FRDC
FISHERIES RESEARCH & DEVELOPMENT CORPORATION

Survey of Foodborne Viruses in Australian Oysters

Valeria Torok, Kate Hodgson, Jessica Tan, Navreet Malhi and Alison Turnbull

December 2015

FRDC Project No. 2013/234

MISA marineinnovationsa

SafeFish

SARDI
SOUTH AUSTRALIAN RESEARCH AND DEVELOPMENT INSTITUTE

Government of South Australia
Primary Industries and Regions SA

ISBN **978-1-921563-87-4**

Survey of Foodborne Viruses in Australian oysters
2013/234

2015

Ownership of Intellectual property rights

Creative Commons licence

Disclaimer

Researcher Contact Details

Name: Dr Valeria Torok
Address: South Australian Research and Development Institute, Food Safety and Innovation
GPO Box 397, Adelaide SA 5001
Phone: (08) 8303 9688
Fax: (08) 8303 9424
Email: valeria.torok@sa.gov.au

FRDC Contact Details

Address: 25 Geils Court
Deakin ACT 2600
Phone: 02 6285 0400
Fax: 02 6285 0499
Email: frdc@frdc.com.au
Web: www.frdc.com.au

In submitting this report, the researcher has agreed to FRDC publishing this material in its edited form.

Foreword

In 2011 “Blue Frontiers” recognised extensive bivalve shellfish farming as one of the few industries that has a net positive impact on the environment.[1] It also produces a product that, provided appropriate food safety controls are maintained, is nutritious and healthy. Bivalve shellfish contribute positively to the health of its consumers (high protein, low saturated fats, source of omega-3 fatty acids and other essential nutrients, such as selenium and iodine that are difficult to obtain through other food products). As such it is an industry well worth supporting.

However, the history of bivalve shellfish consumption is not so rosy. In the early 1900’s the consumption of shellfish was too often implicated in outbreaks of typhoid fever. Responding to this issue was a significant challenge for industry and scientists, as testing methods were limited by the technology of the time. Yet they forged ahead regardless and developed quality assurance programs that drastically reduced the incidence of foodborne illness associated with shellfish consumption. Recently, new food safety challenges have arisen, with human pathogenic viruses one of the most problematic food safety challenges faced by shellfish industries globally.

Since the turn of the century norovirus outbreaks have been a regular occurrence in many developed countries. Australia by comparison has a large oyster industry with a culture of eating oysters raw and lightly cooked, but conversely has observed a relatively low incidence of shellfish related norovirus outbreaks. This epidemiological data is confounded by the low rate of reporting of foodborne illness in Australia. To determine the real incidence of norovirus in Australian shellfish a robust survey was required. The survey undertaken was aimed at determining the prevalence of norovirus and hepatitis A virus in market-ready shellfish from four oyster growing states in Australia (South Australia, Tasmania, New South Wales and Queensland) to determine if the current risk management practices in Australia were adequate.

The challenge of undertaking a robust survey of Australian oyster growing areas over multiple seasons is significant. The industry in the four states sampled consists of hundreds of growers distributed across ~3,000 km of coastline. The researchers are to be commended on their coordination of a large and complex sampling effort on a constrained operating budget.

The results of norovirus surveys in other countries have often yielded high rates of norovirus detection in shellfish. Once published these surveys create significant negative publicity. The decision by the Australian oyster industry to voluntarily undertake an in-depth survey of norovirus prevalence in Australian oysters represents a proactive and forward thinking approach to ensuring product integrity.

Not a single sample analysed during the survey had detectable levels of norovirus or hepatitis A virus. This translates into an exceptionally low prevalence of these viruses (<2%): a commendation to the risk management programs undertaken in accordance with guidelines listed in the Australian Shellfish Quality Assurance Program. The commitment of the industry to ensuring that they supply a safe product to the market place is well known. The efforts of the Australian oyster industry go well beyond the farm. They take a holistic approach to quality assurance by engaging with stakeholders throughout the catchment of their respective growing areas to ensure that the communities surrounding them are active partners in protecting water quality.

The results of this survey bear testament to the success of the Australian oyster industry in their endeavours to protect water quality and the environment in which they operate. The results of the survey provide consumers with confidence in Australian oysters as a safe and nutritious product. The results also provide the Australian oyster industry and food safety regulators with defendable scientific evidence that

[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.

current quality assurance measures being implemented by the industry are adequate and additional requirements such as routine virus monitoring are not required to ensure product safety.

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

Contents

Tables

Figures

Acknowledgments

We would like to acknowledge the steering committee members for their advice and input into the sampling plan design, including assistance with gathering state production data.

We also acknowledge the New South Wales, South Australian, Tasmanian and Queensland oyster industries for their in principle support of the project. Furthermore, we would like to acknowledge the oyster industry for providing time and resources for the survey sample collection.

We acknowledge the technical support of SARDI staff including Linda Friedrich and Joanne Tomkins.

We acknowledge the financial support of the Fisheries Research and Development Corporation, Oysters Australia, Tasmanian Oyster Research Council, Tasmanian Shellfish Executive Committee, South Australian Oyster Research Council, South Australian Shellfish Quality Assurance Program and New South Wales Food Authority.

Abbreviations

Australian Bureau of Agricultural and Resource Economics and Sciences	ABARES
Australian Shellfish Quality Assurance Program	ASQAP
Centre for Environment, Fisheries and Aquaculture Science	CEFAS
Codex Committee of Food Hygiene	CCFH
Colony forming units	CFU
Department of Primary Industries, Parks, Water and Environment	DPIPWE
Digestive tissue	DT
Double stranded deoxyribonucleic acid	dsDNA
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 Safety and Innovation	FSI
Hazard Analysis and Critical Control Points	HACCP
Hepatitis A virus	HAV
Human norovirus	NoV
International Organisation of Standardisation Technical Specification	ISO/TS
Murine norovirus	MNV
New South Wales	NSW
New South Wales Food Authority	NSWFA
Primary Industries and Regions South Australia	PIRSA
Queensland	Qld
Reverse Transcription Polymerase Chain Reaction	RT-PCR
South Australia	SA
South Australian Oyster Growers Association	SAOGA

South Australian Oyster Research Council	SAORC
South Australian Research and Development Institute	SARDI
South Australian Shellfish Quality Assurance Program	SASQAP
Tasmania	Tas
Tasmanian Oyster Research Council	TORC
Tasmanian Shellfish Executive Council	TSEC
United Kingdom	UK
United States of America	USA
Victorian Infectious Diseases Reference Laboratory	VIDRL
World Health Organization	WHO

Executive Summary

What the report is about

The South Australian Research and Development Institute (SARDI) Food Safety and Innovation group, with the support of Oysters Australia, Fisheries Research and Development Corporation (FRDC), New South Wales Food Authority (NSWFA), South Australian Shellfish Quality Assurance Program (SASQAP), South Australian Oyster Research Council (SAORC), Tasmanian Oyster Research Council (TORC) and Tasmanian Shellfish Executive Council (TSEC), undertook a national yearlong survey for foodborne viruses in Australian oysters. The survey was done to investigate the prevalence of human Norovirus (NoV) and Hepatitis A virus (HAV) in Australian grown oysters at production. These human enteric viruses are frequently associated with shellfish related foodborne outbreaks internationally, and occur when shellfish growing areas become contaminated with human sewage. Sampling for the survey was done between July 2014 and August 2015, and represented all the major commercial oyster production areas within Australia, including New South Wales (NSW), South Australia (SA), Tasmania (Tas) and Queensland (Qld). Samples were only collected from growing areas in the *Open Status* for harvest as defined by the Australian Shellfish Quality Assurance Program's (ASQAP) Manual of Operations. For Tasmania, South Australia and Queensland this represented oysters fit for market. For New South Wales, this represented either oysters fit for market or fit for depuration prior to market. Testing for NoV and HAV in oysters was done using a recently published International Organisation of Standardisation Technical Specification (ISO/TS 15216). The prevalence survey for foodborne viruses in oysters reported here is the first national survey undertaken in Australia.

Background

Human enteric viruses are increasingly recognised as important causes of foodborne disease. Globally, shellfish related viral foodborne outbreaks are commonly associated with NoV and HAV in oysters eaten raw. As oysters are filter feeders, they may become contaminated with human enteric pathogens when grown in sewage-contaminated waters. There are currently no effective control measures available to eliminate these viruses from food without changing the characteristics of the product (e.g. cooking). The most effective risk management strategy for NoV and HAV in bivalve shellfish is to prevent contamination of the production areas. The European Union legislation on the microbiological criteria for foodstuff has suggested that "criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently". In 2012 an international standard technical specification (ISO/TS 15216) testing for NoV and HAV in bivalve molluscs by real-time Reverse Transcription Polymerase Chain Reaction (RT-PCR) became available. This method would be suitable for use in legislation and, as a result, risk managers overseas are considering establishing virus limits for high-risk food groups such as live bivalve molluscs. The European Food Safety Association (EFSA) has recommended: the establishment of an acceptable limit for NoV in oysters to be harvested and placed on the market; NoV testing of oysters to verify compliance with the acceptable NoV limits established; and that food businesses verify their Hazard Analysis and Critical Control Point (HACCP) plans demonstrating compliance with established acceptable levels. Furthermore, the European Union Community Reference Laboratory has recommended that if virus standards are introduced then standards for NoV should be quantitative (i.e. a maximum acceptable level be determined) and standards for HAV be qualitative (i.e. presence/absence). As a response to these impending international regulations (noting that some importing nations already require NoV testing), the Australian oyster industry members indicated that they would like a comprehensive evaluation of the prevalence of foodborne viruses in Australian oysters as there is little information on Australian baseline levels.

Aims/objectives

- Design a statistically robust survey to evaluate virus occurrence in oyster harvest areas in New South Wales, South Australia, Tasmania and Queensland.
- Identify the prevalence of Norovirus and Hepatitis A virus associated with Australian oysters at harvest.
- Use the survey results to support trade and market access of Australian oysters.

Methodology

Sampling for the prevalence survey was done over a period of a year in 2014-15. During this period two rounds of sampling were undertaken to capture "peak" and "off-peak" times for NoV circulating within the community. During each sampling round, 150 oyster samples were collected. Collection of 150 samples enabled the detection of one virus positive sample with a probability of 0.95 if ≥2% of the oyster samples were contaminated with the virus. All commercial oyster production areas were included in the survey. Sample numbers were assigned to states and then production regions within each state based on proportional production levels. Within production regions, the allocation of samples to harvest areas was weighted by the proportion of the region's production. Samples were further assigned to active oyster leases producing mature shellfish in an unweighted randomised system. Sampling kits were distributed to industry coordinators in each state who undertook the sampling during the requested periods. All samples were sent to the SARDI Food Safety Laboratory where they were processed and tested for 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*. This is a culture independent method based on the detection and possible quantification of the viral genome.

Results/key findings

A total of 33 oyster production regions in NSW, SA, Tas and Qld were involved in the oyster survey. 149 samples (n=63 NSW, n=32 Tas, n=53 SA and n=1 Qld) were received during sampling round one (July to November 2014). 148 samples (n=60 NSW, n=34 Tas, n=53 SA and n=1 Qld) were received during sampling round two (December 2014 to August 2015). All production areas submitted samples for the survey with the exception of Macleay River (NSW) and Shoalhaven/Crookhaven River (NSW). Both these areas are minor NSW production areas, producing only 0.9% (Macleay River) and 2.5% (Shoalhaven/Crookhaven River) of the state's total annual oyster production.

No NoV or HAV was detected in Australian oysters collected at production in either sampling round during 2014-15. For each of the two sampling rounds this translated into an estimated prevalence for NoV and HAV in Australian oysters of <2% with a 95% confidence interval ranging from 0-2.5% to 0-2.7% in rounds one and two of sampling, respectively.

Implications for relevant stakeholders

This is the first national prevalence survey reported for NoV and HAV in Australian oysters at production. No viruses were detected in 297 oysters received during the survey period (July 2014 to August 2015) resulting in the statistical estimate of prevalence of these viruses in Australian oysters as being <2%. To increase the accuracy in the estimate of the prevalence (below the upper limit of 2%) would require significantly greater sampling and cost. These results indicate that Australian oysters have a very low estimated prevalence for NoV and HAV. This is quite distinct to the situation recently reported in European surveys which indicate presence of NoV in up to 76% of sampled UK oysters and 31% of Irish oysters sampled from Class A waters (<230 *E. coli* CFU/100 g shellfish). These results are approximately equivalent to Australia's Approved areas in the *Open Status*. Samples in this survey were

only collected from shellfish suitable for direct market access or for depuration (i.e. growing areas open for harvest as defined in the ASQAP Manual of Operations). These results that show no detected NoV or HAV (<2% estimated prevalence) during the survey period indicate that the ASQAP is effective in managing food safety risks in shellfish associated with enteric foodborne viruses, as well as complying with the Food Standards Code and Export Orders as they relate to bivalve shellfish and the schedule to Standard 4.2.1 of the Food Standards Code.

There is an inherent risk of foodborne viral illness associated with oysters when the product is eaten raw, especially if grown in water which can be impacted by sewage and environmental run-off. However, the results of this survey show that the Australian oyster industry is producing a safe product with limited risk of viral contamination. The low estimated prevalence (<2%) of foodborne viruses in Australian oysters was also supported by epidemiological evidence, with no oyster related foodborne illness reported in Australia during the survey period.

Results of the survey could be used to expand international market access and provide a scientific argument against the current push to introduce mandatory testing and monitoring as being proposed within the European Union.

Recommendations

Internationally, it is accepted that enteric bacteria (coliforms and *Escherichia coli*), which are present in the intestine of all warm blooded animals, are not good indicators for the presence of human enteric foodborne viruses. Yet within the Australian context, it appears that the use of these bacterial indicators in sanitary surveys, in conjunction with in-depth shoreline surveys as part of the ASQAP, is effective in producing oysters with a low risk of foodborne viral contamination.

The results of the survey can be used to underpin the market access of Australian oysters and market them as world class from an enteric virus food safety perspective.

The results of the survey can be used in evidence against mandatory international viral testing of Australian oysters.

Viral monitoring of oysters should be undertaken in harvest areas which are epidemiologically linked to foodborne viral illness as part of remediation and validation prior to re-opening.

The results of the oyster survey are not transferable to other filter feeding bivalve molluscan shellfish as filtration and virus retention rates vary among species.

Keywords

Norovirus, Hepatitis A virus, Pacific Oyster, Sydney Rock Oyster

1. Introduction

Between 2002–03 and 2012-13 the gross value of Australian aquaculture production has increased by 12% ($108 million) (ABARES, 2014). The largest increase over this period has come from the production value of salmonids and edible oysters, with edible oysters increasing by $14 million (17% and 1852 tonnes). Aquaculture edible oysters dominated the production of molluscs in 2012–13 by value and volume. In 2012–13 edible oysters accounted for 9% of the total value of Australian aquaculture production. Between 2011–12 and 2012–13 edible oysters increased in value by $4 million (5%) to $95 million. This was primarily the result of a 5% increase in the average unit price. In 2012-2013 the major edible oyster producing states by value were: NSW ($35.0 million), SA ($35.0 million) and Tas ($23.1 million) with minor production in Qld ($0.5 million) (ABARES, 2014).

Human enteric viruses are increasingly recognised as important causes of foodborne disease globally, based on the incidence of reported foodborne disease and the severity of disease (including mortality) (FAO/WHO, 2008). International estimates of the proportion of these viruses attributed to food are in the range of approximately 5% for Hepatitis A virus (HAV) and 12-47% for Norovirus (NoV) (FAO/WHO, 2012). Three major sources of viral contamination of foods have been identified: (1) human sewage/faeces; (2) infected food handlers; and (3) animals harbouring zoonotic viruses, although combinations of these have also been described. During the 2008 FAO/WHO expert meeting on "Viruses in Food", the virus-commodity combinations of greatest public health concern selected were NoV and HAV in bivalve molluscs, fresh produce and prepared (ready-to-eat) food (FAO/WHO, 2008). A systematic review of global shellfish related viral foodborne outbreaks between 1980 and 2012 reported NoV (83.7%) and HAV (12.8%) as the most common viral pathogens and oysters (58.4%) as the most frequently consumed shellfish associated with outbreaks (Bellou et al., 2013). The majority of the reported outbreaks were located in East Asia, followed by Europe, America, Oceania, Australia and Africa (Bellou et al., 2013). In Australia, between 2001 and 2010, seventeen cases of suspected shellfish related NoV outbreaks were reported in OzFoodNet (OzFoodNet, 2011). The most recent oyster related outbreak of NoV occurred in 2013 with 525 people affected nationally following consumption of contaminated oysters from Tasmania (Lodo et al., 2014).

Risk management for bivalve shellfish currently relies on the use of enteric bacteria as indicators of faecal contamination. The adoption of regulations to specify acceptable levels of enteric bacterial pathogens in shellfish tissues or in waters where shellfish are grown has led to the classification of production areas for shellfish harvest fit for human consumption. The US and Australia use water-based sampling programs for shellfish developed around indicator bacteria known as coliforms (which include *Escherichia coli*). The EU program is a shellfish sampling based program which classifies shellfish harvesting areas into A, B and C categories, based on *E. coli* levels usually found in the shellfish: A (<230 CFU/100 g), B (230-4,600 CFU/100 g) and C (4,600-46,000 CFU/100 g). Australian and US oyster growing areas which are classified as *Approved* (ASQAP or Food and Drug Administration National Shellfish Sanitation Program) and are in the *Open Status* (i.e. oysters can be harvested for direct human consumption) are approximately equivalent to the EU Class A harvesting areas. Although coliforms and *E. coli* are good indicators of faecal contamination of growing waters by warm blooded animals they are not good indicators of the presence of human enteric viruses. Flannery et al. (2009) reported that 31% of compliant oysters harvested from Class A Irish waters were positive for NoV. Although on an individual sample basis *E. coli* is a poor predictor of NoV risk, on a site-specific basis average *E. coli* levels have been shown to correlate with average NoV levels when UK winter period data was investigated (Lowther et al., 2012).

Enteric viruses are more resistant to wastewater treatment and are more environmentally stable than faecal bacterial indicators. Viruses are also concentrated in higher levels and persist longer in shellfish than bacteria. Various shellfish species have been reported to accumulate and retain enteric viruses differently. This may be due in part to the variation in filtration rate among species. Among shellfish

species the prevalence of NoV has been noted to be significantly higher in mussels than in oysters and clams taken from the same waters (Pavoni et al., 2013; Polo et al., 2015; Seo et al., 2014). Hence, it has been suggested that mussels may be a good indicator species for pre-harvest viral surveillance, while clams may be more suitable for post-harvest surveillance due to their slower depuration rate (Polo et al., 2015).

High-risk factors for contamination of oysters with enteric viruses include low water temperatures (allowing greater persistence of the viruses), prevalence of enteric illness within the community and high rainfall leading to sewage system overflow (CEFAS, 2011). As there are currently no effective control measures available to eliminate these viruses from food without changing the characteristics of the product, the most effective risk management strategy for NoV and HAV in bivalve shellfish is to prevent contamination in production and harvesting areas. Freezing of shellfish does not deactivate foodborne viruses, but rather preserves them.

In 2012 the Codex Alimentarius Commission released guidelines on general principles of food hygiene to control viruses in food with Annex I specifically focussing on control of HAV and NoV in bivalve molluscs (FAO/WHO, 2008). It recommended that countries monitor for NoV and HAV in bivalves following shellfish-related foodborne outbreaks and high-risk pollution events (heavy rainfall and overflow from sewage treatment plants). The EU legislation on the microbiological criteria for foodstuff has suggested that "criteria for pathogenic viruses in live bivalve molluscs should be established when the analytical methods are developed sufficiently" (EC, 2005). With the development of the ISO/TS 15216 method *Microbiology of food and animal feed- horizontal method for the determination of hepatitis A virus and norovirus in food using real-time RT-PCR* virus methods have become available that may be considered suitable for use in legislation (ISO/CEN, 2013). Hence, risk managers are considering establishing virus limits for high-risk live bivalve molluscs. The EFSA Scientific Opinion on NoV in oysters recommended: the establishment of an acceptable limit for NoV in oysters to be harvested and placed on the market; NoV testing of oysters to verify compliance with the acceptable NoV limits established; and for food businesses to verify their HACCP plans and demonstrate compliance with the acceptable levels (EFSA, 2012). In 2012 the EU Community Reference Laboratory recommended that if virus standards are introduced then standards for NoV should be quantitative (i.e. a maximum acceptable level be determined) and standards for HAV be qualitative (i.e. presence/absence) (CEFAS, 2013a). It also considered and made recommendations on possible levels for a NoV standard in the context of both end-product and production area monitoring applications (CEFAS, 2013a).

Prior to the survey conducted in this study there was little information on the baseline levels of NoV in Australian oysters. Although, a small study of six high-risk Australian growing areas had detected NoV in 1.7% of samples (2/120), the study was not representative of oysters which were market-ready as defined by ASQAP (Brake et al., 2014). As a response to the impending international regulations (noting that some importing nations already require NoV testing e.g. Singapore) the Australian oyster industry indicated a desire for a more comprehensive evaluation of the prevalence of enteric foodborne viruses in Australian oysters. Similar surveys have been undertaken worldwide, including in France, Ireland, Italy, Japan, Korea, Morocco, Spain, United Kingdom and United States of America (Benabbes et al., 2013; Costantini et al., 2006; DePaola et al., 2010; Doré et al., 2010; Flannery et al., 2009; Loutreul et al., 2014; Lowther et al., 2012; Maekawa et al., 2007; Moon et al., 2011; Pavoni et al., 2013; Polo et al., 2015; Schaeffer et al., 2013; Seo et al., 2014; Shin et al., 2013; Suffredini et al., 2014). Although the reported data is difficult to compare between nations (varied testing methodologies and sampling approaches) the prevalence of foodborne viruses in oysters obtained in market product was comparable to those observed in commercial harvesting areas (EFSA, 2012).

Data obtained from this project provides a national baseline for NoV and HAV in Australian market-ready oysters. It is substantiated by epidemiological data on Australian oyster-related foodborne

illness during the survey period. The results of the survey may also contribute to the development of market access strategies at the international level.

2. Objectives

- Design a statistically robust survey to evaluate virus occurrence in oyster harvest areas in New South Wales, South Australia, Tasmania and Queensland.
- Identify the prevalence of Norovirus and Hepatitis A virus associated with Australian oysters at harvest.
- Use the survey results to support trade and market access of Australian oysters.

3. Method

3.1 Steering Committee

A steering committee, comprising both Australian oyster industry and shellfish regulatory representatives, was established at the start of the project. This included representation from each of the major oyster growing states within Australia. The steering committee comprised:

Jane Clout	Kooringal Oysters, Queensland (committee member)
Rachel King	Oysters Australia, National (committee member)
Trudy McGowan	SAOGA, South Australia (committee member)
Jon Poke	Bolduans Bay Oysters, Tasmania (committee member)
Rebecca Schofield	SafeFood, Queensland (committee member)
Valeria Torok	Principal Investigator, SARDI (permanent observer and secretariat)
Tony Troup	Camden Haven Oysters, New South Wales (committee member)
Alison Turnbull	SARDI/SafeFish (permanent observer)
Clinton Wilkinson	SASQAP, South Australia (committee member)
Howel Williams	Department of Health and Human Services, Tasmania (committee member)
Anthony Zammit	NSWFA, NSW (committee chair)

The terms of reference for the steering committee were to:

1. Provide strategic oversight of the project to ensure project outputs continue to contribute to enhanced capability for management of foodborne viruses in oysters.
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. Provide guidance to project researchers and industry on protocols for handling commercially or market sensitive information arising from the project.
5. Assist with advice on reporting policy.
6. Provide feedback on sampling design and protocol, and help with sample collection.

3.2 Data Management Plan

In consultation with the steering committee, a data management plan was developed for the project with sign off by all members of the steering committee. The data management plan is attached as Appendix A.

3.3 Survey Design

3.3.1 Determination of sample size

It was determined that a sample size of 150 for each of two sampling rounds would provide a statistical probability of 0.95 of detecting at least one sample with detectable levels of viruses if ≥2% of the samples were contaminated. The sample size calculation was based on the binomial distribution:

$$P(X = x) = \binom{n}{x} p^x (1-p)^{n-x}$$

where X is the discrete random variable representing the number of samples with detected virus out of the total number of samples, x = 0, p = 0.02 (assumed prevalence) and n, the total sample size, is the variable of interest. In addition, the largest margin of error for a prevalence estimate with this sample size is ±8% (for a 95% confidence interval).

3.3.2 Attribution of samples to Australian oyster production and harvest areas

A total of 300 oyster samples were collected in major oyster harvest areas in Australia (NSW, SA, Tas and Qld) over a period of a year. One hundred and fifty samples were collected in each of two sampling rounds representing "peak" (winter/spring) and "off-peak" (summer/autumn) seasons for NoV circulation within the community. The total sample numbers collected per state were informed by five years of national oyster production data from 2007-08 to 2011-12, obtained from the *ABARES Fisheries Production* for edible oysters (ABARES, 2012).

Sampling plans and assignment of sample numbers to production areas within each state were informed by state production data over a five year period, with the exception of SA (three year detailed period 2008-12 available). Data for NSW was obtained from NSW Aquaculture Production Reports (http://www.dpi.nsw.gov.au/fisheries/aquaculture/publications/aquaculture-production-reports). Data for SA was obtained from Primary Industries and Regions South Australia (PIRSA), Aquaculture Policy and Planning Programs. Data for Tasmania was obtained from Department of Primary Industries, Parks, Water and Environment (DPIPWE), Marine Resources.

Further information on production broken down to harvest areas was not possible to obtain in the public domain. Therefore, state industry and regulatory bodies assisted in obtaining estimates of harvest area production to further inform the sampling plan. In NSW, regional industry coordinators provided informed estimates on proportional production from harvesting areas within their production region. In Tasmania, the Tasmanian Shellfish Executive Council (TSEC) coordinated the collection of information on the proportion of production from harvest areas within a production area, as did the South Australian Oyster Growers Association (SAOGA) in SA. In Qld, Aquaculture Policy and Industry Development, Fisheries Queensland provided information on oyster production.

The proportional production per harvesting area was used to weight the probability of assigning a sample to a particular harvest area in a randomised manner. Within each harvesting area the particular site for sample collection was determined by unweighted randomised sampling based on active lease numbers producing mature oysters. The finalised sampling schedule was determined by SARDI using R software (R Core Development Team, version 3.1.3) to avoid any bias.

Samples were only collected from leases that were fit for human consumption from an enteric virus perspective, i.e. in the open status for *Approved* areas, or shellfish suitable for depuration from *Conditionally Approved* or *Restricted* areas in NSW.

All unpublished production information obtained was confidential and only used to inform the sampling regime. Sampling for round one was planned from July through to the end of September

2014. Sampling for round two was planned from January through to the end of March 2015. Samples were randomly allocated to the identified harvest areas in fortnightly blocks.

3.3.3 Sampling kits and sample reception

Sampling kits (polystyrene foam box, freezer pad, clear tape, labelled re-sealable bags, plastic pot for water salinity sampling, sample submission form, sampling guidelines and return courier dockets) were dispatched to growing area coordinators for sample collection and shipment to SARDI Food Safety and Innovation (FSI). On receipt of samples at the FSI laboratories, the condition of the samples were checked, the arrival temperature, date and time logged and samples stored at −80°C until testing.

3.4 Analytical Testing for Foodborne Viruses

The method used for testing for NoV GI, NoV GII and HAV in oysters was as outlined within the IOS/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 instead of Mengo virus as the process control virus (ISO/CEN, 2013).

3.4.1 Sample preparation

Oysters were thawed overnight at 4°C prior to sample preparation. Ideally, a sample comprised 12 individual oysters collected from one oyster lease. Oysters were scrubbed under potable running water prior to being shucked and the oyster meat collected into clean zip locked plastic bags. All oyster samples were physically separated from each other, and shucking knives, gloves and any other equipment was thoroughly cleaned between each sample preparation to avoid cross-contamination. The digestive tissue (DT) from each individual oyster within a sample was carefully dissected out with scalpels, transferred to a clean petri dish and finely chopped to produce a composite sample. A 2 g sub-sample of the DT was taken and 10 µL (6.6×10^4 plaque forming units/µL) of the process control virus (MNV) and 2 mL of Proteinase K solution (3 units/mL) were added to the sample. Samples were mixed and incubated at 37°C shaking (320 rpm) for 60 min. Any residual untreated DT was stored at −20°C for retesting if required. Following the initial incubation, samples were transferred to a water bath and incubated at 60°C for 15 min without shaking. Following the second incubation, samples were centrifuged at 3,000 × g for 5 min and the supernatant recovered; the volume was recorded and retained at −20°C for downstream nucleic acid extraction.

3.4.2 Nucleic acid extraction

Extraction and purification of viral RNA was done using the bioMerieux NucliSENS® Minimag system (bioMerieux Pty. Ltd. Baulkham Hills, NSW), following the manufacturer's recommendation. In brief, RNA was extracted from the entire virus using guanidine isothiocyanate which disrupts the viral coat protein. The viral RNA was adsorbed onto magnetic silica beads, washed with various buffers and released into 100 µL of elution buffer. Each batch of nucleic acid extractions included a negative extract control (sterile water), as well as in-house positive controls (a 10 µL aliquot of the process control virus, MNV).

3.4.3 Detection and quantification by real-time RT-PCR

Real-time RT-PCR for HAV, NoV GI and NoV GII was done using primers and probes as specified in ISO/TS 15216-1 (Table 1) (ISO/CEN, 2013). Primers and probes for real-time RT-PCR of the process control virus (MNV) were those specified by Hewitt et al. 2009 (Table 1) (Hewitt et al., 2009).

The RT-PCR master mix used for all assays was the RNA Ultrasense™ one-step qRT-PCR system (Invitrogen), prepared following the manufacturer's recommendations. Real-time RT-PCR cycling parameters were as specified in ISO/TS 15216-1 and included an initial incubation at 55°C for 1 hr followed by denaturation at 95°C for 5 min and 45 cycles of 95°C for 15 sec, 60°C for 1 min and 65°C for 1 min. Real-time RT-PCR was run in a 384-well format (ViiA™ 7 system, Applied Biosystems), with mastermix and template being dispensed using a Biomek 3000 Laboratory Automation Workstation (Beckman Coulter, Fullerton, CA, USA). The ISO/TS 15216 protocol outlines a number of controls and standards including the use of a process control virus (MNV) to determine virus extraction efficiency and External Control (EC) RNA to determine amplification efficiency. Plasmid standards for NoV GI, NoV GII and HAV were used for quantification of virus detected within a sample. Each sample was assayed for NoV GI, NoV GII, HAV and the process control virus (MNV). Each sample was tested in duplicate neat and diluted 10^{-1}. EC RNA (see below) was added to a third neat and 10^{-1} test reaction to determine amplification efficiency.

3.4.4 Preparation of standards and controls for NoV and HAV real-time RT-PCR

Standards for real-time RT-PCR quantification

The ISO method specifies the use of double stranded DNA (dsDNA) control material prepared from purified plasmids carrying target sequence for each virus of interest: HAV, NoV GI and NoV GII. This material is quantified and used to prepare a standard curve for each virus being tested. The standard curve allows quantification of positive results (template copies/µL). The plasmid is also used to generate the EC RNA (see below).

The HAV, NoV GI and NoV GII control plasmids were provided by James Lowther (CEFAS, UK). These three plasmids were constructed by ligating target DNA sequence (186 bp for HAV; 90 bp for NoV GI; and 95 bp for NoV GII into the pGEM-3Zf(+) (3197 bp) vector (Promega). Each of the three plasmids were propagated by transforming material received from James Lowther into JM109 competent *E. coli* (Promega), plating onto selective media supporting growth of transformed *E. coli* only (LB agar containing 100 µg/mL ampicillin), and growing a single recombinant bacterial colony in LB media containing 150 µg/mL ampicillin overnight at 37°C. The plasmids were extracted from the bacterial host cells and purified using the QIAprep Spin Minikit (Qiagen). Presence of target sequence was confirmed by Sanger sequencing (AGRF, Adelaide).

Quantification of dsDNA plasmid was determined using spectrophotometry (Thermo Scientific Nanodrop) from the absorbance at 260 nm. The concentration of dsDNA in copies/µL was calculated using the following formula:

$$\frac{\text{Concentration of dsDNA (ng/µL)} \times 10^{-9}}{\text{(Plasmid length} \times \text{relative molecular mass of average base pair)/Avogadro constant}}$$

Relative molecular mass of an average base pair = 607.4
Avogadro constant = 6.02×10^{23}

The plasmids were diluted 1×10^4 - 1×10^5 template copies/µL. This stock material was used to generate the dsDNA standard curve by four serial ten-fold dilution (10^5, 10^4, 10^3, 10^2, 10^1 copies/µL) in purified water, dispensed into strip tubes for single use per real-time RT-PCR run and stored at −80±5°C.

External control RNA (EC RNA)

The EC RNA is a reference RNA that can serve as a target for real-time RT-PCR. It is used to control real-time RT-PCR amplification and is spiked into a replicate test sample (containing either neat

extracted sample nucleic acid or extracted nucleic acid which has been diluted 10^{-1}). The purpose of this is to determine if the extracted sample contains any inhibitors to RT-PCR and if dilution of the sample can reduce the inhibitory effect. The amplification efficiency should be greater than 25%. If the undiluted sample has efficiency less than 25%, the results for the 10^{-1} sample are used. If the efficiencies for both the undiluted and 10^{-1} are less than 25%, the results are invalid and the sample should be re-tested. It should be noted that if a sample shows unacceptable amplification efficiency but produces an otherwise valid positive result, it can be reported as positive but not quantifiable.

The EC RNAs are generated using the dsDNA control plasmids (see previous section). The initial step is the linearisation of each plasmid followed by *in vitro* RNA transcription using the SP6/T7 Riboprobe combination system (Promega). The generated RNA is isolated with the RNeasy Minikit (Qiagen). Purified RNA is checked for contamination with DNA using target specific real-time RT-PCR. The RT-PCR mix is divided into two portions, one of which is heated to deactivate the reverse transcription enzyme before RT-PCR. If amplification in the heat treated reaction is <0.1% of the untreated reaction, then this indicates no significant DNA contamination is present. Spectrophotometry (Thermo Scientific Nanodrop) was used to quantify RNA concentration from the absorbance at 260 nm.

The concentration of RNA in copies/µL is calculated using the following formula:

$$\frac{\text{Concentration of dsDNA (ng/µL)} \times 10^{-9}}{(\text{RNA length} \times \text{relative molecular mass of average ribonucleotide})/\text{Avogadro constant}}$$

Relative molecular mass of an average ribonucleotide = 320.5
Avogadro constant = 6.02 × 1023

The EC RNA stocks were diluted in purified water 1×10^6 - 1×10^8 template copies/µL. Single use aliquots were dispensed into strip tubes and stored at −80±5°C.

Table 1: Real-time RT-PCR primers and probes used for NoV, HAV and MNV detection.

Primer/Probe	Sequence	Reference
	NoV GI Assay	
QNIF4 (Fwd primer)	CGC TGG ATG CGN TTC CAT	(Da Silva et al., 2007)
NV1LCR (Rev primer)	CCT TAG ACG CCA TCA TCA TTT AC	(Svraka et al., 2007)
NVGG1p (Probe)	TGG ACA GGA GAY CGC RAT CT	(Svraka et al., 2007)
Probe labelled: 5' end with FAM and 3' end with TAMRA		
	NoV GII Assay	
QNIF2 (Fwd primer)	ATG TTC AGR TGG ATG AGR TTC TCW GA	(Loisy et al., 2005)
COG2R (Rev primer)	TCG ACG CCA TCT TCA TTC ACA	(Kageyama et al., 2003)
QNIFs (Probe)	AGC ACG TGG GAG GGC GAT CG	(Loisy et al., 2005)
Probe labelled: 5' end with FAM and 3' end with TAMRA		
	HAV Assay	
HAV68 (Fwd primer)	TCA CCG TTT GCC TAG	(Costafreda et al., 2006)
HAV240 (Rev primer)	GGA GAG CCC TGG AAG AAA G	(Costafreda et al., 2006)
HAV150 (-) (Probe)	CCT GAA CCT GCA GGA ATT AA	(Costafreda et al., 2006)
Probe labelled: 5' end with FAM and 3' end with MGBNFQ		
	MNV Assay	
MNV (Fwd primer)	TGCAAGCTCTACAACGAAGG	(Hewitt et al., 2009)
MNV (Rev primer)	CACAGAGGCCAATTGGTAAA	(Hewitt et al., 2009)
MNV p (Probe)	CCTTCCCGACCGATGGCATC	(Hewitt et al., 2009)
Probe labelled: 5' end with FAM and 3' end with BHQ1		

Process control virus

Foodstuffs are complex matrices and the target viruses can be present at low concentrations. Furthermore, some target virus can be lost during the virus extraction steps. To determine the virus extraction efficiency, an exogenous virus (process control virus) is spiked into the sample prior to processing. Murine norovirus (MNV) was used as the process control virus with 10 µL containing 6.6×10^5 viruses being added to the samples before processing. This virus was selected as it is not pathogenic to humans and closely resembles the target viruses both morphologically and physicochemically. A working stock of the MNV was prepared, split into single use aliquots and stored at −80±5°C.

The level of MNV recovery was calculated for each sample to determine virus extraction efficiency. It should be noted that the extraction efficiency was only used for quality assurance purposes and not to adjust test results. If virus extraction efficiency is less than 1% (in the absence of inhibitory substances to RT-PCR) the sample results are deemed to be invalid and samples were re-tested. This approach was in line with the ISO/TS 15216-1 standard.

3.4.5 International proficiency trial for detection of foodborne viruses in bivalve molluscs

SARDI has participated in the European Reference Laboratory Proficiency (EURL) testing scheme for shellfish organised by the Centre for Environment, Fisheries and Aquaculture Science, UK (CEFAS-

UK) in 2012 (PT 46) and 2013 (PT 50). On each occasion these trials involved the analysis of four contaminated shellfish samples and two lenticule discs (reference material) for HAV, NoV GI and NoV GII using the laboratory's method of choice. Our testing methodology was as detailed in ISO/TS 15216-1 for bivalve molluscan shellfish.

3.5 Statistical Analysis

The R software (R Core Development Team, version 3.1.3) was used to perform statistical analysis and generate the prevalence estimates including associated 95% confidence intervals for NoV GI, NoV GII and HAV. The estimate of prevalence is the number of samples with detected levels of virus expressed as a proportion of the total number of samples – in the instance of no detections, it can be concluded that the estimated prevalence is low, <2% (with 95% confidence), based on the assumptions of the sample size calculation. The upper bound for the prevalence estimate was calculated based on the sample size and a 95% probability of detecting at least one sample with detectable levels of viruses.

4. Results

4.1 Sampling Plan

4.1.1 National distribution of oyster samples

Between 2007-08 and 2011-12 the annual average production of oysters per state was valued at $40.7 million, $0.5 million, $34.6 million and $21.8 million for NSW, Qld, SA and Tas respectively (ABARES, 2012). The proportion of state oyster production to the overall national annual production was used to allocate sample numbers for collection from each state (Figure 1). For NSW, 63 samples were collected in the first sampling round and 62 in the second to give a total of 125 samples for the overall survey. In Tasmania, 33 and 34 samples were collected in sampling rounds 1 and 2 respectively. In SA, 53 samples were collected in each round and in Qld one sample was collected in each round.

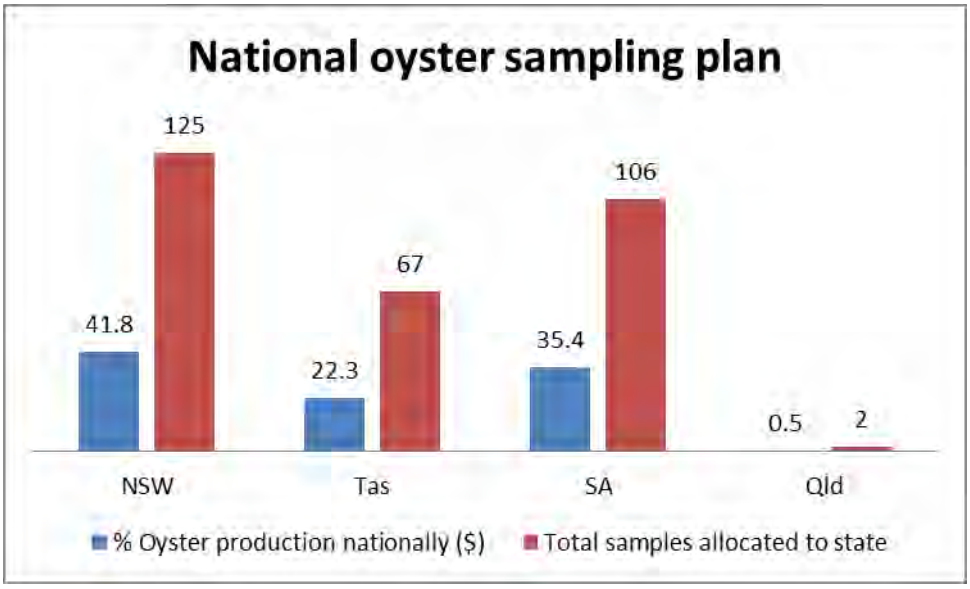

Figure 1: Oyster sampling plan based on state contribution to annual national oyster production valued in $AUD. Sample numbers collected from each state represent the total numbers for the entire survey (rounds one and two).

Locations and numbers of samples to be collected from each production area nationally during each of the two sampling periods are shown in Figure 2.

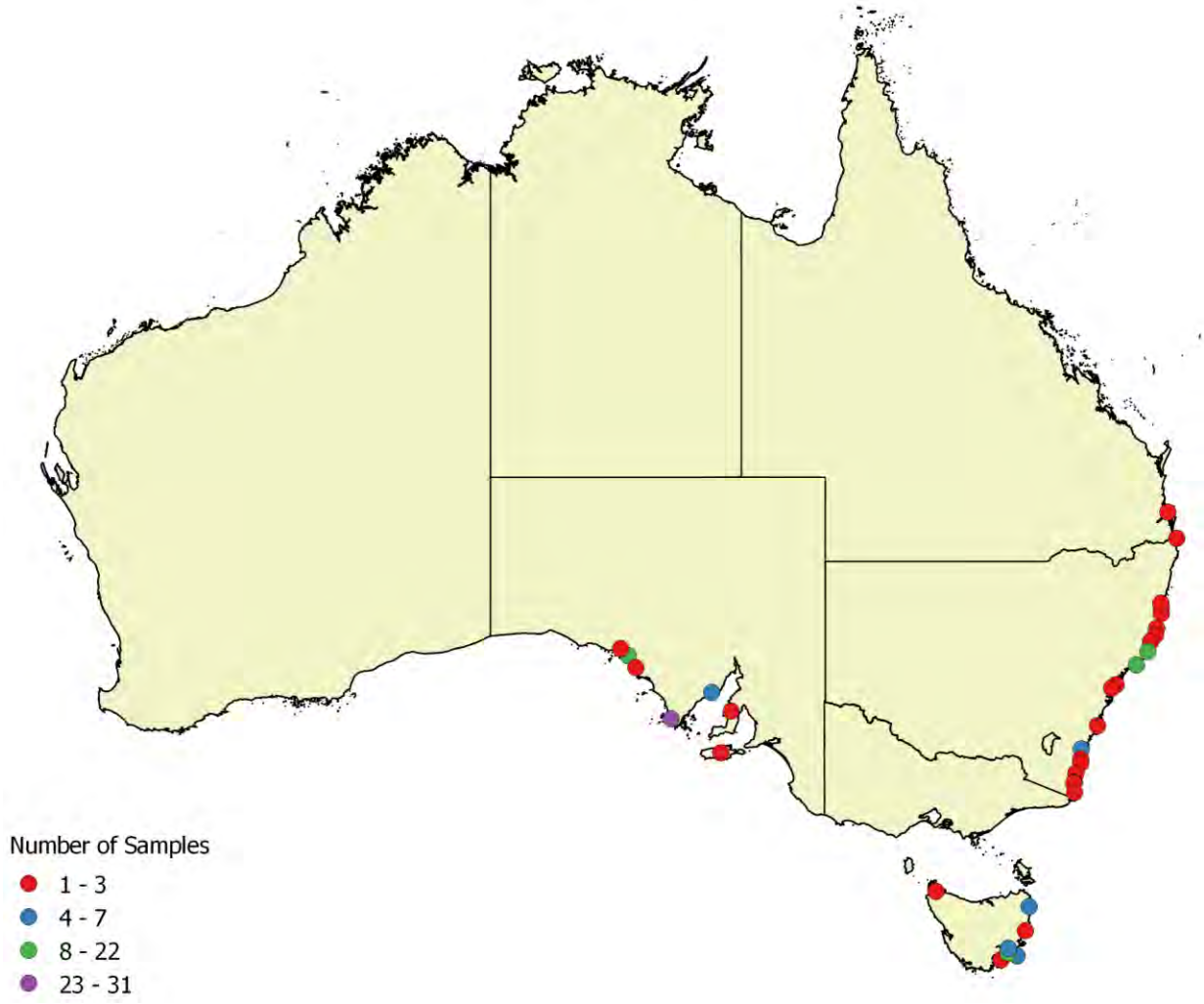

Figure 2: Locations and numbers of oyster samples collected during each sampling round.

4.1.2 State distribution of samples

New South Wales

The anticipated sampling plan for NSW is shown in Figure 3. The actual sample numbers received per sampling round are shown in Figure 4. The reason for the variation in sample numbers was due to the need to reallocate samples if harvest areas were closed, growing areas were unwilling to participate in the survey or requested samples were not submitted. Only the Macleay River growers from NSW were unwilling to participate. One sample per sampling round was originally allocated to this production area. Shoalhaven/Crookhaven River was the only other production area identified which did not submit samples for the survey. Two samples from this area were to be collected per sampling round. Samples from round one were not received due to closures while samples from round two were not submitted. For both sampling rounds these samples were statistically reallocated to other production areas within NSW using the randomised procedures detailed above.

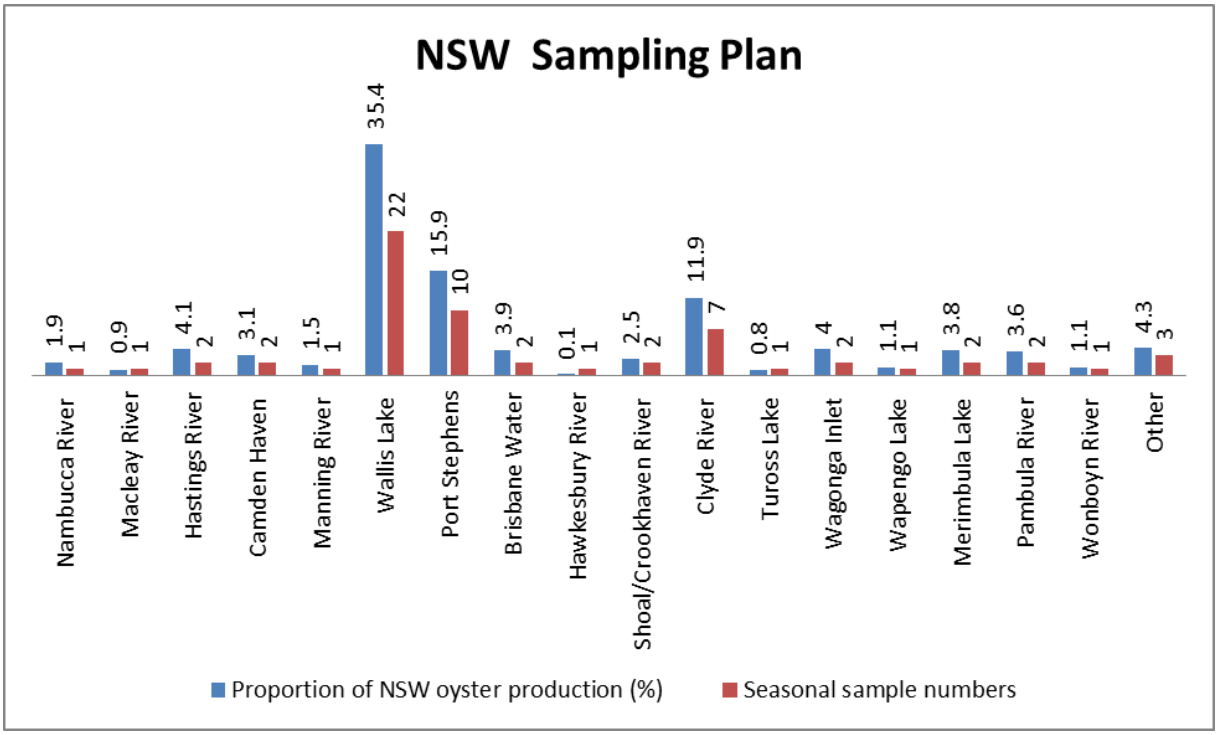

Figure 3: Percentage oyster production for NSW production areas and sample numbers to be collected per sampling round. "Other" growing areas indicate Tweed, Richmond, Clarence, Wooli Wooli, Bellinger and Bermengui Rivers and Nelson Lagoon.

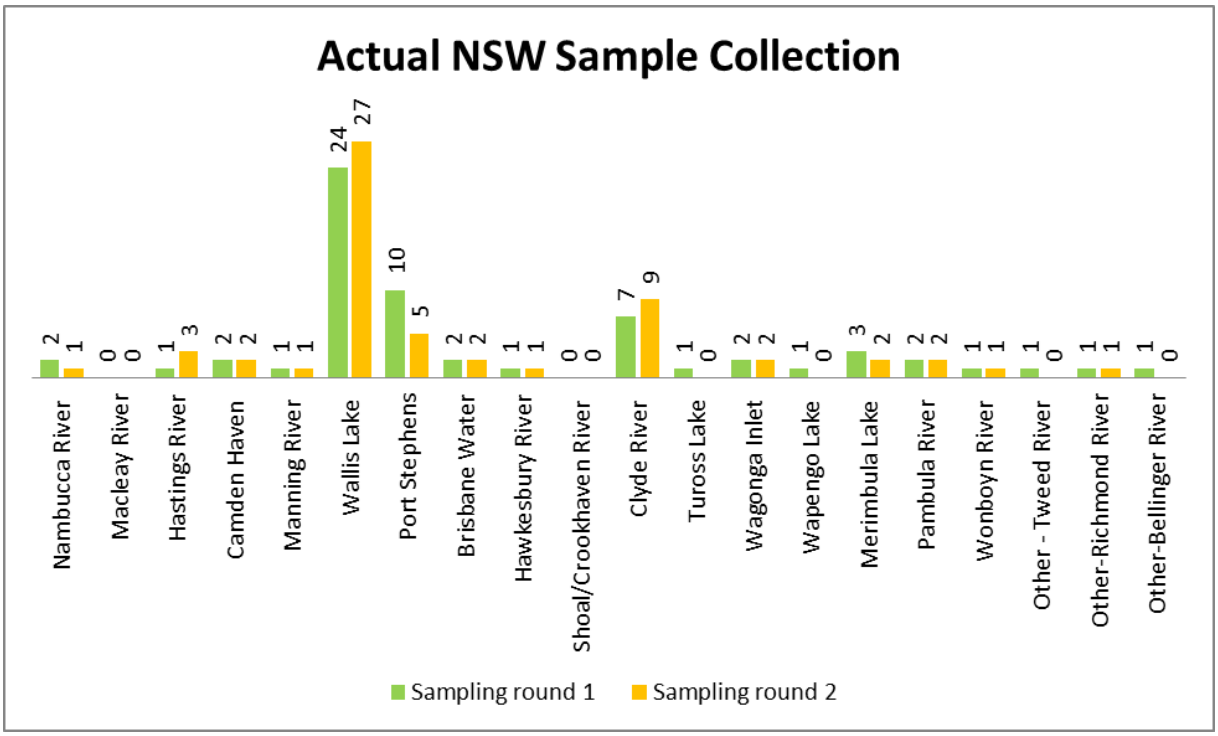

Figure 4: Actual sample numbers collected from NSW oyster production areas per sampling round.

South Australia

Figure 5 shows oyster production for South Australian production areas and sample numbers to be collected per sampling round. The actual number of samples collect per sampling round did not vary from the plan.

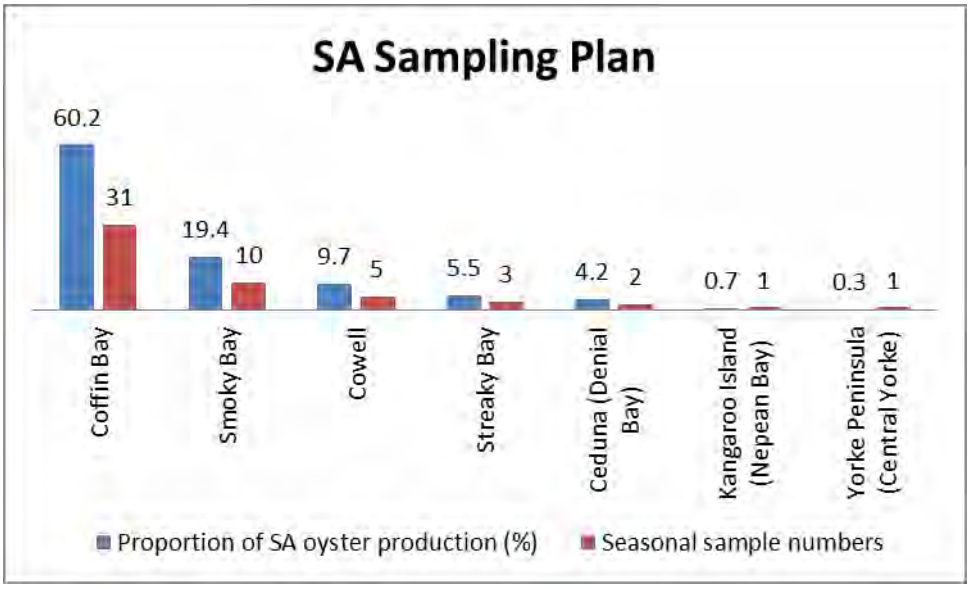

Figure 5: Percentage oyster production for SA production areas and sample numbers collected per sampling round.

Tasmania

Figure 6 shows oyster production for Tasmania production areas and sample numbers to be collected per sampling round. The actual number of samples collect per sampling round did not vary from the plan with the exception of the Far North West production area in round one. Two samples were collected from this production area as planned for round one. However, due to closures one sample could not be collected until January 2015. By this time the second round of sampling had begun and it was decided to include this late round one sample in round two.

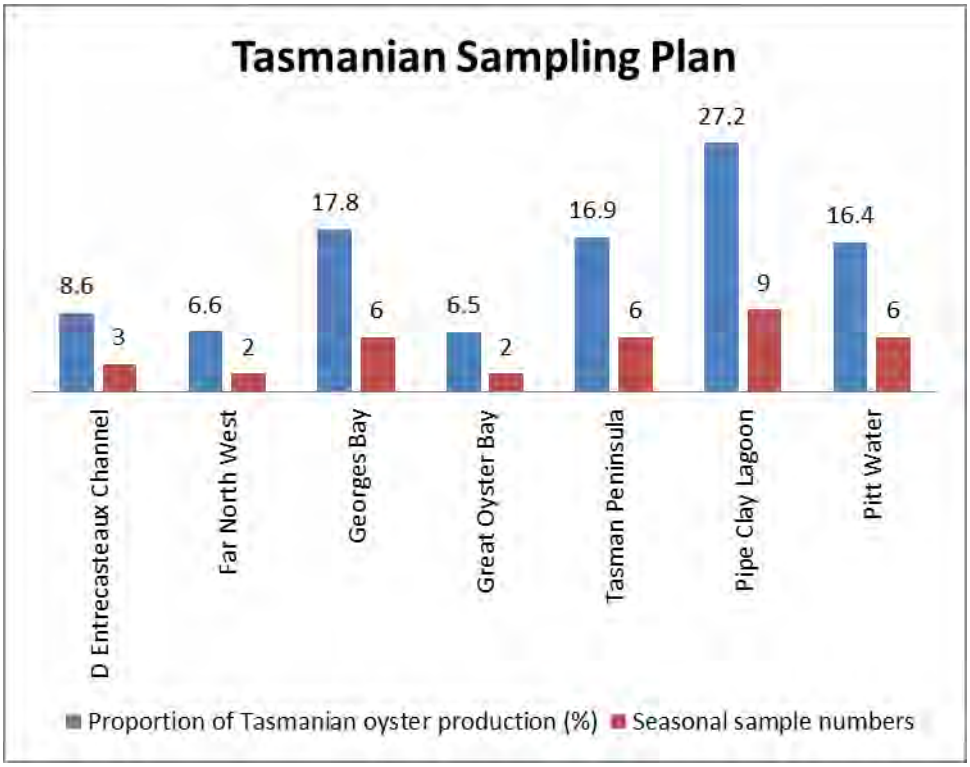

Figure 6: Percentage oyster production for Tasmanian growing areas and sample numbers collected per sampling round.

Queensland

Two samples for the survey (one per sampling round) were collected from Morton Bay.

4.1.3 Timing of sample collection

As a result of numerous harvest area closures during each of the sampling rounds, sample collection was often delayed and in some cases needed to be reallocated. Figure 7 shows actual sample numbers received per month from each state during the survey. As a result, sampling for round one was from July through to the end of November 2014 instead of the planned September 2014. Sampling for round two was from January through to the end of August 2015 instead of the planned March 2015.

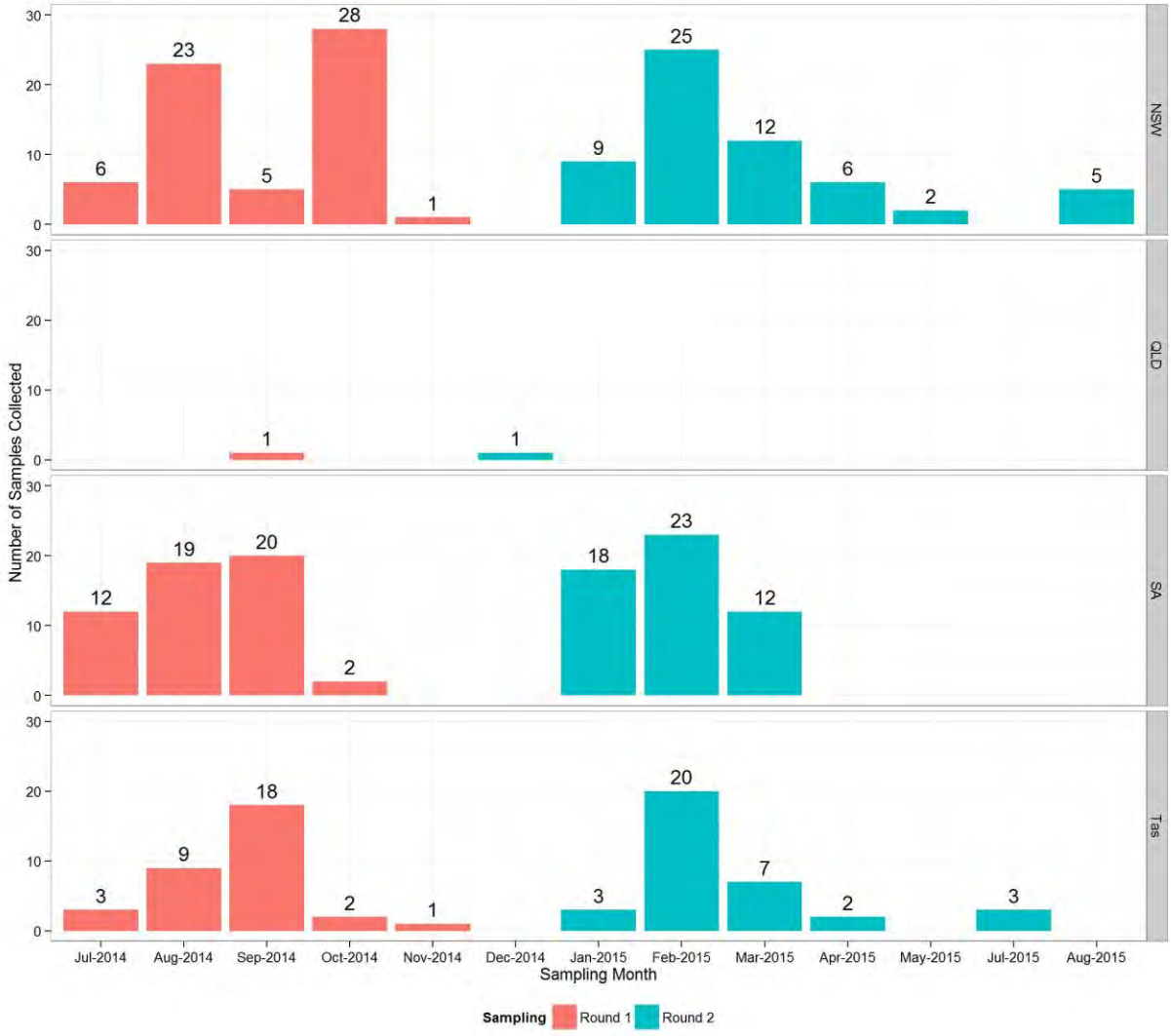

Figure 7: Sample numbers received per month during rounds one and two of the survey.

4.2 Sample Reception

Oyster samples were shipped cold, usually overnight, and arrived in good condition. The average arrival temperature of oysters from round one was 12.7°C (standard deviation; SD of 6.2°C), while the average arrival temperature of oysters from round two was 13.9°C (SD 10.3°C). The average number of days between sample collection and arrival in the laboratory was 2.1 days (SD 1.2 days) during round one, and 3.6 days (SD 2.4 days) during round two, for oysters sent live. A small number of samples were sent frozen and arrived frozen.

4.3 International Proficiency Trial for Detection of Foodborne Viruses in Bivalve Molluscs

In 2012, forty-two international laboratories participated in the CEFAS proficiency trial (PT 46) of which 60% scored an overall accuracy of 100% (CEFAS, 2013b). In 2013 forty-four international

laboratories participated in the proficiency trial (PT 50) of which 79% scored an overall accuracy of 100% (CEFAS, 2014). SARDI FSI laboratories scored 100% in relative accuracy, specificity and sensitivity for both of these trials.

4.4 Foodborne Viruses in Australian Oysters

4.4.1 Virus extraction efficiency

A total of four samples showed unacceptable virus extraction efficiency (<1%) and were excluded from calculation of virus prevalence estimates. Of the remaining samples included in the calculation of the prevalence estimate, 25 samples showed a virus extraction efficiency of ≤10% and 268 had a virus extraction efficiency of >10%.

4.4.2 Amplification efficiency

The amplification efficiencies were used for quality assurance and not to adjust the results. Unacceptable amplification efficiencies (<25%) were obtained for 18 NoV GI, 14 NoV GII and 4 HAV test results. Of these, 12 test samples showed an unacceptable amplification efficiency for NoV GI and GII, one for NoV GI and HAV only and three for all three viruses. If a test sample resulted in unacceptable amplification efficiency the test was deemed invalid and the sample excluded from the calculation of the prevalence estimate.

4.4.3 Detection of norovirus

No norovirus (NoV GI or NoV GII) was detected in oysters during either round of sampling. Of the 149 oyster samples received and analysed during round one of the survey, 141 and 142 samples gave valid test results for NoV GI and GII, respectively. During round two of the survey, 148 samples were received and analysed of which 134 and 137 gave valid test results for NoV GI and GII, respectively.

4.4.4 Detection of Hepatitis A virus

No HAV was detected in oysters during either round of sampling. Of the 149 oyster samples received and analysed in the first round of sampling, 147 gave valid test results. Of the 148 samples received and analysed in the second round of sampling, 142 gave valid test results.

4.5 Prevalence Estimates for Foodborne Viruses in Australian Oysters at Production

Based on the results of the survey for NoV and HAV in Australian oysters at production during 2014-15 the prevalence estimate for these viruses are <2% in both rounds of sampling (Table 2).

Table 2: Prevalence estimates for NoV and HAV in Australian oysters at production.

	Prevalence estimate (95% confidence interval)	Number of virus detections	Number of valid test results
Sampling round 1			
NoV GI	< 2% (0 - 2.6%)	0	141
NoV GII	< 2% (0 - 2.6%)	0	142
HAV	< 2% (0 - 2.5%)	0	147
Sampling round 2			
NoV GI	< 2% (0 - 2.7%)	0	134
NoV GII	< 2% (0 - 2.7%)	0	137
HAV	< 2% (0 - 2.6%)	0	142

5. Discussion

The survey reported here for NoV in Australian oysters is one of only a few internationally that has investigated national production comprehensively (DePaola et al., 2010; Lowther et al., 2012; Pavoni et al., 2013). It is also only the second report of HAV in oysters at a national level (DePaola et al., 2010). Furthermore, to the best of our knowledge this is the first survey of foodborne viruses in oysters where the sampling plan has been statistically designed to estimate prevalence with confidence intervals. The sampling plan was designed to capture national commercial oyster production by production region, of which 33 were identified within Australia in NSW, SA, Tas and Qld. The survey was undertaken in two rounds to enable capture of any seasonal variability in NoV and HAV prevalence. The survey only sampled mature shellfish taken from harvest areas which were open for harvest as defined by the state managed Shellfish Quality Assurance Programs. For Tasmania, South Australia and Queensland this represented oysters fit for market. For New South Wales, this represented either oysters fit for market or fit for depuration prior to market. Sample numbers (n=150) per round were determined so as to enable detection of at least one virus positive sample with probability of 0.95, if the estimated prevalence for that virus was ≥2%. During the 2014-15 survey we did not detect NoV or HAV in oysters during either sampling round, which translated into an estimated prevalence of <2% (95% CI 0-2.7%) for these viruses in Australian market-ready oysters.

To the best of our knowledge this is the first survey of foodborne viruses in oysters where the sampling plan has been statistically designed to estimate prevalence nationally. Samples were only collected from leases that were fit for human consumption from an enteric virus perspective, i.e. in the open status for *Approved* areas, or shellfish suitable for depuration from *Conditionally Approved* or *Restricted* areas in NSW. Depuration is effective in purging shellfish of bacterial contamination but ineffective in significantly reducing foodborne viral contamination (EFSA, 2012). Over the past decade several international studies have investigated the presence of human enteric viruses, including NoV and HAV, in commercial and wild shellfish (oysters, mussels, clams and cockles) from Australia, France, Ireland, Italy, Japan, Korea, Morocco, Spain, United Kingdom and United States of America (Benabbes et al., 2013; Brake et al., 2014; Costantini et al., 2006; DePaola et al., 2010; Doré et al., 2010; Flannery et al., 2009; Loutreul et al., 2014; Lowther et al., 2012; Maekawa et al., 2007; Moon et al., 2011; Pavoni et al., 2013; Polo et al., 2015; Schaeffer et al., 2013; Seo et al., 2014; Shin et al., 2013; Suffredini et al., 2014). Comparison of the various published studies has been difficult for a variety of reasons including: use of varied sample preparation and detection methodologies; surveys not being representative of a country's national production; not discriminating amongst oysters which are deemed fit for human consumption as raw product; absence of a statistically valid sampling plan; and often point-in-time investigations.

The presence of NoV in oysters has been reported in the range of 3.9-20% in the USA (Costantini et al., 2006; DePaola et al., 2010), 14.1-45% in Korea (Moon et al., 2011; Seo et al., 2014; Shin et al., 2013), 4.2% in Japan (Maekawa et al., 2007), 2.4% (Class A harvest area) and 47.8% (Class B harvest area) in Italy (Pavoni et al., 2013; Suffredini et al., 2014), 9% in France (Schaeffer et al., 2013), 2.9% in Morocco (Benabbes et al., 2013), 76.2% in the UK (Lowther et al., 2012) and 31% (Class A harvest area) in Ireland (Flannery et al., 2009). Less information is available on HAV in oysters but the presence is generally much lower, with 4.4% reported in the USA (DePaola et al., 2010) and none detected in Korean oysters (Seo et al., 2014). HAV has been reported in Spanish shellfish (mussels, cockles and clams) from Class B harvesting areas with a prevalence of 10.1% (Polo et al., 2015). Although, these studies have provided information on the level of contamination in shellfish, none have related results back to epidemiological data as a reality check for the risk of shellfish-related illness.

In this national, yearlong survey of foodborne viruses of oysters there were no virus positive samples detected. This translates statistically into an estimated prevalence for NoV and HAV in Australian

oyster of <2%. To increase the accuracy in the estimate of the prevalence (below the upper limit of 2%) would require significantly greater sampling per round. The only other previous study of NoV in Australian oysters investigated limited geographic sites (n=6) in NSW, SA and Tas (two sites/state). Site selection targeted those considered most compromised with respect to the potential for human faecal contamination and did not discriminate amongst harvest site classification or harvest status (Brake et al., 2014). NoV was only detected in 1.7% of sampled oysters (2/120), but it should be noted that these samples were taken from closed growing areas and not representative of oysters fit for market. Despite this the previous study supports our observation of the potential for a very low prevalence of human enteric viruses (<2%) in Australian oysters as managed by the ASQAP. Our results of a very low prevalence compare favourably to available international data. Oysters harvested from European Class A waters, approximately equivalent to the Australia open for harvest classification, have reported varied levels of NoV from 2.4% in Italy to 31% in Ireland.

Virus extraction and detection methodologies reported in prior studies have been varied among and even within investigations (Pavoni et al., 2013). Many studies have failed to use adequate controls for virus extraction and amplification efficiency. Virus extraction methodology can have a major impact on the recovery of foodborne viruses from the oyster matrix and in the ISO/TS 15216 method it is recommended that an exogenous process control virus be used to determine virus recovery (ISO/CEN, 2013). Detection methodology (end-point RT-PCR, quantitative RT-PCR, primers and probes) can all impact on sensitivity and specificity of the assay. With the publication of the ISO/TS 15216 method for the detection of NoV and HAV in foodstuffs, including bivalve shellfish, future studies employing this methodology will enable results to be more comparable among investigations. Although this method does not distinguish between infective and non-infective virus, it does enable both the prevalence and quantification of the virus to be determined and provides a basis for participation in internationally run proficiency trials. SARDI FSI have participated in two of these proficiency trials (2012 and 2013) which have been coordinated by CEFAS and obtained 100% in relative accuracy, specificity and sensitivity.

Although a majority of investigations into NoV and HAV in shellfish have been undertaken over a period of a year or longer, others have been short point-in-time studies which may not reflect the true situation under a variety of environmental and seasonal conditions. This survey was done over a year and included two rounds of sampling to enable capture of seasonal variability in Australian oysters. As we did not detect NoV or HAV in either sampling round we are unable to comment on seasonal variability, although it is known that NoV is more common within the Australian community during winter/spring (VIDRL, 2013). Hence, the potential for contaminated sewage to reach growing waters would be presumed to be greater. Prevalence of NoV and HAV in oysters from the UK, USA and Ireland is reported to be higher in the colder winter months than in summer (DePaola et al., 2010; Flannery et al., 2009; Lowther et al., 2012). Seasonal occurrence of shellfish associated NoV outbreaks in Europe has been noted and is thought to be attributed to several factors including a higher prevalence of the disease within the community during winter, increased stability of the viruses in cold water, reduced solar inactivation and selective bioaccumulation by shellfish. Alternatively, a retail survey of NoV and HAV in Korean shellfish identified no seasonal variability in virus prevalence (Seo et al., 2014), while an 18 month survey of Spanish shellfish (mussels, cockles and clams) found higher viral contamination in the shellfish during the warmer summer months than in the winter (Polo et al., 2015).

Only a few recently published studies have quantified the amount of NoV detected in contaminated shellfish (Lowther et al., 2012; Polo et al., 2015; Schaeffer et al., 2013; Suffredini et al., 2014). A prevalence of 22.1% for NoV in Italian shellfish (mussels and clams) harvested from Class A waters has been reported with a quantifiable geometric mean of 310 RNA copies/g DT (Suffredini et al., 2014). By contrast, 47.8% of oysters collected from Class B Italian waters, prior to depuration, were contaminated with NoV with a geometric mean of 2,000 RNA copies/g DT. Spanish shellfish (mussels, cockles and clams) sampled from Class B harvesting areas were found to be contaminated with NoV GI (32.1%), NoV GII (25.6%) and HAV (10.1%) with reported viral levels of 100-1,000

RNA copies/g DT (Polo et al., 2015). A French retail survey of oysters found that 9% of samples were contaminated with NoV with quantifiable levels ranging from 93-220 RNA copies/g DT (Schaeffer et al., 2013). Moroccan oysters collected on the Atlantic coast had a NoV prevalence of 2.9% with levels below the limit of quantification for the assay (<50 RNA copies/g DT) (Benabbes et al., 2013). Lowther et al. (2012) has undertaken a systematic survey of 39 UK oyster production areas (Class A, B, C) detecting NoV in 76.2% of samples. The majority (52.1%) of these were below the level of quantification for the assay (<100 RNA copies/g DT), while a small number of positive samples had levels >10,000 RNA copies/g DT) (Lowther et al., 2012). NoV was detected in 1.7% of Australian oysters sampled with quantification of 120 and 50,000 RNA copies/g DT (Brake et al., 2014). The variation in the limit of quantification report amongst studies is a result of the variation in detection methodologies employed.

Available data indicate that NoV concentration in shellfish linked to human cases vary greatly from <100 to >10,000 RNA copies/g DT, yet there is growing evidence of a dose response for NoV (EFSA, 2012). It has been suggested that NoV monitoring of at-risk oyster harvesting areas, along with the introduction of an upper limit for NoV in oysters could prevent a significant number of outbreaks associated with oyster consumption in Europe (Doré et al., 2010). However, the lack of suitable data to date has hampered the development of a quantitative risk analysis and definition of microbiological criteria for viruses in shellfish. With the development of the ISO/TS 15216 method and resulting quantitative data it should be possible to more accurately estimate the exposure of consumers to contaminated food and potentially define an acceptable threshold of virus presence.

This national survey for NoV and HAV in Australian oysters at production established that the estimated prevalence for these viruses is <2%, despite no viruses being detected during the survey period. Our results were also supported by epidemiological evidence, as no Australian shellfish-related viral foodborne outbreaks were reported during the survey period. Results of the survey indicate that the ASQAP is effective in managing food safety risks in shellfish associated with enteric foodborne viruses, as well as complying with the Food Standards Code and Export Orders as they relate to bivalve shellfish and the schedule to Standard 4.2.1 of the Food Standards Code (AQIS, 2004; ASQAAC, 2009; FSANZ, 2012). Results of the current survey could be used to support trade, increase market access and potentially improve market value by demonstrating the high quality of Australian oysters and the low risk of foodborne enteric viruses. The results of the survey could also be used to argue against the need for unnecessary, excessive and costly mandatory virus monitoring as being proposed by the EU.

6. Conclusion

An objective of the project was to design a statistically robust survey to evaluate NoV and HAV occurrence in oyster harvest areas in New South Wales, South Australia, Tasmania and Queensland. Two sampling rounds were chosen over a year (July 2104 to August 2015) to enable detection of seasonal variation in virus prevalence. One hundred and fifty samples were collected per sampling round, to enable the detection of at least one positive sample with 95% confidence, if there was a prevalence estimate of ≥2% per sampling round. Sample numbers collected per round were distributed among commercial oyster producing states based on their proportion of national production. Sample numbers were further distributed to production areas within states based on proportion of state production. Samples were finally assigned randomly to active oyster leases within production regions but were weighted by the proportional production of the harvest area within the production region. In total, 33 national production regions were asked to participate in the survey of which two returned no samples. Both these areas were minor NSW production areas supplying only 0.9% (Macleay River) and 2.5% (Shoalhaven/Crookhaven River) of the state's total annual oyster production (in total 1.4% of Australian production). The three samples per season allocated to these production areas were statistically reassigned within NSW. Mature oyster samples were only collected from assigned leases if they were open for harvest as defined by the state shellfish quality assurance programs. Of the 300 samples anticipated for the entire survey, 297 were received. The survey design was representative of Australian commercial oyster production of market-ready product.

Testing of oysters was as outlined 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 MNV was used instead of Mengo virus as the process control virus (ISO/CEN, 2013). SARDI FSI has participated in the European Reference Laboratory Proficiency (EURL) testing scheme for shellfish organised by CEFAS-UK in 2012 (PT 46) and 2013 (PT 50). On each occasion these trials involved the analysis of four shellfish samples and two reference material for HAV and NoV. Testing was done using ISO/TS 15216 and on both occasions SARDI scored 100% in relative accuracy, specificity and sensitivity.

During the 2014-15 survey for NoV and HAV in Australian oysters at production, no NoV or HAV were detected in any of the samples tested during either round of sampling. This resulted in a prevalence estimate of <2% for NoV or HAV in Australian oysters at production during each sampling round.

These results of a very low estimated prevalence for NoV and HAV indicate that ASQAP is effective in managing food safety risks in shellfish associated with enteric foodborne viruses, as well as complying with the Food Standards Code and Export Orders as they relate to bivalve shellfish and the schedule to Standard 4.2.1 of the Food Standards Code. Although there will always be a risk of foodborne viral illness associated with oysters when product is eaten raw, especially if grown in water that can be impacted by sewage and environmental run-off, the results of this survey show that the Australian oyster industry is producing a safe product with respect to viral contamination. Furthermore, the low estimated prevalence (<2%) of foodborne viruses in Australian oysters was supported by epidemiological evidence, with no oyster-related foodborne illness reported during the survey period.

Results of the survey could be used to expand international market access and provide a scientific argument against the current push to introduce mandatory testing and monitoring as being proposed within the European Union.

7. Implications

In Australia it has been estimated that the cost of all foodborne gastroenteritis (bacterial and viral pathogens and all food types) is $811 million annually (Abelson et al., 2006). This is largely due to losses in productivity, lifestyle and premature mortality. Costs, however, to health care services, surveillance programs, primary producers, food industries and trade are also significant. The cost attributed to NoV and specifically to oysters is not available. However, one widely reported case of hepatitis A in 1997 caused by consumption of contaminated oysters from Wallis Lake, NSW was responsible for an estimated 444 cases of illness and one death (Conaty et al., 2000). This outbreak had a multifactorial negative economic impact which included a national health cost of disease estimated at $12.1 million and a 15-20% decrease in the market share for the local oyster industry (net income loss of $500K pa in 1997 and for a few years following). Other broader negative impacts were estimated losses of $0.2 and $1.0 million to the fishing and accommodation sectors respectively in 1997, and reduced public perception of oysters (Handmer et al., 2004; OzFoodNet, 2006). As a result of the Wallis Lake incident and several other NoV outbreaks, NSW significantly changed the risk management of oyster growing areas, in line with the guidelines in the ASQAP Manual of Operations.

This national survey for NoV and HAV in Australian oysters resulted in an estimated prevalence of <2% for these viruses with no virus positive samples detected and no related foodborne illnesses reported. The implication for shellfish regulators, the oyster industry and consumers is positive. The findings demonstrate that the Australian Shellfish Quality Assurance Programs are effective in producing oysters free of enteric viruses. The results for industry could mean improved trade and market access of product shown to be a premium product when it comes to food safety. The results of the survey can also be used in a scientific risk-based argument against mandatory virus testing by demonstrating the Australia oyster industry does not have an ongoing issue with foodborne enteric viruses. Avoiding mandatory virus monitoring would be a significant cost saving to both industry and consumers. Consumers can be confident that Australian oysters pose a low risk for foodborne viruses at production.

8. Recommendations

We recommend that the results of the survey are published in an international journal as a peer reviewed scientific paper. This will raise awareness of the variable risk of enteric viruses internationally, promoting a risk-based approach for viral management at Codex Alimentarius forums. Such an approach is necessary to avoid costly mandatory testing. It will also raise awareness of Australian oysters as a safe product with respect to viral contamination.

The report and any resulting publication should be distributed to Australian delegates to the Codex Committee of Food Hygiene (CCFH) to ensure they are aware of the findings. CCFFH is the forum where mandatory testing of viruses will be debated.

We also recommend that the report be disseminated to:

- the shellfish industry regulators and fisheries managers in shellfish producing states
- Department of Health Food Units (or equivalent) in all states
- Department of Agriculture and Water Resources Exports Division
- OzFood Net
- FSANZ
- and uploaded to the SafeFish website when peer reviewed.

Finally we recommend that the oyster industry use the results of the survey to promote the food safety of their produce domestically and internationally.

9. Extension and Adoption

The project has been orally presented at numerous industry and research fora including:

V. Torok "Current FRDC project on Norovirus and Hepatitis A virus survey of Australian oysters" at the ASQAAC Science Day in Hobart on the 15th October 2014.

V. Torok "National survey of foodborne viruses in Australian oysters" at Shellfish Futures held in Smithton on the 18th October 2015.

V. Torok "National survey of foodborne viruses in Australian oysters" FRDC 2013-234" at the Oysters Australia Meeting held in Sydney on the 21st April 2015.

A. Turnbull "National survey of foodborne viruses in Australian oysters" presentation to a visiting delegation of SAUDI Food and Drug Authority in Adelaide on the 27th July 2015.

A. Turnbull "National survey of foodborne viruses in Australian oysters" at the 37th ASQAAC held in Queenscliff on the 16th September 2015.

V. Torok "Survey for foodborne viruses in Australian oysters and bacteriophage as a shellfish management tool" at Shellfish Futures held in St Helens on the 24th October 2015.

V. Torok "Outcomes of the national survey for foodborne viruses in Australian oyster" at the NSW SQAP meeting in Sydney on the 2nd December 2015.

9.1 Project coverage

Fact sheet on project posted on the Oyster Australia website: http://oystersaustraliablog.org.au/green-light-for-new-rd-projects/

Radio Interview 29th Oct 2015
"Australia has completed its first comprehensive Norovirus and Hepatitis A virus survey. SARDI research scientist Dr Valeria Torok says all the samples tested were free of two highly contagious viruses"
Host: Rosemary Grant
Source: ABC Rural, Duration: 4min 25sec
http://www.abc.net.au/news/2015-10-29/shellfish-oyster-food-poison-survey/6890916

On-line article 29th October 2015
"Australian oyster survey shows low risk of viruses that cause gastro"
Tas Country Hour by Rosemary Grant
http://www.abc.net.au/news/2015-10-29/australian-oyster-food-poison-survey-clean-bill-of-health/6894998

Glossary

Approved harvest area means a shellfish harvest area classified (as *Approved*) for harvesting or collecting shellfish for direct marketing.

Closed status means a condition that may apply to a harvest area where the commercial harvesting of shellfish is temporarily prohibited. A closed status may be placed on any of six classified harvest area categories: *Approved, Approved Remote, Conditionally Approved, Restricted, Conditionally Restricted* or *Off-shore*.

Coliform group includes all of the aerobic and facultative anaerobic, gram negative, non-spore-forming rod shaped bacilli that ferment lactose with gas production within 48 hours at 35 ± 0.5°C.

Conditionally Approved means the classification of a shellfish harvest area which meets Approved harvest area criteria for a predictable period. The period depends upon established performance standards specified in a management plan. A *Conditionally Approved* area is closed when it does not meet the *Approved* harvest area criteria.

Depuration means the process that uses a controlled aquatic environment to reduce the level of certain pathogenic organisms that may be present in live shellfish. In the Australian context depuration of shellfish potentially contaminated with enteric viruses is not permitted. Depuration may only be applied to shellfish potentially contaminated with bacteria at levels that can be effectively reduced to show compliance with the FSANZ Food Standards Code.

***Escherichia coli* (*E.coli*)** are thermotolerant (faecal) coliforms that produce gas from lactose and indole from tryptone after incubation at 44.0 to 44.5 °C for 24 + hours.

Growing area means a marine or enclosed body of water (i.e. bay, harbour, gulf, cove, lagoon, inlet, estuary or river) in which commercial species of bivalve molluscs grow naturally or are grown by means of aquaculture. A growing area may consist of one or more harvest areas.

Harvest area means an area that has been designated by a competent authority for the purpose of growing and/or harvesting commercial quantities of shellstock and may include wildstock or aquacultured shellstock.

Hepatitis A virus (HAV) is a non-enveloped icosahedral shaped virus (27-32 nm) with a positive-sense, single stranded RNA genome. Of the seven genotypes recognised, four (genotypes I–III and VII) infect humans. Despite this genetic variation, human HAV comprises a single serotype. HAV causes acute infectious disease of the liver.

Norovirus (NoV) is a genetically diverse group of single-stranded RNA, non-enveloped icosahedral shaped viruses (27-30 nm). They can be classified into five distinct genogroups of which genogroups I, II and IV most commonly infect humans. Human NoV is the leading cause of non-bacterial gastroenteritis worldwide and is highly contagious with no known treatment for infection. Immunity to the virus is often short lived and new pandemics appear every 2-3 years. A consequence of the genetic variability in NoV strains is that individuals may be repeatedly infected during their lifetime, No effective vaccine to human NoV is currently available.

Open status with respect to:

(a) an *Approved, Approved Remote, Conditionally Approved* or *Off-Shore* harvest area, means that shellfish may be harvested for direct marketing when shellfish harvest waters or shellfish meet harvesting criteria as determined by the SSCA and in the case of a conditional classification, as defined in the harvesting criteria detailed in the management plan for the shellfish growing area.

(b) a *Restricted* or *Conditionally Restricted* harvest area means that shellfish may be harvested for depuration or relaying when the shellfish growing waters and the shellfish meet harvesting criteria as determined by the SSCA and in the case of a conditional classification, as defined in the management plan for the shellfish growing area.

Thermotolerant (faecal) coliforms are those members of the coliform group that ferment lactose with gas production within 48 hours at 44.0 to 44.5°C.

Project Materials Developed

Project fact sheet (Appendix B).

The results of the survey will be submitted to an international peer reviewed journal for publication.

Appendices

Appendix A: Data Management Plan

Survey of Foodborne Viruses in Australian Oysters, (FRDC 2013/234)

1) Samples will be submitted to the SARDI Food Safety and Innovation Laboratory by nominated industry representatives in each state. Sampling kits and instructions will be provided by SARDI.

2) A sample submission form will be completed by the sampler which will note parameters such as: production area, harvest area, lease number, date and time of collection, GPS location of lease, species of oyster, weather conditions, water temperature, salinity of growing water, information on whether lease is actively selling, and if and how long samples have been depurated. All samples are allocated a unique identification number which will be used during the testing procedures undertaken at SARDI. This will ensure that samples are tested blind by staff and protect the identity of any samples testing positive for foodborne viruses.

3) The SARDI laboratory will undertake all testing and collate data into a central database which will record all information submitted with the sample. This database will be maintained and backed up on a secure SARDI network drive with restricted access. Access will only be given to the Principal Investigator, Seafood Program Leader, SARDI Research Officer responsible for entering and maintaining the database and SARDI Statistician.

4) Testing for norovirus (NoV) and hepatitis A virus (HAV) will be done using the international standard for testing (Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification; ISO/TS 15216-1:2013, Corrected Version 2013-05-01). SARDI has participated in several international proficiency trials for NoV and HAV testing in shellfish and has always scored 100% proficiency. These proficiency trials have been coordinated by the Centre for Environment, Fisheries and Aquaculture Science (CEFAS) in the United Kingdom.

5) Samples will be tested in batches within three months of reception of the last sample submitted during the two seasonal sampling periods. There will be a minimum of four weeks between sampling and reporting of results.

6) In the event of the detection of NoV or HAV in oysters, SARDI will inform the appropriate state shellfish regulator and growing area co-ordinator. There is no regulatory limit for NoV and HAV in Australian food law nor is testing a requirement of the shellfish management program. Due to the delay in testing of samples, any positive results will not be a reflection of oysters presently available in the marketplace. The regulator will use the information in conjunction with other public health or management data to determine if any action is required.

7) Publication of final results. The project to determine the prevalence of virus contamination in Australian shellfish is aimed to determine the level of management required for this hazard, and to potentially aid in market access negotiations for the oyster industry. Any publication of results will only include de-identified data, such that positive results will not be able to be attributed to individual growing or harvest areas.

8) The project steering committee will oversight any communications/publications arising from the project in accordance with the terms of reference for the steering committee.

Appendix B: Fact Sheet

Survey of Foodborne Viruses in Australian Oysters

Survey of Foodborne Viruses in Australian Oysters" supported by funding from the FRDC on behalf of the Australian Government

Aims of the project

1 To design a statistically robust survey to evaluate virus occurrence in oyster growing areas in NSW, Qld, SA and Tas
2 To identify the prevalence of human norovirus (NoV) and hepatitis A virus (HAV) associated with Australian oysters at harvest
3 To use the survey results to support trade and market access of Australian oysters

What are the benefits of the project?

Currently there is little information on the baseline level of viruses in Australian oysters, although a pilot project that targeted two high risk areas from each of SA, NSW and Tas indicated a low prevalence (<2%). Similar surveys have been undertaken worldwide, including in the USA, UK, France, China and NZ and have been used to develop or implement regulation.

There is considerable discussion on the international scene for regulation of viruses in bivalves. The European Food Safety Authority has recommended the introduction of an acceptable NoV limit in oysters and the EU Community Reference Laboratory has recommended an absence criterion be applied for HAV in bivalves. Furthermore, an international standard (ISO/TS 15216) was published in 2012 for testing of NoV and HAV in bivalves and the Codex Committee on Food Hygiene released guidelines this year on viruses in foods, with a specific annex on bivalves.

This survey will:

- Provide assurance to overseas markets that we are implementing international best practice by examining the level of risk from enteric viruses in Australia to determine if further management practises are necessary,
- Contribute to the development of market access strategies at the international level,
- Provide an opportunity for pollution source remediation if virus hot spots are identified, hence contributing to reducing cost of impacts.

What will results mean?

The result will provide information on a baseline level of NoV in Australia shellfish growing areas. A low prevalence will support the argument that further regulation (including regular testing) is unnecessary. Higher prevalences will indicate that more work should be conducted to better manage this issue, at least is some areas.

Sample details and time-frame

Sampling will be done of oysters sourced at the production area level and not at retail. Samples are proposed to be taken in each of the main oyster producing states (NSW, SA, Tas and Qld). 300 samples will be taken over a 12 month period in the calendar year of 2014. 150 samples will be taken during summer and autumn and 150 samples during spring and winter to capture both ‘peak’ and ‘off peak’ NoV seasons within the community. Sampling of 150 oysters (in each of the two periods) will ensure that at least one positive NoV result is obtained if >2% of oysters grown in Australia are contaminated with NoV. Conversely, if all results are negative, this will indicate that no more than 2% of oysters produced during the study period were contaminated with NoV.

How will production areas be chosen?

The contribution of each growing area in each state to total oyster production (by volume) will be determined over the previous 5 year period. This proportion of national production will be used to determine the growing areas to be sampled. The production area volumes will inform a randomised sampling program which is weighted for each area by five-year average production volumes. This approach will facilitate representative sampling based on recent production. The sampling program eliminates non-random sampling bias and therefore enhances the scientific robustness of the study. This approach will likely mean that samples are taken from a range of different production area classifications (e.g. approved, conditionally approved, etc.)

What will be done with samples?
Samples will be sent to SARDI for testing. One sample will comprise six individual oysters. Samples can be frozen as this will not affect analysis. Once samples are received they will be stored in a freezer, and batch analysed for NoV and HAV using a molecular method which detects the viral genome. There will be a minimum of one month between sampling and analysis, although this time frame is likley to be greater. Detection is based on an international standard released in 2012 (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). These methods are able to detect and quantify NoV and HAV in shellfish. The limit of detection of the method is approximately 100 viral genomes per gram of shellfish gut.

What happens to positive results?
There are no limits for NoV currently prescribed in the Australian Food Standards Code, or the Australian Shellfish Quality Assurance Operations Manual. Any action on positive results will be determined by the local state regulator. A communication protocol for such events will be developed in consultation with the steering committee, which will include both industry and regulatory representative.

The regulator will need to interpret the results in light of the limits of the test method, the date of sampling, the sanitary survey for the growing area, any other relevant information from the area (recent microbiological results, environmental events, sale history, etc).

Selection and role of steering committee
Members of the steering committee will be established through consultation with the oyster industry stakeholders prior to commencement of the project (if approved). It could include a national oyster industry representative, a state oyster industry representative from each participating state (SA, NSW, Tas, Qld), State regulators and Department of Agriculture representatives. The role of the committee will be to provide strategic oversight of the project, assist with communicating results of the study to industry and other stakeholders and provide guidance to project researchers and industry on protocols for handling commercially or market sensitive information arising from the project. The role of the steering committee will not be to drive the science of the research although feedback on project design will be sought.

State meetings to communicate project
The key researcher will travel to participating states (Sydney, Hobart and Port Lincoln) at the beginning of the project to meet with regulators and shellfish growers with the aim of communicating the project and establishing the sampling plan. Outcomes of the project will also be communicated to both industry and regulators through presentation at annual state shellfish conferences.

Frequently Asked Questions

Survey of Foodborne Viruses in Australian Oysters

Is there any information on the prevalence of NoV in Australia and the risk to shellfish production?
In Australia it has been estimated that 32% of all reported gastroenteritis is foodborne, accounting for 5.4 million cases, 15,000 hospitalisations and 80 deaths annually. Of these, NoV accounts for approximately a quarter of foodborne gastroenteritis annually.

Between 1980 and 2012 there were 368 reported shellfish associated viral gastroenteritis outbreaks reported in the international scientific literature. The most common viral pathogens involved were NoV (84 %) and HAV (13 %) with the most frequent shellfish implicated in outbreaks being oysters (58%).

Between 2001-2010 seventeen Australian cases of suspected shellfish related NoV outbreaks were reported in OzFoodNet. In some cases, where NoV was confirmed as the cause of illness, frozen imported oysters were implicated.

More recently consumption of oysters from Camden Haven (NSW) caused 36 people to suffer from NoV illness (2012) while over 500 people were reportedly affected by NoV following consumption of contaminated oysters from Tasmania (2013).

Is there a link between faecal coliform levels and norovirus?
There is weak correlation between presence of faecal coliforms (*E. coli*) and the presence of NoV in waterways. Faecal coliforms will be present in all faecal contamination events; however, NoV will only be present if it is circulating within the human population. Furthermore, faecal coliforms are less stable in the environment and hence relatively short lived following a contamination events, whereas NoV, survives for longer meaning NoV may still be detectable when faecal coliforms are no longer present. NoV has also been shown to be selectively accumulated and retained within the digestive tissues of oysters, persisting long after bacterial indicators of sewage contamination are no longer detectable. Hence, depuration is not effective in eliminating NoV from shellfish.

Is there a risk of growing waters being contaminated with norovirus?
Faecal contamination of shellfish production areas, especially near highly urbanised locations, results in an increased risk of any NoV that is circulating within the community accumulating in filter feeding shellfish and resulting in foodborne outbreaks. Currently NoV detections do not discriminate between viable (infective) or non-viable (non-infective) virus, but its detection is linked to presence of human sewage in the environment: a high risk situation in oyster growing areas.

Is there a difference between viable and non-viable norovirus and what does that mean?
Yes there is a difference between viable and non-viable NoV as only the viable viruses are able to cause gastroenteritis. NoV is excreted at high levels ($\leq 10^{11}$ virus particles/g faeces) from infected individuals, and it only takes a few virus particles to cause illness. The median infectious dose of NoV is estimated to be as low as18 virus particles, although the probability of becoming ill in susceptible individuals is dose-dependent. Currently the best practice for detecting NoV and HAV in shellfish is based on an international standard (ISO/TS15216). Although this analysis is the most advanced methodology available, it is likely to underestimate presence of both viable and non-viable virus. Therefore, the risk of not detecting NoV when it is present at very low levels is potentially greater than detecting non-infectious NoV.

What is the time frame between sampling and analysis?
The proposed work will be run as a research project and not as a diagnostic service. In our current experimental plan we estimate a month lag between sampling and analysis, although this will be generally longer due to batching of processed samples prior to analysis. The benefits of this are that the costs of undertaking the project remain reasonable due to economy of scale. We do have the capacity to do diagnostic testing with a week turnaround; however such work is only done when oysters have been implicated in a NoV outbreak.

Appendix C: Project Participants

SARDI research staff
Valeria Torok, Kate Hodgson, Jessica Tan, Navreet Mahli, Alison Turnbull, Linda Friedrich and Joanne Tomkins

Steering Committee:

Jane Clout	Kooringal Oysters, Queensland
Rachel King	Oysters Australia, National
Trudy McGowan	SAOGA, South Australia
Jon Poke	Bolduans Bay Oysters, Tasmania
Rebecca Schofield	SafeFood, Queensland
Valeria Torok	Principal Investigator, SARDI
Tony Troup	Camden Haven Oysters, New South Wales
Alison Turnbull	SARDI/SafeFish
Clinton Wilkinson	SASQAP, South Australia
Howel Williams	Department of Health and Human Services, Tasmania
Anthony Zammit	NSWFA, NSW

Assistance with provision of State oyster production data

John Dexter	Department of Agriculture, Fisheries and Forestry Aquaculture Policy and Industry Development Fisheries Queensland
Tim Paice	Department of Primary Industries, Parks, Water and Environment, Marine Resources Tasmania
Benjamin Tanti	Primary Industries and Regions SA, Aquaculture Policy and Planning Programs

Oyster growers and samplers
Tasmania:
Hayden Dyke, Tom Gray, Justin Goc, Craig Lockwood, Jon Poke, Mike Webb, Jeff Whayman.

South Australia:
Clinton Wilkinson

New South Wales:
Bruce Alford, Richard Barrie, Jason Bloomfield, Dominic Boyton, Matt Burgoyne, Ad Ferguson, Brett Harper, Kelvin Henry, Mark Hunter, Geoff Lawler, Bob Lee, Craig Lilley, John Lindsey, Rex Marks, Anthony Munn, Rodney McIntyre, Bruce Redmayne, John Ritchie, Mark Salm, Anthony Sciacca, Bert Sherlock, John Smith, Mick Swanston, Greg Woodford, John Yiannaros.

Queensland:
Tony Carlaw, Pat Verner

References

ABARES. (2012). Table 1: Gross value of fisheries production. http://data.daff.gov.au/data/warehouse/9aam/afstad9aamd003/2012/AFS_Production11Yrs_v1.0.0.xls.

ABARES. (2014). *Australian fisheries and aquaculture statistics 2013*. http://data.daff.gov.au/data/warehouse/9aam/afstad9aamd003/2013/AustFishAquacStats_2013_v1.2.0.pdf.

Abelson, P., Forbes, M., & Hall, G. (2006). The annual cost of foodborne illness in Australia. *Australian Government Department of Health and Ageing*.

AQIS. (2004). Australian Shellfi sh Quality Assurance Program. Export Standards. http://www.agriculture.gov.au/export/food/fish/shellfish-qa: Commonwealth of Australia.

ASQAAC. (2009). Australian Shellfish Quality Assurance Program Operations Manual. http://dpipwe.tas.gov.au/biosecurity/product-integrity/food-safety/seafood/shellfish-quality/australian-shellfish-quality-assurance-program.

Bellou, M., Kokkinos, P., & Vantarakis, A. (2013). Shellfish-Borne Viral Outbreaks: A Systematic Review. *Food and Environmental Virology, 5*(1), 13-23.

Benabbes, L., Ollivier, J., Schaeffer, J., Parnaudeau, S., Rhaissi, H., Nourlil, J., & Le Guyader, F. S. (2013). Norovirus and Other Human Enteric Viruses in Moroccan Shellfish. *Food and Environmental Virology, 5*(1), 35-40. doi: 10.1007/s12560-012-9095-8

Brake, F., Ross, T., Holds, G., Kiermeier, A., & McLeod, C. (2014). A survey of Australian oysters for the presence of human noroviruses. *Food Microbiology, 44*, 264-270. doi: 10.1016/j.fm.2014.06.012

CEFAS. (2011). Investigation into the prevalence, distribution and levels of norovirus titre in oyster harvesting areas in the UK (Vol. C3027): CEFAS Weymouth Laboratory.

CEFAS. (2013a). Discussion paper on live bivalve molluscs (LBM) and human enteric virus contamination: options for improving risk management in EU food hygiene package (Vol. WS12/15). Weymouth, UK: European Union Reference laboratory for monitoring bacteriological and viral contamination of bivalve molluscs.

CEFAS. (2013b). European Union Reference Laboratory (EURL) Proficiency Testing Scheme. Norovirus (Genogroup I and II) and hepatitis A virus proficiency testing 2013. EURL proficiency testing reference number: PT 46. https://eurlcefas.org/public-documents/proficiency-testing/pt-reports.aspx.

CEFAS. (2014). European Union Reference Laboratory (EURL) Proficiency Testing Scheme. Noroviruses and hepatitis A virus. EURL PT reference number: PT 50. https://eurlcefas.org/public-documents/proficiency-testing/pt-reports.aspx.

Conaty, S., Bird, P., Bell, G., Kraa, E., Grohmann, G., & McAnulty, J. M. (2000). Hepatitis A in New South Wales, Australia from consumption of oysters: the first reported outbreak. *Epidemiol Infect, 124*(1), 121-130.

Costafreda, M. I., Bosch, A., & Pintó, R. M. (2006). Development, evaluation, and standardization of a real-time TaqMan reverse transcription-PCR assay for quantification of hepatitis A virus in clinical and shellfish samples. *Applied and Environmental Microbiology, 72*(6), 3846-3855.

Costantini, V., Loisy, F., Joens, L., Le Guyader, F. S., & Saif, L. J. (2006). Human and animal enteric caliciviruses in oysters from different coastal regions of the United States. *Applied and Environmental Microbiology, 72*(3), 1800-1809. doi: 10.1128/AEM.72.3.1800-1809.2006

Da Silva, A. K., Le Saux, J. C., Parnaudeau, S., Pommepuy, M., Elimelech, M., & Le Guyader, F. S. (2007). Evaluation of removal of noroviruses during wastewater treatment, using real-time reverse transcription-PCR: Different behaviors of genogroups I and II. *Applied and Environmental Microbiology, 73*(24), 7891-7897.

DePaola, A., Jones, J. L., Woods, J., Burkhardt, W., 3rd, Calci, K. R., Krantz, J. A., Bowers, J. C., Kasturi, K., Byars, R. H., Jacobs, E., Williams-Hill, D., & Nabe, K. (2010). Bacterial and

viral pathogens in live oysters: 2007 United States market survey. *Appl Environ Microbiol, 76*(9), 2754-2768. doi: 10.1128/aem.02590-09

Doré, B., Keaveney, S., Flannery, J., & Rajko-Nenow, P. (2010). Management of health risks associated with oysters harvested from a norovirus contaminated area, Ireland, February-March 2010. *Eurosurveillance, 15*(19), 1-4.

EC. (2005). COMMISSION REGULATION (EC) No 2073/2005 on microbiological criteria for foodstuffs. *Official Journal of the European Union*(L 338/1).

EFSA. (2012). Scientific Opinion on Norovirus (NoV) in oysters: methods, limits and control options. *EFSA journal, 10*(1:2500).

FAO/WHO. (2008). Viruses in Food: Scientific advice to support risk management activities: meeting report. Microbiological Risk Assessment Series. No. 13. *Microbiological Risks Assessment Series*: FAO/WHO.

FAO/WHO. (2012). Guidelines on the Application of General Principles of Food Hygiene to the Control of Viruses in Food *Microbiological Risks Assessment Series* (Vol. CAC/GL 79-2012). Rome: Food and Agriculture Organization - World Health Organization.

Flannery, J., Keaveney, S., & Dore, W. (2009). Use of FRNA bacteriophages to indicate the risk of norovirus contamination in Irish oysters. *J Food Prot, 72*(11), 2358-2362.

FSANZ. (2012). Australia New Zealand Food Standards Code - Standard 4.2.1 - Primary Production and Processing Standard for Seafood (Australia Only). https://www.comlaw.gov.au/Details/F2012C00775.

Handmer, J., & Hillman, M. (2004). Economic and financial recovery from disaster. *The Australian Journal of Emergency Management, 19*, 44-50

Hewitt, J., Rivera-Aban, M., & Greening, G. E. (2009). Evaluation of murine norovirus as a surrogate for human norovirus and hepatitis A virus in heat inactivation studies. *Journal of Applied Microbiology, 107*(1), 65-71. doi: ; Wobus, C.E., Thackray, L.B., Virgin IV, H.W., Murine norovirus: A model system to study norovirus biology and pathogenesis (2006) Journal of Virology, 80 (11), pp. 5104-5112. , DOI 10.1128/JVI.02346-05

ISO/CEN. (2013). Microbiology of food and animal feed - Horizontal method for determination of hepatitis A virus and norovirus in food using real-time RT-PCR - Part 1: Method for quantification ISO/TS 15216-1:2013, Corrected Version 2013-05-01 *CEN/TC 275 - Food analysis - Horizontal methods*.

Kageyama, T., Kojima, S., Shinohara, M., Uchida, K., Fukushi, S., Hoshino, F. B., Takeda, N., & Katayama, K. (2003). Broadly reactive and highly sensitive assay for Norwalk-like viruses based on real-time quantitative reverse transcription-PCR. *Journal of Clinical Microbiology, 41*(4), 1548-1557.

Lodo, K. L., Veitch, M. G. K., & Green, M. L. (2014). An outbreak of norovirus linked to oysters in Tasmania. *Communicable diseases intelligence, 38*(1).

Loisy, F., Atmar, R. L., Guillon, P., Le Cann, P., Pommepuy, M., & Le Guyader, F. S. (2005). Real-time RT-PCR for norovirus screening in shellfish. *Journal of Virological Methods, 123*(1), 1-7.

Loutreul, J., Cazeaux, C., Levert, D., Nicolas, A., Vautier, S., Le Sauvage, A. L., Perelle, S., & Morin, T. (2014). Prevalence of Human Noroviruses in Frozen Marketed Shellfish, Red Fruits and Fresh Vegetables. *Food and Environmental Virology, 6*(3), 157-168. doi: 10.1007/s12560-014-9150-8

Lowther, J. A., Gustar, N. E., Powell, A. L., Hartnell, R. E., & Lees, D. N. (2012). Two-Year Systematic Study To Assess Norovirus Contamination in Oysters from Commercial Harvesting Areas in the United Kingdom. *Applied and Environmental Microbiology, 78*(16), 5812-5817. doi: 10.1128/aem.01046-12

Maekawa, F., Miura, Y., Kato, A., Takahashi, K. G., & Muroga, K. (2007). Norovirus contamination in wild oysters and mussels in Shiogama Bay, northeastern Japan. *Journal of Shellfish Research, 26*(2), 365-370. doi: 10.2983/0730-8000(2007)26[365:nciwoa]2.0.co;2

Moon, A., Hwang, I. G., & Choi, W. S. (2011). Prevalence of Noroviruses in Oysters in Korea. *Food Science and Biotechnology, 20*(4), 1151-1154. doi: 10.1007/s10068-011-0157-8

OzFoodNet. (2006). The annual cost of foodborne illness in Australia - 7.2 industry costs of contaminated oysters in Wallis Lake *http://www.ozfoodnet.gov.au/internet/ozfoodnet/publishing.nsf/Content/annual-cost-foodborne-illness.htm~annual-cost-foodborne-illness-07.htm~annual-cost-foodborne-illness-07.2.htm*.

OzFoodNet. (2011). Outbreaks of norovirus, hepatitis A or unknown aetiology associated with bivalves, Australia, January 2001 to September 2010.

Pavoni, E., Consoli, M., Suffredini, E., Arcangeli, G., Serracca, L., Battistini, R., Rossini, I., Croci, L., & Losio, M. N. (2013). Noroviruses in Seafood: A 9-Year Monitoring in Italy. *Foodborne Pathogens and Disease, 10*(6), 533-539. doi: 10.1089/fpd.2012.1399

Polo, D., Varela, M. F., & Romalde, J. L. (2015). Detection and quantification of hepatitis A virus and norovirus in Spanish authorized shellfish harvesting areas. *International Journal of Food Microbiology, 193*, 43-50. doi: 10.1016/j.ijfoodmicro.2014.10.007

Schaeffer, J., Le Saux, J. C., Lora, M., Atmar, R. L., & Le Guyader, F. S. (2013). Norovirus contamination on French marketed oysters. *International Journal of Food Microbiology, 166*(2), 244-248. doi: 10.1016/j.ijfoodmicro.2013.07.022

Seo, D. J., Lee, M. H., Son, N. R., Seo, S., Lee, K. B., Wang, X., & Choi, C. (2014). Seasonal and regional prevalence of norovirus, hepatitis A virus, hepatitis E virus, and rotavirus in shellfish harvested from South Korea. *Food Control, 41*, 178-184. doi: 10.1016/j.foodcont.2014.01.020

Shin, S. B., Oh, E. G., Yu, H., Son, K. T., Lee, H. J., Park, J. Y., & Kim, J. H. (2013). Genetic Diversity of Noroviruses Detected in Oysters in Jinhae Bay, Korea. *Food Science and Biotechnology, 22*(5), 1453-1460. doi: 10.1007/s10068-013-0237-z

Suffredini, E., Lanni, L., Arcangeli, G., Pepe, T., Mazzette, R., Ciccaglioni, G., & Croci, L. (2014). Qualitative and quantitative assessment of viral contamination in bivalve molluscs harvested in Italy. *International Journal of Food Microbiology, 184*, 21-26. doi: 10.1016/j.ijfoodmicro.2014.02.026

Svraka, S., Duizer, E., Vennema, H., De Bruin, E., Van Der Veer, B., Dorresteijn, B., & Koopmans, M. (2007). Etiological role of viruses in outbreaks of acute gastroenteritis in The Netherlands from 1994 through 2005. *Journal of Clinical Microbiology, 45*(5), 1389-1394.

VIDRL. (2013). The VIDRL Norovirus Reprot July 2013. www.vidrl.org.au: Victorian Infectious Diseases Reference Laboratory.