucidated but may be birds and/or domestic and agricultural run-off. A conservative approach would be to establish the 60 pfu/100 g shellfish flesh level year round in these three areas, however, the potential for maintaining a closure based on high FRNA phage titres in winter is recognised. Consequently, it may be worth collecting more data on the levels and sources of phage in these growing areas during winter in order to consider setting a higher baseline for during this season.

The aim of the adverse sewage event investigation was to confirm the validity of using FRNA phages as indicators of human enteric viruses in bivalve shellfish. A conservative indicator may or may not be present in the absence of the pathogens, however, it should always be detected if the pathogen is detected. FRNA phages were below the level of detection (30 pfu/100 g shellfish flesh) for 53 of the total 76 adverse event samples. FRNA phages were detected on 16 occasions in the absence of human enteric viruses. In one adverse event, a trace level of NoV was detected in one day 1 sample with a corresponding phage titre of 150 pfu/100 g. NoV was detected at very low levels in 6 samples where FRNA phages were below the level of detection, that is <30 pfu/100 g shellfish flesh. The results have not corroborated the presence of FRNA phages in all samples with detectable levels of NoV viral genomes.

The molecular method for human enteric viruses, qRT-PCR, detects the viral genome which may be naked non-encapsulated or degraded viral RNA and viruses with damaged capsids that cannot initiate infection. There is no method available at this stage to differentiate between infective and non-infective viruses. FRNA phages are detected and enumerated by plaque assay resulting in an infective titre. PCR and the plaque assay are not directly comparable. No comment can be made regarding the infectivity of the NoV detected in those shellfish. Two growing areas were re-opened in under 21 days using the FRNA phage data. NoV was detected in day 1 samples from one growing area. No illnesses have been reported.

The guidance document for regulators and industry on appropriate implementation of this method has been compiled. It outlines design of adverse event investigations including recommendations on the investigation design, appropriate sample numbers and interpretation of results.

Laboratory training in the FRNA phage procedures was held on the 7th and 8th November 2017 at the SARDI FSI Laboratories in Adelaide with representatives from the Port Macquarie Hastings Environmental

Laboratory, TasWater and SARDI in attendance. A detailed laboratory workshop manual was prepared including relevant appendices.

Implications for relevant stakeholders

The application of FRNA phages as virus indicators has the potential to significantly reduce losses to the oyster industry due to sewage spills. The option to reopen growing areas in under 21 days, provided all parameters considered by the regulators are acceptable, should limit the financial impact of an adverse sewage event. For example, one three week closure in the Pittwater growing area was estimated to cost in the range of $250-$400k combined sales, depending on the season.

The industry has taken a conservative approach to shellfish production which has been responsible, in part, for the very low prevalence of human viruses in shellfish at production. FRNA phage data should complement this approach providing added confidence to industry and consumers of the safety of the product.

At the same time consideration must be given to the potential cost to industry if a growing area is reopened in under 21 days on the basis of FRNA phage results and then implicated in an outbreak of foodborne viral illnesses.

Recommendations

Expansion of background studies to other “at risk” oyster growing areas is recommended. Ideally a growing area will have determined the background phage level prior to applying FRNA phage testing after an adverse event. There will be a cost associated with the testing necessary to establish the background, however, a growing area could be disadvantaged if the background is higher than 60 pfu/100 g shellfish flesh and results after a spill could lead to an unnecessarily extended closure.

We also recommend further analysis of samples for FRNA phages, NoV and HAV after adverse sewage events to provide more information on the effectiveness of this technique. The number and types of events investigated was limited, therefore, more data relating to a variety of scenarios including large spills are required. 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 by the application of the ASQAAC guideline.

Investigation of the FRNA phage sources isolated from background and adverse event samples to confirm whether the origin of the phage isolates is human or animal is also recommended. Brisbane Water and Pittwater are recommended due to occasional elevated background phage levels. There are a number of published methods for source tracking and development of source tracking capability in an Australian laboratory would be valuable.

Keywords

Shellfish, Sewage, Norovirus, Hepatitis A virus, Indicator, Bacteriophage

Introduction

The consumption of raw or lightly cooked shellfish contaminated with human enteric viruses is associated with risks to human health. The most commonly reported foodborne viral pathogens are Norovirus (NoV) (83.7%) and Hepatitis A virus (HAV) (12.8%) with oysters (58.4%) the most frequently implicated shellfish (Bellou et al. 2013). The contamination of foods can occur at any stage of the process from production to consumption, however, the major source of viruses in bivalve molluscs is pre-harvest contamination of growing waters with human sewage (FAO/WHO 2012). The control of viruses in shellfish needs to focus on prevention of contamination rather than removal or inactivation through processing as the only practical post-harvest option for reducing virus numbers in bivalve shellfish is cooking.

Internationally, there were 368 foodborne viral outbreaks associated with shellfish reported in the scientific literature between 1980 and 2012 (Bellou et al. 2013). In Australia, recent outbreaks associated with oysters include NoV infections linked to the consumption of oysters from Camden Haven NSW in October-November 2012 (n=8) and Dunalley, Tasmania in March 2013 (n=525) (OzFoodNet 2013, Lodo et al. 2014). No subsequent outbreaks associated with oysters have been reported in OzFoodNet quarterly reports from March 2014 to September 2017 which cover the period from April 2013 to September 2015: http://www.health.gov.au/internet/main/publishing.nsf/Content/cdi4102-1.

Risk management for bivalve shellfish has traditionally relied on enteric bacteria, the coliforms and *E. coli*, as indicators of faecal contamination. International regulations have been developed to specify acceptable levels of enteric bacterial pathogens in shellfish tissues or in waters where shellfish are grown (FAO/WHO 2008). Bacteria, however, are recognised to be poor indicators of viral contamination and do not reflect the presence or absence of enteric viruses (Doré and Lees 1995, Flannery et al. 2009). Viruses are morphologically different to bacterial cells and exhibit significantly diverse resistance and susceptibility responses to environmental conditions including sewage treatment processes (Blatchley et al. 2007, Stewart et al. 2008). The Codex Alimentarius Commission has recommended that NoV and HAV levels in bivalves be monitored following adverse sewage events and foodborne outbreaks with the aim of establishing virus limits once the analytical methods have been validated and verified (FAO/WHO 2012). Polymerase chain reaction (PCR) detection is the best methodology available for foodborne virus detection at this stage, however, the infectious risk associated with NoV and HAV detected in environmental or shellfish samples is uncertain due to the limitations of the assay (EFSA 2011, Liu et al. 2011, Stals et al. 2012).

Bacteriophages (phages) have been proposed as indicators or surrogates for human enteric viruses due to similarities in morphology and survival dynamics (Havelaar et al. 1986, Doré and Lees 1995, Hodgson et al. 2017). Phages are viruses specific for only bacteria. Male-specific coliphages (MSC), FRNA phages or F-specific phages have been the most extensively studied and recommended as indicators. A critical characteristic of indicator organisms is that they should always be present when pathogens from similar origins are present. The US Interstate Shellfish Sanitation Conference (ISSC) officially recognised the application of phages as indicators of viral contamination of bivalve shellfish from sewage in 2009 (FDA 2009). The NSSP allows the obligatory three week closure following a sewage spill to be reduced if samples collected at least 7 days post contamination with raw untreated sewage discharge have FRNA phage levels below the critical limit of 50 pfu/100 g shellfish, or below background FRNA phage levels (FDA 2015). The critical limit was based on data from a UK study which showed when mean levels of FRNA phages were below 50 pfu/100 g no NoV were detected (Doré et al. 2000, FDA 2009).

In 2014, SARDI conducted a review of published literature for the NSWFA on the use of phages in shellfish risk management and recommended that the application of FRNA phage detection and enumeration in the Australian context be further investigated. This was the basis for an extensive review publication in *Food Microbiology* (Hodgson et al. 2017). The review of published data focussed on the validity of using phage levels as indicators of the risk of human enteric viruses in shellfish, specifically examining the question of whether phages are always present when human viruses are detected. Generally the data supported the use of phages as indicators in contaminated sites and post adverse sewage events with only one of eleven publications not supportive. Under normal growing conditions the data was variable and did not substantiate the use of phage data for assessment of risk.

SARDI also undertook a national prevalence survey for foodborne viruses, NoV and HAV, in Australian oysters at production between July 2014 and August 2015 (Torok et al. 2015, Torok et al. 2018). No NoV or HAV were detected resulting in an estimated prevalence for NoV and HAV of <2% with a 95% confidence interval of 0-2.5% to 0-2.7%, respectively.

Standard risk management practice in Australia following potential contamination of growing areas due to adverse sewage events or overflows is to instigate a mandatory 21 day closure (ASQAAC 2016). This approach is conservative and in some instances may result in closure of growing areas not impacted by the sewage leading to significant losses in production, market share and reputation. In the period between July 2016 and December 2017, NSW harvest areas initiated 21 closures (personal communication, Anthony Zammit NSWFA) and in Tasmania the Pittwater, Island Inlet and Moulting Bay harvest areas underwent 10 closures (personal communication, Megan Burgoyne DPIPWE) related to sewage spills.

Prior to 2016, only the US approved the use of phage levels in management of bivalve shellfish production. However, in 2016, the ASQAP Operations Manual was reviewed leading to the inclusion of FRNA phage levels in shellfish to re-open after sewage spills (ASQAAC 2016). The most recent version of the manual (ASQAAC 2018) states in section 6.1.10 c):

> "A harvest area temporarily placed in the closed status is reopened only when:
>
> c) for closures associated with an untreated or partially treated sewage discharge or an untreated sewage discharge from a community sewage system:
>
> i. at least 21 days have passed since the end of the contamination event;
>
> OR
>
> ii. Shellstock samples, collected from representative locations in each harvest area (no sooner than seven days after the contamination has ceased), are found to have Male Specific Coliphage levels which do not exceed background levels or a level of 50 Male Specific Coliphage per 100 grams"

It is anticipated that this method will contribute additional information for regulators and growers to assess whether viral contamination from sewage has impacted a growing area. Prior to this study, there have been no investigations into levels of phages in Australian growing areas or shellfish.

Objectives

- Establish baseline levels of FRNA bacteriophages in “at risk” Australian growing areas.
- Determine appropriate sampling plans for FRNA bacteriophages in shellfish following sewage incidents.
- Enable implementation of FRNA phage levels as a management tool for use following adverse sewage incidents in bivalve shellfish growing waters.
- Train laboratories to be competent in using appropriate testing methodologies for FRNA phages in shellfish.

Methods

1. Steering committee

A steering committee was established at the start of the project. The committee included representatives from the Australian oyster industry in NSW and Tas, contributing water authorities and councils, and shellfish regulatory representatives.

Chair:

Anthony Zammit	NSWFA, NSW

Members:

Maree Smith	Port Macquarie-Hastings Council, NSW
Wayne Hutchinson	Oysters Australia
Joanna Waugh	Gosford City Council, NSW
or Stephen Shinners	Gosford City Council, NSW
Andrew McVey	Shoalhaven City Council, NSW
David Holmes	TasWater, Tas
Kate Wilson	Tasmanian Shellfish Quality Assurance Program, Tas
Phil Baker	NSWFA, NSW
Justin Goc	Barilla Bay Oysters, Tas
Brandon Armstrong	Armstrong Oysters, NSW

Permanent Observers and Secretariat:

Kate Hodgson	SARDI, Principal Investigator
Alison Turnbull	SARDI/SafeFish
Valeria Torok	SARDI
Navreet Malhi	SARDI

The terms of reference were to:

1. Provide strategic oversight of the project to ensure project outputs are in line with the stated objectives.
2. Assist with communicating research to regulators and growers.
3. Assist with communicating results of the study to industry and other stakeholders to ensure successful delivery of research outcomes.
4. Assist with advice on reporting policy.
5. Provide feedback on sampling design and protocol, and help with organisation of sample collection.

2. Background Study Design

Shellfish from five "at risk" growing areas were sampled on a monthly schedule for 18 months, from July 2016 to December 2018 inclusive, to determine the baseline FRNA phage levels. Three areas in NSW and two in Tasmania were selected on the basis of their history of previous sewage spills. The reliability of oyster samplers and ease of transport of the shellfish to the SARDI FSI Laboratory in Adelaide, SA in a timely manner, were also taken into consideration. The growing areas, harvest areas and leases selected are detailed in Table 1. Maps of each of the background study sites are shown in Figure 1 (Camden Haven, NSW), Figure 2 (Brisbane Water, NSW), Figure 3 (Shoalhaven, NSW), Figure 4 (Pittwater, Tas) and Figure 5 (Island Inlet, Tas). The environmental and bacterial contamination triggers for closure of these growing areas are outlined in Table 2 for NSW and Table 3 for Tasmania. Tables 2 and 3 also include the growing areas sampled for adverse sewage events that had not been included in the background study.

2.1. Sample collection

The samples for the background study, each consisting of a minimum of 12 shellfish, were collected according to the sample plan, packed into doubled plastic press seal bags in a polystyrene box with chill wraps and transported to the SARDI Food Safety and Innovation laboratory. Samples were processed on arrival, or stored at 4˚C for no more than 24 hrs then processed in batches, and analysed for FRNA phages. The method was as described in the Centre for Environment, Fisheries and Aquaculture Science (Cefas) standard operating procedure 'Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish' Issue 7, 2007 (Cefas 2007) and outlined in detail within the training manual "FRNA bacteriophage testing in bivalve molluscan shellfish" (Appendix E).

Table 1: Growing and harvest areas investigated in FRNA phage background study

Growing area, Harvest area	Classification	Depuration	Lease number	Pollution sources	Sewage treatment
Camden Haven, Hanleys Point, NSW	Conditionally restricted [1]	Yes	AL00/050	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: >10km via ocean Type of receiving waters: complex system including estuarine
Brisbane Water, Murphys Bay, NSW	Conditionally restricted	Yes	OL79/137	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: 18,036 ML to 19,746 ML (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]	Occasional	OL06/007	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 at Penguin Head > 9 km from Goodnight Island
Shoalhaven, Berrys Bay, NSW	Conditionally restricted	Yes	OL58/019	Underperforming OSMS from 24 houses, large drain with high *E. coli* counts from unknown source (agricultural/domestic)	As for Goodnight Island

Pittwater, Zone 3, Tas	Conditionally approved	No	001	Industrial & agricultural catchment, high risk SPS drains, outfall further away, birds	Level of treatment: tertiary, Membrane Bioreactor (MBR), Membrane filtration Type of disinfection: chlorination & 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, Tas	Conditionally approved	No	007	Sewage outfall, some small boating, stormwater, very good flushing	Level of treatment: tertiary, Membrane Bioreactor (MBR), 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

[1] The ASQAP operations manual 2016 (ASQAAC 2016) states for conditionally restricted growing areas:

3.6.1 A comprehensive sanitary survey 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 determining this period are known, predictable and are not so complex as to preclude a reasonable management approach."

[2] and for Conditionally Approved growing areas:

"3.4.1 A comprehensive sanitary survey 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 known, predictable and are not so complex as to preclude a reasonable management approach."

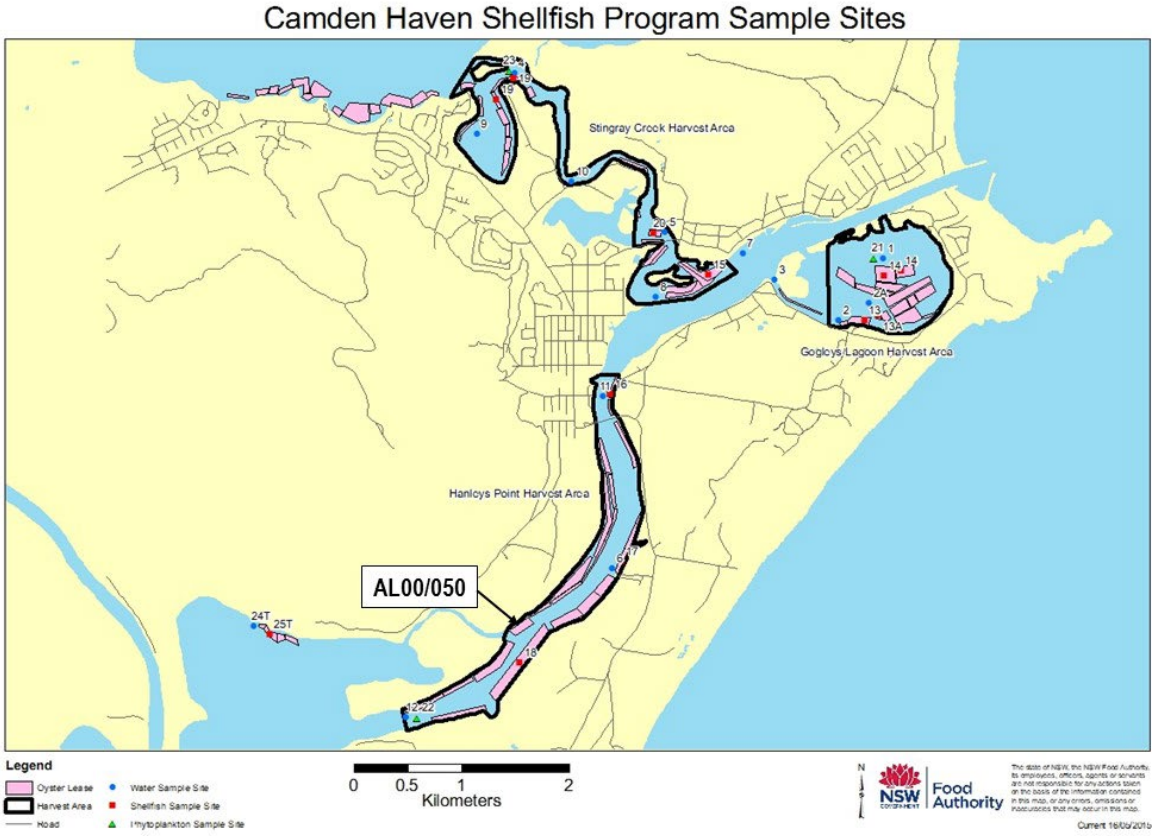

Figure 1: Camden Haven NSW growing area

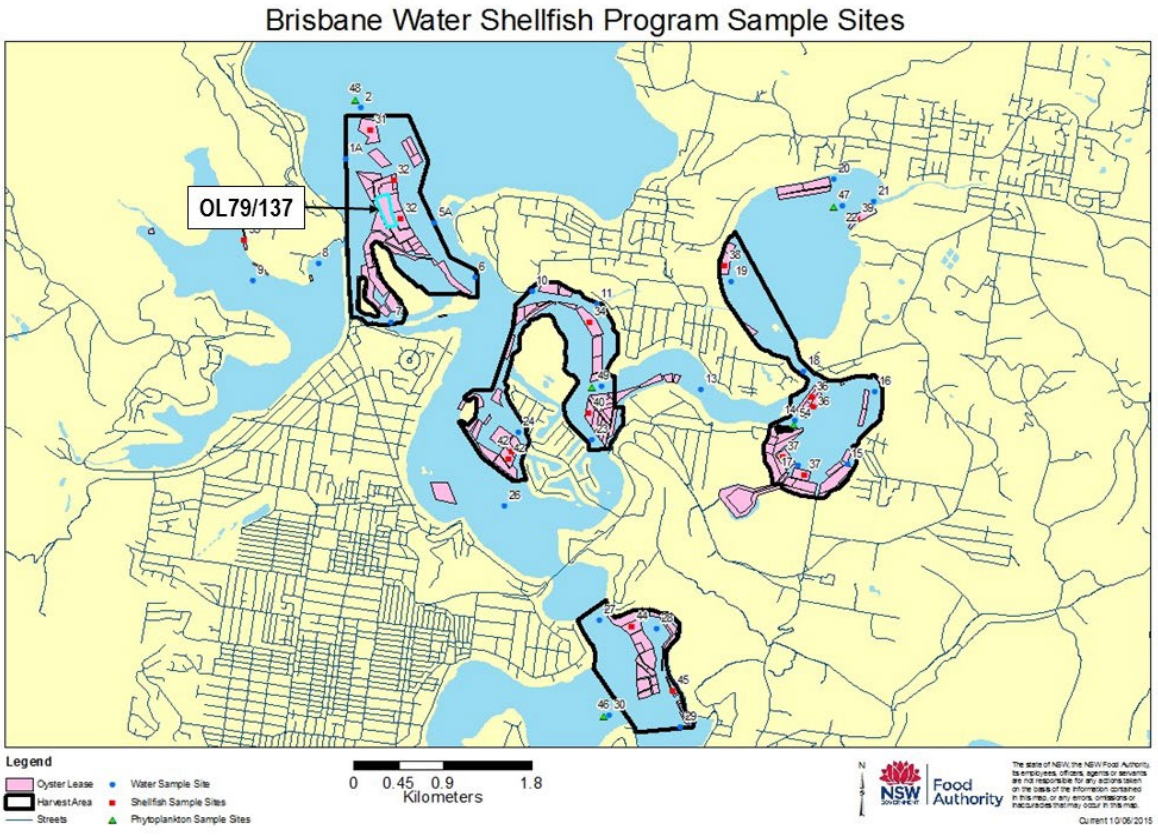

Figure 2: Brisbane Water NSW growing area

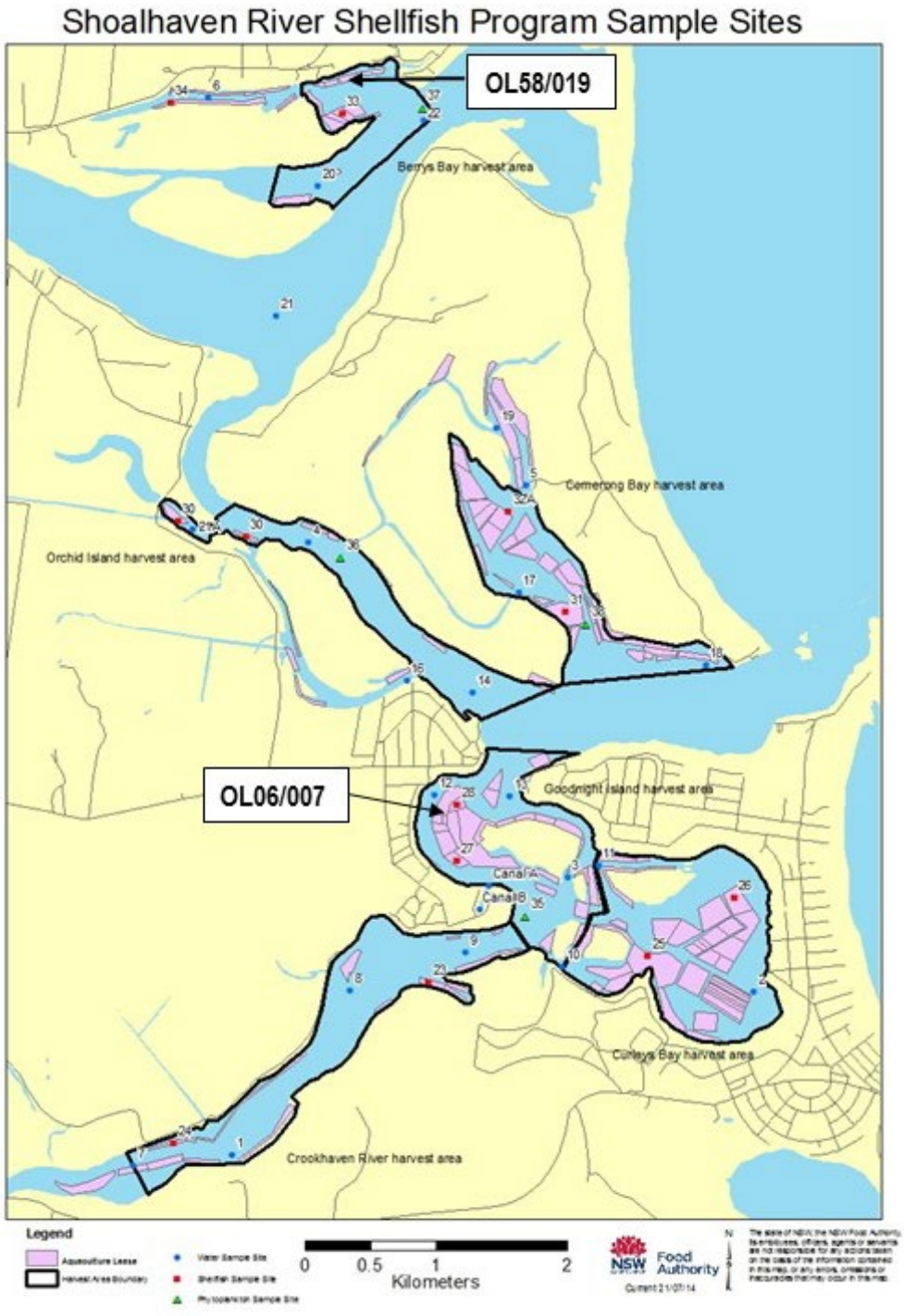

Figure 3: Shoalhaven NSW growing areas

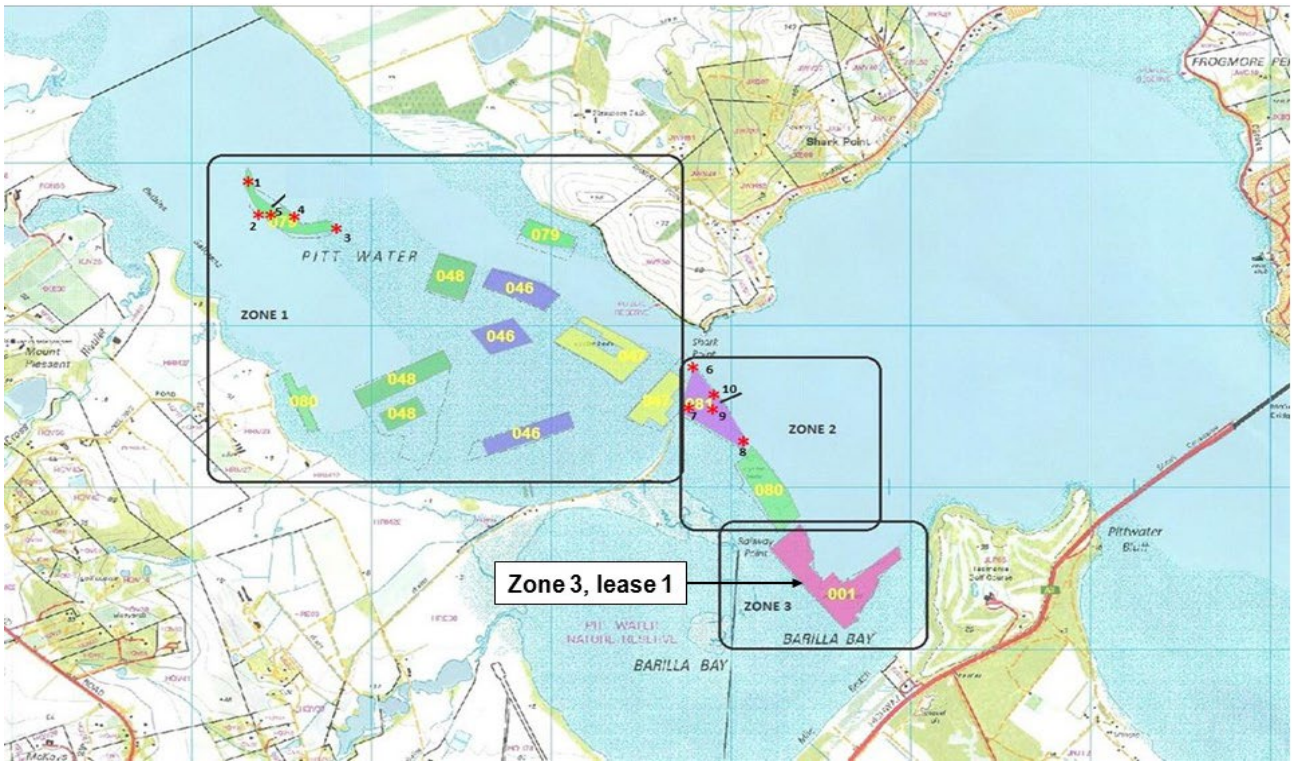

Figure 4: Pittwater Tasmania growing area

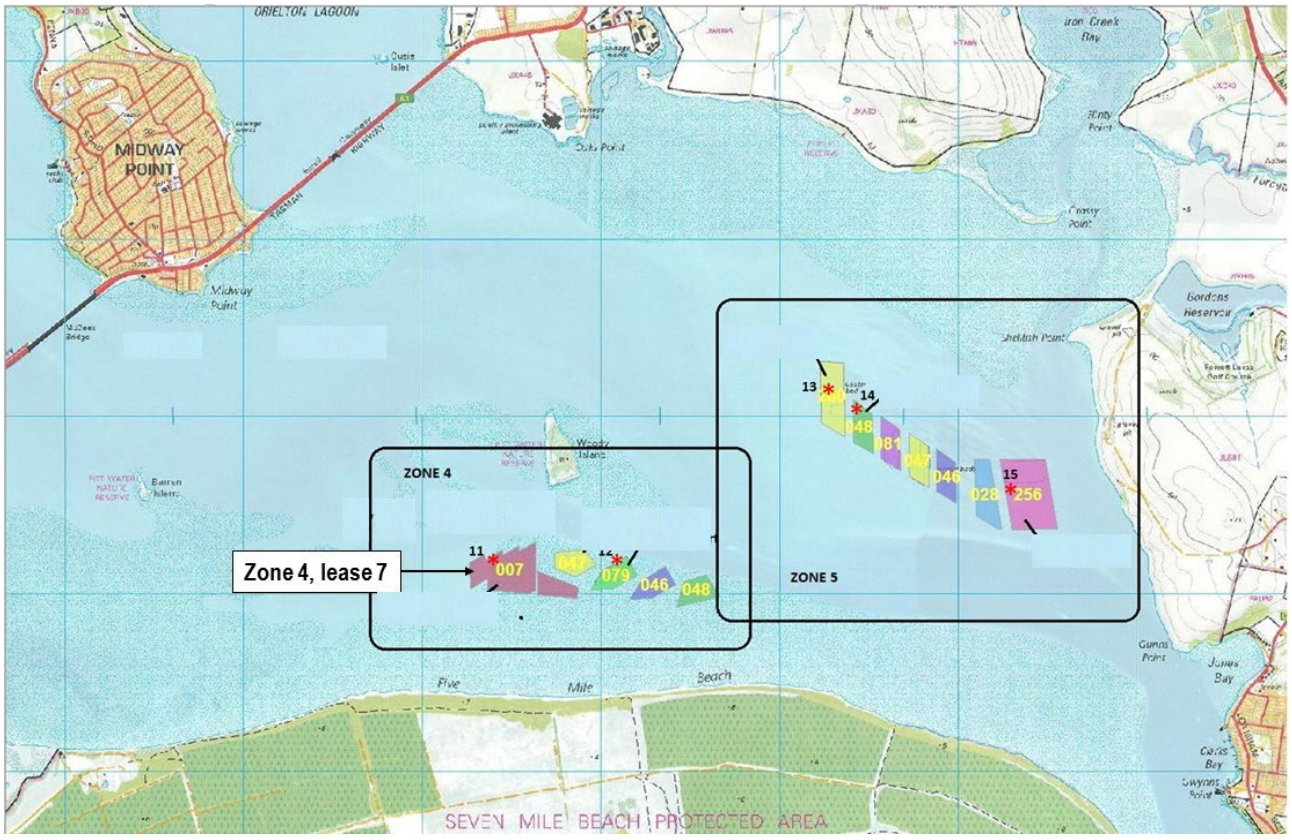

Figure 5: Island Inlet Tasmania growing area

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

NSW	Classification	Environmental triggers	Bacterial contamination trigger	Shellfish Species	Potential pollution sources
Shoalhaven, Goodnight Island (Background and adverse event study)	Conditionally Approved dual-management since 2015	**Approved conditions when**: • <40 mm of rainfall occurred within 48 hours, • <60 mm of rainfall occurred within 7 days, • salinity measured and averaged at all sites at mid-ebb tide was >26 ‰ [2]. **Restricted conditions** when: • <50 mm of rainfall occurred within 48 hours, • salinity measured and averaged at all sites at mid-ebb tide was >22 ‰. **Closed** when: • ≥50 mm of rainfall occurred within 48 hours, • salinity measured and averaged between all sites at mid-ebb tide was <22 ‰	Closed when: level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters* OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	• Sydney Rock Oyster (*Saccostrea glomerata*) • Pacific Oyster (*Crassostrea gigas*)	18 PPS[1]: Low, low-medium or medium n=13 Medium-high n=3 High n=2
Shoalhaven, Berrys Bay (Background and adverse event study)	Conditionally Restricted	**Closed when**: • ≥40 mm of rainfall occurred within 48 hours, • salinity measured at site 33 at mid-ebb tide was <18 ‰	Closed when: level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	• Sydney Rock Oyster (*Saccostrea glomerata*) • Pacific Oyster (*Crassostrea gigas*)	32 PPS: Low, low-medium or medium n=29 Medium-high n=2 High n=1
Camden Haven, Hanleys Point (Background and adverse event study)	Conditionally Restricted	**Closed when:** • >40 mm of rainfall occurred within 48 hour • >100 mm of rainfall occurred within 7 days • salinity measured at sites 11 or 12 at mid-ebb tide was <18 ‰	Closed when: level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	• Sydney Rock Oyster (*Saccostrea glomerata*) • Angasi Oyster (*Ostrea angasi*)	53 PPS: High n=7

Camden Haven, Stingray Creek **(Adverse event study)**	Conditionally Restricted Mandatory depuration	**Closed when:** • >40 mm of rainfall occurred within 48 hour • >100 mm of rainfall occurred within 7 days • salinity measured at any water site at mid-ebb tide was <18 ‰	Closed when: level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	• Sydney Rock Oyster (*Saccostrea glomerata*) • Angasi Oyster (*Ostrea angasi*)	53 PPS: High n=7
Camden Haven, Gogleys Lagoon **(Adverse event study)**	Conditionally Approved dual-management since 2010	**Approved conditions when:** • <20 mm of rainfall occurred within 24 hours • <50 mm of rainfall occurred within 7 days • salinity measured at any site at mid-ebb tide was >20 ‰ **Restricted conditions when:** • <50 mm of rainfall occurred within 48 hours • <100 mm of rainfall occurred within 7 days • salinity measured at any site at mid-ebb tide was >20 ‰ **Closed when:** • >50 mm of rainfall occurred within 48 hours • >100 mm of rainfall occurred within 7 days • salinity measured at any site at mid-ebb tide was <20 ‰	Closed when: level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	• Sydney Rock Oyster (*Saccostrea glomerata*) • Angasi Oyster (*Ostrea angasi*)	53 PPS: High n=7
Brisbane Water, Murphys Bay **(Background and adverse event study)**	Conditionally Restricted	**Closed when:** • >40 mm of rainfall occurred within 48 hours, • salinity measured at sites 31 or 32 at mid-ebb tide was <21 ‰.	**Closed when:** level of faecal coliforms at any sample site exceeds Restricted harvest area water quality parameters OR *E. coli* in any shellfish sample exceeds 10.0 *E. coli* per gram	Sydney Rock Oyster (*Saccostrea glomerata*)	100 PPS: Most rated low risk High n=9

[1] PPS Potential pollution sources

[2] ‰ = per-mille e.g. 1 ‰ = 0.1

Table 3: Environmental and bacterial triggers for Tasmanian growing areas involved in background study and adverse sewage events

Tasmania	Classification	Environmental triggers	Bacterial contamination trigger	Shellfish Species	Potential pollution sources
Pittwater, Zone 3 **(Background and adverse event study)**	Conditionally Approved	**Closed when:** • ≥70mm rain in 7 days @ Hobart Airport • salinity ≤29‰	**Water**: • TC[1] confirmed >14 cfu/100mL •TC presumptive >21 cfu/100ml **Shellfish meat**: •1 dozen whole shellfish: FAIL if > 2.3 *E. coli* per gram•5 dozen whole shellfish: FAIL if 2 of 5 dozen > 2.3 *E. coli* per gram OR FAIL if 1 of 5 dozen > 7 *E. coli* per gram.	• Pacific Oyster (*Crassostrea gigas*)	High risk: concentrated surface water runoff Med-high risk: sewerage system failure, stormwater runoff, dispersed surface water runoff Multiple low-med risk: e.g. septic systems, stormwater runoff, wildlife areas
Island Inlet, Zone 4 **(Background and adverse event study)**	Conditionally Approved	**Closed when:** • ≥20mm rain in 3 days @ Hobart Airport if no salinity data • salinity ≤32.8‰	**Water**: • TC confirmed >14 cfu/100mL •TC presumptive >21 cfu/100ml **Shellfish meat**: •1 dozen whole shellfish: FAIL if > 2.3 *E. coli* per gram •5 dozen whole shellfish: FAIL if 2 of 5 dozen > 2.3 *E. coli* per gram OR, FAIL if 1 of 5 dozen > 7 *E. coli* per gram.	• Pacific Oyster (*Crassostrea gigas*)	As for Pittwater

Moulting Bay, Zone 1, 2, 4 and 5 **(Adverse event study)**	Conditionally Approved	**Closed when:** **Zone 1 and 2** • ≥40mm rain in 3 days @ Pyengana or St Helens • salinity ≤30‰ • Georges River ≥12 cumecs[3] for zone 1 or ≥8 cumecs for zone 2 **Zone 4 and 5** • ≥35mm rain in 3 days @ Pyengana or St Helens • salinity ≤30‰ • Georges River ≥8 cumecs for zone 4 or ≥12 cumecs for zone 5	**Water**: • TC confirmed >14 cfu/100mL •TC presumptive >21 cfu/100ml **Shellfish meat**: •1 dozen whole shellfish: FAIL if > 2.3 *E. coli* per gram •5 dozen whole shellfish: FAIL if 2 of 5 dozen > 2.3 *E. coli* per gram OR, FAIL if 1 of 5 dozen > 7 *E. coli* per gram.	• Pacific Oyster (*Crassostrea gigas*)	High risk: sewerage system failure, potential overflow from wastewater treatment plant, concentrated surface water runoff Medium: stormwater, urban runoff, port, slipway, moorings, wildlife-bird breeding habitat Med-low risk: septic systems, domestic wastes-campgrounds.

[1] TC thermotolerant coliforms

[2] ‰ = per-mille e.g. 1 ‰ = 0.1

[3] cumecs = cubic metre per second

3. Adverse Sewage Event Design

Five adverse sewage events were investigated. The aim was to examine a mixture of large (>1000 kL/day) and small (<1000 kL/day) events, ideally in growing areas included in the background study. In the event that suitable spills did not occur within the project timeline in the background study sites, other growing areas would be sampled. Each case study aimed to collect samples from up to five sites impacted by the event based on criteria such as the water flow, geography and tides. The environmental and bacterial contamination triggers for closure of the areas impacted are outlined in Table 2 for NSW and Table 3 for Tasmania. The first event investigated was in Moulting Bay Tasmania in January 2017 which is a growing area not included in the background study. The remaining sewage events; Camden Haven, NSW and Brisbane Water, NSW both in March 2017; Camden Haven, NSW and Pittwater and Island Inlet, Tasmania both in December 2017 were all included in the background study.

3.1. Sample collection and preparation

The samples were collected as soon as possible once the sewage event ceased (day 1), 7 days post the event (day 7) and 21 days post the event (day 21). The samples, each consisting of a minimum of 24 shellfish, were collected according to the sample plan, packed into doubled plastic press seal bags in a polystyrene box with chill wraps and transported to the SARDI FSI Laboratory. Samples were processed on arrival, or stored at 4±2˚C then processed in batches within 24 hrs of receipt, and analysed for *E. coli* (section 3.3) and FRNA phages (section 3.2). The remaining shellfish (n=12) were stored at -80˚C for NoV and HAV detection by quantitative reverse transcriptase polymerase chain reaction (qRT-PCR) (section 3.4).

3.2. Analysis for FRNA phages by soft agar overlay

3.2.1. Sample preparation

Damaged or dead shellfish were discarded. Each sample was washed and shucked. The shellfish flesh and liquor were collected and weighed then homogenised with 2 mL of 0.1% peptone water (PW) per 1 g of shellfish at high speed (four bursts for 15 sec with 5 sec between each burst). All oyster samples were prepared using sterile shucking knives and homogeniser jars with equipment cleaned between each sample to avoid cross-contamination. Homogenates were assayed in batches and could be stored for up to 48 hr at 4˚C prior to analysis. The homogenate, 30-50 mL, was centrifuged at 2000 × *g* at room temperature for 5 minutes. The supernatant was decanted into a clean tube for analysis.

3.2.2. Agar overlay assay

The samples were expected to have low levels of FRNA phages, therefore, 10 replicate agar overlay plates were prepared for each sample as specified in the Cefas protocol (CEFAS 2007). The overlay of soft tryptone yeast extract glucose agar 1% (TYGA1) was inoculated with one mL of the *Salmonella typhimurium* strain WG49 (WG49) host bacteria in early log phase and one mL of the sample supernatant. Each overlay also contained 0.1% nalidixic acid to inhibit potential background bacteria in the sample. The overlay was gently mixed, poured onto the surface of the base tryptone yeast extract glucose agar 2% (TYGA2) agar plate and incubated at 37±1˚C for 18±4 hrs. Following incubation the number of plaques were counted. Plaques exhibiting typical DNA phage morphology (clear lysis centres and approximately 6 mm diameter) were noted but not counted in the total.

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

$$C_{pfu} = \left[\frac{N\text{-}N_{RNase}}{n} \times F \right] \times 300$$

C_{pfu} confirmed number of FRNA phages

N total number of plaques counted

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

n number of replicates

F dilution factor

Each batch of samples tested included various controls including a blank of 0.1% PW and two positive MS2 phage controls, one at the start of the assay and another at the end, to confirm host bacteria WG49 susceptibility to FRNA phages. If no phage were detected, the result was expressed as <30 pfu/100 g shellfish flesh (the limit of detection for this assay).

The adverse sewage event study included the addition of RNase overlay agar plates for each sample. This involved the inoculation of a parallel series of overlay agar plates with the addition of 100±1 µL RNase solution (1 mg/mL) to each overlay. The addition of RNase facilitates confirmation of the results. The plaques on RNase plates are DNA phages which are subtracted from the total number of plaques on the standard overlay plates.

The phage titres using this method are multiples of 30 pfu. The ASQAP operations manual states 'Shellstock samples, collected from representative locations in each harvest area (no sooner than seven days after the contamination has ceased), are found to have Male Specific Coliphage levels which do not exceed background levels or a level of 50 Male Specific Coliphage per 100 grams'.

This will need to be revised to 60 pfu/100g shellfish flesh if the method employed by SARDI is applied. The method for FRNA phage enumeration at SARDI is based on the Cefas standard procedure adapted from the ISO standard for water (ISO 1995, CEFAS 2007). The limit of detection is 30 pfu/100 g shellfish flesh and phage titres are multiples of 30 pfu. SARDI developed this method as the host bacterial culture and MS2 control phage were readily available. The US FDA ISSC method differs in relation to the host bacterial species, host volume in overlays, sample preparation and volume of shellfish supernatant tested resulting in a limit of detection of approximately 7 pfu/100 g shellfish depending on the shellfish species and initial mass of shellfish.

3.2.3. Preparation of WG49 host bacteria working culture

One vial of *Salmonella typhimurium* strain WG49 phage type 3 Nal^r (F' 42 lac:Tn5) - NCTC 12484 (WG49) stock culture was thawed, streaked onto a cysteine lactose electrolyte deficient agar (CLED) agar plate and incubated at 37±1°C for 18±2 hrs. After incubation, 5-7 lactose-positive colonies (yellow) were selected, inoculated into pre-warmed tryptone yeast extract glucose broth (TYGB) and incubated for 5±1 hrs at 37±1°C with shaking until the optical density (OD) corresponded to 2.5-15x10^8 cfu/mL. Following incubation sterile glycerol was added and mixed thoroughly. Vials of approximately 1.2 mL were dispensed and stored at <-80°C.

3.2.3.1. Incubation period of WG49 host bacteria working culture

One vial of WG49 working culture was thawed and 500±5 µL inoculated into 50±0.5 mL of pre-warmed TYGB. Approximately 2.5 mL of inoculated broth from the flask was sampled immediately (time 0 sample).

The OD was determined at 600 nm and 100±1 µL of 10^{-4}, 10^{-5} and 10^{-6} dilutions of the culture were spread in duplicate onto TYGA2 plates. The culture was incubated at 37±1°C with shaking at 100±10 rpm for 4±2 hrs with samples taken and analysed every 30 min throughout the incubation period. The TYGA2 plates were incubated at 37±1°C for 24±2 hrs. After incubation, colonies on each plate with 30-300 colonies were counted and the number of colony forming units (cfu) per mL calculated. This procedure was repeated on three separate occasions.

From the results the OD range and incubation time corresponding to a cell concentration between 7-40x10^7 cfu/mL was determined.

3.2.3.2. Quality control of WG49 host bacteria working culture

3.2.3.2.1. Plasmid segregation

Duplicate CLED plates were spread with 100±1 µL volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of WG49 and incubated at 37±1°C for 24±2 hrs. The percentage of lactose negative (blue) colonies was calculated from the colony counts and the WG49 host culture was accepted if lactose negative colonies were <8% of the total.

3.2.3.2.2. Nalidixic acid and kanamycin resistance

Duplicate CLED plates were spread with 100±1 µL volumes of the 10^{-2} dilution of WG49. Two nalidixic acid (30 µg, 6 mm) and two kanamycin antibiotic (30 µg, 6 mm) discs were placed on each plate and incubated at 37±1°C for 24±2 hrs. The diameter of any zones of inhibition were measured. The WG49 host culture was accepted if there was no inhibition zone around the nalidixic acid disk and the inhibition zone for kanamycin was <15 mm.

3.2.3.2.3. Bacteriophage susceptibility

Bacteriophage susceptibility was determined by inoculating overlay agar plates with one mL of the WG49 host bacteria in early log phase and one mL of MS2 phage control. The plaques were counted after incubation at 37±1˚C for 18±4 hrs. The WG49 host culture was accepted if the plaque count was within the MS2 control limits as previously determined (section 2.3.1).

3.2.4. Preparation of MS2 phage working culture

Prewarmed TYGB 50±0.5 mL was inoculated with 500±5 µL of WG49 working culture and incubated at 37±1°C for 18±2 hrs with shaking at 100±10 rpm. Following incubation, 50±0.5 mL of fresh prewarmed TYGB was inoculated with 500±5 µL of the WG49 overnight culture and incubated at 37±1°C for 90±10 min. The 90 min WG49 culture was then inoculated with 500±5 µL of stock MS2 and incubated at 37±1°C for 5±1 hrs. Following incubation 5.0±0.1 mL of chloroform was added, mixed thoroughly and stored at 4±2˚C for 18±2 hrs. The culture was aspirated from the chloroform and centrifuged at 3000±200 × *g* for 20±5 min. The supernatant was serially diluted to 10^{-10} in 0.1% PW for titration as per the standard agar overlay method. The working culture was adjusted to a final concentration of 50-200 pfu/ml using 0.1% PW and 3±0.1 ml aliquots stored at <-80°C.

3.2.4.1. Determination of MS2 control limits

The MS2 working culture was assayed for FRNA phage levels by the standard overlay agar method (section 2.1.2). This was repeated twenty times on ten separate occasions. From these observations, the control limits for the MS2 were determined. Control charts were constructed incorporating warning and action limits defined as: warning limits: mean ± 2SD, action limit: mean ± 3SD. A new stock of MS2 would be cultured if the phage stock was not within the control limits.

3.3. *E. coli* by Most Probable Number (MPN)

3.3.1. Sample preparation

The method was as described in the New Zealand Food Safety Authority Guidelines 'Enumeration of *Escherichia coli* in Bivalve Molluscan Shellfish, Version 6, 17 November 2006' with the modification that the homogenate prepared in 0.1% PW for the FRNA phage analysis was used. The homogenate had been prepared as a 1/3 dilution (2 mL 0.1% PW for each 1 g shellfish). This was further diluted to 10^{-1} by the addition of 70 mL of Peptone saline solution (PSS) to 30 mL of homogenate followed by preparation of a 10^{-2} dilution in PSS (NZFSA 2006). The MPN test was inoculated as follows: five tubes of double strength minerals modified glutamate broth (MMGB) with 10±0.2 mL of the 10^{-1} homogenate, five tubes of single strength MMGB with 1±0.1 mL of the 10^{-1} homogenate and five tubes of single strength MMGB with 1±0.1 mL of the 10^{-2} homogenate and appropriate controls, specifically the positive control inoculated with *E. coli* ATCC 25922 and MMGB only. Tubes were incubated at 37±1°C for 24±2 hrs. The presence of *E. coli* in tubes positive for acid was confirmed by subculturing onto tryptone bile X-glucuronide medium (TBX) agar plates and incubation at 44±1°C for 22±2 hrs. *E. coli* colonies were identified by the characteristic blue or blue-green colour. The MPN of *E. coli* was then determined according to the appropriate probability table (USFDA 2010).

3.4. Analytical testing for foodborne viruses

The samples were analysed for NoV GI, NoV GII and HAV as described in the ISO/TS 15216 method *Microbiology of food and animal feed – horizontal method for determination of HAV and NoV in food using real-time RT-PCR* with the exception that murine norovirus (MNV) was used as the process control virus instead of Mengo virus (ISO/CEN 2013, Torok et al. 2015).

3.4.1. Sample preparation

The oyster samples, ideally 12 individuals, were thawed overnight at 4±2°C then scrubbed under potable running water and shucked with the oyster meat collected into clean zip locked plastic bags. All oyster samples were prepared using sterile shucking knives with other equipment cleaned between each sample to avoid cross-contamination. Each oyster was dissected with sterile scalpels to remove the digestive tissue (DT) which was transferred to a clean petri dish, finely chopped and combined with DT from other oysters in that sample. A 2±0.2 g aliquot of the DT was taken, mixed with 10±0.5 µL (3.0×10^4 pfu/µL) of the process control virus, murine norovirus (MNV) and virus extraction done as outlined for bivalve molluscs (ISO/CEN 2013, Torok et al. 2015).

3.4.2. Nucleic acid extraction

The bioMerieux NucliSENS® Minimag system (bioMerieux Pty. Ltd. Baulkham Hills, NSW) was used to extract and purify the viral RNA following the manufacturer's protocol. RNA was extracted from the viruses using guanidine isothiocyanate then adsorbed onto magnetic silica beads, washed with several buffers and released into 100±1 µL of elution buffer. Each batch of nucleic acid extractions included a negative extract control (ultrapure water) and an in-house positive control (10±0.5 µL aliquot of the process control virus, MNV).

3.4.3. Detection by qRT-PCR

Quantitative RT-PCR for HAV, NoV GI and NoV GII was done using primers and probes as specified in ISO/TS 15216-1:2013. Primers and probes for real-time RT-PCR of the process control virus (MNV) were those specified by Hewitt et al. 2009 (Hewitt et al. 2009) and methodology including controls are outlined in Torok et al 2015 (Torok et al. 2015). 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 qRT-PCR was repeated for confirmation.

4. Laboratory training in the procedures for FRNA phage enumeration in bivalve shellfish

State based regulators and laboratories were invited to nominate appropriate staff to participate in training in the procedures for FRNA phage enumeration in bivalve shellfish. The invitation was extended to ALS Global, Central Coast Council, MidCoast Water, Port Macquarie-Hastings Council, Shoalhaven City Council and TasWater. The training was scheduled for dates suitable for those expressing interest and was held at the SARDI FSI laboratories in Adelaide on the 7th and 8th November 2017.

Results

1. FRNA phage background study

Samples for the background baseline study came from the five growing areas: Camden Haven, Hanleys Point, NSW; Brisbane Water, Murphys Bay, NSW; Shoalhaven, Goodnight Island, NSW; Shoalhaven, Berrys Bay, NSW; Pittwater, Zone 3, Tas; and Island Inlet, Zone 4, Tas. Samples were usually collected in the third week of each month from July 2016 to December 2017, with 18 rounds and a total of 100 samples analysed (Appendix D). All samples were received at SARDI FSI Laboratories within five days of collection. Arrival temperatures ranged from 6.6°C to 28.6°C. No samples were provided by the growers from the Shoalhaven area (Goodnight Island and Berrys Bay) in the December 2016 round and Camden Haven in the October 2017 round. No background samples were taken from Camden Haven or Shoalhaven, Goodnight Island in the March 2017 round and Camden Haven, Pittwater and Island Inlet in the December 2017 round due to adverse sewage events. A summary of sample collection per round is presented in Appendix D.

All 100 samples were homogenised within 24 hrs of receipt and all sample homogenates were analysed for FRNA phages within 48 hrs of preparation. The results of the analysis are presented in Figure 6.

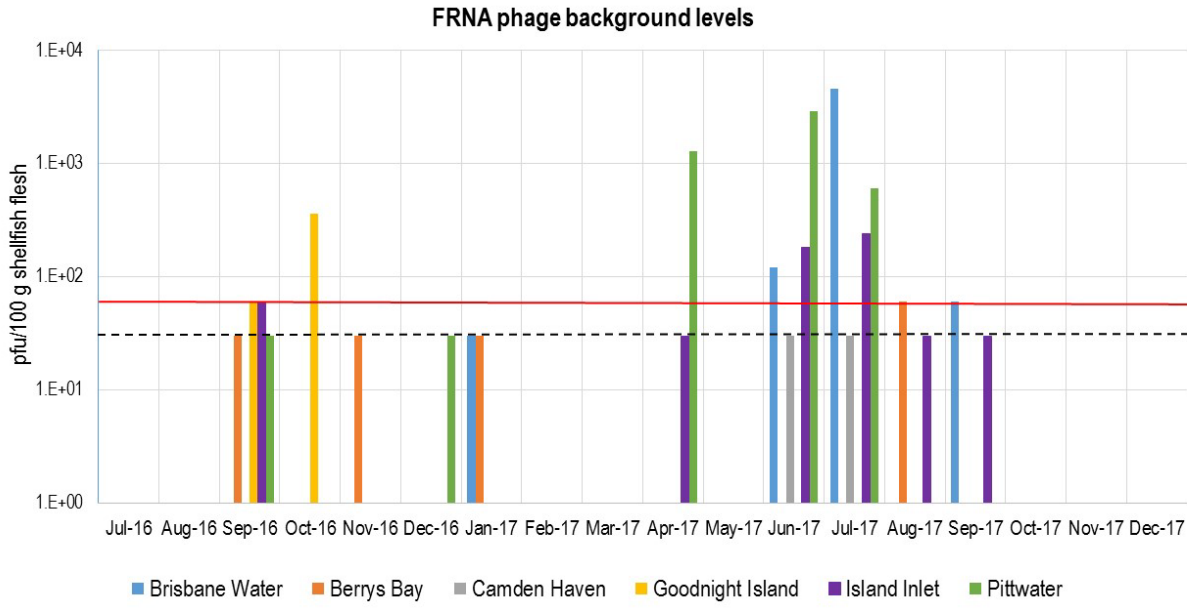

Figure 6: FRNA phage background levels over 18 months in three New South Wales and two Tasmanian oyster growing areas. The red line corresponds to the proposed cut-off for re-opening after an adverse sewage event of 60 pfu/100 g shellfish flesh. The dashed black line corresponds to the limit of detection of 30 pfu/100 g shellfish flesh. Only samples with ≥30 pfu/100 g are shown, 77 samples were <30 pfu/100 g shellfish flesh.

The results are expressed as plaque forming units (pfu) per 100 g of shellfish flesh. The ASQAP Manual 2018 cut-off for re-opening after an adverse event is 50 pfu/100 g shellfish flesh[2], unless previously established background levels for the specific harvest area have been identified as valid and safe by extensive sampling during open for harvest periods.

Of the 100 samples analysed, phages were detected in 23 samples with the remaining 77 samples reported as having <30 pfu/100 g shellfish flesh. Spikes in FRNA phage levels, ≥1000 pfu/100 g of shellfish flesh, were

[2] The method for FRNA phage enumeration at SARDI is based on the Cefas standard procedure, refer to methods, section 3.2. The limit of detection is 30 pfu/100 g shellfish flesh and phage titres are multiples of 30 pfu. The ASQAP manual will need to be revised to 60 pfu/100g shellfish flesh if the method employed by SARDI is applied.

detected in two samples from Pittwater, Tas (1290 pfu/100 g shellfish flesh in April 2017 and 2880 pfu/100 g shellfish flesh in June 2017) and one sample from Brisbane Water, NSW (4590 pfu/100 g shellfish flesh in July 2017).

The Pittwater sample collected on the 19th April was taken from an open lease where no rainfall had been recorded for the previous five days with only 1.4 mm logged over two days on the 13th and 14th April and salinity was 36.8 PSU. Similarly the sample collected on the 20th June was from an open lease, no rain had been recorded for the previous 15 days and salinity was 35.8 PSU. The environmental triggers for closure in Pittwater are when rainfall ≥70 mm in seven days at Hobart airport or salinity ≤29 PSU, hence neither rainfall nor salinity were approaching the closure limits (refer to Table 3 for environmental and bacterial triggers for Pittwater).

The Brisbane Water sample taken on the 16th July was taken from sub-tidal floating baskets in an open lease. This lease is conditionally restricted with closure triggered when rainfall is >40 mm within 24 hrs or salinity measured at sites 31 or 32 (Figure 2), adjacent to this lease at mid-ebb tide is <21 PSU. No rainfall had been recorded since 2.6 mm on the 13th July and salinity at this lease was 30.7 PSU (refer to Table 2 for environmental and bacterial triggers for Brisbane Water).

The remaining samples with elevated levels were also taken from open leases that did not approach environmental trigger levels of rainfall or salinity. The phage titres in pfu/100 g shellfish flesh ranged from 180 (19th June 2017, Island Inlet, 36.2 PSU and no rain the preceding 13 days), 240 (17th July 2017, Island Inlet, 36.0 PSU and only 0.2 mm rain on 16th July and none the preceding 11 days), 360 (16th Oct 2016, Goodnight Island, 31 PSU and only 1.8 mm rain on the 13th Oct), and 120 (19th June 2017, Brisbane Water, salinity level not reported and a total of 2.6 mm rain over the preceding 7 days).

The environmental and bacterial triggers for closure of the growing areas investigated in the background phage study, as well as those investigated in the adverse sewage events are outlined for NSW (Table 2) and Tasmania (Table 3).

2. Adverse sewage events

2.1. Moulting Bay, Tasmania (January 2017)

In January 2017, localised heavy rain resulted in pump station sewage discharge into Moulting Bay in Tasmania. This site was not included in the background study. The event started, was first reported and ended on the 20th January. Approximately 37mm of rain fell at St Helens to 9 am on 20th January 2017. A further 52mm fell between 9 am and 12 pm that same day. High tide was recorded at 4:45 am and 16:43 pm on the 20th January and the period included incoming and outgoing tides. The volume was estimated to be >100 kL and consisted of untreated sewage and stormwater. Stormwater infiltration of the sewerage network resulted in the overflow of two sewage pump stations located on the Esplanade and Jason St in St Helens. The overflow was estimated to persist for 4 hrs at Jason St and 6 and ¾ hrs at the Esplanade area. The Esplanade sewage pump station (SPS) is marked as SPS1 and Jason St SPS is marked as SPS2 in Figure 7. The environmental and bacterial contamination triggers for closure of this growing area are detailed on Table 3.

Figure 7: Esplanade (SPS1) and Jason St (SPS2) sites

Receiving waters and wastewaters were tested on the 20th and 21st January with the results detailed in Table 4.

Table 4: Moulting Bay water samples and results

Sample	date	Thermotolerant coliforms cfu/100 mL
Beauty Bay upstream	20/01/2017	350
Jason St/Beauty Bay SPS2 outfall	20/01/2017	280
Beauty Bay downstream	20/01/2017	350
Esplanade upstream	20/01/2017	12,000
Esplanade SPS1 outfall	20/01/2017	9,600
Esplanade downstream	20/01/2017	8,000
Esplanade SPS1 - Upstream	22/01/2017	100
Esplanade SPS1 - Outfall	22/01/2017	130
Esplanade SPS1 - Downstream	22/01/2017	140

Five sites were selected for shellfish sampling shown in figures 8 and 9 with the first samples of Pacific oysters collected on the 23rd January (day 3), then samples on the 30th January (day 10) and the 13-14th January 2017 (day 24).

Figure 8: Moulting Bay spill and sample sites

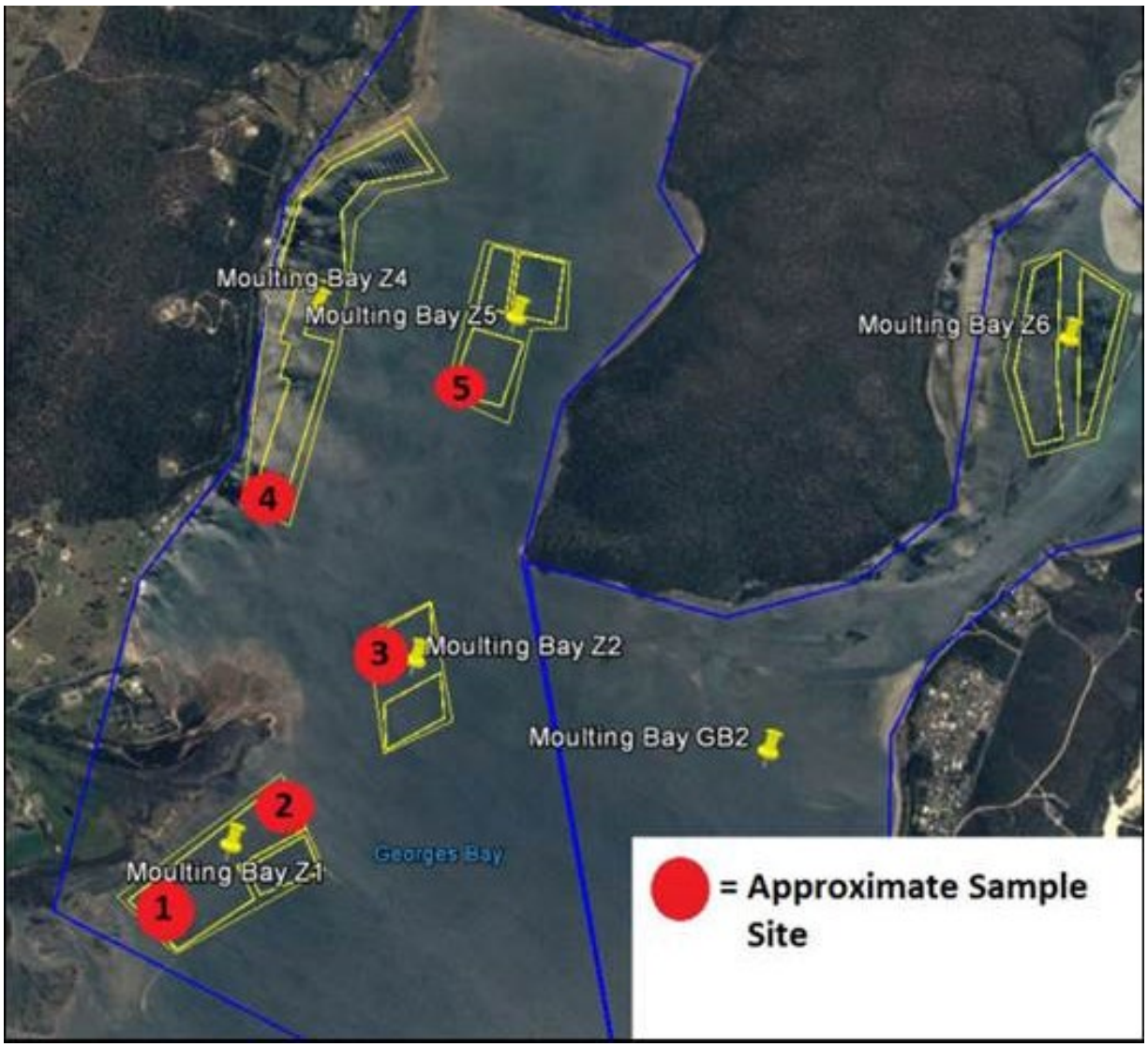

Figure 9: Moulting Bay sample sites

The distance from the SPSs to sample sites are estimated to be 2.9 km for sample 1, 2 km for sample 2, 5.9 km for sample 3, 5.9 km for sample 4 km and 6.0 km for sample 5 as detailed on Figure 8 and Figure 9.

The results of phage, *E. coli* and foodborne virus testing are summarised in Table 5.

The extraction and amplification efficiencies for the enteric virus assays of all samples were acceptable. NoV or HAV were not detected. Levels of FRNA phages were 30 pfu or below. *E.coli* levels were high at all sites initially, and persisted at high levels in at least one site across all sampling events.

Table 5: Moulting Bay, Tasmania, adverse sewage event January 2017

Sample ID	Date sampled	Date tested	FRNA phage pfu/100 g shellfish flesh	*E. coli* MPN/100 g shellfish flesh	NoV GI	NoV GII	HAV
Day 3 samples:							
Zone 1 sample 1	23/01/2017	27/01/2017	<30	70	ND[1]	ND	ND
Zone 1 sample 2	23/01/2017	27/01/2017	<30	430	ND	ND	ND
Zone 2 sample 3	23/01/2017	27/01/2017	30	700	ND	ND	ND
Zone 4 sample 4	23/01/2017	27/01/2017	<30	700	ND	ND	ND
Zone 5 sample 5	23/01/2017	27/01/2017	<30	310	ND	ND	ND
Day 10 samples:							
Zone 1 sample 1	30/01/2017	2/02/2017	<30	160	ND	ND	ND
Zone 1 sample 2	30/01/2017	2/02/2017	<30	750	ND	ND	ND
Zone 2 sample 3	30/01/2017	2/02/2017	<30	20	ND	ND	ND
Zone 4 sample 4	30/01/2017	2/02/2017	<30	20	ND	ND	ND
Zone 5 sample 5	30/01/2017	2/02/2017	<30	40	ND	ND	ND
Day 24 samples:							
Zone 1 sample 1	13/02/2017	16/02/2017	<30	90	ND	ND	ND
Zone 1 sample 2	13/02/2017	16/02/2017	<30	700	ND	ND	ND
Zone 2 sample 3	13/02/2017	16/02/2017	<30	40	ND	ND	ND
Zone 4 sample 4	14/02/2017	16/02/2017	<30	<20	ND	ND	ND
Zone 5 sample 5	13/02/2017	16/02/2017	<30	<20	ND	ND	ND

[1] ND - not detected

2.2. Camden Haven, NSW (March 2017)

In NSW in March 2017, a pump station spill in heavy rainfall impacted the Camden Haven growing area. At 8:00 am on the 16th March, the council noted evidence of a surcharge from the reticulated sewerage network in McLellan St, Laurieton, from infiltration and inflow into the reticulation system due to heavy rain. The overflow had ceased when council staff arrived onsite to clean up and disinfect. The tide was incoming and high tide was recorded at 11:49 am on the 16th March. The volume was unknown, however, the overflow discharged into the Camden Haven River via a stormwater drain, see Figure 10. The Bureau of Meteorology (BOM) station at Laurieton recorded 203.0 mm rain accumulated over three days from the 14th - 16th March. This resulted in a 21 day closure for all three harvest areas in the estuary; Gogleys Lagoon, Hanleys Point and Stingray Creek. The environmental and bacterial contamination triggers for closure of this growing area are detailed on Table 2.

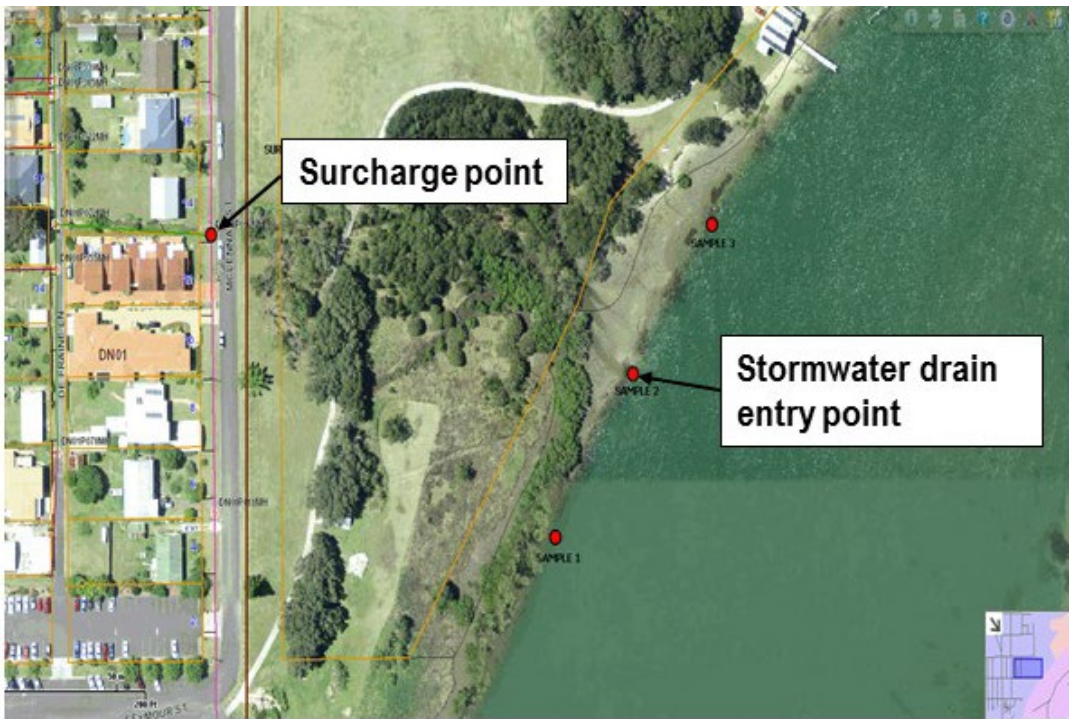

Figure 10: Location of spill manhole and drain entry point, Laurieton impacting Camden Haven growing area

Samples of Sydney Rock oysters were taken from four sites (see Figure 11), two at Stingray Creek and two at Hanleys Point, as soon as possible after the event on the 20th March, day 11 samples on the 27th March and day 25 samples on the 10th April 2017. Hanleys Point site 16 was closest to the surcharge point at only 215 m away, in contrast to the remaining sites Hanleys Point site 6, Stingray Creek site 15 and Stingray Creek site 20 at approximately 1.7 km, 1.23 km and 1.7 km, respectively.

In addition, water samples were taken from Camden Haven River at the three sites shown in Figure 10 and tested for thermotolerant coliforms with the results detailed on Table 6.

Table 6: Camden Haven March 2017 water samples

Thermotolerant coliforms (presumptive) cfu/100 mL			
Sample		16/03/2017	17/03/2017
Sample 1	50 m upstream	3900	5200
Sample 2	Entry point to drain	780	4300
Sample 3	50 m downstream	540	3300

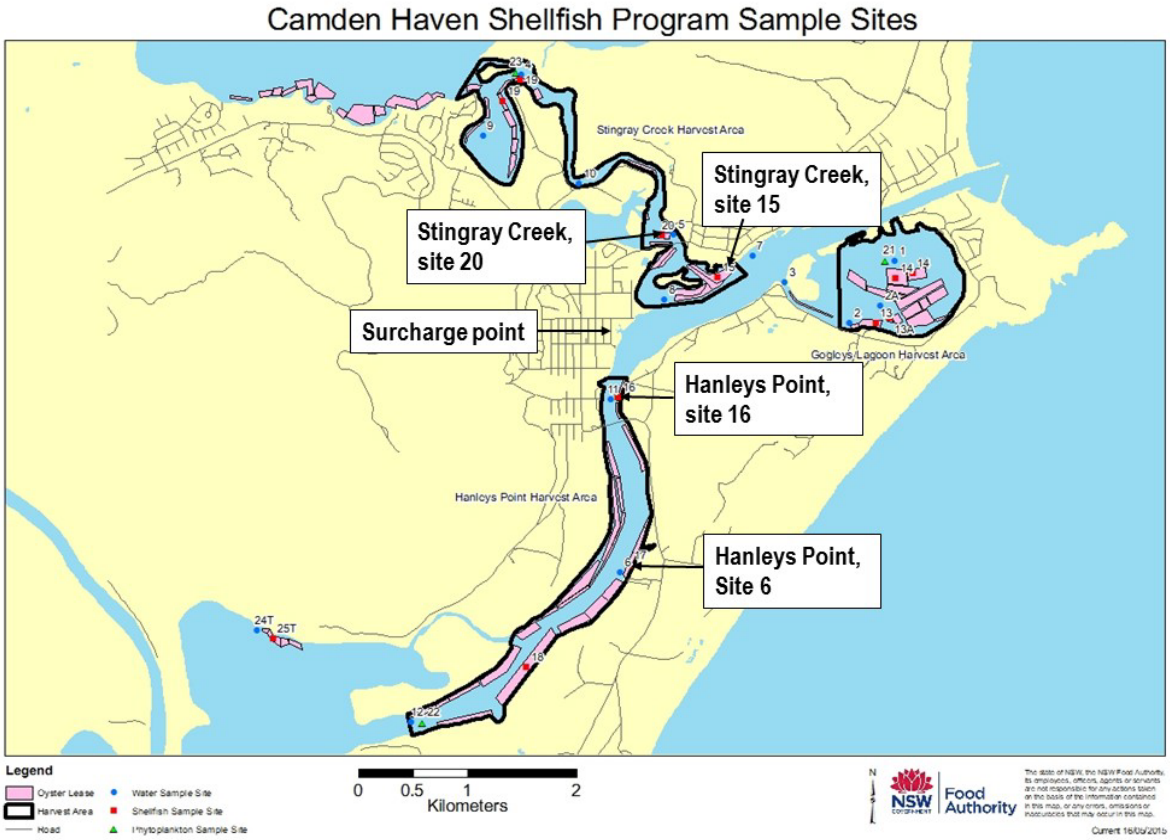

Figure 11: Camden Haven, March 2017 adverse sewage event sample sites

The shellfish microbiological and enteric virus results are summarised in Table 7.

Table 7: Camden Haven, NSW, adverse sewage event March 2017.

Camden Haven site	Date sampled	Date tested	FRNA phage pfu/100 g shellfish flesh	*E. coli* MPN/100 g shellfish flesh	NoV GI	NoV GII	HAV
Day 4 samples:							
Hanleys Point site 17[1]	20/03/2017	22/03/2017	<30	750	ND[2]	ND	ND
Stingray Creek site 15	20/03/2017	22/03/2017	60	110	ND	ND	ND
Hanleys Point site 16	20/03/2017	22/03/2017	<30	750	ND	ND	ND
Stingray Creek site 20	20/03/2017	22/03/2017	30	430	ND	ND	ND
Day 11 samples:							
Hanleys Point site 6	27/03/2017	28/03/2017	<30	700	ND	ND	ND
Stingray Creek site 15	27/03/2017	28/03/2017	<30	220	ND	ND	ND
Hanleys Point site 16	27/03/2017	28/03/2017	60	700	ND	ND	ND
Stingray Creek site 20	27/03/2017	28/03/2017	<30	500	ND	ND	ND
Day 25 samples:							
Hanleys Point site 6	10/04/2017	12/04/2017	<30	<20	ND	ND	ND
Stingray Creek site 15	10/04/2017	12/04/2017	<30	20	ND	ND	ND
Hanleys Point site 16	10/04/2017	12/04/2017	<30	20	ND	ND	ND
Stingray Creek site 20	10/04/2017	12/04/2017	<30	310	ND	ND	ND

[1] Day 11 and 25 samples taken from site 6, adjacent to site 17
[2] ND - Not detected

The extraction and amplification efficiencies for the enteric virus assays of all samples were acceptable, no NoV or HAV were detected. FRNA phage levels were 60 pfu/100 g or lower for all samples. *E. coli* levels were elevated at all sites until day 25.

2.3. Brisbane Water, NSW (March 2017)

A second adverse event in March 2017 in NSW involved a total volume of 2.4 ML into Murphys Bay in the Brisbane Water growing area, see Figure 12 and Figure 13. The spill comprised an estimated 1.4 ML of untreated raw sewage plus 1 ML of stormwater. The spill started at 7:08 pm on the 22nd March 2017, as reported by the SCADA alarm, and ceased at 10:48 pm that evening. The tide was outgoing as high tide was recorded at 7:00 pm. It was caused by an intense rainfall event of 38 mm from 3:50 to 4:30 pm combined with a power outage resulting in shutting down the sewer pump stations. Samples of Sydney Rock oysters were taken from five sites: two sites with subtidal floating baskets, two sites with intertidal cultivation trays and one site of wild caught oysters, see Figure 14. Samples were collected on the 24th March, day 7 samples on 29th March and day 21 samples on the 12th April 2017. The environmental and bacterial contamination triggers for closure of this growing area are detailed on Table 2.

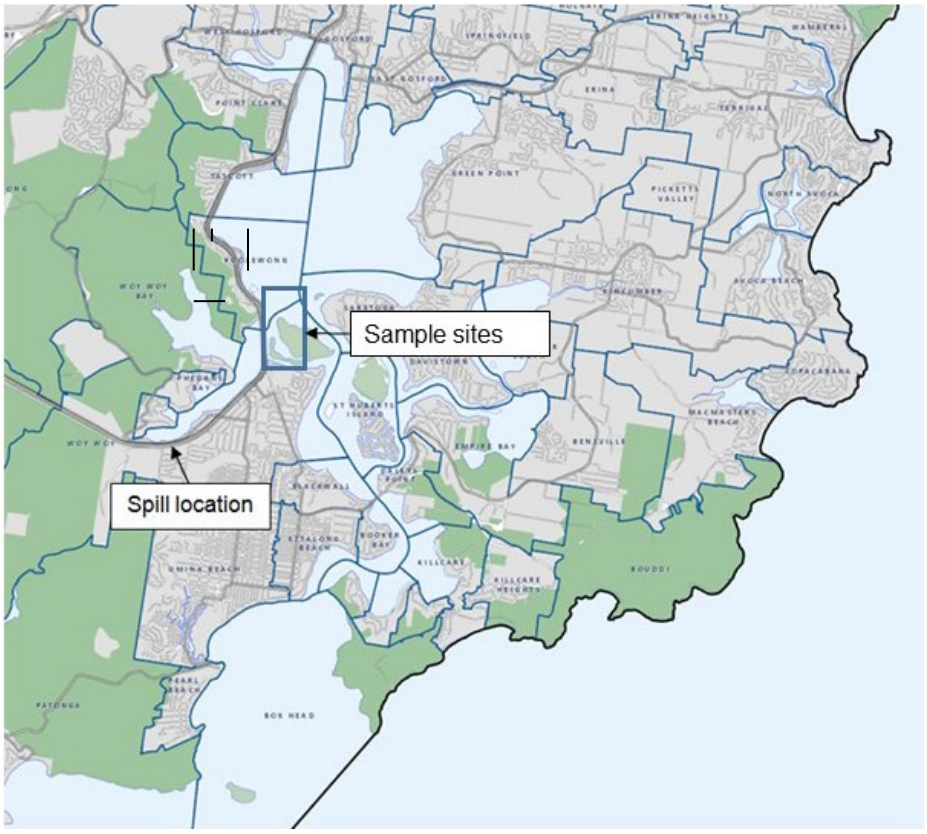

Figure 12: Brisbane Water adverse event spill location and sample sites

Figure 13: Sewer pump station WWMJ and discharge

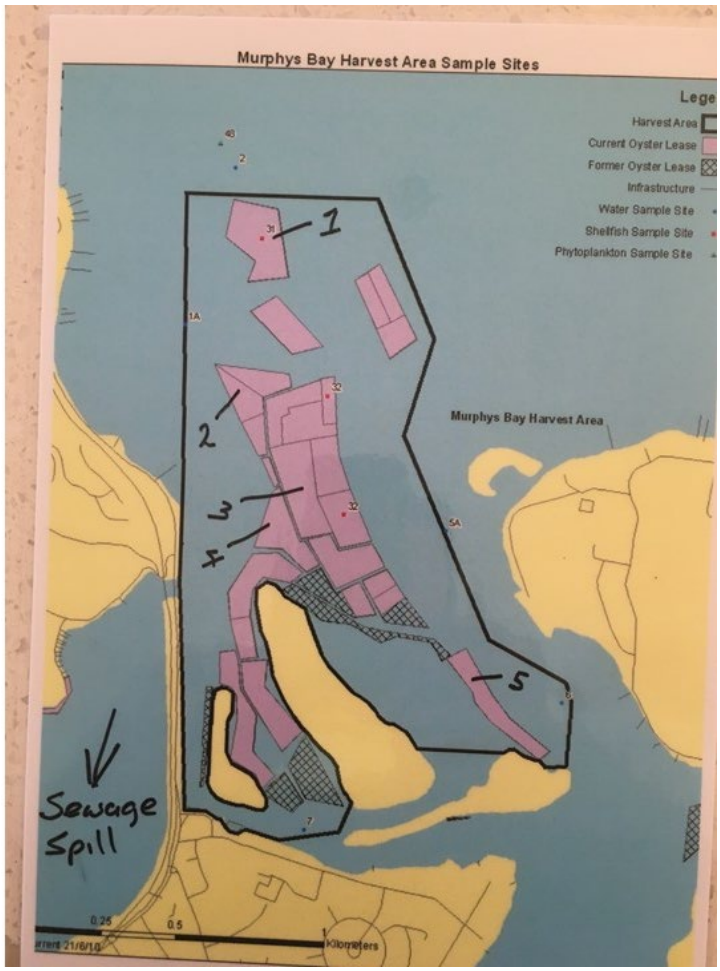

Figure 14: Brisbane Water, Murphys Bay adverse event sample sites

The distances from the discharge site to each sample site were approximately 4.2 km for site 1, 3.7 km for site 2, 3.5 km for site 3, 3.4 km for site 4 and 3.9 km for site 5.

The results of microbiological and enteric virus testing are summarised in Table 8.

Table 8: Brisbane Water, NSW, adverse sewage event March 2017 shellfish test results.

Brisbane Water site	Date sampled	Date tested	FRNA phage pfu/100 g shellfish flesh	*E. coli* MPN/100 g shellfish flesh	NoV GI	NoV GII Ct values	HAV
Day 2 samples:							
site 1: intertidal cultivation tray	24/03/2017	28/03/2017	180	310	ND[1]	ND	ND
site 2: subtidal floating baskets	24/03/2017	28/03/2017	150	>18000	ND	(44.364, ND)[2] (38.607, ND)	ND
site 3: intertidal cultivation tray	24/03/2017	28/03/2017	480	1,300	ND	ND	ND
site 4: wild caught oysters	24/03/2017	28/03/2017	420	3,500	ND	ND	ND
site 5: subtidal floating baskets	24/03/2017	28/03/2017	<30	750	ND	ND	ND
Day 7 samples:							
site 1: intertidal cultivation tray	29/03/2017	30/03/2017	<30	220	ND	ND	ND
site 2: subtidal floating baskets	29/03/2017	30/03/2017	<30	>18000	ND	ND	ND
site 3: intertidal cultivation tray	29/03/2017	30/03/2017	<30	110	ND	ND	ND
site 4: wild caught oysters	29/03/2017	30/03/2017	<30	110	ND	ND	ND
site 5: subtidal floating baskets	29/03/2017	30/03/2017	<30	160	ND	ND	ND
Day 21 samples:							
site 1: intertidal cultivation tray	12/04/2017	13/04/2017	<30	220	ND	ND	ND
site 2: subtidal floating baskets	12/04/2017	13/04/2017	<30	>18000	ND	ND	ND
site 3: intertidal cultivation tray	12/04/2017	13/04/2017	<30	90	ND	ND	ND
site 4: wild caught oysters	12/04/2017	13/04/2017	<30	110	ND	ND	ND
site 5: subtidal floating baskets	12/04/2017	13/04/2017	<30	500	ND	ND	ND

[1] Not detected
[2] Ct values are for neat samples in duplicate. Positive PCRs were repeated to confirm the result.

The extraction and amplification efficiencies for the enteric virus assays of all samples were acceptable. A trace level of NoV GII was detected in one sample on day 2. FRNA phage levels were elevated on day 2 at 4 of the 5 sites but returned to background levels by day 7. The *E. coli* levels were raised at all sites on day 2 and persisted at high levels at least one site on all occasions.

2.4. Camden Haven, NSW (December 2017)

On the 6th December 2017, a raw sewage leak from a new line being laid in the Camden Haven area in NSW was identified and continued for an estimated 12 hrs covering both incoming and outgoing tides with high tide at 11:33 am. The leak was initially noted at 7:30 am, although it was presumed to have started the previous day with an estimated 24 hrs duration. The location was between Bay St and The Boulevarde, Dunbogan, see Figure 15. The volume of this spill was estimated at 1500 kL caused by failure of a sewer rising main pipe. The environmental and bacterial contamination triggers for closure of this growing area are detailed on Table 2. Sand was used to barricade the area to prevent the rising tide from inundating the affected area. Approximately 385 kL of sewage contaminated salt water was removed from the site using vacuum tankers.

Figure 15: Surcharge and sand plug locations, Camden Haven

Samples of Sydney Rock oysters were collected from four sites shown in Figure 16. The distance from the surcharge and sample sites was approximately 300 m to Hanleys Point site 16, 1.1 km to Stingray Creek site 15, 3 km to Gogleys site 13 and 3.3 km to Gogleys site 14. The first samples were taken on 6th December, day 6 samples on the 11th December and day 23 samples on 28th December 2017. The day 23 samples were further impacted by heavy rainfall of 177.0 mm recorded at the Laurieton weather station the previous 24 hrs.

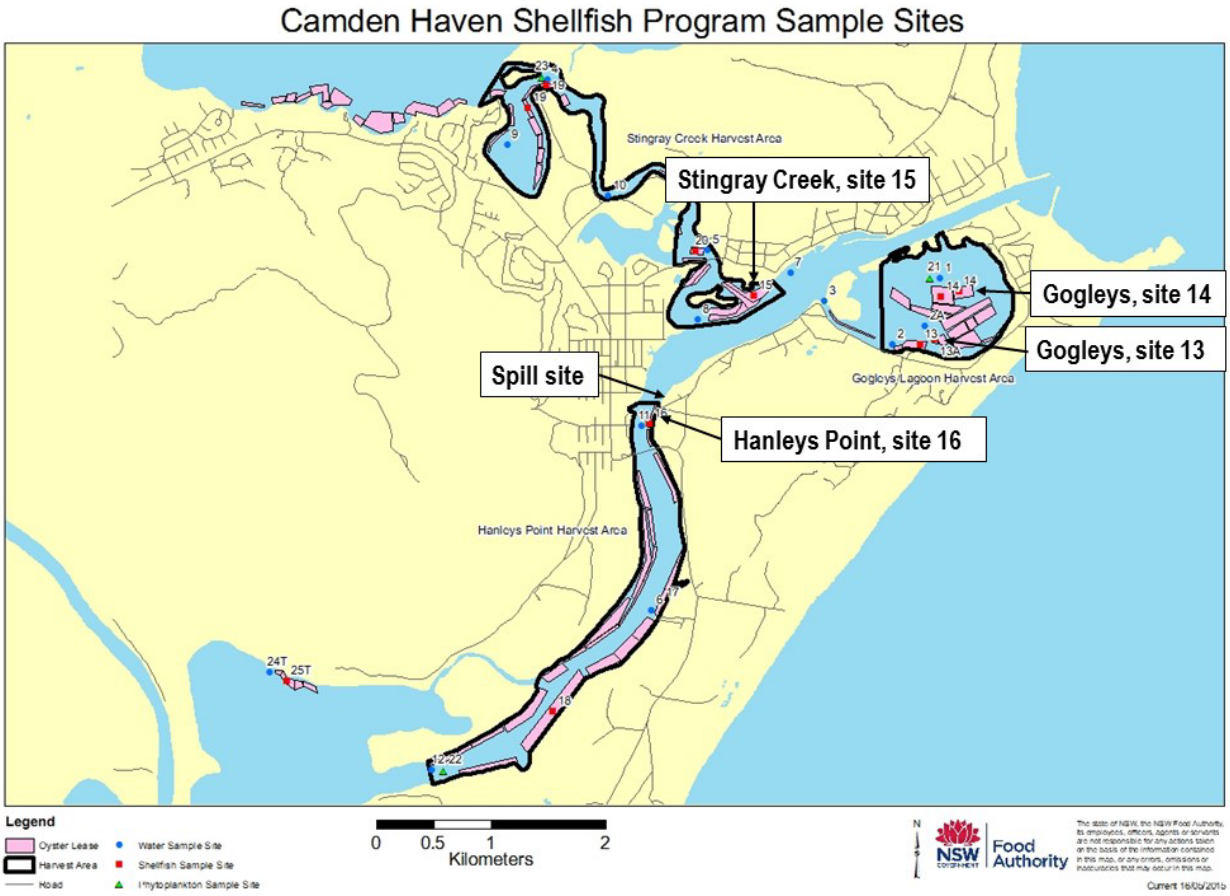

Figure 16: Camden Haven sample sites, December 2017

Water samples were taken near the affected area at the sites located on Figure 17 and analysed for thermotolerant coliforms.

Figure 17: Location of water sample sites, Camden Haven Dec 2017

The results of water thermotolerant coliform testing are detailed on Table 9.

Table 9: Camden Haven December 2017 water samples results

Thermotolerant coliforms (presumptive) cfu/100 mL				
Sample		6/12/2017	7/12/2017	8/12/2017
Sample 1	50 m upstream	10,000	18	11
Sample 2	In front	7900	27	~5
Sample 3	50 m downstream	6,100	90	14

The results of FRNA phage, *E. coli* and enteric virus testing are summarised in Table 10.

Table 10: Camden Haven, NSW, adverse sewage event December 2017 shellfish test results.

Camden Haven site	Date sampled	Date tested	FRNA phage pfu/100 g shellfish flesh	*E. coli* MPN/100 g shellfish flesh	NoV GI Ct values	NoV GII Ct values	HAV
Day 1 samples:							
Gogleys site 13	6/12/2017	8/12/2017	<30	160	(37.875, 36.866)[1] (36.455, 35.748)	ND[2]	ND
Gogleys site 14	6/12/2017	8/12/2017	<30	40	(36.962, 38.343) (35.540, 36.209)	ND	ND
Stingray Creek site 15	6/12/2017	8/12/2017	<30	1700	(36.458, 38.281) (35.928, 37.713)	41.676, 41.755 36.539, 37.329	ND
Hanleys Point site 16	6/12/2017	8/12/2017	<30	1300	(36.580, 39.151) (34.963, ND)	(40.785, 41.853) (37.164, ND)	ND
Day 6 samples:							
Gogleys site 13	11/12/2017	15/12/2017	30	<20	ND	ND	ND
Gogleys site 14	11/12/2017	15/12/2017	<30	20	ND	ND	ND
Stingray Creek site 15	11/12/2017	15/12/2017	<30	20	ND	ND	ND
Hanleys Point site 16	11/12/2017	15/12/2017	<30	90	(40.175, 41.126) (36.077, 36.841)	ND	ND
Day 23 samples:							
Gogleys site 13	28/12/2017	29/12/2017	<30	4800	ND	ND	ND
Gogleys site 14	28/12/2017	29/12/2017	<30	720	ND	ND	ND
Stingray Creek site 15	28/12/2017	29/12/2017	<30	330	ND	ND	ND
Hanleys Point site 16	28/12/2017	29/12/2017	<30	390	ND	ND	ND

[1] Ct values are for neat samples in duplicate. Positive PCRs were repeated to confirm the result.
[2] Not detected

The extraction and amplification efficiencies for the enteric virus assay of all samples were acceptable. NoV was detected in oysters from all four sites on day 1, but only at site 16 on day 6. There were no elevated FRNA phage levels from any samples. *E. coli* levels were raised in the day 1 samples, had dropped by day 6, but had increased even higher in the day 23 samples than compared to day 1 samples in response to the second rainfall event. The FRNA phage and the *E. coli* levels of the day 6 sample positive for NoV were not raised.

2.5. Pittwater and Island Inlet, Tasmania (December 2017)

The second adverse event in Tasmania occurred in December 2017 due to sewage overflows into the Pittwater and Island Inlet growing areas in Tasmania during a rainfall event. Rainfall of 66 mm to 9am was recorded on the 4th December at Hobart airport with an additional 86 mm of rain during the same period at Richmond. The environmental and bacterial contamination triggers for closure of this growing area are detailed on Table 3. Stormwater infiltration of the sewerage system led to three confirmed untreated or partially treated sewage discharges from the following locations as shown in Figure 16; Bilney Street SPS (SPS3) in Richmond, the two Cambridge Park SPSs (SPS4 and SPS5) and the Cambridge sewage treatment plant (STP).

The Bilney St SPS discharge started at 6:15 pm on 3rd December when the pump station was taken offline due to flooding and risk of electrical problems during an incoming tide, as high tide was recorded at 9:42 pm. An undetermined amount of raw sewage was discharged and flowed into the Coal River upstream of the Pittwater growing area. The Cambridge Park SPS failures resulted in raw sewage overflowing from the lids and into the stormwater system discharging into the growing areas. The Cambridge STP went onto bypass due to the excess amount of stormwater in the sewage system resulting in an estimated volume of 1.2 ML of partially treated sewage (screened and chlorinated only) bypassing the treatment plant and being discharged into St Clair's Creek at the western end of the Island Inlet growing area.

In addition, sewage discharge overflowed from sewer lines at Mid-Way Point due to a tree root blockage on the 8th December, see Figure 18. Tides were incoming and outgoing as high tides were recorded at 2:00 am and 12:40 pm on the 8th December. TasWater estimated that approximately 2-3 kL of raw sewage had reached the stormwater drain. This led to a new 21 day closure period on the 8th December.

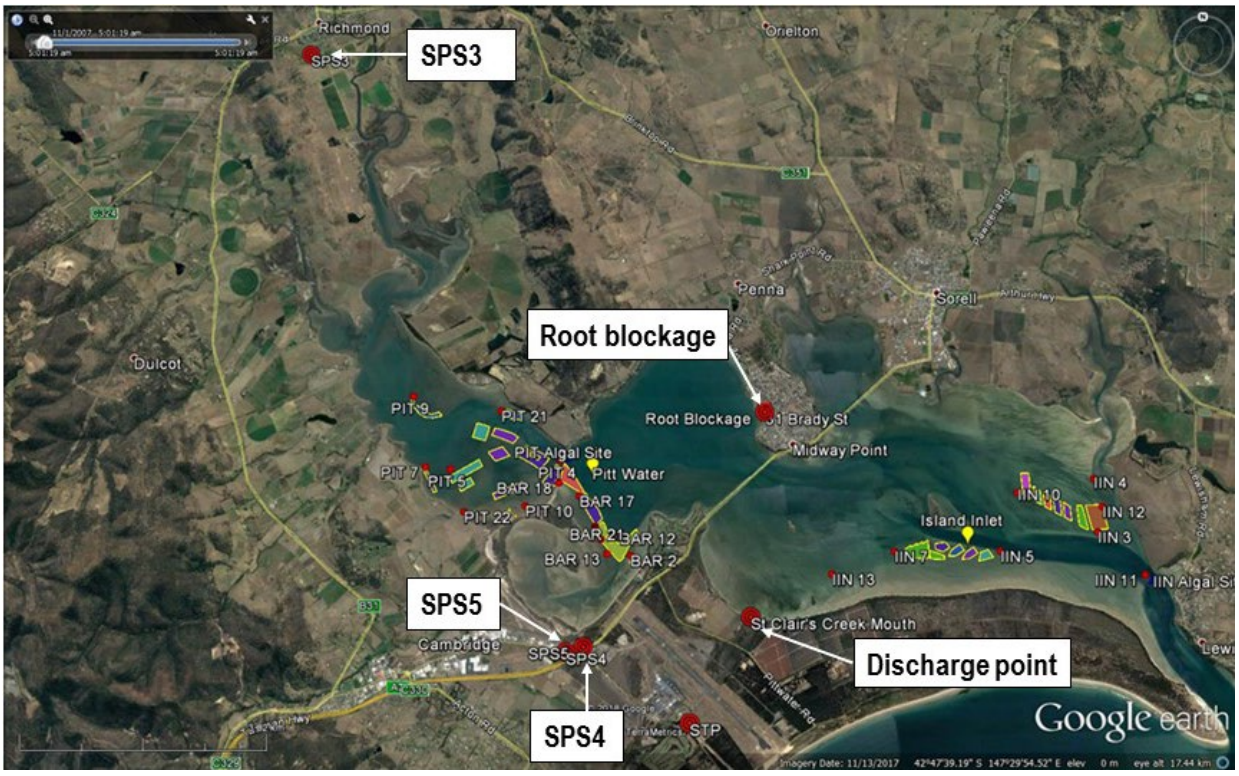

Figure 18: Bilney Street SPS (SPS3) in Richmond, Cambridge Park SPS's (SPS4 & 5), root blockage and discharge point

Pacific oysters were collected from seven sites (4 leases in Pittwater and 3 leases in Island Inlet) on day 5 (6-7th December), eight sites (4 leases in Pittwater and 4 leases in Island Inlet) on day 7 (11th December) and seven sites (3 leases in Pittwater and 4 leases in Island Inlet) on day 23 (27th December), see Figure 19 and Figure 20.

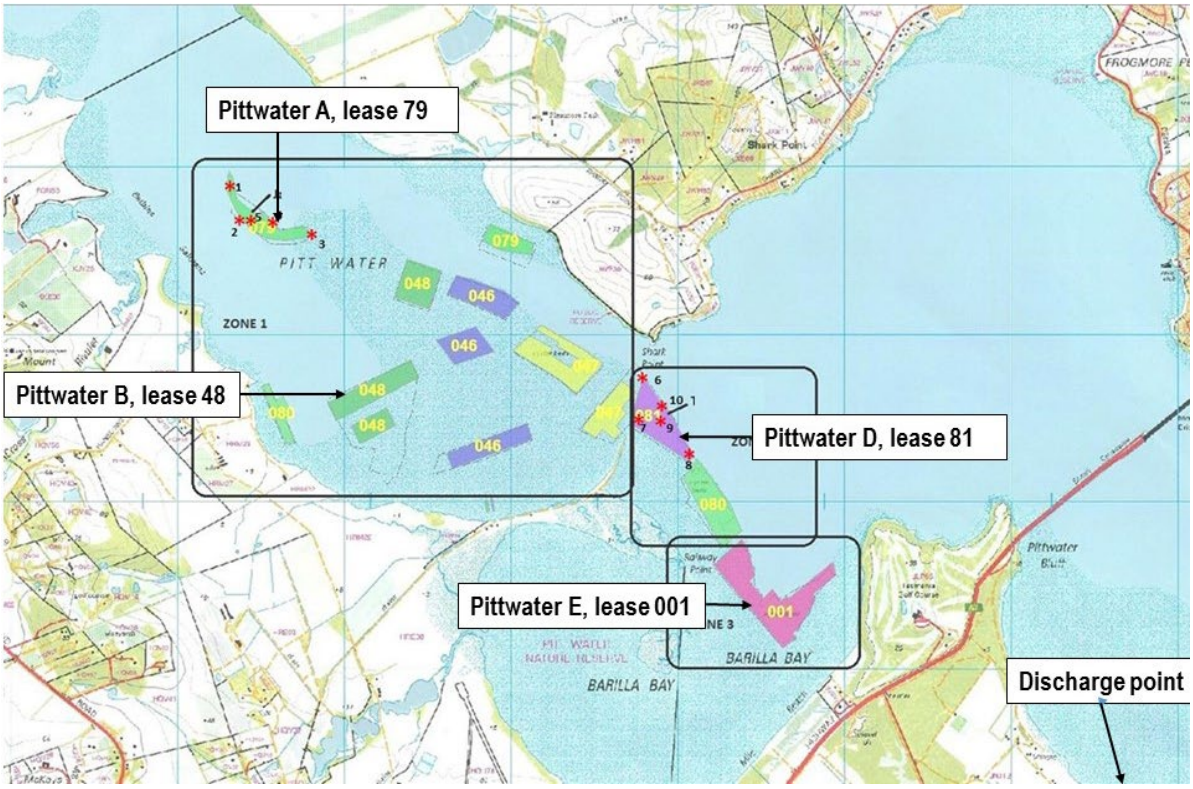

Figure 19: Pittwater sample sites for December 2017 adverse sewage event

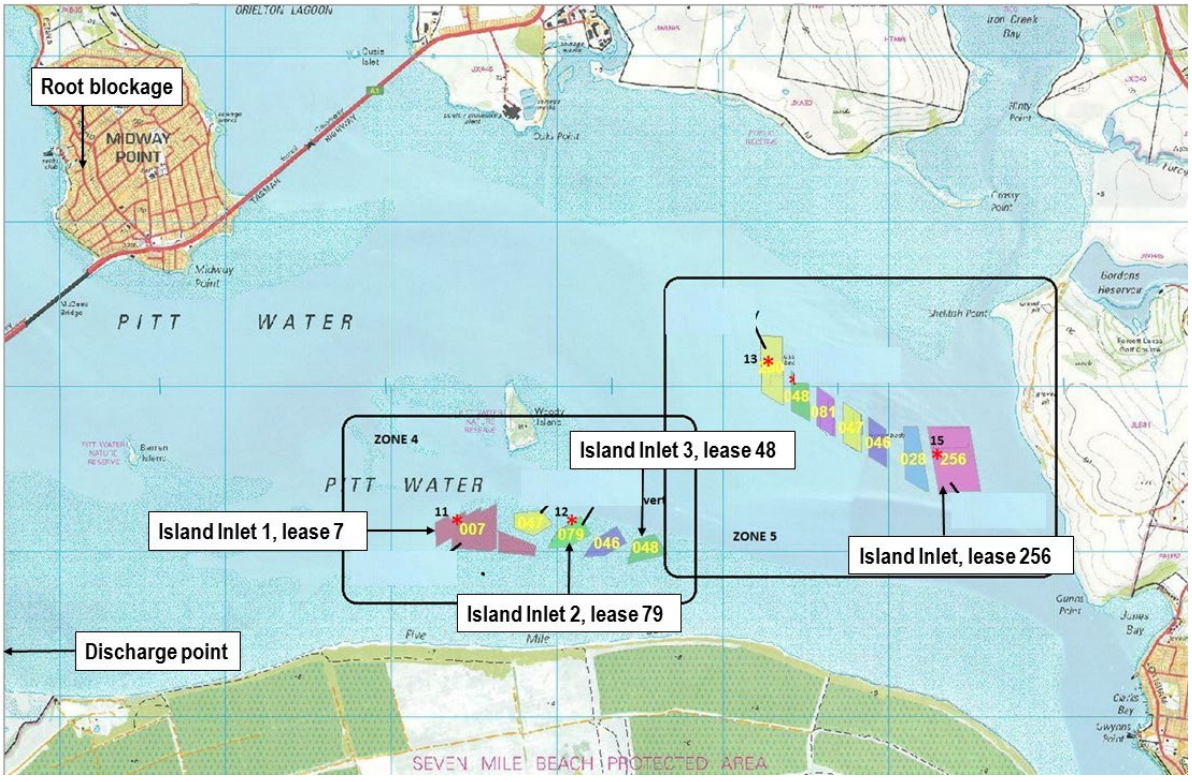

Figure 20: Island Inlet sample sites for December 2017 adverse sewage event

Pittwater sites A, B, D and E were 7.6 km, 6.7 km, 4.6 km and 4.1 km from the discharge point, respectively. Island Inlet sites 1, 2, 3 and 5 were 2.75 km, 3.5 km, 3.9 km and 5.8 km, respectively.

The results of FRNA phage, *E. coli* and enteric virus testing are summarised in Table 11.

Table 11: Pittwater and Island Inlet, Tasmania, adverse sewage event December 2017 shellfish test results.

Pittwater/Island Inlet site	Date sampled	Date tested	FRNA phage pfu/100 g shellfish flesh	*E. coli* MPN/100 g shellfish flesh	NoV GI	NoV GII Ct values[1]	HAV
Day 3 samples:							
Pittwater A, lease 79	7/12/2017	8/12/2017	120	2,400	ND[1]	ND	ND
Pittwater B, lease 48	7/12/2017	8/12/2017	120	500	ND	ND	ND
Pittwater D, lease 81	6/12/2017	8/12/2017	30	1,300	ND	ND	ND
Pittwater E, lease 001	7/12/2017	8/12/2017	30	160	ND	ND	ND
Island Inlet 2, lease 79	7/12/2017	8/12/2017	<30	500	ND	ND	ND
Island Inlet 3, lease 48	7/12/2017	8/12/2017	30	500	ND	ND	ND
Island Inlet 5, lease 256	7/12/2017	8/12/2017	<30	500	ND	(38.669, 37.874)[2] (42.823, 35.628)	ND
Day 7 samples:							
Pittwater A, lease 79	11/12/2017	14/12/2017	30	220	ND	ND	ND
Pittwater B, lease 48	11/12/2017	14/12/2017	<30	<20	ND	ND	ND
Pittwater D, lease 81	11/12/2017	14/12/2017	30	<20	ND	ND	ND
Pittwater E, lease 001	11/12/2017	14/12/2017	<30	220	ND	ND	ND
Island Inlet 1, lease 7	11/12/2017	14/12/2017	<30	40	ND	ND	ND
Island Inlet 2, lease 79	11/12/2017	14/12/2017	<30	40	ND	ND	ND
Island Inlet 3, lease 48	11/12/2017	14/12/2017	<30	310	ND	ND	ND
Island Inlet 5, lease 256	11/12/2017	14/12/2017	<30	<20	ND	ND	ND
Day 23 samples:							
Pittwater A, lease 79	27/12/2017	28/12/2017	<30	<20	ND	ND	ND
Pittwater B, lease 48	27/12/2017	28/12/2017	<30	36	ND	ND	ND
Pittwater E, lease 001	27/12/2017	28/12/2017	<30	<20	ND	ND	ND
Island Inlet 1, lease 7	27/12/2017	28/12/2017	<30	<20	ND	ND	ND
Island Inlet 2, lease 79	27/12/2017	28/12/2017	<30	<20	ND	ND	ND
Island Inlet 3, lease 48	27/12/2017	28/12/2017	1,260	3,150	ND	ND	ND
Island Inlet 5, lease 256	27/12/2017	28/12/2017	<30	36	ND	ND	ND

[1] Not detected
[2] Ct values are for neat samples in duplicate. Each PCR was repeated to confirm the result.

The extraction and amplification efficiencies for the enteric virus assay of all samples were acceptable. Trace levels of NoV were found in oysters from one site (Island Inlet 5) on day 3 only. FRNA 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 day 7 samples. The day 23 sample with high FRNA phage levels also had a very high *E. coli* level.

3. Guidance document

A guidance document to assist regulators and growers to implement this methodology has been prepared and attached to this report as Appendix G. The guidance document considers adverse event investigations including recommendations on the investigation design, appropriate sample numbers and interpretation of results.

4. Laboratory training in the procedures for FRNA phage enumeration in bivalve shellfish

State based regulators and laboratories including ALS Global, Central Coast Council, MidCoast Water, Port Macquarie-Hastings Council, Shoalhaven City Council and TasWater were invited to nominate appropriate staff to participate in training in the procedures for FRNA phage enumeration in bivalve shellfish. The training was presented on the 7th and 8th November 2017 at the SARDI FSI Laboratories in Adelaide. The Port Macquarie Hastings Environmental Laboratory and TasWater each sent one representative. The

remaining councils and laboratories declined the offer. Two SARDI members also participated in the training which included an overview and comparison of the Cefas (shellfish), US FDA (shellfish) and ISO (water) FRNA phage enumeration methods, media and sample preparation, storage of stock cultures and considerations for NATA accreditation. Participants undertook a practical component comprising sample preparation of bivalve oysters (Pacific oysters), enumeration of FRNA phages, analysis of data and expression of results. A detailed training manual with associated appendices was prepared and presented to each participant (Appendix E).

Discussion

The baseline level of FRNA phages in five 'at-risk' growing areas in NSW and Tasmania has been established for the period between July 2016 and December 2017 inclusive. The growing areas were selected on the basis of a history of previous mandatory 21 day closures as a consequence of adverse sewage events. The most frequent FRNA phage titre for all sites investigated was <30 pfu/100 g shellfish flesh indicating that phages were not detected (i.e. absent or present at very low levels) for most sampling periods. Based on this data, a baseline of 60 pfu/100 g shellfish flesh is reasonable for these growing areas. The number of occasions when FRNA phage titres were above 60 pfu/100 g shellfish flesh for each site are worth noting: Brisbane Water 11% (2/18), Berrys Bay 0% (0/17), Camden Haven 0% (0/15), Goodnight Island 5.8% (1/17), Island Inlet 13% (2/15) and Pittwater 20% (3/15). The significance of the occasional higher titre and the very high spikes detected in Pittwater (1290 pfu/100 g in April 2017 and 2880 pfu/100 g in June 2017) and Brisbane Water (4590 pfu/100 g in July 2017) in the absence of reported sewage spills and environmental triggers is unknown. The water quality over this period is recognised to be adversely affected by the presence of birds and high levels of agricultural and urban run-off. Consequently, it may be prudent to set the baseline at 60 pfu/100 g shellfish flesh for the Brisbane Water, Island Inlet and Pittwater growing areas but more data may lead to consideration of a higher seasonal baseline for the winter months.

It is possible these phages are not from human sewage or wastewater. They may be associated with animal sources such as agricultural stock or birds. It is not uncommon for birds, including migratory birds, to frequent waterways where shellfish are farmed leading to very high numbers at various times of the year. This could be investigated using microbial source tracking. Techniques include genotyping FRNA phage isolates using PCR, serotyping and F^+ phage latex agglutination and typing (CLAT) (Osawa et al. 1981, Long et al. 2005, Love and Sobsey 2007, Mieszkin et al. 2013). Studies have indicated that phages in the genogroup II and III FRNA phages are likely from human or wastewater sources, whereas phages from genogroup I and IV are predominantly associated with other animals.

The aim of the adverse event study was to confirm the validity of using FRNA phages as indicators 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 always 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 sampled for this project; Moulting Bay, Tas in January 2017 and Camden Haven, NSW in March 2017. It is worth noting these two events were similar, both due to pump station spills in heavy rainfall, leading to elevated *E. coli* levels and low phage titres of <30, 30 or 60 pfu/100 g shellfish flesh in samples taken at day 1 or 7. Moulting Bay was not included in the background study but 14 of the 15 adverse event samples had titres of <30 pfu/100 g indicating the background level is likely to be very low. The oyster leases tested in these two events do not appear to have been impacted by the sewage spills, possibly due to the dilution effect of the rain, the local water movement, or a combination of both.

TSQAP re-opened Moulting Bay zones 2, 4 and 5 at 1:30pm on 6th February 2017 based on the acceptable phage levels and other sampling and environmental criteria (salinity, rainfall and the Georges River flow rate). This was 4 days earlier than 21 days and was the first area in Australia re-opened under the phage protocol in ASQAP 2016 (ASQAAC 2016). The *E. coli* result for Zone 1 did not meet requirements despite the acceptable phage result and was not re-opened until 8th February 2017, once the *E. coli* result and environmental conditions were acceptable.

The third adverse sewage event into Brisbane Waters, NSW in March 2017 was considerably larger resulting in higher *E. coli* and FRNA phage levels in day 2 samples. The background levels of phages in Brisbane Water were generally low, <30 to 60 pfu/100 g shellfish flesh, however, a higher count of 120 pfu/100 g and very high spike of 4,590 pfu/100 g were noted in June and July 2017 respectively. Despite this, it is most likely the day 2 phage titres ranging from 60 to 480 pfu/100 g shellfish flesh were due to the sewage spill and the intense local stormwater run-off. The day 7 and day 21 phage titres were reduced to <30 pfu/100 g for all sites. *E. coli* levels were also raised on day 2 from between 310 to >18000 MPN/100 g and were lower for days 7 and 21, except in the shellfish from subtidal floating baskets in site 2 where the *E.*

coli remained very high at >18000 MPN/100 g throughout. A low level of NoV GII RNA was detected in shellfish from this site on day 2 and the corresponding phage titre was 150 pfu/100 g. The qRT-PCR was repeated, each with duplicate aliquots of the sample, resulting in high threshold cycle (Ct) levels in only one of each aliquot, specifically 44.364 and 38.607. Cts are inversely proportional to the amount of target RNA, therefore a high Ct means the sample has a low level of the target RNA. A single copy of the target RNA should, theoretically, have a Ct of 40 if the amplification efficiency of the assay is 100%. A difference in Ct of 3.2 units indicates a ten-fold difference in genome copies. NoV and HAV RNA were not detected in any other sample. FRNA phages were detected in 4 of the 5 day 2 samples where no human virus RNA was detected and significant FRNA phages were detected at higher (150 pfu/100 g) than 'normal' background levels in the one sample where NoV GII was detected.

The fourth adverse sewage event was a leak of an estimated 1500 kL of raw sewage in Camden Haven, NSW in December 2017, and is the only event sampled that is not related to a rainfall event. The results of samples from this event are concerning as low levels of NoV GI and NoV GII RNA were detected in all day 1 samples (4/4) and one day 6 sample (1/4), yet FRNA phage titres were all <30 or 30 pfu/100 g shellfish flesh. The Cts of these samples were high, ranging from 34.963 (NoV GI, Hanleys Point, site 16, day 1) to 41.853 (NoV GII, Hanleys Point, site 16, day 1) indicating low levels of NoV GI RNA and NoV GII RNA. The amplification efficiency of these assays were acceptable (equal to or greater than 71.33%). The day 6 sample positive for NoV came from the closest site to the sewage spill, only 330 m compared to between 1.1 and 3.3 km for the remaining samples. The *E. coli* was marginally higher than the other sites, 90 MPN/100 g compared to <20 and 20 MPN/100 g. No NoV or HAV RNA was detected in day 23 samples and FRNA phage titres were <30 pfu/100 g shellfish flesh. These day 23 samples were impacted by very heavy rainfall in the preceding 24 hrs leading to elevated *E. coli* levels. The risk of infection with NoV and HAV from shellfish is difficult to determine using molecular methodologies such as PCR which also detects naked non-encapsulated or degraded viral RNA and viruses with damaged capsids that cannot initiate infection. In contrast, the agar overlay plaque assay for detection and enumeration of phages is an infective titre as the plaques are produced by individual phages infecting and amplifying within a host cell. PCR and the plaque assay are not directly comparable. The NoV RNA detected may be from non-infective viruses, however, there is currently no method to differentiate between intact infective and non-infective viruses. It is important to comment that Gogleys Lagoon growing area was re-opened at 1.30 pm on the 17th December, 11 days after the spill. No NoV was detected in the day 6 samples from Gogleys Lagoon. Multiple factors were taken into account in a tool-kit approach by the regulator, the NSWFA, to re-open the leases. Previous hydrological studies combined with the *E. coli* levels in water and the phage data were considered and led to the conclusion that it was appropriate to re-open. This was the second area in Australia re-opened under the phage protocol in ASQAP 2016 (ASQAAC 2016). No foodborne viral illnesses have been reported. Hanleys Point and Stingray Creek growing areas remained closed until the 2nd January 2018.

Finally, the fifth adverse sewage event in Pittwater and Island Inlet, Tasmania, December 2017, resulted in raised *E. coli* and FRNA phage levels in some day 3 samples. Day 7 and day 23 sample indicators were not raised except one site on day 23 with unexplained spikes in both FRNA phage and *E. coli*. Of concern was the detection of a low level of NoV GII in one day 3 sample (1/7 total) where the FRNA phage titre was <30 pfu/100 g shellfish flesh. The Cts of this sample ranged from 35.628 to 42.823, with an amplification efficiency of 95.94%, indicating a low level of NoV GII RNA. As discussed above, infectivity is not known.

There is currently no established acceptable limit for NoV in oysters (EFSA 2012). As the viral load increases, the probability of infection increases. Published data from outbreaks associated with oysters have detected NoV concentrations of less than 100 to more than 10,000 genome copies per gram of material tested (EFSA 2012). EFSA has recommended that an acceptable limit for NoV in oysters be established with testing using the standardised CEN method to verify compliance with the limit. The level of risk determined using quantitative PCR results is indirect as this data does not indicate infectivity. The EU Community Reference Laboratory has recommended if virus standards are introduced the standard for NoV be quantitative (genome copies/g with a maximum acceptable level to be determined) and the standard for HAV be qualitative (presence or absence) (CEFAS 2013). The levels for NoV under consideration are a standard of approximately less than 1,000 copies/g as an absolute upper limit for production areas and an end product limit of around less than 200 virus genome copies/g. This document also emphasises that virus controls not be seen as alternatives to sanitary measures and avoidance of contamination of growing areas

(CEFAS 2013). In this study, NoV was detected at a level higher than 200 genome copies in one day 1 sample and no day 7 samples. However, evidence has shown that there is a risk of infection at any level of NoV contamination. For example, an investigation into the NoV outbreak in 2013 involving Tasmanian oysters, which led to 525 identified cases, unquantifiable trace levels of NoV GII RNA were detected in two un-shucked oyster samples (Lodo et al. 2014).

This investigation has identified that during adverse events the number of sites contaminated varies, as would be expected, considering the multifactorial nature of each incident. The number of sites sampled for each adverse event varied between 4 and 8 leases. On occasion there was no significant difference in the phage, *E. coli* and enteric virus levels in oysters from the selected sites, specifically samples from Moulting Bay in January 2017 and Camden Haven in March 2017, where the impact of the spills were minimal. The Brisbane Water spill in March 2017 was larger in volume leading to contamination in 4 of the 5 samples tested as indicated by the phage and *E. coli* data. These samples were also closer to the spill site than the uncontaminated sample, with highest phage levels detected in the two leases closest to the spill location. The Pittwater /Island Inlet adverse event highlights the fact that the leases closest to the discharge site may not be those most contaminated. This is likely due to the nature of the harvest areas and complexity of water movements combined with incoming and outgoing tides. The most contaminated samples were in the north and centre of Pittwater, well away from of the discharge points, whilst sites closer to the discharges showed less contamination. A previous investigation of the spatial and temporal distribution of NoV, HAV and *E. coli* in oysters after a contamination event in NSW also resulted in the detection of NoV in the sample furthest (8.2 km) from the spill location and no NoV detected in a sample 6.82 km from the spill although the source may have been outfall from other STPs (Brake et al. 2018). In instances of spills involving multiple harvest areas and complex water movements predicting potential contamination is difficult so we would recommend sampling more than 5 leases and covering all harvest areas. At a minimum, for less complex growing areas and situations, no fewer than 5 samples are recommended.

In conclusion, the adverse sewage event data is not comprehensive enough in relation to the usefulness of FRNA phages as indicators of human enteric viruses. Four of the spill events occurred during heavy storm events and were accompanied by significant run-off. Three of the five sewage spills occurring during the study period were in the small to medium size range <1000 kL. Two were > 1000 kL with the volumes estimated as 1400 kL at Brisbane Water in March 2017 and 1200 kL at Pittwater/Island Inlet in December 2017. FRNA phages were below the level of detection (30 pfu/100 g shellfish flesh) on 53 of the total 76 sampling occasions. FRNA phages were detected on 16 occasions in the absence of human enteric viruses. FRNA phages were detected with NoV on one occasion. Low levels of human enteric virus RNA were detected in the absence of FRNA phages on 6 occasions associated with two sewage events, however, we do not know how this relates to risk of infection to the consumer. NoV RNA was detected in adverse sewage event day 1 to 6 oyster samples at very low levels (high Ct values roughly corresponding to levels in the hundreds of genome copies/g or less). There is insufficient data from this study to state whether phage is an effective indicator and further investigations should be undertaken.

Conclusion

An objective of this project was to establish background FRNA phage levels in 'at-risk' growing areas. This has been achieved, with a background level of 60 FRNA phage/100 g shellfish flesh being recommended for these areas. This level is similar to that suggested in the ASQAP Manual as the default baseline. The background studies should be extended to all harvest areas considering applying the ASQAP operations guideline related to FRNA phages. The 2016 version of the manual (ASQAAC 2016) states in section 6.1.10 c):

"A harvest area temporarily placed in the closed status is reopened only when:

c) for closures associated with an untreated or partially treated sewage discharge or an untreated sewage discharge from a community sewage system:

i. at least 21 days have passed since the end of the contamination event; OR

ii. Shellstock samples, collected from representative locations in each harvest area (no sooner than seven days after the contamination has ceased), are found to have Male Specific Coliphage levels which do not exceed background levels or a level of 50 Male Specific Coliphage per 100 grams"

The few unexplained spikes in phage titres that were detected raise questions regarding the source of contamination. Further investigations would be valuable.

The adverse sewage event investigations have neither supported nor refuted the use of FRNA phages as indicators of human enteric viruses in bivalve shellfish in the limited Australian oyster growing areas investigated. The results do not confirm the presence of FRNA phages in all samples with detectable levels of NoV viral genomes. The fact that the FRNA phage plaque assay enumerates infective bacterial viruses means the PCR assay for detection of viral RNA is not directly comparable. In order to confirm the validity of using FRNA phages as indicators of human viral risk in bivalve shellfish following adverse sewage events more data is required. This study investigated only five events of which three were small to medium in size, <1000 kL and involved rainfall events. The 2017 review publication noted that there is more evidence supporting the use of FRNA phages as virus indicators in shellfish from contaminated sites but less for sewage spills associated with rainfall events (Hodgson et al. 2017).

Each event is unique with multiple factors impacting on the level of contamination in the shellfish including the spill volume, level of treatment of the sewage, dilution in receiving waters, distance of leases from the source and incoming or outgoing tides. Additional data from large sewage spills, particularly those not involving rainfall events, is necessary. In addition, epidemiological data on illnesses and outbreaks in the community implicating oysters must be included. The national prevalence survey undertaken by SARDI for NoV and HAV in Australian oysters detected no NoV or HAV resulting in a very low prevalence estimate of <2% for both viruses with a 95% confidence interval of 0-2.5% for NoV and 0-2.7% for HAV (Torok et al. 2015, Torok et al. 2018). Under current conservative management practices in Australia the risk of illness due to enteric viruses from shellfish is low. If risk managers choose to use FRNA phage testing to obtain information to assess the impact of sewage spills, it should be used to complement the other parameters, for example *E. coli* levels, used by regulators and growers, not replace it.

The guidance document for appropriate implementation of this method has been compiled and will be made available to industry and regulators. The guidance document is designed to assist in the application of the phage method to adverse event investigations and includes recommendations on the investigation design, appropriate sample numbers and interpretation of results.

Laboratory training in the FRNA phage procedures was held in November 2017 with representatives from the Port Macquarie Hastings Environmental Laboratory, TasWater and SARDI in attendance. A detailed laboratory workshop manual was prepared and can be made available for future reference.

Implications

The implementation of the FRNA phage indicators has the potential to significantly reduce losses after sewage spills to industry. The option to re-open earlier than the obligatory 21 days if FRNA phage levels can be shown to be <60 pfu/100 g shellfish flesh or at background levels can reduce the financial impact on industry. If FRNA phages can be shown to be appropriate indicators there should be increased confidence for risk managers as it provides additional information for consideration prior to officially reopening growing areas. For some high risk growing areas there would be a cost to industry to determine the FRNA phage baseline levels, however, this should be balanced with the reduced cost of closures due to sewage spills provided, on occasion, growing areas are approved for re-opening in under 21 days.

The number of growing area closures related to sewage spills and overflows and associated cost to industry is significant. In New South Wales, over the period of this project, July 2016 to December 2017, harvest areas were closed on 21 occasions. One growing area, Gogleys Lagoon, closed in December 2017 was reopened after 11 days using, among other factors, the FRNA phage data. The use of FRNA phages as proposed has the potential to reduce the number of days closed and consequently the number of days of lost sales. In Tasmania, there were 10 harvest area closures in the Pittwater, Island Inlet and Moulting Bay growing areas of greater than 21 days related to sewage spills over the same time period. A three week closure in the Pittwater growing area in 2013 was estimated to cost $250-$400k in combined sales. In addition, the cumulative impact of these spills has been estimated to reduce the value of businesses in this growing area by a combined value of $12 million across the bay.

These values do not take into account the loss of reputation and that closures may lead to a delay in recovery of consumer confidence in the product. The potential reduction in costs must also be balanced with the cost to industry if a growing area is reopened in under 21 days on the basis of FRNA phage results and then implicated in an outbreak of viral illnesses.

Recommendations and Further development

We recommend expansion of background studies to other oyster growing areas. Application of the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) guideline related the FRNA phages without determination of the background phage levels could disadvantage a growing area if the normal titre is higher and results in an unnecessarily extended closure after a sewage spill.

We also recommend further analysis of samples after adverse events to consolidate the application of this methodology. The data from this investigation should be built on in order to validate the method. In addition it will be critical to track epidemiological data to confirm or not the safety of shellfish from growing areas where the reopening has been brought forward following an adverse event by the application of the ASQAAC guideline.

Investigation of the sources of FRNA phages isolated from background samples and adverse events to confirm whether the origin of the phage isolates is human or animal is also recommended. Brisbane Water and Pittwater are specifically recommended due to the occasional elevated background phage levels. There are a number of published methods for source tracking and to develop the capability in an Australian laboratory would be valuable for future investigations.

Extension and Adoption

The project has been presented at various meetings and conferences, specifically:

K. Hodgson, A. Turnbull, V. Torok "The use of FRNA bacteriophage for rapid re-opening of growing areas after sewage spills" at the ASQAAC Science Day in Sydney on the 3rd November 2016.

K. Hodgson, A. Turnbull, V. Torok "FRNA bacteriophages for risk management of Australian oysters" for the SARDI Waite Seminar Series on Thursday 17th August 2017.

A. Turnbull, K. Hodgson, and V. Torok "Improving Risk Management of Enteric Viruses in Australian Oysters" at ICMSS in Galway, Ireland in May 2017.

A workshop coordinated by K. Hodgson "FRNA bacteriophages in bivalve shellfish – Laboratory training" was conducted at the SARDI Food Safety and Innovation laboratories, Adelaide on the 7th and 8th November 2017.

A poster was presented at the Australasian Virology Society conference in Adelaide on the 5th – 8th Dec 2017: K. Hodgson, A. Turnbull, V. Torok "FRNA bacteriophages for risk management of Australian oysters"

K. Hodgson, A. Turnbull, V. Torok "2015-037 Oysters Australia IPA: The use of FRNA bacteriophages for rapid re-opening of growing areas after sewage spills" at the Oysters Australia meeting in Sydney on the 9th April 2018.

A guidance document on application of this methodology has been compiled for industry and managers.

Project materials developed

- Project Fact Sheet, Appendix A
- Training Manual “FRNA bacteriophage testing in bivalve molluscan shellfish” Laboratory Training Program, Appendix E.
- Guidance Document, Appendix G

Appendices

Appendix A: Fact Sheet

Fact Sheet

FRDC PROJECT NUMBER: 2015/037

Oysters Australia IPA: "The use of FRNA bacteriophage for rapid re-opening of growing areas after sewage spills" supported by funding from the FRDC, Port Macquarie Hastings Council, Central Coast Council, Shoalhaven City Council and TasWater

Background:

Bivalve molluscan shellfish feed on particulates in the surrounding water resulting in the accumulation and concentration of contaminants, including pathogenic micro-organisms, in their soft tissue. The human health risks associated with consumption of raw or lightly cooked shellfish containing human enteric viruses, such as norovirus (NoV) and hepatitis A virus (HAV), are well recognised with numerous foodborne outbreaks documented. Traditionally indicator bacteria, the coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish. Numerous studies have established that they are inadequate as indicators of human enteric viruses. Bacteriophages (phages) have been identified as potential indicators for human enteric viruses due to their similarities in morphology, behaviour in water environments and survival dynamics. The US has used phage detection and enumeration for regulation in shellfish production since 2009 when the Interstate Shellfish Sanitation Conference (ISSC) officially recognised the use of phages as indicators of viral contamination of bivalve shellfish.

This project follows a review funded by SafeFish and the NSW Food Authority in 2014 entitled "The potential use of bacteriophages to aid the management of sewage outputs in shellfish growing areas: Systematic review of current literature". The review was conducted by SARDI and presented at the Australian Shellfish Quality Assurance Advisory Committee (ASQAAC) Science Day, ASQAAC meeting and Shellfish Futures in Tasmania in October 2014.

Aims of the project:

- Establish baseline levels of FRNA phages in 'at risk' growing areas
- Determine appropriate sampling plans for FRNA phages in shellfish following sewage incidents
- Enable implementation of FRNA phage levels as a management tool for use following adverse sewage incidents in bivalve shellfish growing waters

- Train laboratories to be competent in using appropriate testing methodologies for FRNA phages

What are the benefits of the project?

Current standard risk management practice in the event of an overflow or sewage spill from reticulated sewage and/or waste water treatment plants is to instigate a 21 day closure of affected oyster growing areas. This can lead to significant losses in production and market share and damage to reputation. This project will investigate the use of FRNA phages as indicators of the risk of human enteric viruses in bivalve shellfish after adverse sewage events.
Closure times following sewage spills may be reduced when a low risk of contamination can be demonstrated by FRNA phage levels. If these levels show contamination is negligible, regulators may allow re-opening of growing areas as early as 10 days after the spill following results from shellfish sampled 7 days after the event has ceased, significantly decreasing the cost of spills to growers.

In addition, FRNA phage testing has the potential to provide the industry and regulators with greater confidence in risk management after adverse sewage events and reduce adverse consequences to public health.

Sample details and time-frame

The baseline survey will cover 5 growing areas, 3 in New South Wales and 2 in Tasmania. The growing areas will be selected where sewage incidents have occurred and are classified as 'at-risk'. Each will be sampled monthly for 18 months. Each sample will consist of 12 oysters from 4 sites within the lease. They will be chilled to 4˚C, transported to the SARDI Food Safety laboratory and analysed within 24 hr of receipt for FRNA phages. This data will establish the normal background level of FRNA phages when the risk of contamination of shellfish with human enteric viruses is minimal.

Five sewage event case studies will be investigated. The 5 events will ideally be located in the baseline study growing areas, however if not feasible in the time-frame of the project, alternative areas will be sampled. The events should include large (>1000 kL/d) and small (<1000 kL/day) spills. Shellfish will be collected from up to 5 sites per event that are selected on the basis of factors including the geography, tides and water flow. The shellfish samples will be collected as soon as possible after the event, 7 days post the event and 21 days post the event. The samples, 24 oysters per site, will be chilled to 4˚C and transported to the SARDI Food Safety laboratory as soon as possible. They will be analysed for *E. coli* (AS 5013.15 - 2006: Microbiology of food and animal feeding stuff – Horizontal method for the detection and enumeration of presumptive Escherichia coli – Most probable number technique) and FRNA phages (Cefas, 2007: Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish) within 24 hr of receipt. The remainder will be stored at -20˚C for NoV and HAV analysis by qRT-PCR (ISO/TS15216: Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR). The data from these case studies will contribute to the verification or otherwise of a relationship between FRNA phages and human enteric viruses in faecally contaminated shellfish and the validity of the use of phages as viral indicators.

Guidance document

The guidance document will be produced in collaboration with wastewater authorities and risk managers to facilitate implementation of the ASQAAC policy on the use of FRNA phages in risk management. It will cover recommendations on appropriate use of the policy, limitations on the use of FRNA phages, shellfish sampling plans and interpretation of the data from FRNA phage testing.

Laboratory training

Representatives from state based laboratories will be invited to attend a training day at the SARDI Food Safety laboratory in Adelaide on the methodology of FRNA phage testing. This will include the maintenance of cultures, media preparation, appropriate controls, procedures and interpretation of results. The training will equip the laboratories for all aspects of the methodology.

Selection and role of the steering committee

The steering committee will be established prior to commencement of the project. Representatives will be invited from the oyster industry, the contributing councils, FRDC, TasWater and the NSW Food Authority.

The role of the committee is to provide strategic oversight of the project, assist with communicating the research to regulators and growers and the results of the study to industry and other stakeholders.

It will also provide advice on reporting policy and feedback on the sampling design, protocol and organisation of sample collection.

Project outcome

The results of the investigation and guidance document will be presented at an ASQAAC meeting to industry and risk managers.

Frequently asked questions

The use of FRNA bacteriophage for rapid re-opening of growing areas after sewage spills

What are bacteriophages?

Bacteriophages (phages) are viruses that are specific for only bacteria. Phages cannot infect other organisms including plants and animals. They co-exist and co-evolve with bacteria in a dynamic predator-prey relationship. They infect and replicate in the bacterial host and are generally specific to species level. Those that infect *E. coli* and closely related coliforms are called coliphages.

Phages have been proposed as indicators or surrogates for human enteric viruses due to fact that their morphology and survival dynamics resemble those of human enteric viruses. The group of phages of most commonly applied for shellfish testing are the FRNA phages (also known as male-specific RNA coliphages).

Are phages used in testing in Australia?

Phage testing and monitoring is used by a number of Australian water authorities for the validation of waste-water treatment processes and as indicators of human enteric viruses. The Australian National Water Quality Management Strategy Drinking Water Guidelines 6 outlined by the NHMRC in 2011 recommends the use of coliphage detection and enumeration for validation of treatment processes. Phage testing is routinely applied by various water authorities and commercial laboratories in the ACT, SA, WA, NSW and Qld.

Does the presence of FRNA phage correlate with coliforms in shellfish?

The coliforms & *E. coli* are useful as indicators of faecal pollution in shellfish as they inhabit the intestinal tract of animals including humans in large numbers and will be present in sewage. FRNA phages are also routinely found in sewage. Shellfish are filter-feeders and concentrate both bacterial and viral contaminants from the surrounding water in their soft tissue. Studies have shown that bacteria are depurated from faecally contaminated shellfish more rapidly than viruses including FRNA phages. As a result FRNA phages can be detected in shellfish when coliforms or *E. coli* are no longer present.

Do FRNA phage levels correlate with human enteric virus levels in shellfish?

In contrast to FRNA phages, human enteric viruses may not be present in sewage unless there has been a disease outbreak or there are individuals excreting the viruses. Shellfish grown in sewage contaminated waters would be expected to accumulate FRNA phages. Human enteric viruses may or may not be present. The detection of FRNA phages would indicate that there is a risk of human enteric viruses in the shellfish. FRNA phages may be detected in the absence of human enteric viruses.

Will FRNA phage testing replace coliform testing in shellfish?

Coliforms and *E. coli* will continue to be useful as indicators of faecally polluted shellfish. The addition of FRNA phage testing has the potential to complement these and provide a more accurate indication of the risk of human enteric viruses in shellfish.

Can FRNA phages be used for routine monitoring of growing areas?

There are limited studies at this stage supporting the use of phages as indicators during normal growing conditions. Further investigations should be completed to confirm and validate their use during routine monitoring.

Appendix B: Project Participants

SARDI research staff:

Dr Kate Hodgson, Mrs Navreet Malhi, Ms Alison Turnbull, Dr Valeria Torok, Ms Jessica Tan and Ms Linda Friedrich

Steering Committee

Chair:

Anthony Zammit	NSWFA, New South Wales

Members:

Brandon Armstrong	Armstrong Oysters, New South Wales
Phil Baker	NSWFA, New South Wales
Justin Goc	Barilla Bay Oysters, Tasmania
David Holmes	TasWater, Tasmania
Wayne Hutchinson	Oysters Australia, National
Stephen Shinners	Central Coast Council, New South Wales
Maree Smith	Port Macquarie-Hastings Council, New South Wales
Andrew McVey	Shoalhaven City Council, New South Wales
Joanna Waugh	Central Coast Council, New South Wales
Kate Wilson	Tasmanian Shellfish Quality Assurance Program, Tasmania

Permanent Observers and Secretariat:

Kate Hodgson	Principal Investigator, SARDI, South Australia
Alison Turnbull	SARDI, South Australia
Valeria Torok	SARDI, South Australia
Navreet Malhi	SARDI, South Australia

Oyster growers and samplers

New South Wales:
Brandon Armstrong, Wayne Moxham, Anthony Munn
Tasmania:
Justin Goc, Josh Poke, James Calvert, Peter Kosmeyer, Tony Byrne

Appendix C: Steering Committee Terms of Reference

Project 2015/137: "Oysters Australia IPA: the use of FRNA bacteriophages for rapid re-opening of growing areas after sewage spills"

Steering Committee Terms of Reference

Project description

This project is jointly funded by the Fisheries Research and Development Corporation (FRDC), South Australian Research and Development Corporation, Gosford City Council, Port Macquarie-Hastings Council, Shoalhaven City Council, and TasWater, with support from the New South Wales Shellfish Program and the Tasmanian Shellfish Quality Assurance Program (TSQAP). Australian shellfish quality assurance programs use coliform bacteria and *Escherichia coli* (*E. coli*) as indicators of faecal pollution in water and shellfish however they are acknowledged to be poor indicators of enteric virus risk. Studies have indicated that FRNA bacteriophages can be useful for determining the risk of contamination with enteric viruses if sewage spills have contaminated oyster growing areas, potentially allowing earlier re-opening of areas shown to be not impacted. This method is currently applied in the US in shellfish production.

The overall aims of the project are to:

- Establish baseline levels of FRNA bacteriophage in two Tasmanian and three NSW growing areas known to be impacted by sewage incidents on occasion.
- Evaluate of the use of FRNA bacteriophages in adverse sewage events from Tasmania and NSW in five case studies.
- Develop a guidance document for sampling and management following sewage spills to allow easy implementation of the new ASQAAC policy (yet to be ratified).
- Train laboratories in each state in the detection of FRNA bacteriophages in shellfish.

The project involves four stages.

Stage 1 is the determination of baseline levels of FRNA phages in shellfish from 5 "at risk" growing areas (2 in Tas, 3 in NSW) by sampling monthly for 18 months. This data will establish the background levels of FRNA phages indicating no appreciable risk to consumers. In the US this is 50 male-specific coliphage (FRNA phages) per 100 grams shellfish. Work will also be undertaken with the waste water authorities to determine the efficacy of sewage treatment for the removal of human enteric viruses in waste waters entering these growing areas.

Stage 2 will investigate the use of FRNA phages following sewage incidents to identify the validity of correlating FRNA phage numbers with human enteric viruses. Five case studies, including large (>1000 kL/d) and small events (<1000 kL/d) will be selected and tracked by sampling five sites at 3 time periods (time 0 or as soon as logistically feasible, 7 and 21 days). Samples will be analysed for *E. coli*, FRNA phages and human enteric viruses (Norovirus and Hepatitis A virus) by qRT-PCR.

Stage 3 will be the production of a guidance document for sampling and management following sewage incidents to allow implementation of the method.

Stage 4 will be training sessions for laboratories in each state hosted by SARDI on the methodology for FRNA phage testing in shellfish.

The results of the project will:

1. Provide the shellfish industry with an improved risk management option that has the potential to reduce closure times after an adverse sewage event.
2. Provide water authorities with data related to the efficacy of treatment processes and the impact on shellfish growing areas in relation to the removal of potentially pathogenic viruses.
3. Equip state laboratories for FRNA phage enumeration in shellfish samples.

Terms of Reference:

1. Provide strategic oversight of the project to ensure project outputs are in line with the stated objectives.
2. Assist with communicating research to regulators and growers.
3. Assist with communicating results of the study to industry and other stakeholders to ensure successful delivery of research outcomes.
4. Assist with advice on reporting policy.
5. Provide feedback on sampling design and protocol, and help with organisation of sample collection.

Meetings:

Meetings will be held by teleconference.

Appendix D: FRNA phage baseline monthly sampling

Date sampled	No of samples	Date received	Date tested
Round 1: July 2016			
18.07.16	3	20.07.16	22.07.16
18.07.16	1	22.07.16	
21.07.16	2	22.07.16	
Round 2: Aug 2016			
15.08.16	4	17.08.16	18.08.16
15.08.16	1	18.08.16	
16.08.16	1	18.08.16	
Round 3: Sept 2016			
19.09.16	3	20.09.16	23.09.16
19.09.16	2	23.09.16	
20.09.16	1	21.09.16	
Round 4: Oct 2016			
16.10.16	2	18.10.16	20.10.16
17.10.16	1	18.10.16	
18.10.16	3	19.10.16	
Round 5: Nov 2016			
21.11.16	2	22.11.16	24.11.16
21.11.16	2	23.11.16	
22.11.16	2	23.11.16	
Round 6: Dec 2016			
19.12.16	2	22.12.16	22.12.16
20.12.16	1	21.12.16	
21.12.16	1	22.12.16	
Round 7: Jan 2017			
16.01.17	1	18.01.17	19.01.17
16.01.17	3	19.01.17	
17.01.17	1	18.01.17	
18.01.17	1	19.01.17	
Round 8: Feb 2017			
16.02.17	2	21.02.17	22.02.17
20.02.17	3	22.02.17	
21.02.17	1	22.02.17	
Round 9: March 2017			
17.03.17	2	21.03.17	22.03.17
20.03.17	1	22.03.17	
21.03.17	1	22.03.17	

Date sampled	No of samples	Date received	Date tested
Round 10: April 2017			
18.04.17	1	19.04.17	21.04.17
18.04.17	2	20.04.17	
19.04.17	1	20.04.17	
19.04.17	2	21.04.17	
Round 11: May 2017			
15.05.17	2	17.05.17	19.05.17
16.05.17	4	18.05.17	
Round 12: June 2017			
19.06.17	2	21.06.17	23.06.17
19.06.17	3	23.06.17	
20.06.17	1	21.06.17	
Round 13: July 2017			
16.07.17	1	19.07.17	20.07.17
17.07.17	3	19.07.17	
18.07.17	2	19.07.17	
Round 14: August 2017			
21.08.17	4	23.08.17	25.08.17
21.08.17	1	22.08.17	
22.08.17	1	23.08.17	
Round 15: September 2017			
17.09.17	1	19.09.17	22.09.17
18.09.17	2	20.09.17	
19.09.17	1	20.09.17	
19.09.17	2	22.09.17	
Round 16: October 2017			
16.10.17	3	18.10.17	22.10.17
17.10.17	2	19.10.17	
Round 17: November 2017			
20.11.17	6	23.11.17	24.11.17
Round 18: December 2017			
11.12.17	1	13.12.17	15.12.17
15.12.17	2	20.12.17	20.12.17

Appendix E. FRNA bacteriophage testing in bivalve molluscan shellfish training manual

FRNA bacteriophage testing in bivalve molluscan shellfish

Laboratory Training Program

November 2017

FRNA bacteriophage testing in bivalve molluscan shellfish

Information current as of 7th November 2017

Disclaimer

Manual prepared by Kate Hodgson and Valeria Torok

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Background

This procedure has been based on ISO 10705 - 1 "Water quality - Detection and enumeration of bacteriophages – Part 1: Enumeration of F-specific RNA bacteriophages (Anon 1996) (Appendix A) and the Cefas Generic Standard Operation Procedure 1671 version 7 2007: Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish (Appendix B). The United States Food and Drug Administration Interstate Shellfish Sanitation Conference (USFDA ISSC) approved the application of phage detection and enumeration for shellfish management in 2009. The US ISSC method "Modified double agar overlay method for determining male-specific coliphage in soft-shelled clams, American oysters, and quahogs (*M. mercenaria*)" (Nov 2013) (Appendix C) has been modified several times and varies in a number of details as outlined in the table comparing the three procedures (Appendix D).

This procedure describes the preparation of samples and a double agar overlay method for enumeration of FRNA bacteriophages (phages) in shellfish flesh. Procedures for production and quality control of *Salmonella* Typhimurium WG49 host cells and FRNA bacteriophage MS2 positive control are critical for the validity of results.

An aliquot of the host WG49 bacteria in early log phase is inoculated into a soft agar overlay with an aliquot of shellfish homogenate and gently mixed. This overlay is poured and spread onto the surface of a base agar plate and incubated at 37˚C for 18 hours. The host WG49 bacteria multiply producing a lawn of confluent growth within the overlay. Phages in the homogenate will produce plaques identified as clear or turbid zones of bacterial lysis within the confluent bacterial lawn. Each plaque is derived from one phage. The results are expressed as the number of plaque forming units (pfu) per 100 g of shellfish. The theoretical limit of detection is 30 pfu per 100 g of shellfish flesh.

FRNA phages (also referred to as male-specific coliphages) have been recognized as potential indicators of pathogenic human enteric viruses e.g. hepatitis A virus and norovirus. They are phage infecting the *Enterobacteriaceae* and belong to the family *Leviviridae* and demonstrate similar size, shape and single-stranded RNA genome to human enteric viruses. They infect the host cell via attachment to the fertility (F) fimbriae on the *Escherichia coli* (*E. coli*) host. The F-fimbriae can only be produced by cells carrying a plasmid encoding this factor and only when the cells are in the logarithmic growth phase at temperatures above 30°C.

FRNA phages replicate in the gastrointestinal tract of mammals, but are unlikely to multiply in water environments where the conditions are unsuitable. The host bacteria used in the method, *Salmonella* Typhimurium WG49, is a strain containing a plasmid encoding F-pili production making it suitable for the detection of FRNA phages. The use of *S.* Typhimurium WG49 as host removes potential interference from somatic coliphages commonly found in sewage. Somatic phages have a single or double stranded DNA genome and initiate infection by attaching to specific receptors on the cell wall of the bacterial host not the F-fimbriae. The use of bacteriophages as indicators of human enteric viruses specifically in the management of bivalve shellfish has been reviewed in a recent publication (Appendix E).

Abbreviations

Ca/glu	Calcium glucose solution
Cefas	Centre for Environment, Fisheries and Aquaculture Science
CLED	Cysteine lactose electrolyte deficient agar
OD	Optical density
pfu	plaque forming units
PW	Peptone water
RO	Reverse osmosis
SD	Standard deviation
TYGA	Tryptone yeast extract glucose agar
TYGB	Tryptone yeast extract glucose broth

Equipment and consumables

Balance
Bunsen burner
Centrifuge (2,000 × g and capable of accommodating 50 mL centrifuge tubes)
50 mL centrifuge tubes (conical base and chloroform resistant)
Cuvettes
Disposable sterile pipette tips
Freezer (-80˚C)
Incubator set at 37˚C
Laminar air flow cabinet (Class ll)
Microwave oven or steamer
Orbital shaker
Pipettes (single channel)
- 100 µL
- 1 mL

Spectrophotometer (OD at 600 nm)
Sterile 1.5 mL Eppendorf tubes
Sterile 0.22 µm membrane filters (Millex-GV PVDF SLGV033RS)
Sterile plastic 90 mm (diameter petri plates)
Sterile pots, various volumes 30 -120 mL
Sterile plastic vials, 1.5-2.0 mL
Refrigerator at 3 ± 2°C
Stainless steel metal mesh glove
Sterile shucking knife
Waring blender and 1L jars
Water bath set at 48-50˚C

Media and reagents

Suppliers of media and reagents listed are for informational purposes. Alternative suppliers of equivalent reagents may be sourced.

0.1% Peptone water

Trypticase peptone (BD BBL catalogue number 211921)	1.0 g
RO water	1000 mL

Dissolve trypticase peptone in the water. Adjust to pH 7.0 ± 0.1 if necessary. Dispense convenient volumes into Schott bottles with loose caps. Sterilise by autoclaving at 121˚C for 15 minutes. Store at room temperature.

1% Calcium glucose solution (Ca/glu)

Calcium chloride dehydrate ($CaCl_2.2H_2O$; MW 147.01 g/mol)	3.0 g
D-glucose ($C_6H_{12}O_6$; 180.16 g/mol)	10.0 g
RO water	100 mL

Dissolve solids in the water while heating gently. Cool to room temperature and filter sterilize through a 0.22 µm membrane filter. Store in the dark at 4°C ± 2°C for up to 6 months.

Tryptone yeast extract glucose broth (TYGB)

Trypticase peptone (BBL BD catalogue number 211921)	10.0 g
Yeast extract (Oxoid catalogue number LP0021)	1.0 g
Sodium Chloride (NaCl; MW 58.44 g/mol)	8.0 g
RO water	1000 mL

Basal medium
Add all solids to required volume of water. Mix thoroughly and heat to dissolve. Adjust pH to 7.2 ± 0.1 at 25°C. Dispense 200 mL into Schott bottles and sterilise by autoclaving at 121°C for 15 minutes. Store sterile broth at 4°C ± 2°C for up to 6 months.

Complete medium
Aseptically add 2 mL of the Ca/glu solution to 200 mL of the basal medium and mix well. If not for immediate use, store at 4°C ± 2°C for up to 6 months.

1% Tryptone yeast extract glucose agar (TYGA1 – Overlay agar)

Trypticase peptone (BBL BD catalogue number 211921)	10.0 g
Yeast extract (Oxoid catalogue number LP0021)	1.0 g
Sodium Chloride (NaCl; MW 58.44 g/mol)	8.0 g
Agar (BD Difco granulated agar catalogue number 214530)	10.0 g
RO water	1000 mL

Basal medium
Add solids to required volume of water. Mix thoroughly and boil to dissolve. Adjust pH to 7.2 ± 0.1 at 25°C. Dispense 50 mL into Schott bottles and sterilise by autoclaving at 121°C for 15 minutes. Store at 4°C ± 2°C for up to 6 months.

Complete medium
Melt the basal medium and cool to between 45°C and 50°C. Aseptically add 500 µL of 1% Ca/glu and 200 µL nalidixic acid solution per 50 mL and mix gently. Hold at 45°C to 50°C for pouring onto TYGA2 base plates.

2% Tryptone yeast extract glucose agar (TYGA2 – Base agar)

Trypticase peptone (BBL BD catalogue number 211921)	10.0 g
Yeast extract (Oxoid catalogue number LP0021)	1.0 g
Sodium Chloride (NaCl; MW 58.44 g/mol)	8.0 g
Agar (BD Difco granulated agar catalogue number 214530)	20.0 g
RO water	1000 mL

Basal medium
Add solids to required volume of water. Mix thoroughly and boil to dissolve. Adjust pH to 7.2 ± 0.1 at 25°C. Dispense 50-200 mL into Schott bottles and sterilise by autoclaving at 121°C for 15 minutes. Store at 4°C ± 2°C for up to 6 months.

Complete medium
Melt the basal medium and cool to between 45°C and 50°C. Aseptically add 500 µL of 1% Ca/glu per 50 mL and mix gently. Hold at 45° to 50°C and pour approximately 15 mL into 90 mm petri dishes. Store at 4°C ± 2°C for up to 6 months.

Cysteine lactose electrolyte deficient agar (CLED)

CLED powder (BBL™ catalogue number 212218)	36.0 g
RO water	1000 mL

Dissolve powder in water. Heat to boiling with frequent stirring. Autoclave at 121°C for 15 minutes. Cool to 50°C and adjust pH to 7.3 ± 0.2. Pour into 90 mm petri dishes. Store at 4°C ± 2°C for up to 6 months.

Nalidixic acid solution

CAUTION:

Nalidixic acid is a teratogen. Refer to MSDS (Appendix J) for precautions.
Sodium hydroxide is a corrosive. Refer to MSDS (Appendix K) for precautions.

Nalidixic acid ($C_{12}H_{12}N_2O_3$; MW 232.24 g/mol)	2.5 g
1M Sodium hydroxide solution (NaOH; 1 mol/L)	20.0 mL
RO water	80 mL

Dissolve nalidixic acid in 1M NaOH solution. Add required volume of water and mix well. Filter sterilize through a 0.22µm membrane filter. Store at 4ºC ± 2ºC for not longer than 8 hours OR store at -20ºC ± 2ºC for not longer than 6 months.

RNase solution

RNase A (Astral Scientific catalogue number BIORB-0473)	100 mg
RO water	100 mL

Dissolve the RNase in required volume of water with heating for 10 minutes at 100˚C. Dispense 500 µL aliquots into sterile eppendorfs and store at -20˚C for up to 12 months.

Antibiotic discs

Kanamycin 30 µg, diameter 6 mm (ThermoFisher catalogue number CT0026B)

Nalidixic acid 30 µg, diameter 6 mm (ThermoFisher catalogue number CT0031B)

Chloroform

Chloroform (ChemSupply catalogue number CA038)

Sterile glycerol

Glycerol (BDH Analar catalogue number 10118)

Distribute in 20 mL volumes. Autoclave at 121°C for 15 minutes. Store in the dark for up to 12 months.

Microbiological Reference Cultures

Salmonella Typhimurium strain WG49

Phage type 3 Nal^r (F' 42 lac:Tn5)

NCTC 12484 or ATCC 700730 (Appendix F).

Salmonella Typhimurium WG49 is a mutant of low pathogenicity and should be handled in accordance with PC2 guidelines (Appendix G).

Escherichia coli bacteriophage MS2

NCTC 12487 or ATCC 15597-B1 (Appendix H)

FRNA phages are non-pathogenic for humans, animals and plants, however, care must be taken to prevent cross-contamination of samples when using the reference phage (Appendix I).

Methodology

Stocks and working cultures

Host bacteria: *Salmonella* Typhimurium strain WG49

Host bacteria WG49 stock culture

Dispense 50 mL of TYGB, add 500 µL of 1% Ca/glu and warm to 37˚C. Rehydrate the lyophilised ampoule of host strain WG49 (NCTC 12484) as per the NCTC guide.

Aseptically transfer the suspension to the TYGB supplemented with Ca/glu and incubate at 37°C shaking at 100 rpm for 18 ± 2 hours.

Add 10 mL sterile glycerol and mix. Aliquot 1.2 mL into culture tubes and store at -80˚C.

Growth rate of host bacteria WG49 stock culture

Thaw one vial of host strain WG49 stock culture and streak for single colonies onto CLED agar. Incubate at 37°C for 18 ± 2 hours.

Warm 50 mL of TYGB supplemented with 500 µL 1% Ca/glu to 37°C. Inoculate with 5-7 lactose positive colonies (yellow) from the CLED plate and incubate for 5 ± 1 hours at 37°C with shaking at 100 rpm.

At time 0 take a 2.5 mL aliquot. Determine the optical density (OD) at 600 nm and serially dilute to 10^{-6} in 0.1% PW. Spread 100 µL aliquots of 10^{-4}, 10^{-5} and 10^{-6} in duplicate onto TYGA2 plates.

Repeat at 30 minute intervals throughout the incubation period. Incubate plates for 18 hours at 37°C.

After incubation, count colonies on plates with 30-300 colonies. Calculate the number of cfu/mL and establish the OD corresponding to 2.5 - 15 × 10^8 cfu/mL.

Quality control check for bacteriophage MS2 susceptibility

Melt 50 mL TYGA1 and equilibrate in the waterbath at 48 - 50°C. Aseptically add 500 µL of 1% Ca/glu per 100 mL TYGA1. Mix gently and dispense 2.5 mL aliquots into sterile tubes.

Inoculate and pour overlays with 1 mL host bacteria WG49 stock culture and 1 mL phage MS2, refer to p 17 and p 18.

Also inoculate control overlay plates with 1mL 0.1% PW and host strain WG49 stock culture only. Incubate plates for 18 hours at 37°C.

Confirm bacteriophage susceptibility: check for overlay plates for plaques. Count and calculate pfu/mL refer to p 19.

Host bacteria WG49 working culture

Thaw one vial of host strain WG49 stock culture and streak onto a CLED plate for single colonies. Incubate at 37°C for 18 ± 2 hours.

Add 500 µL of Ca/glu solution to 50.0 mL of TYGB and pre-warm to 37°C. Select 5-7 lactose-positive colonies (yellow) from the CLED plate and inoculate the pre-warmed Ca/glu supplemented TYGB.

Incubate for 5 ± 1 hours at 37°C with shaking until an OD corresponding to 2.5 - 15 × 10^8 cfu/mL has been reached.

Following incubation add 20 mL of sterile glycerol and mix thoroughly. Aliquot approximately 1.2 mL into vials and store at -80°C. Use for maximum 2 years.

Growth rate of host bacteria WG49 working culture

Thaw one vial of host strain WG49 working culture. Add 500 µL of 1% Ca/glu solution to 50 mL of TYGB and pre-warm in an incubator at 37°C. Inoculate 500 µL of *S.* Typhimurium WG49 working stock into the pre-warmed TYGB and incubate for 5 ± 1 hours at 37°C with shaking at 100 rpm.

At time 0 take a 2.5 mL aliquot. Determine the OD at 600 nm and serially dilute to 10^{-6} in 0.1% PW. Spread 100 µL aliquots of 10^{-4}, 10^{-5} and 10^{-6} in duplicate onto TYGA2 and CLED plates.

Repeat sampling every 30 minutes throughout the incubation period.

Incubate the plates at 37°C for 24 hours. After incubation, count the total number of colonies on each plate yielding 30-300 colonies and calculate the number of cfu/mL.

Accept if numbers are between 7 - 40 × 10^7 cfu/mL within 4 ± 2 hours. Repeat this procedure on three separate occasions.

From these results, determine the optical density range corresponding to a cell concentration between 7 - 40 × 10^7 cfu/mL.

Quality control of host bacteria WG49 working culture

Plasmid segregation

Inoculate duplicate CLED spread plates with 100 ± 1 µL volumes of the 10^{-4}, 10^{-5} and 10^{-6} dilutions of host strain WG49 working culture taken at time 0, 180 and 210 minutes. Incubate at 37 ± 1°C for 24 ± 2 hours.

From all CLED plates yielding 30-300 colonies, count the number of lactose negative (blue) and positive (yellow) colonies. Calculate the percentage of lactose negative colonies. Accept the host strain if lactose negative colonies are <8% of the total.

Nalidixic acid and kanamycin resistance

At each of the times 0, 180 and 210 minutes spread 100 ± 1 µL of the 10^{-2} dilution onto two CLED plates. Aseptically dispense two nalidixic acid and two kanamycin antibiotic discs onto each plate.

Incubate at 37°C for 24 hours. Following incubation, measure the diameter of any zones of inhibition. Accept if the inhibition zone around the kanamycin disc is <15 mm and no zone around the nalidixic acid disc.

Bacteriophage MS2 susceptibility

To assess phage MS2 susceptibility, inoculate and plate overlays with 1 mL log phase host bacteria WG49 working stock with 1 mL phage MS2 working stock. Incubate at 37°C for 18 ± 4 hours. All procedures using phage MS2 must be performed in the biosafety cabinet.

Accept if characteristic plaques are present and the titre is within the accepted quality control range as determined by the control limits. The diameter of the plaques is variable and dependent on the host cell density and thickness of the overlay. Typically bacteriophage MS2 produces small-medium (0.2-1.0 mm) opaque, circular plaques.

Positive control *E. coli* bacteriophage MS2

Phage MS2 Stock culture

All procedures using bacteriophage MS2 must be performed in the biosafety cabinet.

Add 500 µL of 1 % Ca/glu solution to 50 mL of pre-warmed TYGB. Inoculate with 500 µL of host bacteria WG49 working culture and incubate at 37°C for 18 ± 2 hours with shaking at 100 rpm.

Following incubation of overnight culture, inoculate fresh 50 mL pre-warmed TYGB containing 500 µL of 1 % Ca/glu solution with 500 µL of the host bacteria WG49 overnight culture. Incubate at 37°C for 90 ± 10 minutes.

Rehydrate an ampoule of phage MS2 with 0.1% PW following suppliers recommended method. Inoculate the 90 minute host bacteria WG49 culture with 1.0 mL of the rehydrated phage MS2. Incubate at 37°C for 5 ± 1 hours.

NOTE: If possible, the following steps should be carried out in a laboratory other than that used for routine sample analysis or in a biosafety cabinet.

Following incubation, add 5.0 mL of chloroform to the 50 mL culture and mix thoroughly. Refrigerate at 3 ± 2°C for 18 ± 2 hours.

Carefully aspirate the supernatant and centrifuge at 3,000 x g for 20 minutes. Carefully pipette the supernatant into a 100 mL bottle.

Titration of bacteriophage MS2 stock

Serially dilute the phage MS2 stock culture to 10^{-10} in 0.1% PW. Plate overlays in duplicate of each dilution using the standard method outlined on p. 18 and 19. Calculate the bacteriophage MS2 titre in pfu/mL.

Adjust the volume of stock solution added to give a final phage MS2 concentration of 10^7 pfu/mL. Add sterile glycerol equal to 5% of the total volume and dispense 1 mL aliquots into plastic vials and store at <-70°C.

Bacteriophage MS2 Working culture

Repeat the procedure for phage MS2 stock culture preparation, substituting the addition of rehydrated reference material with 500 µL of stock phage MS2. Titrate the working culture and adjust to a final concentration of 50-200 pfu/mL using 0.1% PW. Do not add glycerol.

Aliquot in 5 mL volumes and store at <-70°C. The ISO 10705-1 standard for water quality includes storage at -20˚C. The phage MS2 titre may decrease more quickly over time at -20˚C, however, this can be monitored using the control limits.

Bacteriophage MS2 control limits

Thaw a vial of phage MS2 working culture and assay for FRNA phage by the standard overlay method. Repeat twenty times on ten separate occasions.

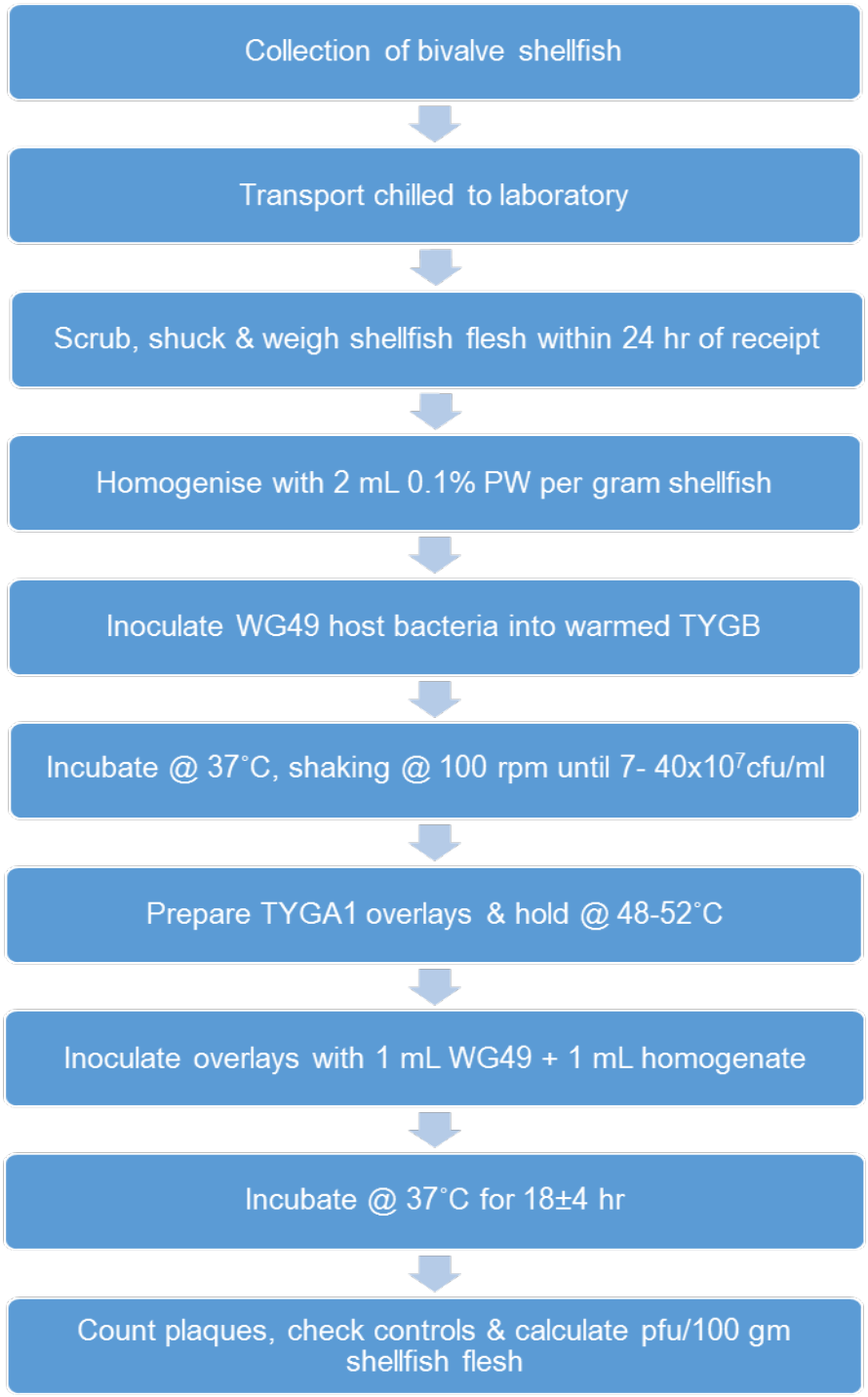
The control limits for the phage MS2 working culture can be determined from these data. Construct control charts of log 10 titres using the mean values and standard deviation. Warning and action limits are defined as:

Warning limit: Mean ± 2 SD

Action limit: Mean ± 3 SD

FRNA bacteriophage testing in bivalve molluscan shellfish

Flowchart



Shellfish sample preparation

Shellfish should be kept at 4˚C once harvested and delivered to the laboratory as quickly as possible under refrigeration or chilled with ice packs (sample size for oysters should be 10-12 shellfish).

On arrival to the testing laboratory the shellfish should be stored at 4˚C until processed which should be with 24 hours of receipt.

Shellfish should be rinsed and scrubbed with a sterile brush under cold, running tap water of potable quality and drained on clean paper towel. Discard damaged or opened shellfish. They should not be re-immersed in water as this may cause them to open.

Open shellfish with a sterile shucking knife (wear a mesh safety glove) and empty meat and liquor into a pre-weighed homogeniser jar.

Add 2.0 mL of 0.1% PW per 1 g of shellfish. Blend on high speed for 4 bursts of 15 sec with at least 5 sec between each burst.

Centrifuge 30-50 mL of shellfish homogenate at 2,000 × g for 5 minutes. Decant the supernatant into a new tube. The homogenate may be stored at 4˚C for up to 48 hours prior to analysis.

Equilibrate supernatant to room temperature before plating. Make decimal dilutions of the supernatant as required in 0.1% PW. The dilutions will depend on the expected levels of faecal contamination of the sample. If the sample is likely to have low levels of phage then 10 replicate plates using the neat sample must be analysed.

Host bacterial culture inoculation

Aseptically dispense TYGB into a sterile container – allow for 10 mL per sample plus 20 mL for controls/extra OR 20 mL per sample if RNase confirmation is included. Aseptically add 100 µL of 1% Ca/Glu per 10 mL of TYGB. Mix and warm to 37˚C.

Thaw one vial of the host bacteria WG49 working culture and inoculate with 100 µL of WG49 per 10 mL TYGB.

Incubate at 37˚C with shaking at 100 rpm for the time required to achieve a cell concentration of 7 - 40 × 10^7 cfu/mL (usually 3 - 3.5 hours).

TYGA1 overlay preparation

Prepare complete TYGA1 overlay medium.

Allow for:

25 mL per sample plus 20 mL for controls/extra

OR

50 mL per sample if RNase confirmation is included.

Equilibrate in the water bath at 48 t- 50°C and dispense 2.5 mL volumes into tubes. Hold at 48 - 50°C.

Procedure

When the host bacteria WG49 has reached the desired cell concentration of 7 – 40 × 10^7 cfu/mL, add 1 mL to each TYGA1 overlay.

Then add 1 mL of the shellfish sample supernatant and gently mix contents by inversion and rolling the tube between palms.

Pour the inoculated overlay over the surface of a TYGA2 base agar and distribute evenly by tilting the plate.

Also inoculate positive (bacteriophage MS2) and blank (0.1% PW) controls at the start and end of testing.

> If RNase confirmation is included also inoculate a parallel series of overlay plates with 100 µL RNase solution added to each overlay. Additional controls of overlays with phage MS2 and RNase should be inoculated and poured. All procedures using phage MS2 must be performed in the biosafety cabinet.
>
> RNase confirmatory tests should be prepared when large numbers of somatic phages (large, circular, clear plaques with smooth edges) are likely, for example in the event of a sewage spill or on occasion as desired to confirm results.

Allow plates to solidify at room temperature for approximately 20 minutes (dry in the biosafety cabinet) and incubate at 37°C for 18 ± 4 hours.

Following incubation, count all plaques on each plate. Exclude those plaques exhibiting typical DNA phage morphology, i.e. plaques of approximately 6 mm diameter with a clear lysis zone in the centre. However, if RNase plates have been inoculated count all plaques on both series.

Expression of results

Calculate the results using the following equation:

$$Cpfu = [(N - N_{RNase}) / n \times F] \times 300$$

Cpfu is the confirmed number of FRNA phages expressed as plaque forming units (pfu) per 100 g shellfish flesh.

N = total no of plaques counted

N_{RNase} = total number of plaques counted on RNase plates

n = no of replicates

F = dilution factor

The limit of detection of the assay is 30 pfu/100g.

If a sample result is negative, and 10 replicates have been carried out in the neat series, the result is expressed as <30 pfu/100 g shellfish flesh.

The positive controls must fall within the limits specified in the phage MS2 control charts (see phage MS2 control limits).

Appendices

Appendix A: ISO 10705 - 1 “Water quality; detection and enumeration of bacteriophages – Part 1: Enumeration of F-specific RNA bacteriophages (Anon 1996)

Appendix B: Cefas Generic Standard Operation Procedure SOP 1671 version 7 2007: Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish:

https://eurlcefas.org/media/6222/crl_sop_phage_17_11_07.pdf

Appendix C: U.S. National Shellfish Sanitation Program (NSSP): Modified double agar overlay method for determining male-specific coliphage in soft-shelled clams, American oysters, and quahogs (*M. mercenaria*) (Nov 2013)

http://www.issc.org/Data/Sites/1/media/2013%20summary%20of%20actions/2013%20issc%20summary%20of%20actions%20website%2006-12-2014.pdf

Appendix D: Comparison of bacteriophage methods

Appendix E: Review publication: Hodgson, K. R., V. A. Torok and A. R. Turnbull (2017). "Bacteriophages as enteric viral indicators in bivalve mollusc management." Food Microbiol 65: 284-293.

Appendix F: *Salmonella* Typhimurium strain WG49 NCTC information sheet

Appendix G: *Salmonella* Typhimurium strain WG49 MSDS

Appendix H: *Escherichia coli* bacteriophage MS2 ATCC information sheet

Appendix I: *Escherichia coli* bacteriophage MS2 MSDS

Appendix J: Nalidixic acid MSDS

Appendix K: Sodium hydroxide MSDS

Appendix L: SARDI FRNA phage results worksheet

Appendix M: SARDI FRNA phage results summary

Appendix N: SARDI results summary sheet

Most of these appendices have not been included in this report but are available on request. Appendix E the review publication is attached to this document as Appendix F.

Appendix F: Review publication: Hodgson, K. R., V. A. Torok and A. R. Turnbull (2017). "Bacteriophages as enteric viral indicators in bivalve mollusc management." Food Microbiol 65: 284-293.

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Review

Bacteriophages as enteric viral indicators in bivalve mollusc management

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ABSTRACT

Human enteric viruses, such as norovirus and hepatitis A virus, are spread by a variety of routes including faecal-oral transmission. Contaminated bivalve shellfish are regularly implicated in foodborne viral disease outbreaks internationally. Traditionally indicator bacteria, the coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish. However, studies have established that they are inadequate as indicators of the risk of human enteric viruses. Bacteriophages 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. The somatic coliphages, male-specific RNA coliphages (FRNA coliphages) and the bacteriophages of *Bacteroides* are the groups recognised as most suitable for water and shellfish testing. In this review, we discuss the rationale and supporting evidence for the application of bacteriophages as surrogates for human enteric viruses in shellfish under a variety of conditions. There is some evidence to support the validity of using bacteriophage levels to indicate viral risk in shellfish in highly contaminated sites and following adverse sewage events.

Contents

1. Introduction

The human health risks associated with consumption of raw or

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http://dx.doi.org/10.1016/j.fm.2017.03.003
0740-0020/

Appendix G: Guidance document

SOUTH AUSTRALIAN RESEARCH & DEVELOPMENT INSTITUTE
PIRSA

Guidelines for the application of FRNA bacteriophages in bivalve mollusc risk management after adverse sewage events

4TH JUNE 2018

Guidelines for the application of FRNA bacteriophages in bivalve mollusc risk management after adverse sewage events

Information current as of 8 May 2018

Disclaimer

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Scope

The Australian Shellfish Quality Assurance Program (ASQAP) Operations Manual was revised in 2016 to include the use of FRNA bacteriophage (phage) levels to potentially re-open leases earlier than the obligatory 21 days after adverse sewage events (ASQAAC 2016). Traditionally, indicator bacteria, including the coliforms and *Escherichia coli*, have been used to detect faecal pollution in growing waters and shellfish, however many studies have established that they are inadequate indicators of human enteric viruses. The addition of FRNA phage testing can be used to demonstrate whether viral contamination of the growing site has occurred. This permits regulators to re-open growing areas earlier than 21 days, if results from testing of samples collected no earlier than 7 days after the event has ceased are appropriate.

Section 6.1.10 of the manual specifies:

"A harvest area temporarily placed in the closed status is reopened only when:

a) the original classification criteria are satisfied;

b) sufficient time has elapsed to allow the shellstock to reduce to acceptable levels: pathogens, indicator organisms, biotoxins or other deleterious substances that may be present in the shellstock, and the shellstock have been demonstrated to be safe;

c) for closures associated with an untreated or partially treated sewage discharge or an untreated sewage discharge from a community sewage system:

i. at least 21 days have passed since the end of the contamination event; OR

ii. Shellstock samples, collected from representative locations in each harvest area (no sooner than seven days after the contamination has ceased), are found to have Male Specific Coliphage levels which do not exceed background levels or a level of 50 Male Specific Coliphage per 100 grams[1]; and

d) supporting information is documented in a central file."

These guidelines contain information designed to assist in the application of this methodology.

1. Design of a post adverse event investigation

The aim of a post adverse event investigation is to determine the impact on shellfish within affected growing areas. The ASQAP Operations Manual specifies that the growing area remain under an obligatory 21 day closure until pathogens in shellstock reduce to an acceptable level. The FRNA phage assay provides an option to assess the potential level of viral contamination of shellfish. If the FRNA phages are at background levels, or below 60 pfu/100 g shellfish flesh in samples collected 7 days or more after the event has ceased, and all other water quality parameters are suitable, the regulators have the option to reopen the growing area earlier. It should be noted that phage investigation does not replace *E.coli* or other water and shellfish testing programs, but should be used in conjunction with these to provide additional information.

[1] The level of 50 pfu/100 g shellfish flesh will need to be revised to 60 pfu/100g shellfish flesh if the method for phage enumeration employed is based on the Cefas standard procedure. The limit of detection is 30 pfu/100 g shellfish flesh and phage titres are multiples of 30 pfu.

The investigation should be designed to maximize information regarding leases in growing areas that may have been impacted by the spill. At a minimum, shellfish samples should be taken for *E.coli* and bacteriophage, and waters tested for the standard measurements conducted following adverse events (e.g. salinity, thermo-tolerant coliforms).

Historical information is valuable when designing a post adverse event investigation. Information such as flushing rates, water currents, previous dye tracker or desk-top analyses of spill events should be considered in the design and interpretation phases.

2. Sampling strategy

Section 6.1.10.ii of the manual states that shellstock samples should be collected from representative locations in each harvest area, no sooner than seven days after the contamination has ceased. Samples may be taken at any time after the seven days has passed. The sampling strategy must aim to effectively assess the impact of the adverse event on the growing area. The sampling should not be random but target leases most likely to be affected. Shellfish Quality Assurance programs may offer additional advice or have specific requirements with regards to sampling.

Factors to consider:

- Source of pollution
- Volume of pollution
- Period of the adverse event
- Type of pollution e.g. overflow of raw or partially treated sewage
- Hydrographic characteristics e.g. tides, river discharge
- Meteorological characteristics e.g. rainfall, wind
- Number of leases in growing area
- Accessibility
- Shellstock levels

3. Number of samples

The strategy must consider the number of samples that will adequately reflect the likely impact of the sewage spill. A minimum of one dozen shellfish samples for each site is required.

The number of sites will depend on the size of the growing area, the number of leases and the complexity of the water movements in the growing area.

There will be inherent variability between individual samples on a lease and variation between leases. Results from the recent research study (FRDC Final Report 2015-037) found that not all samples were contaminated and the samples closest to the spill location were not necessarily those most contaminated. We would recommend a minimum of 5 samples be taken in a growing area, and in instances where there are multiple harvest areas or complex water movements more samples should be analysed.

The capacity of the participating laboratory must also be taken into account. The laboratory should be advised of the likelihood of samples to allow time for preparation of media and staffing, to ensure it is able to analyse the samples by preparing the shellfish homogenates within the designated 24 hours after receipt.

4. Transport and storage of samples

The handling of samples must be conducted to ensure the integrity of the samples is maintained. This includes packing to minimize damage to the samples, temperature control and timely transport to the laboratory.

- Do not freeze samples.
- Pack samples into clearly labelled resealable plastic bags e.g. zip-lock bags, double bag.
- Chill samples as soon as possible.
- Dead or damaged shellfish must be discarded.
- Pack into foam boxes or eskys with chill packs to maintain refrigerated conditions.

Arrange for courier pick-up and transport to the participating laboratory, ideally within 24 hours but 48 hours as applied for *E. coli* testing is acceptable. Samples arriving beyond this time frame may not be suitable for analysis as the validity of the results is in doubt. Preferably resample and ensure that samples are delivered to the laboratory chilled and in good condition within 48 hours.

5. FRNA phage testing method

The ASQAP Operations Manual does not specify which FRNA phage method is applied.

This guideline has been based on ISO 10705 - 1 Water quality - Detection and enumeration of bacteriophages – Part 1: Enumeration of F-specific RNA bacteriophages (ISO 1995) and the Cefas Generic Standard Operation Procedure 1671 version 7 2007: Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish (Cefas 2007).

The United States Food and Drug Administration Interstate Shellfish Sanitation Conference (USFDA ISSC) approved the application of phage detection and enumeration for shellfish management in 2009.

The US ISSC method “Modified double agar overlay method for determining male-specific coliphage in soft-shelled clams, American oysters, and quahogs (*M. mercenaria*)” (FDA 2015) is the method used in the US and varies in a number of details including the bacterial host strain.

The Cefas and US ISSC methods are compared on Table 1.

SARDI has developed the Cefas method in the Food safety and Innovation laboratory in Adelaide as the host bacterial culture *Salmonella typhimurium* strain WG49 and phage control *Escherichia coli* bacteriophage MS2 were available. In addition, this method includes the option of inoculating RNase confirmation plates which can be used to remove potential interference from DNA phages.

Table 1. Comparison of bacteriophage methods

Method	Sample size	Sample preparation		Phage stock culture strain	Working host strain	Working host strain QC	Phage positive control	Phage QC	Negative control	Limit of detection
FDA US[1]	Minimum 12 shellfish	2:1 growth broth: shellfish tissue (v:w) Homogenised Centrifuge 33 g @ 9000×*g*, 15min, 4°C Retain supernatant & weigh	10 plates: 2.5 mL supernatant & 200µL host per plate	*E. coli* F_{amp} ATCC 700891	*E.coli* F_{amp} ATCC 700891		Type strain MS2 (ATCC 15597)		Growth broth: Tryptone 1% Dextrose 0.1% NaCl 0.5% Plated at beginning & end of sample set.	Approx. 9 pfu/100 g American oysters (varies with shellfish species and weight of sample supernatant)
Cefas[2]	12-18 oysters 18-35 mussels Other species also specified	2:1 peptone water: shellfish tissue (v:w) Homogenised Centrifuge 30-50 mL @ 2000×*g*, 5min, RT Retain supernatant	10 plates: 1 mL supernatant & 1 mL host per plate 10 RNase plates: 1 mL sample & 1 mL host per plate Confirmatory test	*S.typhimurium* strain WG49 phage type 3 Nal^r (F' 42 lac:Tn5) – NCTC 12484	*S. typhimurium* strain WG49 phage type 3 Nal^r (F' 42 lac:Tn5) – NCTC 12484	1. Plasmid segregation 2. Nalidixic acid and kanamycin resistance 3. Bacteriophage susceptibility	Type strain MS2 (ATCC 15597)	Determination of MS2 control limits (20 times on 10 separate occasions)	Peptone water	30 pfu/100 g shellfish flesh

[1] Modified Double Agar Overlay Method for Determining Male-specific Coliphage in Soft Shelled Clams and American Oysters. ISSC Summary of Actions 2009. Proposal 05-114, Page 50. (Type IV) and 2013 modification.

[2] The Centre for Environment, Fisheries & Aquaculture Science, Cefas Standard Operating Procedure - Enumeration of male-specific RNA bacteriophages in bivalve molluscan shellfish 17.11.07

5.1. Cefas method

Sample preparation, assay procedure, quality control parameters, storage and procedures for safely working with the reference cultures are described in detail in the Cefas standard operating procedure (Cefas 2007).

5.1.1. Quality control

Quality control of WG49 working culture

The WG49 working culture should be checked for plasmid segregation, nalidixic acid and kanamycin resistance and bacteriophage susceptibility as described in the Cefas method. Plasmid segregation and antimicrobial susceptibility controls should be inoculated when the WG49 working stock is initially prepared and then intermittently as an internal laboratory control at the discretion of the laboratory manager. The bacteriophage susceptibility must be confirmed in each assay with MS2 control plate inoculated at the beginning and on completion of the assay. This will ensure validity of the test as the host bacteria is confirmed to be susceptible to the phages throughout analysis of the samples. This should be included in the SQAP report.

Buffer control

At least one 0.1% peptone water buffer control must be inoculated with each assay. This is the buffer used to prepare the shellfish homogenates and must be inoculated into the overlay with the WG49 host bacteria and without shellfish material as a negative control. There should be no plaques after incubation. The result should be included in the SQAP report.

MS2 control limits

MS2 phage working stock control limits are determined by calculating the concentration of the MS2 phage working culture twenty times on at least 10 occasions as described in the Cefas method. A control chart is constructed incorporating warning and action limits. If the number of plaques on the MS2 positive control plates inoculated with each assay are outside the action limits, the laboratory should prepare a new MS2 working culture. If the number of plaques is greater than the upper control limit, the result of the assay should still be valid for the samples. If the number of plaques is below the lower control limit, the number of plaques detected for each sample may not reflect the actual number present. Retesting may be necessary. The laboratory should include this in the SQAP report.

5.1.2. Analysis of results

The results are calculated using the following equation:

$$Cpfu = [(N - N_{RNase}) / n \times F] \times 3$$

Cpfu is the confirmed number of FRNA phages expressed as plaque forming units (pfu) per 100 g shellfish flesh.

N = total no of plaques counted

N_{RNase} = total number of plaques on RNase plates

n = no of replicates

F = dilution factor

The limit of detection of the assay is 30 pfu/100g.

The background level of phages in shellfish from growing areas in Australia appears to be low, generally less than 60 pfu/100 g shellfish flesh. It is recommended that 10 replicate overlays should be inoculated for each sample. In addition, to ensure no interference from DNA phages, a duplicate set of 10 RNase overlay plates should be inoculated in parallel as described in the Cefas method.
If a sample result is negative, and 10 replicates have been carried out in the neat series, the result is expressed as <30 pfu/100 g shellfish flesh.

The positive controls must fall within the limits specified in the phage MS2 control charts.

The blank buffer controls must have no plaques.

5.1.3. Reporting results

The report for SQAP managers must include the following:

- sample collection date and time
- sample arrival date and time
- temperature of sample on arrival
- time for transport
- date of analysis
- method used
- sample results
- control results
- limit of detection (LOD)

The report should also comment on the condition of the samples on arrival, for example, any dead or damaged shellfish and any other information deemed appropriate.

5.1.4. Interpretation of results

The titre of FRNA phages in each sample should be considered in relation to the pre-determined background study or the 60 pfu/100 g shellfish flesh cut-off. If samples are within these limits no sooner than 7 days after the event ceased, the regulator may consider re-opening the growing area provided all other parameters such as *E. coli* levels are acceptable. FRNA phage detection and enumeration does not replace coliform and *E. coli* testing. The coliforms & *E. coli* are indicative of faecal pollution and may not correlate with the presence of viruses. The regulator should document all evidence available when making a decision to shorten the 21 day closure period.

How to interpret the data if there is no background phage level

A background study of growing areas is recommended, however if samples are analysed from harvest areas where this has not been conducted, some conclusions can be made depending on the result. If the phage titres are low (<60 pfu/100 g shellfish flesh), it would be reasonable to conclude there is no or minimal contamination. No definitive conclusion can be made for samples with phage titres >60 pfu/100 g shellfish flesh.

How to interpret the data if there is a low phage level

A low level of phages (<60 pfu/100 g shellfish flesh) indicates that there is no or a very low level of contamination from sewage pollution and the shellfish are not likely to be a risk to consumers.

How to interpret the data if there is a high phage level

A high level of phages (>60 pfu/100 g shellfish flesh or above the background phage level) indicates that there is contamination from sewage pollution and a risk of human enteric viruses in the shellfish. If any samples from a growing area have phage levels above the limit, even if only one sample is above the limit, the growing area should remain closed. There is inherent variability in phage and virus levels between individual shellfish in a sample, between samples from a lease and between leases. Any sample with unacceptable phage levels indicates there is a risk of human viruses in the shellfish from that growing area. In this case, the growing area should not be reopened even if other parameters such as *E. coli* are acceptable. The regulator needs to consider all other information as listed above – volume and treatment level of effluent, volume of receiving waters, *E. coli* in shellfish, thermotolerant coliforms in water, salinity measurements, water movements e.g. tides, currents, prevailing winds, historical information on spills.

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For further information

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