# Analytical techniques for assessment of water quality, contamination and quality assurance of farmed pacific oysters in South Australia

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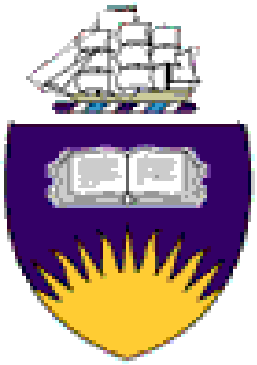
Australian Government

Fisheries Research & Development Corporation



**The Flinders University of South Australia**

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# Analytical techniques for assessment of water quality, contamination and quality assurance of farmed pacific oysters in South Australia

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**Non-technical Summary**

**2000/257** **Analytical techniques for assessment of water quality, contamination and quality assurance of farmed pacific oysters in South Australia**

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**OBJECTIVES:**

1. To develop sensitive tests using polymerase chain reaction (PCR) to detect a range of viruses of direct significance to human health directly from shellfish. The range would include hepatitis A virus, Norwalk virus, Astrovirus, and Adenovirus.

2. Assessment of coliphage concentrations, and indicator microorganisms as reliable tools for assessment of faecal contamination (coliforms, enterococci) in oyster tissues, and harvest waters.

3. Assessment of faecal origin and contamination of oysters and harvest waters by Gas-Chromatography – Mass Spectrometry analysis of faecal steroid profiles.

4. To adapt developed biochemical markers in blue mussel (*M. edulis*) to oysters as indicators of stress from adverse environmental conditions of microbial and chemical origin.

5. Combination and correlation of the above analytical techniques to provide a comprehensive assessment of the extent of contamination of oysters and their harvest waters by pathogens of faecal origin. These techniques would be more reliable than current faecal indicator techniques and allow accurate determination of closure safety zones.

6. Identification and validation of reliable, cost effective monitoring tools for quality assurance of farmed oysters, lease and closure safety zones applicable to South Australian waters.

7. To develop methodologies for quality assurance with national applicability to the oyster farming industry.

8. Assessment of the correlation between the presence of enteric virus nucleic acid with coliphage determinations from shellfish tissue and harvest water.

**NON TECHNICAL SUMMARY**

These investigations focused on methods of assessing the contamination of oysters by human faecal contamination. Current methods using tests for coliform bacteria have been demonstrated to be unreliable indicators of enteric virus contamination. In these investigations the comparison of a range of new techniques was attempted aimed at identifying a single simple and reliable test for oyster microbial quality. New techniques applied were Polymerase Chain Reaction (PCR) methods for enteric viruses, coliphage plaque assays and determinations of faecal sterols. These test were developed and conducted firstly on homogenized oyster tissues and then using naturally bioaccumulated viruses in seeded tank experiments.

As a result of these investigations a new and more rapid method for faecal sterol analysis was developed. This method was as sensitive as the current method using Gas-Chromatography-Mass Spectrometry, but used Gas Chromatography only, and reduced sample processing time from 5 days to 24hrs. This new technique was able to detect reproducibly faecal sterols in oysters at concentrations of less than 1µg $g^{-1}$ of tissue.

Reproducible PCR methods were developed for Hepatitis A, Norovirus (G2), Enterovirus, Adenovirus and Astrovirus. Comparisons between the PCR methods and plate assay

methods using bacteriophage ΦX 174 suggested that a limit for detection of a single infectious particle of virus per oyster was achievable. This detection limit has obvious implications for the ability to provide quantitative risk assessment of market product.

Statistical interpretation of the comparison of results using different methods was limited. This was due to a lack of supply of naturally contaminated oysters. Results indicate that positive coliform estimations are reliable indicators of faecal contamination, and that presence of coliphage in determinations are reliable indicators of the presence of enteric viruses. It was also clear that negative test results for either coliforms of coliphage were not reliable predictors of the absence of enteric viruses. Ultimately analysis for specific viral contamination would be necessary to guarantee shellfish quality for export markets.

**KEYWORDS: Oysters, faecal contamination indicators, viral particles, PCR, coliforms, coliphage, Gas Chromatography .**

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## Background

Currently quality assurance programs for farmed oysters in Australia use faecal coliforms as indicator organisms. However absence of these organisms does not ensure absence of other human pathogens such as enteric viruses. As such the validity of these techniques is questionable, and there are few data available regarding the survival or persistence of these organisms in the oysters or in the harvest waters. There is a need in a National Monitoring Program to implement a test that is cost effective, valid, specific and which can be routinely applied. Work in the European Union suggests that the coliphage test may be a good candidate once validated for Oyster species and water catchments. Feedback from export markets suggests that direct screening for enteric viruses is becoming a key quality assurance issue in shellfish. Current research work on microbial contamination of Sydney rock oysters in NSW is concerned solely with depuration processes and does not address harvest waters contamination *in situ.* Work to date in Australia has only addressed *E.coli* and total heterotrophic plate counts in line with the current code of practice (Buckle – pers. Comm.).

The recently released National Environmental Health Strategy also suggest that a wide ranging review of oysters safety be conducted, including monitoring and maintenance of harvest water quality, and shellfish quality assurance. This report highlights the problems associated with land run-off and faecal contamination in estuarine oyster catchments in New South Wales and the implications for long term industry viability and sustainability. In other states, such as Tasmania and South Australia, catchments are not confined to estuarine locations impacted by land run-off. Assessment of water quality and microbial status of the oysters in these relatively pristine conditions have not been undertaken. There is a need for regulatory authorities eg SASQAP to be able to set reliable boundaries around outfalls, and establish closure safety zones (CSZ). Currently CSZ are determined by subjective judgments based on coliforms counts. Development of a suite of sensitive tests would permit accurate establishment of CSZ in existing and proposed lease areas.

PCR detection, identification and enumeration of viruses in shellfish meats is a reliable but expensive technique, and is yet to be established as a routine protocol. Development of this technique will permit an absolute enumeration of viral particles in oyster flesh and harvest waters. This would be a complementary technique to the depuration assessments carried out in FRDC project 98/319 which did not include the direct detection of specific viral pathogens of humans (Hepatitis A, Norwalk etc.). The detection of coliphage as a monitoring technique may be more applicable than PCR but needs to be validated by correlation with the survival of enteric viruses in harvest water and shellfish meat. Experience in Europe has shown that this correlation is variable and dependent on ambient temperatures, oxygen availability and other factors. In addition other indicators such as the presence of faecal sterols, or stress in shellfish may be useful in determination of water quality and shellfish cleanliness. Faecal sterol detection has a much greater sensitivity than coliform counts and may be a valuable technique in assessing water catchments.

The applicability of these analytical parameters needs validation for both the harvest water and the oyster species being farmed. It is expected that there will be substantial variations in survival rates of microorganisms in harvest waters between different geographical locations. Survival rates in estuarine waters will be quite different to those in other coastal environments where water quality parameters such as salinity turbidity and land run-off are not comparable. The species of farmed oyster (Sydney Rock or Pacific) can also be expected to greatly affect microorganism contamination and survival rates, as well as recovery efficiencies in shellfish meat. An understanding of these variations between species and environmental conditions will aid the development of monitoring tools for water and shellfish quality specific to individual growing areas.

This proposal aims to develop a range of analytical tests for assessing the degree of contamination of both oysters and their harvest waters in South Australia. These tests include detection of viral nucleic acid, indicator bacteria, coliphage enumeration, faecal sterol profiles, and the evaluation of environmental stress in the oysters. The broader application of these techniques to the national industry is likely to enhance the research efforts of assessing depuration efficiencies (FRDC 98/319). They would also meet a number of high priority needs of the SARFB R&D 5 year strategy (see **Need)**.

Work to date has involved use of the enterovirus Coxsackievirus B4 as the model in a pilot project utilizing nucleic acid methodology (PCR testing) to detect the virus in oyster flesh. The virus is similar to Hepatitis A virus but can be readily grown and quantitated in the laboratory. PCR assays for the viruses of interest will be developed and applied to oyster tissue. Viruses to be assayed will include Hepatitis A, Norwalk, Astrovirus and Adenovirus. Appropriate PCR assays have been published for all of these agents, and adopting them for use in this context will be relatively straight-forward, given our extensive experience in establishing in-house PCR's for clinical diagnostic use.

Assessment of the origin of faecal contamination in oysters and their harvest waters is essential in any attempts to manage risk effectively. Identification of the pollutant source will enable management mechanisms to be put in place to minimize both the sources of the pollutants and their effects. Analysis of faecal steroids by this technique allows a discrete profile to be generated which is unique to each pollutant source and is much more sensitive than traditional coliform assessments. The technique is currently being used routinely in our laboratories, and has been applied to catchments, sediments and harvest waters from oyster catchments (Suprahatin 2005, Ralph *et al* 1997, 1998, 1999). This technique when used in conjunction with the other microbiological analyses suggested will provide a very powerful tool for the industry and regulators to minimize and manage risk associated with farming shellfish in potentially polluted areas. The technique will also provide invaluable advice on selection of potential sites for leasing and together with stress evaluations would go a long way to meeting the need for assessment of pollution from terrestrial inputs.
It is known that human faecal sterols like coprostanol can be found in polluted surface waters with decreasing concentration downstream (Walker *et al* ?). The most widespread method for determining contamination is at present is the use of coliform bacterial biomarkers. There are limitations as to the reliability of this method to monitor fecal

contamination due to bacterial death caused by environmental stress in for example the transport and storage conditions of the oysters from the lease to the laboratory (Brown *et al* 1984). Coprostanol has been widely looked at as an indicator of faecal contamination in waste water and marine sediments and is seen to be an absolute indicator of faecal pollution which is not affected by environmental stresses (Fergason *et al* 1996, Dutka et *al* 1978, Leeming *et al* 1996, Brown *et al* 1984, Chan *et al* 1998, Krahn *et al* 1989, Writer *et al* 1995, Edwards *et al* 1998, Walker *et al* 1982). For example the proportion of coprostanol to 24-ethylcoprostanol can give a sterol profile that can be used to indicate the source of the faecal contamination (Idler *et al* 1971, Teshima *et al* 1980, Bereberg *et al* 1981, Leeming *et al* 1996). Coprostanol can constitute up to 65% of the total sterol content excreted by humans (3432 µg $g^{-1}$ dry weight) and along with 24-ethylcoprostanol (1245 µg $g^{-1}$ dry weight) constitute the majority of excreted human sterols. Although these sterols are also found in other higher animals the ratios between the two differ significantly 353µg $g^{-1}$ dry weight and 334 µg $g^{-1}$ dry weight for coprostanol and 24-ethylcoprostanol respectively in pigs (Ferezou 1978, Leeming *et al* 1996). The Pacific Oyster (*Crassostrea gigas*) has eight predominant sterols however 5β-stanols like Coprostanol, *epi*coprostanol, 24-ethylcoprostanol and 24-ethyl*epi*coprostanol do not occur naturally in *C. gigas* or in the water column of marine waters. Oyster sterols reflect to a certain degree their diet due to an absence of *de novo* sterol synthesis and limited dietary sterol bioconversion (Knauer *et al* 1999). Therefore the presence of these sterols within the oyster can indicate that it has come into contact with human faecal contamination.

Existing methods for the extraction of sterols often have (i) a large number of steps increasing the sample preparation time and increasing the opportunity of experimental error in a routine analysis or (ii) extraction steps that are lengthy, 24 hours or greater (Borgjesson, Ghoshi, Chan *et al* 1998, Knauer *et al* 1999 & Korpela *et al* 1982). This method has been developed with a number of factors in mind (i) to reduce the time needed for the analysis from sample receipt to results generation, (ii) to reduce the cost both financially and environmentally with respect to solvent waste and (iii) to not reduce reproducibility or the limit of detection.

A short sample preparation and analysis time is necessary. If the oysters are to be sampled from an area about to be harvested an increase in sample handling time of even a few days will increase the opportunity of the oysters of becoming contaminated post-sampling/pre-harvesting through heavy rain causing stormwater discharges or accidental septic tank discharges, for example.

This protocol has been developed from methods utilized by Knaeur (1999), looking at sterols of oyster spat, Lee *et al* (2001) looking at total lipids in fish tissue and Bligh and Dyer's (1959) method of total lipid extraction.

A range of indicators have been used to evaluate nutritional conditions, growth rate, and health status in a variety of fish and shellfish species. These indicators have then been used to determine improvements and decrements in quality or health. However, the majority of studies examining negative impacts on health have concentrated on crude markers that indicate that detrimental effects are already in evidence.

AEC (Adenylate Energy Charge) is indicative of the metabolic energy available to the organisms from the adenosine nucleotide pool. Assessment of this metabolic energy can be used to assess stress of the organism related to unfavourable environmental conditions. This assessment of stress may detect harvest water and shellfish contamination including heavy metals and pesticides from land run-off not detectable by other microbial methods. Development of analytical techniques which would determine chemical water quality on stress in shellfish would be complementary to the microbial analyses. The combination of these techniques with microbial analyses would permit a comprehensive assessment of water quality for CSZ establishment.

Development and assessment of the above tests could be used to validate current quality assurance monitoring techniques and assess the viability of new more rigorous alternatives. Such tests would provide for increased marketability of oysters to overseas markets through reliable health and safety and quality assurance provision. These tests will also facilitate continuous monitoring of water and product quality in oyster catchments. By using a range of monitoring techniques applied to both harvest waters and oyster flesh it will be possible to identify a technique or techniques which are most valid and amenable for use as reliable routine quality assurance tools.

The establishment of these techniques as tools for the absolute determination of CSZ would be invaluable in the monitoring and assessment of existing and future lease allocations. These techniques (eg coprostanol determinations, PCR) would provide greater sensitivity than existing faecal coliform indicators used for this purpose. Their development may find application in catchments where depuration processes are not used, but may also enable better monitoring of depuration conditions and effectiveness.

**Need**

1. 2nd highest priority (environment) of Aquaculture industry sector in SAFRAB 5 year R &D strategy, development of analytical methods to enhance shellfish quality assurance.

2. 6th highest priority (quality assurance) of Aquaculture industry sector in SAFRAB 5 year R &D strategy, assessment of suitable sites for coastal aquaculture.

3. National research Priorities for Australian Fisheries and Aquaculture; program 2: Environmental Management, sub-program D: Pollution – impacts of terrestrial input.

4. National research Priorities for Australian Fisheries and Aquaculture; program 3: Aquaculture Development, sub-program A: Aquaculture and the Environment, improved site selection issue.

5. FRDC's program 3 – Industry Development, Aquaculture Development, Market Development, and Quality.

**Objectives**

1 To develop sensitive tests using polymerase chain reaction (PCR) to detect a range of viruses of direct significance to human health directly from shellfish. The range would include hepatitis A virus, Norwalk virus, Astrovirus, and Adenovirus.

2 Assessment of coliphage concentrations, and indicator microorganisms as reliable tools for assessment of faecal contamination (coliforms, enterococci) in oyster tissues, and harvest waters.

3 Assessment of faecal origin and contamination of oysters and harvest waters by Gas-Chromatography – Mass Spectrometry analysis of faecal steroid profiles.

4 To adapt developed biochemical markers in blue mussel (*M.edulis*) to oysters as indicators of stress from adverse environmental conditions of microbial and chemical origin.

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6 Identification and validation of reliable, cost effective monitoring tools for quality assurance of farmed oysters, lease and closure safety zones applicable to South Australian waters.

7 To develop methodologies for quality assurance with national applicability to the oyster farming industry.

8 Assessment of the correlation between the presence of enteric virus nucleic acid with coliphage determinations from shellfish tissue and harvest water.

## Methods

### 1.0 Collection and Provision of Oysters

Oysters were received from a number of commercial sources in NSW and SA and transported on ice to the laboratory. Oysters were provided to the laboratory by two means. For seeding experiments and laboratory test validations oysters were provided as live, cleaned, table oysters. These oysters were transferred to aquaria and maintained in clean seawater at 15ºC with daily algal feeds for a minimum 5 days prior to use. Dead oysters were removed daily from the aquaria.

Field samples were transported in two forms. Wherever possible live oysters were transported on ice to the laboratory within 24 hours of collection. Sydney Rock oysters were supplied in refrigerated containers by the NSW Shellfish Quality Assurance program. Care was taken in packing to avoid direct contact between oysters and ice to avoid fatalities in transit. Alternatively, when SASQAP laboratory tests indicated the presence of faecal coliform contamination frozen samples of the homogenised oyster flesh were transported to the laboratory on ice. The delivery of these samples was necessarily after the testing regime was complete (3 days).

### 2.0 Analysis of Faecal Sterol Content

### 2.1 Systematic Names

Systematic names of sterols, (trivial name in brackets); 5β-colestan-3β-ol (coprostanol), 5β- cholestan-3α-ol (*epi*-coprostanol), cholest-5-en-3β-ol (cholesterol), 5α-cholestan-3β-ol (cholestanol), 24-ethyl-5β-cholestan-3β-ol (24-ethylcoprostanol), 24-ethyl-5β-cholestan-3α-ol (24-ethyl-*epi*-coprostanol), 24-methylcholest-5-en-3β-ol (campesterol), 24-ethylcholesta-5,22E-dien-3β-ol (stigmasterol), 24-ethyl-5α-cholest-22E-en-3β-ol (stigmastanol), 24-ethylcholest-5-en-3β-ol (β-Sitosterol), 24-methylcholesta-5,22-dien-3β-ol (brassicasterol), cholesta-5,22E-dien-3β-ol (*trans*-22-dehydrocholeserol), 24-methylcholesta-5,24(28)-dien-3β-ol (24-methylenecholesterol).

### 1.2 Samples

Upon receipt the oyster meat was dissected out and the extra-pallial fluid drained, there was no discrimination of organs as sterols accumulated from the environment are universally distributed throughout the oyster (Gordon *et al* 1982). Approximately 250g of oyster meat was collected from each sample site. 50g aliquots were homogenized in a Waring blender for 30secs at low speed to form a rough paste and stored at –80°C until sterol extraction was performed.

### 1.3 Reagents

A Methanol / chloroform solution was prepared in the ratio 2:1, previous work has shown that this ratio is optimal for lipid extraction in fish tissue with a similar fat content (<2%) to oysters meat (1.46-2.84%) (Lee *et al* 1996, King *et al* 1990). A Hexane

/ chloroform mixture was prepared in a 4:1 ratio. 10% Potassium hydroxide solution (w:v) was prepared in 20% distilled water 80% methanol for saponification. The silyation reaction mixture was 5 aliquots of Dimethyl-formaldehyde (DMF) and 15 aliquots of bis(trimethylsily) triflouroacetamide (BSTFA). All solvents were Analar grade and supplied by Sigma-Aldrich, as were all other reagents and sterol standards. 24-ethylcoprostanol and 24-ethyl*epi*coprostanol (1:9 ratio) were supplied by Barry Dent Global.

### 1.4 Standards

A 100mg / 100ml (1000ppm) 5α-Cholestane in chloroform was prepared as an internal standard. A separate calibration solution of 10 sterols was prepared in chloroform at a 10mg / 100ml (100ppm) concentration. These were diluted down to 5, 10, 15, 20, 25, 30, 40 & 50ppm for calibration.

### 1.5 Sterol extraction, saponification and silyation

5±0.05g of the oyster flesh homogenate was placed into a 250ml Ebertech homogenizer container, 45ml of the Chloroform:Methanol solution was added and 25µl of the 5α-Cholestane internal standard solution. The mixture was homogenized using a Waring blender (model no.91-358) for 1 minute at low speed. The homogenate was centrifuged at 1500rpm for 5 min, the resulting supernatant was transferred to a 250ml separating funnel and 20ml 0.5% NaCl added to separate the lipid containing chloroform fraction from the methanol. The solution was mixed by gently inverting the funnel five times then allowed to stand until a clear separation was visible. The bottom chloroform fraction was collected into a 250ml distillation flask, 5ml of Methanol added and then reduced *en vacuo* to 2ml using a rotary evaporator and transferred to a 10ml sample tube.

2ml 10% Potassium hydroxide solution was added to the reduced chloroform solution and the tube sealed and heated for 2 hours (80˚C) with regular shaking. When cool, 4ml distilled water and 2ml of the Hexane:Chloroform solution was added and mixed thoroughly. The bottom sterol containing chloroform layer was collected and cleaned by passing through a silica / alumina column and then dried under Nitrogen.

Just prior to analysis the samples were reconstituted in 250µl of chloroform. A 100µl aliquot was silyated with 5µl of DMF and 15µl BSTFA, heated at 60˚C for one hour.

### 1.6 Chromatography

Gas chromatography was performed on a Varian 3600CX GC using a DB-5 capillary column 30m x 0.25mm i.d. x 25µm film thickness (J & W Scientific), a splitless injector (set at 275˚C) and a Flame Ionization Detector (set at 300˚C). Nitrogen (99.9997%) was used as the carrier gas with a head pressure of 10psi. 1µl samples were injected with the initial oven temperature of 90˚C held for 1 minute, it was then increased to 220˚C at 15 ˚C/min, 275˚C at 5˚C/min and held for 10 minutes. Finally the oven was increased to

300˚C at 5˚C/min and held for 5 minutes. Peak area was calculated by Varian Star software.

Mass spectrometry was performed on a Varian Saturn 2000 MS with a Varian 3400CX GC using a DB-5MS capillary column 30m x 0.25mm i.d. x 25µm film thickness (J & W Scientific) with a splitless injector (set at 250˚C). Helium (99.9997%) was used as the carrier gas with a head pressure of 10psi. 1µl samples were injected with the initial oven temperature of 90˚C held for 1 minute, it was then increased to 220˚C at 30 ˚C/min then to 300˚C at 5˚C/min and held for 10 minutes. Peak area was calculated by Varian SaturnWS software. Sterols were quantified using selected ion profiles to reduce result inaccuracy by possible co-eluting compounds; cholestane (*m/z* =217), *epi*coprostanol (*m/z* =215), campesterol (*m/z* =129 & 382), ethyl*epi*coprostanol (215 & 216), stigmasterol (*m/z* =55), cholesterol and β-Sitosterol (*m/z* =129), coprostanol, cholestanol and stigmastanol (*m/z* =75).

**2.0 Faecal Coliform Estimations**

Initially faecal coliform determinations were undertaken using the IDEXX Colilert™ most probable number system. This system was rapid and effective for catchment waters. When applied to oyster flesh the system gave all wells as positive, indicating cross reaction with the reagents. This was discontinued for determinations in oyster flesh and the Australian Standard AS 4276.8 (method 8: Fecal streptococci – Estimation of most probable number MPN) was employed in its place.

**3.0 Bacteriophage Enumeration**

The detection and enumeration of coliphage in water samples during this study used a standard double agar overlay method (Green *et al* 2000). The method involved addition of a 1ml volume of the test sample and 250µl of host *Escherichia coli* to a test tube containing 4mls of 2.8% molten nutrient agar (≤ 50°C). The agar was then vortex mixed. Contents of test tube were pored evenly over the surface of a 2.8% nutrient agar plate. Positive results or phage growth were shown by plaques (zones of clearing) on the agar plate, the number of plaques were counted and reported as plaque forming units (PFU) per ml of the original sample.

Extracts were also assayed for coliphage by propagation on *E. coli* host strain WG5. The assay is described in the *Standard Methods for the Examination of Water and Wastewater* (CD) with the modification that 60 µg/ml nalidixic acid is added to all solid media and 30 µg/ml is added to liquid media.

**4.0 PCR methods for viruses.**

The detailed methods for the enterovirus and PhiX174 are described in the appendices. Briefly the enterovirus method involved : (1) extraction of the RNA from the final oyster PEG precipitate with Trizol or a Qiagen RNeasy Plant Minikit, (2) reverse transcription of the viral RNA with a Life Technologies Reverse Transcription kit, (3) amplification of the cDNA by either single round or nested PCR, using a Perkin Elmer Thermal

Cycler and (4) detection of the PCR product by gel electrophoresis using ethidium bromide staining. A visible band under UV light, corresponding to a PCR product of the appropriate size (determined by reference to a 50bp molecular weight ladder) was taken to be a positive result.

For PhiX174 the DNA was extracted from the PEG precipitate with a Qiagen DNeasy Plant Minikit. The extracted DNA was amplified by nested PCR and the product detected by gel electrophoresis as described above.

Additional PCR's were developed during the course of this project for Adenovirus, Astrovirus, Hepatitis A virus and Norovirus Gp.2. There was insufficient time to test them with macerated oyster flesh, and biosafety concerns by the Institutional Biosafety Committee prevented their use in tank experiments with live oysters too.

## 5.0 Concentration of virus from oysters

### 5.0.1 Whole oyster determinations

The initial extraction procedure was a modification of a method previously described by Traore *et al*. (1998). Oysters were shucked and drained. 120 g of oyster meat, or approximately six oysters, were homogenised in 100 ml of 0.05 M glycine – 0.15 M NaCl buffer (pH 9) by blending for 60 sec in a Waring commercial homogeniser.

The suspension was agitated for 15 min and then clarified by centrifugation at 5,000 *g* for 10 min at 4ºC. 50% w/v polyethylene glycol 6000 (PEG 6000) in 0.05 M glycine – 0.15 M NaCl buffer (pH 9) was added to the supernatant such that 10% w/v PEG 6000 was achieved. The tubes were inverted several times to ensure a homogenous mixture and the pH adjusted to 7.3 prior to incubation at 4ºC for 18 hrs.

Precipitated material was collected by centrifugation at 10,000 *g* for 90 min at 4ºC and resuspended in 4 ml 0.15 M $Na_2HPO_4$ (pH 9). Further centrifugation at 1,500 *g* for 20 min at 4ºC removed any remaining particulate matter, then the final extract was adjusted to pH 7.2 prior to further analysis.

### 5.0.2 Dissected oyster tissue determinations

Oyster digestive tissues were initially dissected from whole oysters and retained while the remaining tissues were discarded. 80g of oyster digestive tissues, approximately corresponding to 12 whole oysters, were homogenised in 100 ml 0.05 M glycine – 0.15 M NaCl buffer (pH 9) by blending for 60 sec in a Waring commercial homogeniser. The suspension was agitated for 15 min and then clarified by centrifugation at 5,000 *g* for 10 min at 4ºC. 50% w/v polyethylene glycol 6000 (PEG 6000) in buffer ) was added to the supernatant such that 10% w/v PEG 6000 mixture was achieved. The tubes were inverted several times to ensure a homogenous mixture, and pH adjusted to 7.3 prior to incubation at 4ºC for 2 hrs. Precipitated material was collected by centrifugation at 10,000 *g* for 90 min at 4ºC and resuspended in 4 ml 0.15 M $Na_2HPO_4$ (pH 9). A further centrifugation at 1,500 *g* for 20 min at 4ºC removed additional particulate matter. A

second volume of 50% w/v polyethylene glycol 6000 (PEG 6000) in 0.05 M glycine – 0.15 M NaCl buffer (pH 9) was added to the supernatant to achieve 10% total w/v PEG 6000. Again tubes were inverted and the pH adjusted to 7.3 prior to incubation at 4$^{o}$C, this time for 18 hrs. The precipitate was collected by centrifugation at 10,000 *g* for 90 min at 4$^{o}$C and resuspended in 1 ml 0.15 M $Na_2HPO_4$ (pH 9). Further centrifugation at 1,500 *g* for 20 min at 4$^{o}$C removed any remaining particulate matter. The final concentrate was adjusted to pH 7.2 prior to further analysis.

**6.0 Adenylate charge (AEC) determinations**

Adenylate energy charge (AEC) were intended to be analysed in all oyster flesh samples obtained from field sample sites. AEC was to be performed using methods described in the FRDC Project Report '*Feasibility study to evaluate non-lethal measurements of health of farmed tuna using biochemical methods and surrogate species – Project Nos 95/082 and 97/307.* Soole KL *et al* 2002. However, sampling protocols from field locations were not able to be adapted sufficiently to ensure stability of tissue adenylates following sampling and during transport. This part of the project was abandoned (See Discussion Section 8.4).

**7.0 Oyster Sampling methodology**

**7.1 Seeded Oyster Flesh**

All successful analytical techniques (1-4 above) were initially applied to homogenized oyster flesh seeded with bacteria, virus or sterols. Additions of virus, bacteria or sterol were made prior to blending of the oyster flesh to maximize homogeneity (section 4.1). Virus additions were made from estimated dilutions of viral cultures. Simultaneous evaluation of viral concentration via plaque assays was undertaken to establish concentration of delivered inoculum. A similar protocol of culture of inoculum was used for bacterial determinations. Delivered sterol concentrations were calculated from supplied chemical standard content. From these determinations initial viability of the methods was confirmed. Results from these analyses are presented below (Section 7)

**7.2 Naturally Accumulated Oyster Contamination.**

Once determinations from seeded homogenized oyster flesh had been completed it was decided to trial the methods on viral markers in oysters contaminated via natural accumulation. This was undertaken as there was uncertainty about the survival and attachment of microorganisms in living tissues which may affect the sensitivity of the assays.

Natural accumulation experiments were conducted in seawater tanks, and the microorganisms were introduced with an algal feed to ensure uptake by the oysters.

Prior to experimentation, all whole oysters (*Crassostrea gigas*) were rinsed in running distilled water and any attached debris remove by scrubbing with a sterile, soft bristled brush.

Seeded tank experiments were performed by placing oysters in shallow 20 litre aquaria filled with natural seawater and seeded with the necessary inoculum. Oysters were added at a ratio of no less than five litres of seawater per oyster and maintained at room temperature for 18-24 hours.

Only live oysters were submitted for experimentation, dead or unhealthy oysters were omitted. All oyster meat was processed immediately without freezing or prolonged storage.

Seeded experiments were performed using Poliovirus type 1 (vaccine strain) and coliphage PhiX174. Poliovirus was propagated by inoculating a confluent monolayer of buffalo green monkey kidney cells (BGMK) grown in Eagles Medium Base supplemented with 2% v/v foetal calf serum and 0.25 µg/ml fungizone. Infected cells were tripsinized using 1% v/v trypsin versene in PBS and resuspended in 5 ml of the same medium. Suspended poliovirus was clarified by centrifugation at 900 *g* for 5 min.

Extracts were assayed for poliovirus by propagating on BGMK cells. Infected cells were identified by staining with Monoclonal Mouse Anti-Enterovirus antibody (Dako), followed by fluorescein-conjugated Anti-Mouse Immunoglobulin (Chemicon). Infected cells were visualised by immunofluorescence microscopy.

Coliphage PhiX174 was propagated by excising plaques generated by the phage and resuspending in tryptone water (Oxoid). Suspensions were clarified by centrifugation at 10,000 *g* for 5 min.

### 7.3 Field Sampling

As part of the routine development of analytical techniques oysters were sourced and sampled from the South Australian Oyster Growers Association leases throughout the project. These oysters were routinely subjected to all available analytical techniques. It became clear early in the project that contaminated shellfish were uncommon in the South Australian leases. The routine field sampling of oyster leases was not undertaken, as it was unlikely to produce useful data. As a result attempts were made to source contaminated shellfish from other States, including other shellfish species.

Further samples were routinely analysed to validate developed techniques. These were either sourced from retail outlets (as random samples from the SA growers) or from Bob Simmonds, as a local oyster retailer.

Oysters were also provided by SASQAP when faecal coliform determination indicated that they were contaminated. These oysters were then analysed for the full range of tests. In addition SASQAP provide samples of Blue Mussels which had also recorded positive faecal coliform test results.

Sydney rock oysters were provided in response to a request via FRDC for provision of contaminated shellfish from other States.

Pearl oysters were provided in response to request from Martyn Kirk of NSW health department, who was conducting an investigation of a Norovirus disease outbreak associated with imported product.

## 8.0 Results/Discussion

### 8.1 Faecal sterol determinations

#### 8.1.1 Extraction methods.

The new extraction method gave higher sterol concentrations ($p<0.05$, paired t-test) compared with results using the method employed by Knauer *et al* 1999 (Table 1.) in spiked oyster homogenates. Although 5α-Cholestane was added to the samples as an internal standard for quantitation purposes to calculate the percentage recovery total tissue sterol extraction was assumed. The increase in sterol concentrations in this study seem to indicate that the previous method was not fully extracting all the sterols from the samples as the modified method provided significantly greater recovery from paired samples undergoing identical analytical techniques (Tables 8.1.1 and 8.1.2). Most work to date has looked at the use of sterol biomarkers in water, sludge and sediments (Writer *et al* 1995, Chan *et al* 1998 and Brown and Wade 1984). Although these sterols are known to associate with particulate matter (Ngyun *et al* 1995) the sterols will still be freely available for extraction. In the oyster tissue the sterols are bound in organic matrices within the cells and therefore will be less readily available to be extracted fully. The internal standard added to the samples prior to extraction will be more available for extraction unlike sterols in the oyster flesh. The increase in sterol concentrations detected seems to show a more complete extraction from the oyster. This will increase the level of detection for samples with low sterol contents. The increased sterol extraction in this experiment may lead not only to recovery of higher concentrations with the new method but also to the detection of sterols in samples that were below the level of detection using the ultasonication extraction method of Knauer *et al* (1998).

#### 8.1.2 Chromatographic techniques.

The GC-FID gave higher sterol concentrations than the GC-MS (Table 8.1.2.) when samples were compared using both GC-MS and GC-FID analysis. There was significant difference ($p<0.05$, paired t-test) for cholesterol and 24-ethyl*epi*coprostanol, but two of the main 5β-stanols, coprostanol and *epi*coprostanol showed no statistical difference between the two analytical tools. Certain sterols particularly coprostanol and *epi*coprostanol are being detected where on the GC-MS no quantifiable peaks are being detected. This resulted in a lower limit of detection on the GC-FID, coelution of compounds is unlikely as no other spectra are being detected on the GC-MS at retention times relating to the selected sterols. Reducibility was significantly better on the GC-FID over the GC-MS, with the standard deviations within samples greatly reduced (up to 63%).

Coprostanol, *epi*coprostanol and 24-ethyl*epi*coprostanol were found in oysters from all four sampling sites (Table 8.1.2), whether this is from human contamination at present is unclear. Coprostanol is known to be produced by cetaceans and some seals (Edwards *et al* 1998) but the effect of these animals seems unlikely due to their low population densities. Seals may inhabit areas close to oyster leases attracted by a possible increase of fish around these artificially nutrient rich waters. Seagulls are also known to produce

coprostanol and these may also be attracted to the leases for the same reasons, and using the leases structures as perches may cause coprostanol levels to be elevated in these areas compared with control areas. Further work needs to be done comparing of 5β-stanols concentrations with microbiological comparisons to check for human pathogens. The potential for seagulls to act as vectors for transmission of human disease must also be considered.

The importance of this method is that for routine analysis of shellfish a reliable analysis tool is needed. The GC-FID gave much more reproducible results over the GC-MS and also GC-FID is a much more accessible, affordable tool for the large proportion of laboratories. The method could lead to quick, reliable and cost effective biomarker monitoring for pathogenic indicators caused by human fecal contamination. This test could be run over a much shorter time period 24 hours from sample receipt to results generation as opposed to 5 days for some comparison methods used in this research. The change from GC-MS and Helium as a carrier gas to Nitrogen on the GC-FID has significantly cut the financial cost in addition to a 66% reduction in solvent usage.

There is a limitation to this method, three of the important 5β-stanols, (see Figs 8.1.3-5 coprostanol, *epi*coprostanol & 24-ethyl*epi*coprostanol) can be quantified whereas 24-ethylcoprostanol appears to be co-eluting with another sterol. Previous work by Teshima *et al* (1980) showed that the four major sterols of the oyster *C. gigas* are (*trans*)-22-dehydrocholesterol, cholesterol, brassicasterol and 24-methylenecholesterol (72% of total sterols). No trimethylsily standards of these sterols were available for a comparison of spectral profiles to confirm this. If these are the four major sterols in theses oysters then 24-methylenecholesterol is co-eluting with any 24-ethylcoprostanol that may be present. Coprostanol to 24-ethylcoprostanol has been used for sourcing faecal pollution in water (Leeming *et al* 1998) although it may be possible to use a ratio of coprostanol with one of the other 5β-stanols that are able to be quantified.

**Table 8.1.1. Selected sterol concentrations of *C. gigas* (µg $g^{-1}$ wet wt±SD)**

| Sample | Coprostanol | | *Epi*coprostanol | | Cholesterol | | 24-Ethyl*epi*coprostanol | |
|---|---|---|---|---|---|---|---|---|
| | **a** | **b** | **a** | **b** | **a** | **b** | **a** | **b** |
| **1** | 0.68±0.22 | 1.56±0.81 | 0.00±0.00 | 0.09±0.29 | 292.5±20.3 | 415.8±137.8 | 1.77±0.18 | 2.85±0.85 |
| **2** | 0.58±0.33 | 1.57±0.51 | 0.00±0.00 | 0.17±0.44 | 332.6±42.3 | 444.9±79.9 | 1.72±0.75 | 3.16±0.67 |
| **3** | 0.03±0.04 | 0.08±0.17 | 0.00±0.00 | 0.00±0.00 | 123.7±8.81 | 180.1±15.9 | 0.25±0.05 | 0.23±0.30 |

a 5±0.05g of the oyster homogenate prepared using the method described by Knauer *et al* 1998

b 5±0.05g proposed method

**Table 8.1.2 Comparison of selected sterol concentrations (µg $g^{-1}$ wet wt±SD) for *C. gigas* using the new protocol**

| Sample | Coprostanol | | *Epi*coprostanol | | Cholesterol | | 24-Ethyl*epi*coprostanol | |
|---|---|---|---|---|---|---|---|---|
| | **GC-MS** | **GC-FID** | **GC-MS** | **GC-FID** | **GC-MS** | **GC-FID** | **GC-MS** | **GC-FID** |
| **1** | 1.56±0.81 | 1.85±0.47 | 0.09±0.29 | 0.34±0.35 | 415.8±137.8 | 422.5±126.8 | 2.85±0.85 | 13.39±3.54 |
| **2** | 1.57±0.51 | 1.70±0.35 | 0.17±0.44 | 0.82±0.49 | 444.9±79.9 | 411.7±85.0 | 3.16±0.67 | 14.84±1.40 |
| **3** | 0.08±0.17 | 0.34±0.16 | 0.00±0.00 | 2.06±1.93 | 180.1±15.9 | 191.29±18.9 | 0.23±0.30 | 3.15±0.30 |
| **4** | 1.58±0.38 | 1.31±0.24 | 0.21±0.09 | 0.73±0.29 | 291.0±41.3 | 208.8±14.9 | 0.83±0.26 | 3.92±0.91 |

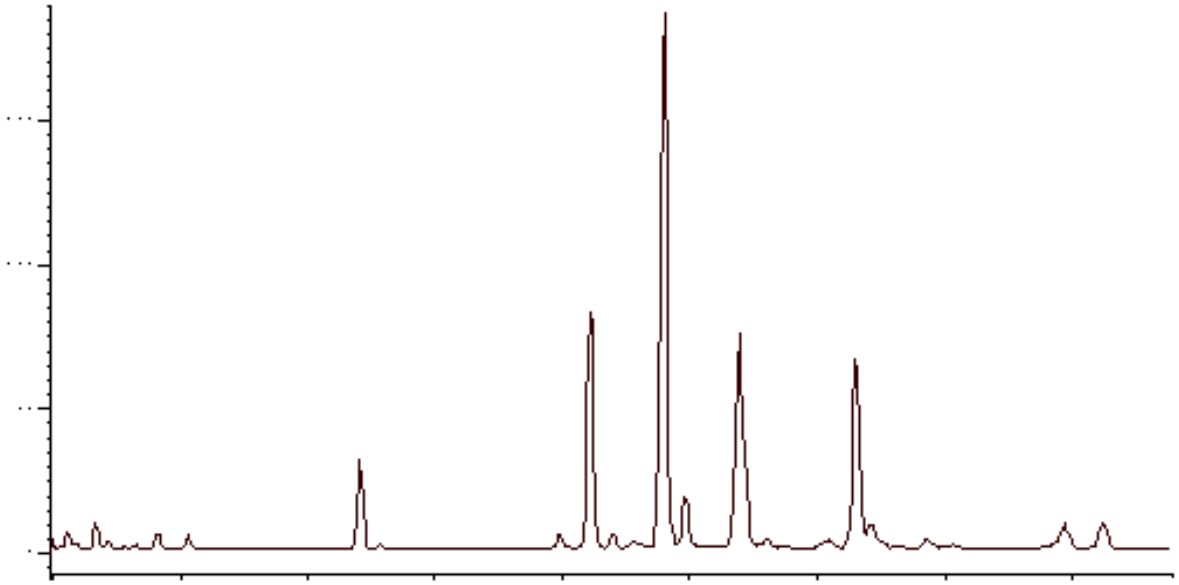

**Figure 8.1.1. GC-MS chromatogram of sterols in *C. gigas* (**1) 5α-Cholestane, (2) Coprostanol, (3) *Epi*coprostanol, (4) Cholesterol, (5) Cholestanol, (6) 24-ethyl*epi*coprostanol

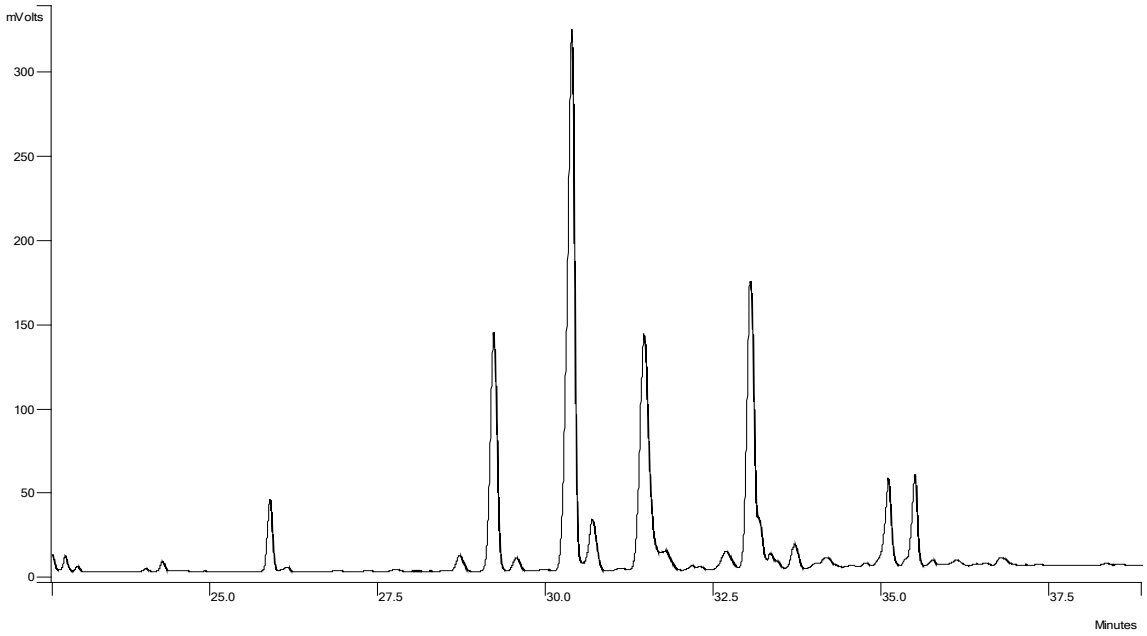


**Figure 8.1.2 GC-FID chromatogram of sterols in *C. gigas*** (1) 5α-Cholestane, (2) Coprostanol, (3) *Epi*coprostanol, (4) Cholesterol, (5)Cholestanol, (6) 24-ethyl*epi*coprostanol

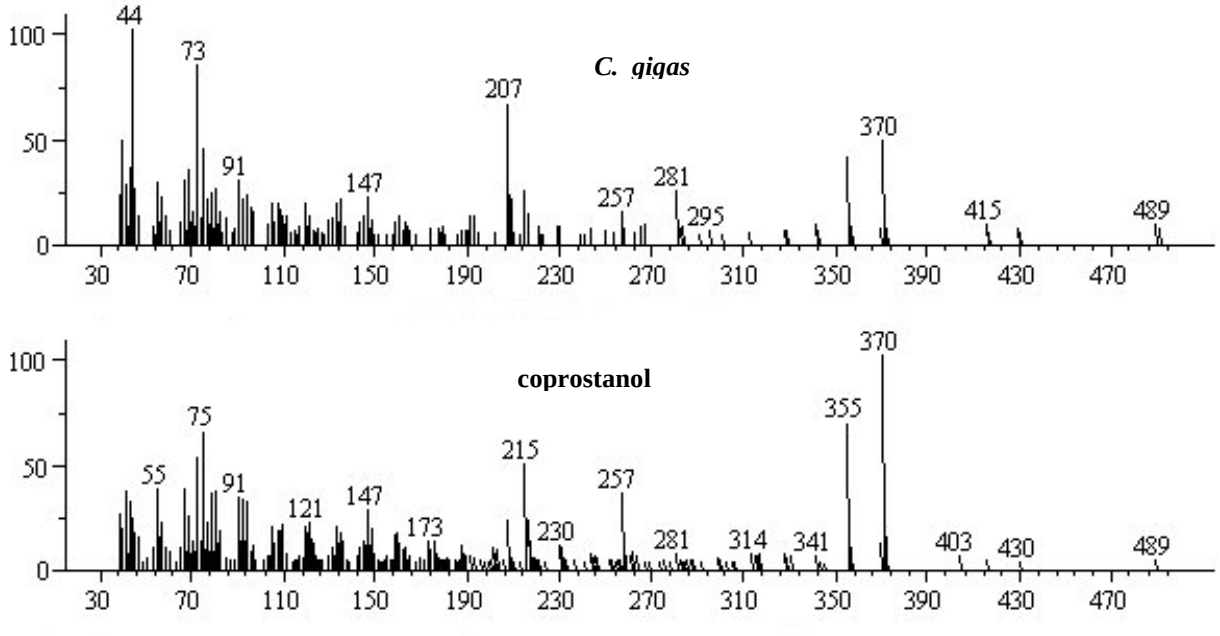


**Fig 8.1.3. Mass spectra of *Crassostrea gigas* sterol and reference sterol, coprostanol**

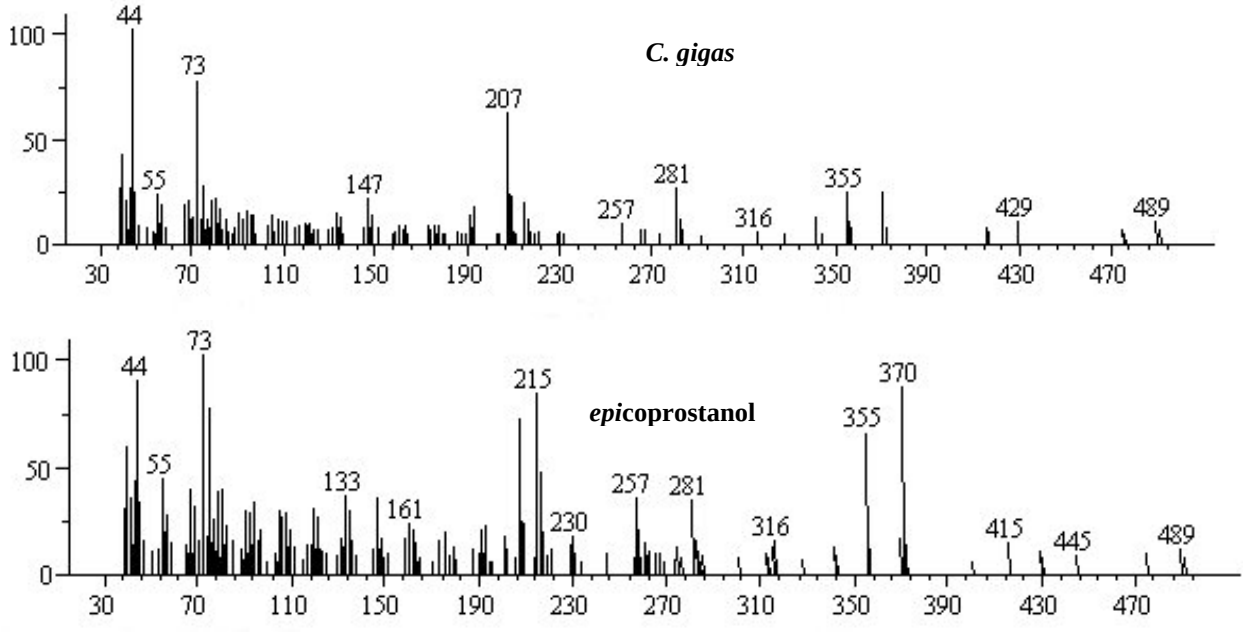


**Fig 8.1.4. Mass spectra of *Crassostrea gigas* sterol and reference sterol, *epi*coprostanol**

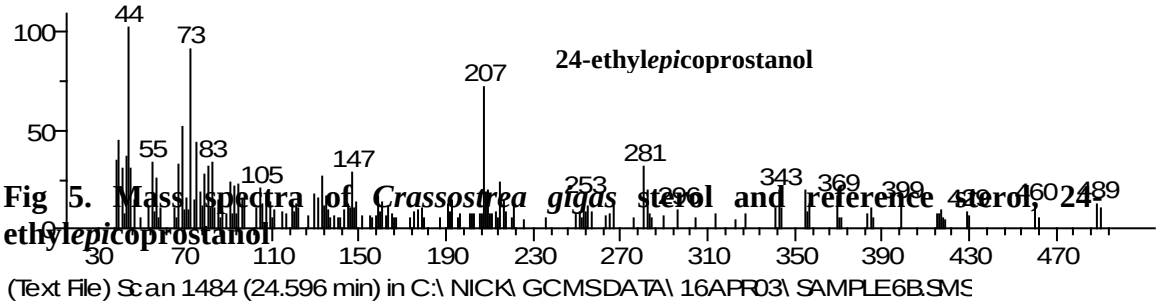


**Fig 5. Mass spectra of *Crassostrea gigas* sterol and reference sterol, 24-ethy*lepic*oprostanol**

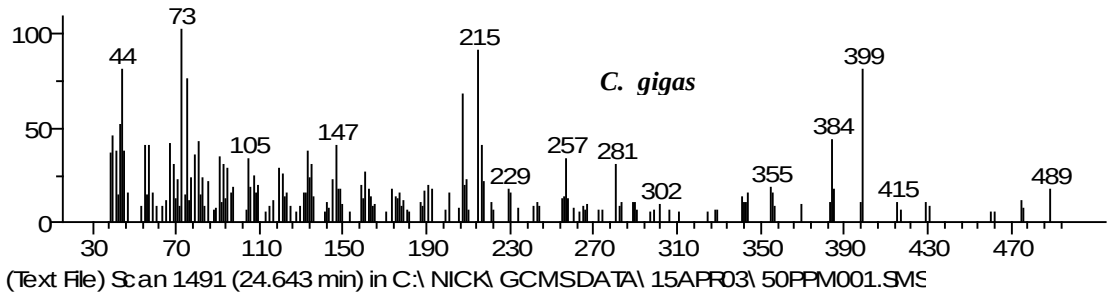


**Fig 8.1.5. Mass spectra of *Crassostrea gigas* sterol and reference sterol, 24-ethy*lepic*oprostanol**

## 8.2 Virus extractions from oyster tissues

A considerable amount of experimental time was spent on investigating alternative extraction methods (1) to maximise the yield of virus from macerated oyster flesh and (2) to produce virus as free as possible from extraneous material which might interfere with subsequent nucleic acid extraction and PCR reactivity.

The initial method by Traore et al (1998) gave reasonably clean preparations of viral RNA, as judged by gel electrophoresis of the poliovirus PCR product (Fig 8.2.1), but extraction from more than a minimal amount of oyster flesh produced heavily-smeared gels which reduced assay sensitivity (Fig 8.2.2). Attempts to make cleaner extracts with increased centrifugation speeds (to 10,000g) were unsuccessful (Fig 8.2.3). Similarly detection of the PCR product by Enzyme Immunoassay, using a Boehringer Digoxygenin labelling kit, instead of gel electrophoresis failed to improve matters (Fig 6). Passing the resuspended PEG precipitate through a Sephacryl S-300 column to exclude macromolecules did not make any difference to the clarity of the gels post PCR either (Fig 8.2.4).

Major progress with this bottleneck was finally made when the RNA extraction protocol was switched from Trizol to a proprietary silica gel process (Qiagen RNeasy Minikit), in which the crude material is passed through a column of immobilised silica, allowing RNA specifically to bind to it. The RNA is then eluted off the column, relatively free of contaminants. This allowed a 14-fold increase in the amount of oyster flesh which could be extracted and still produce clean gels (Fig 8.2.5)

Further improvements in the extraction protocol were suggested in a paper by a New Zealand group (Lewis *et al* 1996) which found that repeating the PEG precipitation allowed for a greater amount of flesh to be extracted initially without any loss of virus from the additional clean-up step. This was tested with both poliovirus and phiX174. Alternative incubation times for the precipitation steps were also examined, as there was evidence that virus would dissociate from the precipitate if it were left for too long. The polio data was hard to interpret because both intermediate and final products were frequently toxic to the cell culture in which the infectivity assays were performed. However it appeared that with 2 hour and 18 hour incubations for the first and second precipitations respectively, there was minimal loss of virus from the second PEG precipitation, while the amount of flesh being extracted which still gave a good band on the gel could be increased about 3-fold (Fig 8.2.6). For phiX174 the second precipitation resulted in a loss of about 60%, based on plaque assays of retained virus (Table 8.2.1).

The final refinement to the extraction protocol was to identify where virus became localised following bioaccumulation, so that only potentially infected tissue is used in testing. Oysters were exposed to phiX174 in a tray of sea water and harvested after 3 days. Plaque assay on the dissected internal structures showed that about 85% of detectable virus had been retained in the mantle and adductor muscle which together represented about 27% of total oyster tissue (Table 8.2.2). This dissection step therefore produced an effective increase in sensitivity of a further 3-fold by concentrating on relevant tissue only.

Putting these various steps together, recovery of these two viruses from oysters that had been allowed to bioaccumulate them in tank experiments was tested. In the case of phiX174, 60-65% of virus present in the initial dissected tissue was present in the final extract (by plaque assay) and a detection limit of 2-3 Plaque-Forming Units per oyster was determined by nested PCR. For poliovirus, the cell culture assays were again unreliable because of toxicity problems, but the limit of detection by nested PCR is less than 1 tissue culture infectious unit per oyster, based on the infectivity count from the initial oyster homogenate (Table 8.2.3).

Fig 8.2.1 : Gel photo of poliovirus PCR product from single-round PCR, comparing different amounts of virus in buffer and seeded in macerated oyster flesh. Outer lanes contain 50 base-pair molecular weight ladder for size determination; lanes next to them contain positive and negative controls.

+ve Oyster Buffer -ve

50bp PCR Oys Buff $10^3$ $10^2$ 10 1.2 0 $10^3$ $10^2$ 10 1.2 0 QC PCR 50bp

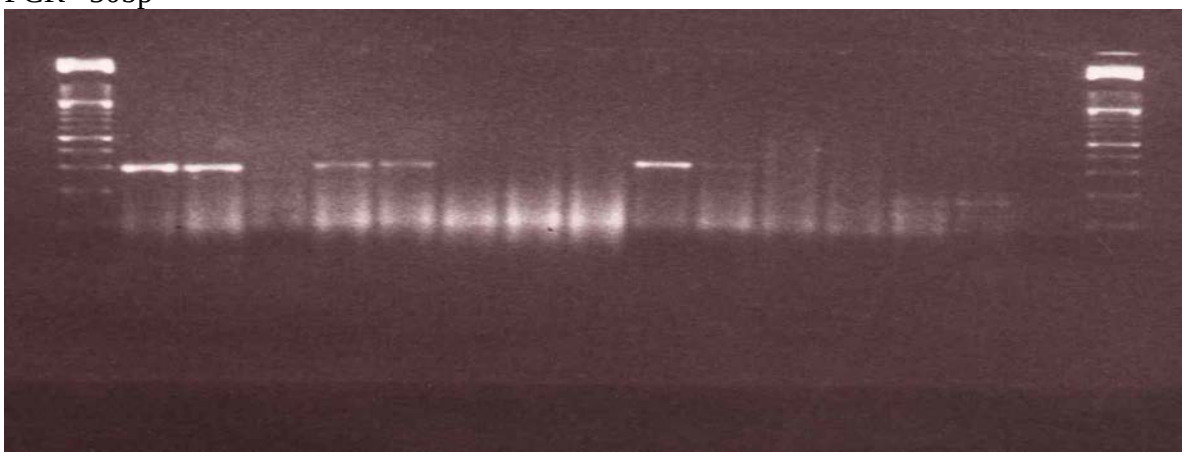

Fig 8.2.2 : Gel photo of poliovirus PCR product from single-round PCR, showing effect of extracting different amounts and volumes of oyster homogenate on PCR assay. Controls and M.W. ladders as before

50bp PCR+ 0.18g 1.06g 1.24g oys- 5ml 30ml 40ml buf- PCR- 50bp

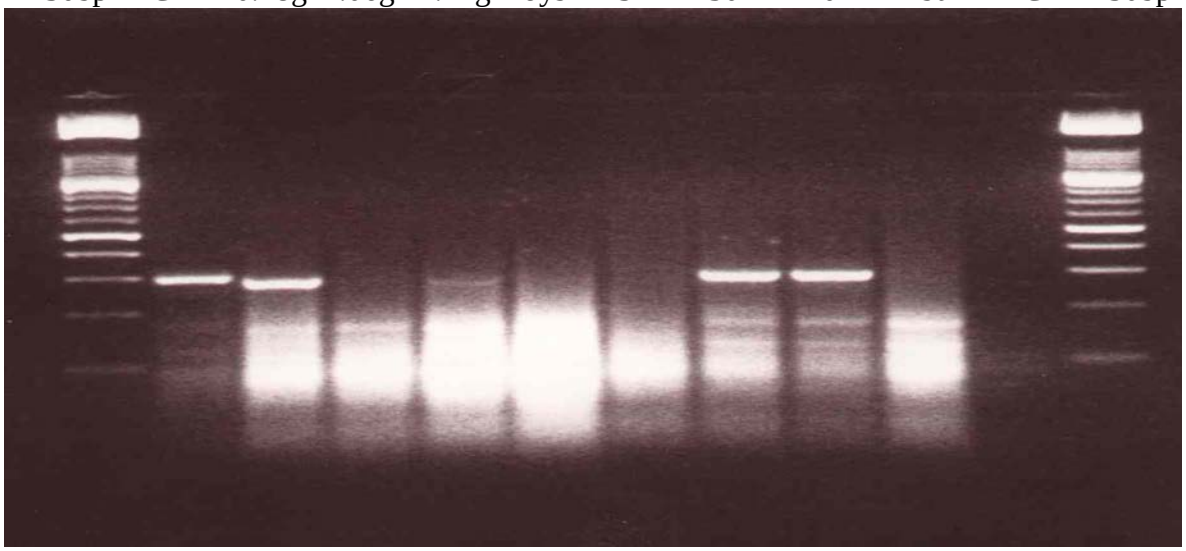

Fig 8.2.3 : Gel photo of poliovirus PCR product from single-round PCR, showing effect of changing centrifugation speeds during extraction on PCR assay with differing amounts of virus added Controls and M.W. ladders as before.

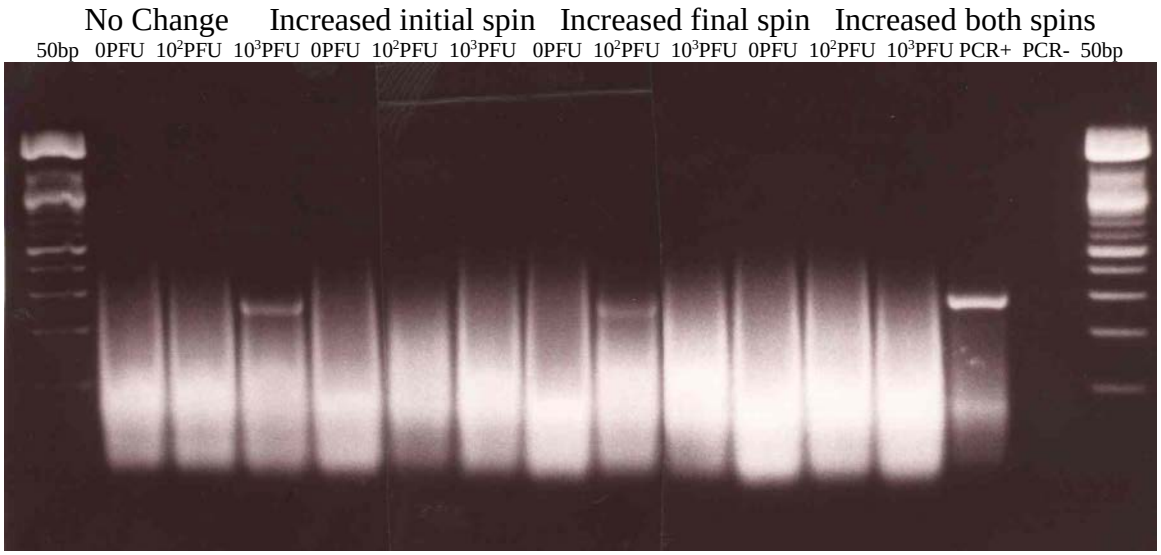


Fig 8.2.4 : Gel photo of poliovirus PCR product from single-round PCR, showing effect of passing oyster extract (containing different amounts of virus) through Sephacryl S-300 column prior to submitting to PCR assay Controls and M.W. ladders as before.

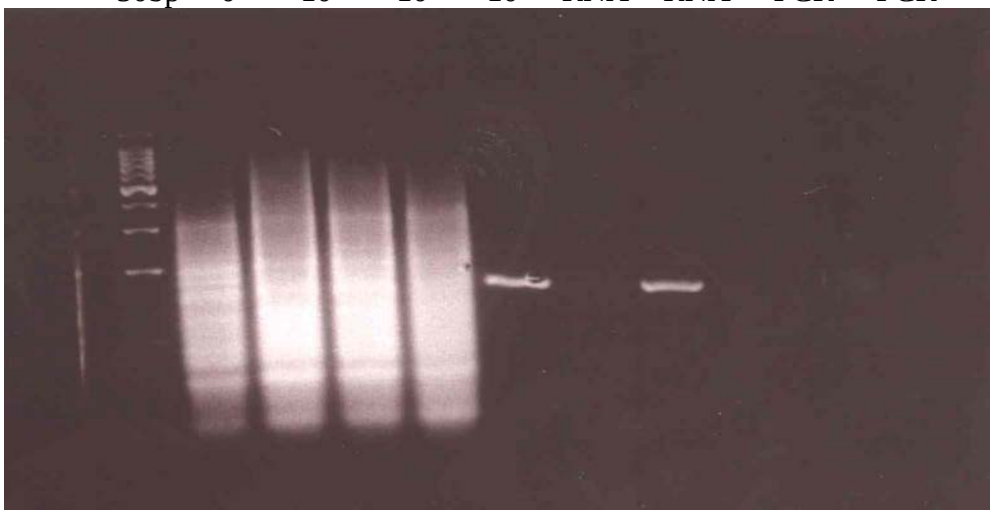

Fig 8.2.5 : Gel photo of poliovirus PCR product from single-round PCR, showing effect of extracting different amounts of oyster flesh with Qiagen RNeasy Plant Minikit. 2mL lane represents 5gm of oyster flesh. Controls and M.W. ladders as before.

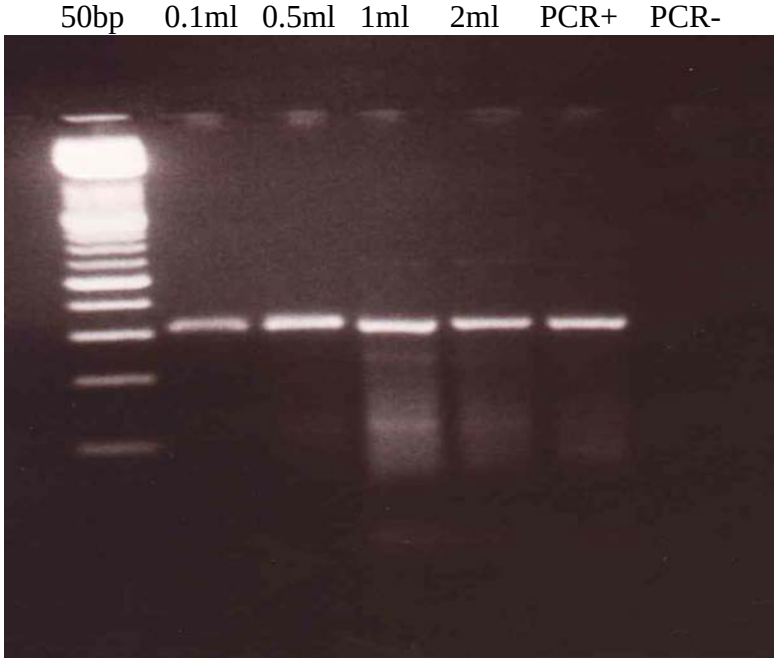


Fig 8.2.6 : Gel photo of poliovirus PCR product from single-round PCR, showing permissible load of double PEG precipitated oyster extract following extraction with RNeasy Plant Minikit. Volume of 0.5mL represents 13.3g of extract. Controls and M.W. ladders as before.

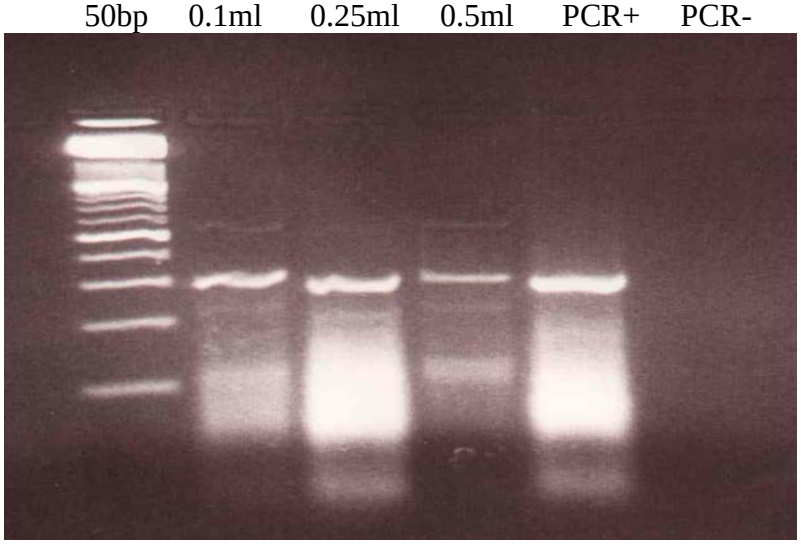

Table 8.2.1 : Oyster homogenate was seeded with a known concentration of phiX174. Virus was extracted with double PEG precipitation, aliquots being collected at each step of the process. Each sample was tested in triplicate by plaque assay to determine amount of virus present. (discard = supernatant, Retain = retained pellet).

| Extraction stage | Raw data – Total number of PFU remaining in extract (PFU * $10^5$) | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | A | | | B | | | C | | |
| | Discard | Retain | Total | Discard | Retain | Total | Discard | Retain | Total |
| Initial | **0** | **20.7** | **20.7** | **0** | **26.9** | **26.9** | **0** | **20** | **20** |
| Spin 1 | **1.24** | **21** | **22.24** | **0.222** | **13** | **13.222** | **1.05** | **16.1** | **17.15** |
| PEG ppt 1 | **0.0134** | **16.8** | **16.8134** | **0.055** | **16.4** | **16.455** | **0** | **17** | **17** |
| Spin 2 | **3.35** | **17.6** | **20.95** | **2.33** | **14** | **16.33** | **6.55** | **15.7** | **22.25** |
| PEG ppt 2 | **0.229** | **6.33** | **6.559** | **0.145** | **5.34** | **5.485** | **0.021** | **6.3** | **6.321** |

Table 8.2.2 : 10 oysters were placed in 10L of sea water (containing 2x $10^5$ PFU) in a tray and allowed to bioaccumulate the virus for 3 days. Three oysters were dissected (in triplicate) and amount of virus in each organ was determined by plaque assay.

| **Oyster tissues** | **Total number of PFU in dissected oyster tissues** | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | **A** | | **B** | | **C** | | **Mean %** | **Standard Deviation** |
| | **PFU*$10^4$** | **% of total** | **PFU*$10^4$** | **% of total** | **PFU*$10^4$** | **% of total** | | |
| **Gills** | **0.25** | **1.59** | **0.24** | **1.39** | **0.28** | **1.64** | **1.54** | **0.18** |
| **Mantle** | **10.39** | **65.26** | **11.75** | **68.12** | **11.14** | **65.34** | **66.24** | **1.97** |
| **Internal organs** | **2.25** | **14.13** | **2.39** | **13.86** | **1.99** | **11.67** | **13.22** | **1.55** |
| **Adductor** | **3.03** | **19.02** | **2.87** | **16.64** | **3.64** | **21.35** | **19** | **3.33** |
| **Total oyster** | **15.92** | **100** | **17.25** | **100** | **17.05** | **100** | **100** | **0** |

Table 8.2.3 : 9 oysters were added to each of 7 trays containing 10L of sea water containing serial 10-fold dilutions of poliovirus. Oysters were harvested after 1 day, digestive diverticulum was dissected out and extracted with double PEG precipitation. Viral extract was assayed by both cell culture and nested RT-PCR. Virus was detected by PCR in only the most dilute sample for reasons that are unclear. This does not affect interpretation of the result.

| **Sample** | **Total Poliovirus infectious units in oyster tissue** | | | **PCR Result** |
|---|---|---|---|---|
| | **Inoculum (water)** | **Initial oyster** | **Final eluate** | |
| **C** | **0** | **0** | **0** | **-** |
| **$10^{-5}$** | **4.64** | **0** | **1700** | **+** |
| **$10^{-4}$** | **46.4** | **0** | **2700** | **-** |
| **$10^{-3}$** | **464** | **0** | **83700** | **-** |
| **$10^{-2}$** | **4640** | **0** | **551700** | **-** |
| **$10^{-1}$** | **46400** | **0** | **590400** | **-** |
| **Neat** | **464000** | **643500** | **576900** | **-** |

### 8.3 Bacteriophage assays

Results of bacteriophage plaque culture assays are detailed in section 8.5 below and in Appendix 6

### 8.4 Adenylate Charge Investigations

Previous studies of bivalves (*M. edulis*, blue mussel) colonizing tuna pens at Port Lincoln, SA, revealed significant stress-response impacts on energy balance, notably adenylate energy charge and inosine monophosphate load (IMPL). These indices were found to be sensitive to poor water quality in tuna pens characterized by accumulated ammonium derived from tuna excreta and decomposed feed. These findings were able to be replicated in aquarium experiments in which mussels were exposed to ammonium added to water. In the current project we had intended to examine the impact of degraded water quality, particularly those with faecal inputs, on energy balance indictors in farmed oysters. The sampling protocol required fresh oyster samples to be immediately frozen in liquid nitrogen-cooled tongs, and to be packaged and stored in liquid nitrogen prior to sample preparation and analysis by high performance liquid chromatography. Oyster samples in this project were obtained opportunistically from field sites at Port Lincoln, SA. These were then transported on ice (4C) to our laboratory for microbial analysis. While this sampling protocol was satisfactory for the reliable enumeration of bacteria and virus, we were unable to provide for field sampling facilities adequate for sample preparation to prevent the degradation of tissue adenylates during sampling and transport. Aquarium studies planned for this project would have involved the application of faecal-contaminated water supplies to oysters. However, Occupational Health and Safety considerations (relating to the potential exposure of personnel to aerosolized pathogens from aquaria) did not permit this part of the project to proceed. For these reasons, the energy balance/stress

response components of this project were abandoned. We hope that this part of the project design may be further developed in a subsequent research proposal.

### 8.5 Field Sampling and predictive capacity of PCR methods for potentially infective shellfish

The results of field sampling on shellfish are presented at Appendix 6. A key aim of this project was to establish techniques for the accurate determination of microbial and viral contamination in farmed oyster. The sensitive tests developed for testing oyster contamination were analysed together with the standard indicators of risk to determine whether risk of enterovirus exposure was predicted by microbial assays.

Contingency tables were completed for associations between presence or absence of any enteric virus (including Norwark, hepatitis A, astrovirus, adenovirus) by PCR and presence of absence of *E. coli*, bacteriophage, faecal coliforms, enterococci. Positive or negative outcomes on microbiological testing was based on arbitrary cut-off points for number of colonies identified. For example, the industry standard for *E. coli* contamination is 7 MPN/g. Presence or absence of *E. coli* is defined as $\geq$7 MPN/g and <7 MPN/g respectively. In this way, the predictive capacity of the enteric virus PCR method is also examined with more stringent microbiological cut-off values, e.g. >1 MPN/g and <1 MPN/g.

The data analysis format is shown in the Table 8.5.1. below, using *E. coli* and viral contamination as an example. The figures represent the number of oyster samples yielding positive and negative results in each of the indicator tests. The contingency test used was the Fisher Exact Test, since in all cases, at least one expected observation was less than 5. Sensitivity, specificity, positive and negative predictive values and the likelihood ratio were also determined from tests yielding a significant association in the contingency table. Notes defining these parameters are included in Appendix 4. Complete tables are included in Appendix 5.

**Table 8.5.1 Fisher Exact Test correlation tables.**

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥7MPN** | 2 | 2 | *4* |
| **E. coli <7MPN** | 3 | 24 | *27* |
| *column totals* | *5* | *26* | *31* |

Fisher Exact Test, two-sided, p=0.112 – poor association

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥1MPN** | 3 | 3 | *6* |
| **E. coli <1MPN** | 2 | 23 | *25* |
| *column totals* | *5* | *26* | *31* |

Fisher Exact Test, two-sided, p=0.038 – good association
sensitivity – 0.600
specificity – 0.885
positive predicitive value – 0.500
negative predictive value – 0.920
Likelihood ratio – 5.200

**Table 8.5.2 Associations between positive tests of enteric viruses and microbiological oyster quality indices**

| **Microbiological endpoint** | **Fisher Exact Test p** | **Sensitivity** | **Specificity** | **Predictive value** | | **Likelihood ratio** |
|---|---|---|---|---|---|---|
| | | | | **+ve** | **-ve** | |
| *E. coli* (7 MPN/g) | 0.112 | 0.400 | 0.923 | 0.50 | 0.89 | 5.200 |
| *E. coli* (7 MPN/g) (excluding adenovirus) | 0.349 | 0.333 | 0.893 | 0.25 | 0.93 | 3.111 |
| *E. coli* (1 MPN/g) | 0.038 | 0.600 | 0.885 | 0.50 | 0.92 | 5.200 |
| *E. coli* (1 MPN/g) (excluding adenovirus) | 0.088 | 0.667 | 0.857 | 0.33 | 0.96 | 4.667 |
| Phage (50 pfu/g) | 0.172 | 0.200 | 1.000 | 1.00 | 0.86 | * |
| Phage (50 pfu/g) (excluding adenovirus) | 0.103 | 0.333 | 1.000 | 1.00 | 0.93 | * |
| Faecal coliform (1 MPN/g) | 0.060 | 0.800 | 0.692 | 0.33 | 0.95 | 2.600 |
| Faecal coliforms (1 MPN) (excluding adenovirus) | 0.049 | 1.000 | 0.679 | 0.25 | 1.00 | 3.111 |

These data show that the measurement of *E. coli* and faecal coliforms are the most associated with the detection of enteric viruses in oysters (Table 8.5.2). When the Food

Standards cut-off for *E. coli* in oysters (7 MPN/g) is used, there is a poor association, but when a more stringent arbitrary cut-off is applied (1 MPN/g) there is a strong association. This association remains relatively strong when adenovirus assessment is omitted from the enteric virus measurements. The PCR primers used for adenovirus detect all adenovirus forms, including those causing gastrointestinal infection and conjunctivitis in bathers. We have not enough information to confirm whether PCR detection of adenovirus using these primers should be considered a predictor of potential pathogenic virus or as a test confirming presence of pathogen and hence risk.

While sensitivities and specificities of the *E.coli* (1 MPN/g) and faecal coliforms predictors are reasonably high, they each have weak predictive value, successfully identifying virus positive oysters in 50% or fewer of cases. However, they successfully confirm the absence of virus in negative oysters in 90-100% of cases This may be a consequence of the small number of samples overall (31) and the small number of virus positive samples available for this analysis (up to 5). These data are promising, and further work is necessary to establish the utility of these tests for predicting viral contamination of oysters.

## Benefits and adoption

This research specifically benefits commercial growers and exporters of pacific oysters (*Crassostrea gigas*). A quantifiable market value is not determinable at this point in time. Recent outbreaks of disease in Japan and South East Asia have prompted importers of oysters to request evidence of the microbial quality of the product. It is anticipated that in the near future this may become a significant hurdle for growers and exporters alike in maintaining their international markets. Since commencement of this project the value of coliform estimations, or surrogates for viral contamination to the market has diminished in favour of reliable and sensitive techniques for detection of specific viral pathogens. This project has successfully developed techniques which are both sensitive and specific for viral pathogens. Limits of detection established so far suggest that quantitative human health risk assessment for shellfish may be made in the near future.

South Australia and Tasmania are the principal growers and exporters of Pacific oysters. This research has provided quality assurance tools that may be adopted to meet the requirements of their export markets. Further refining and confirmatory testing of this work will be necessary before a quality assurance program can be fully developed and applied.

**Further Development**

A number of issues regarding this research remain unresolved:

1) Analysis of Sydney Rock oysters and Pearl Oysters with known contaminations were negative for viruses using the methods applied to Pacific oysters. In one instance the PCR method was able to detect Norovirus in a human faecal specimen, but not in a pearl oyster associated with a foodborne disease outbreak. PCR analyses on Blue Mussels were unsuccessful. It seems likely that the failure of the PCR method was due to a sub-optimal DNA/RNA extraction procedure. Further work is required to develop procedures that will permit successful identification and quantitation of virus in these species.

2) Successful and sensitive detection of viral pathogens has broader application than the shellfish program, and application of these techniques to other export produce may be worthwhile in the future.

3) The Coliform and Phage sampling date suggested that current (coliform based) methods are reasonably sensitive predictors of viral contamination when positive test results are recorded. The data suggest that negative test results will not pr3dict the absence of viral particles. The data also suggested that phage estimations were even more sensitive positive predictors and even less sensitive as negative predictors. Development of a standard phage estimation method may be an extremely useful and simple supplement to routine quality assurance tool. The relative merits of somatic and $HF^{+}$ phage in predicting viral contamination needs to be assessed. These methods must also be validated for the various catchment waters (estuarine or oceanic) where oysters are cultivated.

4) Faecal sterol determinations on contaminated oysters provided insufficient data to evaluate the merits of the method. A more rapid, technically less complex and more sensitive method was developed. Estimation of the value of this method was hampered by the inability to source contaminated oysters. Correlations between these analyses and microbial contaminants were not possible. A useful further development would be to analyse the correlations and sensitivities of the entire suite of techniques using artificially contaminated shellfish in controlled aquarium conditions. Dilutions of wastewater as contamination inocula could be used to estimate relative sensitivities and predictive powers of each technique.

5) This project was limited by the lack of access to contaminated shellfish. It would be valuable to the industry to investigate detection of viruses in deliberately contaminated oysters under controlled conditions. Facilities with suitable conditions and provisions for the containment of virus would be necessary.

**Planned Outcomes**

**Outcome 1. Development of sensitive and specific analytical techniques for risk assessment of proposed sites for oyster cultivation.**

This project successfully developed faecal sterol and viral detection methods. Both of these methods provided greater sensitivity in detection of contamination than has previously been available. The project also identified that current coliform estimations are generally reliable predictors, and that positive coliphage estimations may be an even stronger predictor of enteric virus contamination.

**Outcome 2. Development of rapid cost effective and robust analytical tools as alternatives to PCR for routine quality assurance of farmed oysters and thereby facilitate industry development and product safety.**

This project successfully identified that current coliform methods were sensitive predictors of viral contamination. The project also identified that positive coliphage test results were the most sensitive predictor of the presence of enteric viruses.

**Outcome 3. A suite or tests for rapid identification of water catchments suitable for oyster farming and identification of closure safety zones.**

This project successfully developed a suite of test which could be used to identify the water quality of proposed catchments. Sensitivity of these tests was such that it they could be used in conjunction to rapidly identify suitability of proposed leases.

**Outcome 4. Validation of monitoring techniques such as coliphage enumeration, as routine quality assurance tools specific to Pacific oysters grown in South Australian waters**

This project successfully identified the coliphage plaque assay as a strong predictor of the presence of enteric viruses. The poor sensitivity of the method made it a less reliable quality assurance tool than coliform tests. Changes in the requirements of the export markets may mean that these tests, though valid, will be insufficient as stand alone quality assurance tools.

**Outcome 5. Development of routine sensitive analytical techniques to detect the presence of enteric viruses in water and shellfish from shellfish harvesting areas in South Australia.**

This project successfully developed PCR methods for detection of Hepatitis A, Norovirus (G2), Enterovirus, Adenovirus and Astrovirus in pacific oysters. Provisional determinations indicate that a single viral particle per oyster is a realistic detection limit of these methods. Detection limits in seawater may be enhanced further by ultrafiltration.

**Outcome 6. Provide overseas markets assurance that shellfish from South Australia are free from enteric viruses.**

Further work will be necessary to provide a reliable quality assurance program that would satisfy the requirements of overseas markets. However, the techniques are now available that would permit development of such quality assurance program.

## Conclusions

This project successfully developed a suite analytical techniques for the detection of faecal contamination in farmed pacific oysters. Novel detection methods for faecal sterols, and a range of enteric viruses were major outputs from the research. Detection limits for both sterols and virus were low enough for these techniques to be used for routine quality assurance. These outcomes met the original objectives 1, 3 and 7 (p12).

Field sampling of a limited number of contaminated shellfish indicated that the current faecal coliform methods are weak predictors of the presence of enteric viruses in positive samples. However, negative coliform test results are strong predictors of the absence of enteric viruses. Coliphage estimations had a low sensitivity of detection of enteric virus, but positive results were strongly correlated to enteric virus detection. As a result, the coliform estimations appear a more suitable routine testing method. These outcomes met original objectives 2, 5, 6 and 8 (p12). It can be concluded that the most reliable indicator for the presence of enteric viruses would e direction detection of the organisms. This work provided methodology that may achieve this goal at detection limits relevant to human health risk criteria.

The research identified that the biochemical stress marker (adenylate charge) was probably not applicable as a routine assessment due to the technical requirements in sampling collection and processing. This research direction was discontinued in the project which meant that objective 4 (p12) was not addressed.

The project met all of planned outcomes in varying degrees. The problems of sourcing contaminated shellfish for statistical and analytical validation of techniques meant that further work will be necessary to develop the tools to the extent where they could be applied in quality assurance. Future work involving direct contamination of oysters or various species is likely to yield valuable validation techniques for the aquaculture and export industries. Such work was not within the scope of this project, as the initial focus was on naturally contaminated shellfish.

## References


Abbaszadegan, M., P. Stewart, et al. (1999). "A strategy for detection of viruses in groundwater by PCR." Appl Environ Microbiol 65(2): 444-9.

Allard, A., B. Albinsson, et al. (1992). "Detection of adenoviruses in stools from healthy persons and patients with diarrhea by two-step polymerase chain reaction." J Med Virol 37(2): 149-57.

Allard, A., R. Girones, et al. (1990). "Polymerase chain reaction for detection of adenoviruses in stool samples." J Clin Microbiol 28(12): 2659-67.

Ando, T., S. S. Monroe, et al. (1995). "Detection and differentiation of antigenically distinct small round-structured viruses (Norwalk-like viruses) by reverse transcription-PCR and southern hybridization." J Clin Microbiol 33(1): 64-71.

Arnal, C., V. Ferre-Aubineau, et al. (1999). "Comparison of seven RNA extraction methods on stool and shellfish samples prior to hepatitis A virus amplification." J Virol Methods 77(1): 17-26.

Arnal, C., V. Ferre-Aubineau, et al. (1999). "Quantification of hepatitis A virus in shellfish by competitive reverse transcription-PCR with coextraction of standard RNA." Appl Environ Microbiol 65(1): 322-6.

Atmar, R. L., F. H. Neill, et al. (1995). "Detection of Norwalk virus and hepatitis A virus in shellfish tissues with the PCR." Appl Environ Microbiol 61(8): 3014-8.

Atmar, R. L., F. H. Neill, et al. (1996). "Collaborative evaluation of a method for the detection of Norwalk virus in shellfish tissues by PCR." Appl Environ Microbiol 62(1): 254-8.

Avellon, A., P. Perez, et al. (2001). "Rapid and sensitive diagnosis of human adenovirus infections by a generic polymerase chain reaction." J Virol Methods 92(2): 113-20.

Avery, E. L., R. H. Dunstan, et al. (1998). "The use of Lipid Metabolic Profiling to Assess the Biological Impact of Marine Sewage Pollution." Archives of Environmental Contamination and Toxicology 35: 229-235.

Belliot, G., H. Laveran, et al. (1997). "Detection and genetic differentiation of human astroviruses: phylogenetic grouping varies by coding region." Arch Virol 142(7): 1323-34.

Berenberg, C. J. and G. W. Patterson (1981). "The Relationship between Dietary Phytosterols and the Sterols of Wild and Cultivated Oysters." Lipids 16(4): 276-278.

Bligh, E. G. and W. J. Dyer (1959). "A rapid method for total lipid extraction and purification." Canadian Journal of Biochemistry and Physiology 37: 911-917.

Bon, F., P. Fascia, et al. (1999). "Prevalence of group A rotavirus, human calicivirus, astrovirus, and adenovirus type 40 and 41 infections among children with acute gastroenteritis in Dijon, France." J Clin Microbiol 37(9): 3055-8.

Börjesson, E., A. Sundin, et al. (1998). "New method for determination of fecal sterols in urine using non-chlorinated solvent." Journal of Chromatography 713: 438-442.

Brown, R. C. and T. L. Wade (1984). "Sedimentary coprostanol and hydrocarbon distribution adjacent to a sewage outfall." Water Research 18(5): 621-632.

Buonagurio, D. A., J. W. Coleman, et al. (1999). "Direct detection of Sabin poliovirus vaccine strains in stool specimens of first-dose vaccinees by a sensitive reverse transcription-PCR method." J Clin Microbiol 37(2): 283-9.

Burkhardt, W., 3rd, G. M. Blackstone, et al. (2002). "Applied technique for increasing calicivirus detection in shellfish extracts." J Appl Microbiol 93(2): 235-40.

Cabrini, L., L. Landi, et al. (1992). "Extraction of Lipdis and Lipophillic Anitoxidants from Fish Tissues: A Comparison Among Different Mehtods." Comparative Biochemisrty & Physiology 101B(3): 383-386.

Casas, N. and E. Sunen (2001). "Detection of enterovirus and hepatitis A virus RNA in mussels (Mytilus spp.) by reverse transcriptase-polymerase chain reaction." J Appl Microbiol 90(1): 89-95.

Castignolles, N., F. Petit, et al. (1998). "Detection of adenovirus in the waters of the Seine River estuary by nested-PCR." Mol Cell Probes 12(3): 175-80.

Cauchi, M. R., J. C. Doultree, et al. (1996). "Molecular characterization of Camberwell virus and sequence variation in ORF3 of small round-structured (Norwalk-like) viruses." J Med Virol 49(1): 70-6.

Centers for Desease Control and Prevention, C. (2001). "Norwalk-like Viruses: Public Health Consequences and Outbreak Management." Morbidity and Mortality Weekly Report 50(RR-9).

Chan, K.-H., M. H. W. Lam, et al. (1998). "Application of sedimentary fecal stanols and sterols in tracing sewage pollution in coastal waters." Water Research 32(1): 225-235.

Christie, W. W. (1993). "Extraction of lipids from samples." Lipid Technology 5: 18-19.

Chung, H., L. A. Jaykus, et al. (1996). "Detection of human enteric viruses in oysters by in vivo and in vitro amplification of nucleic acids." Appl Environ Microbiol 62(10): 3772-8.

Cohen, J. I., J. R. Ticehurst, et al. (1987). "Complete nucleotide sequence of wild-type hepatitis A virus: comparison with different strains of hepatitis A virus and other picornaviruses." J Virol 61(1): 50-9.

Conaty, S., P. Bird, et al. (2000). "Hepatitis A in New South Wales, Australia from consumption of oysters: the first reported outbreak." Epidemiol Infect 124(1): 121-30.

Croci, L., D. De Medici, et al. (2000). "Determination of enteroviruses, hepatitis A virus, bacteriophages and Escherichia coli in Adriatic Sea mussels." J Appl Microbiol 88(2): 293-8.

Cromeans, T. L., O. V. Nainan, et al. (1997). "Detection of hepatitis A virus RNA in oyster meat." Appl Environ Microbiol 63(6): 2460-3.

Csallany, S. A. and L. K. Ayaz (1976). "Quantitative Determination of Organic Solvent Soluble Lipofuscin Pigments in Tissues." Lipids 11(5): 412-417.

Csallany, S. A., S. E. Kingdom, et al. (1989). "HPLC Method for Quantitation of Cholesterol and Four of its Major Oxidation Products in Muscle and Liver Tissues." Lipids 24(7): 645-651.

Desenclos, J. C., K. C. Klontz, et al. (1991). "A multistate outbreak of hepatitis A caused by the consumption of raw oysters." Am J Public Health 81(10): 1268-72.

Dore, W. J., K. Henshilwood, et al. (2000). "Evaluation of F-specific RNA bacteriophage as a candidate human enteric virus indicator for bivalve molluscan shellfish." Appl Environ Microbiol 66(4): 1280-5.

Dore, W. J. and D. N. Lees (1995). "Behavior of Escherichia coli and male-specific bacteriophage in environmentally contaminated bivalve molluscs before and after depuration." Appl Environ Microbiol 61(8): 2830-4.

Dougan, J. and L. Tan (1973). "Extraction and Quatitative Measurement of Fecal Water Pollution Using a Solid-Injection Gas Chromatographic Technique and Fecal Sterols as a Chemical Index." Journal of Chromatography 86: 107-116.

Dutka, B. J. and A. El-Shaarawi (1975). "Relationship between various bacterial populations and coprostanol and cholesterol." Canadian Journal of Microbiology 21: 1386-1398.

Edwards, D. D., G. A. McFeters, et al. (1998). "Distribution of Clostridium perfringens and fecal sterols in a benthic coastal marine environment influenced by the sewage outfall from McMurdo station, Antarctica." Applied and Environmental Microbiology 64(7): 2596-2600.

Enriquez, R., G. G. Frosner, et al. (1992). "Accumulation and persistence of hepatitis A virus in mussels." J Med Virol 37(3): 174-9.

Estes, M. K., J. M. Ball, et al. (2000). "Norwalk virus vaccines: challenges and progress." J Infect Dis 181 Suppl 2: S367-73.

Estes, M. K. and M. E. Hardy (1995). Norwalk Virus and Other Entric Caliciviruses. Infections of the Gastrointestinal Tract. M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg and R. L. Guerrant. New York, Raven Press, Ltd.: 1009-1034.

Farkas, T., X. Jiang, et al. (2000). "Prevalence and genetic diversity of human caliciviruses (HuCVs) in Mexican children." J Med Virol 62(2): 217-23.

Farrah, S. R., S. M. Goyal, et al. (1978). "Concentration of poliovirus from tap water onto membrane filters with aluminum chloride at ambient pH levels." Appl Environ Microbiol 35(3): 624-6.

Férézou, J., E. Gouffier, et al. (1978). "Daily Elimination of Faecal Sterols by Humans." Digestion 18: 201-212.

Ferguson, C. M., B. G. Coote, et al. (1996). "Relationship between indicators, pathogens and water quality in an estuarine system." Water Research 30(9): 2045-2054.

Folch, J., M. Lees, et al. (1957). "A Simple Method for the Isolation and Purification of Total Lipides from Animal Tissues." Journal of Biological Chemistry 226: 497-509.

Gee, A. J., L. A. Groen, et al. (2000). "Ion trap mass spectrometry of trimethylsilyamides following gas chromatography." Journal of Mass Spectrometry 35: 305-310.

Ghosh, P., G. W. Patterson, et al. (1997). "Use of an Improved Internal-Standard Method in the Quatitative Sterol Analysis of Phytoplankton and Oysters." Lipids 32(9): 1011-1014.

Goh, E. H., S. M. Colles, et al. (1989). "HPLC Analysis of Desmosterol, 7-Dehydrocholesterol and Cholesterol." Lipids 24(7): 652-655.

Gordon, D. T. and N. Collins (1982). "Anatomical Distribution of Sterols in Oysters (Crassostrea gigas)." Lipids 17(11): 811-817.

Goswami, B. B. (1995). "Detection and Quantitation of Hepatitis A Virus in Shellfish by the Polymerase Chain Reaction." Bacteriological Analytical Manual, 8th Edn. Chapter 26.

Goswami, B. B., W. H. Koch, et al. (1994). "Competitor template RNA for detection and quantitation of hepatitis A virus by PCR." Biotechniques 16(1): 114-5, 118-21.

Goyal, S. M. and C. P. Gerba (1983). "Viradel method for detection of rotavirus from seawater." J Virol Methods 7(5-6): 279-85.

Green, J., K. Henshilwood, et al. (1998). "A nested reverse transcriptase PCR assay for detection of small round-structured viruses in environmentally contaminated molluscan shellfish." Appl Environ Microbiol 64(3): 858-63.

Greening, G. E., J. Dawson, et al. (2001). "Survival of poliovirus in New Zealand green-lipped mussels, Perna canaliculus, on refrigerated and frozen storage." J Food Prot 64(6): 881-4.

Greening, G. E., M. Mirams, et al. (2001). "Molecular epidemiology of 'Norwalk-like viruses' associated with gastroenteritis outbreaks in New Zealand." J Med Virol 64(1): 58-66.

Greening, G. E., L. Woodfield, et al. (1999). "RT-PCR and chemiluminescent ELISA for detection of enteroviruses." J Virol Methods 82(2): 157-66.

Gregor, J., N. Garrett, et al. (2002). "Use of classification and regression trees (CART) analysis with chemical faecal indicators to determine sources of contamination." New Zealand Journal of Marine and Freshwater Research 36: 387-398.

Grohmann, G., R. I. Glass, et al. (1991). "Outbreak of human calicivirus gastroenteritis in a day-care center in Sydney, Australia." J Clin Microbiol 29(3): 544-50.

Hafliger, D., M. Gilgen, et al. (1997). "Seminested RT-PCR systems for small round structured viruses and detection of enteric viruses in seafood." Int J Food Microbiol 37(1): 27-36.

Hara, A. and N. S. Radin (1978). "Lipid Extraction of Tissues with a Low-Toxicity Solvent." Analytical Biochemistry 90: 420-426.

Heath, P. L. and J. H. Wilson (1999). "Assessment of pacific oyster, Crassostrea gigas (Thurnberg), size and quality using a computer-based shape analysis technique." Aquaculture Research 30: 299-303.

Henshilwood, K., L. Cross, et al. "Development of quantitative RT-PCR assay for detection of Norwalk-like virus in wastewaters and application."

Herrmann, J. E. and N. R. Blacklow (1995). Enteric Adenoviruses. Infections of the Gastrointestinal Tract. M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg and R. L. Guerrant. New York, Raven Press, Ltd.: 1047-1053.

Honma, S., S. Nakata, et al. (2000). "Evaluation of nine sets of PCR primers in the RNA dependent RNA polymerase region for detection and differentiation of members of the family Caliciviridae, Norwalk virus and Sapporo virus." Microbiol Immunol 44(5): 411-9.

Huang, P. W., D. Laborde, et al. (2000). "Concentration and detection of caliciviruses in water samples by reverse transcription-PCR." Appl Environ Microbiol 66(10): 4383-8.

Idler, D. R. and P. Wiseman (1971). "Sterols Of Molluscs." International Journal of Biochemistry 2: 516-528.

Jaykus, L. A., R. De Leon, et al. (1996). "A virion concentration method for detection of human enteric viruses in oysters by PCR and oligoprobe hybridization." Appl Environ Microbiol 62(6): 2074-80.

Jiang, S., R. Noble, et al. (2001). "Human adenoviruses and coliphages in urban runoff-impacted coastal waters of Southern California." Appl Environ Microbiol 67(1): 179-84.

Jiang, X., P. W. Huang, et al. (1999). "Design and evaluation of a primer pair that detects both Norwalk- and Sapporo-like caliciviruses by RT-PCR." J Virol Methods 83(1-2): 145-54.

Kamenarska, Z. G., S. D. Dimitrova-Konaklieva, et al. (2003). "A Comparative Study on the Sterol Composition of Some Brown Algae from the Black Sea." Journal of the Serbian Chemical Society 68(4-5): 269-275.

Kanazawa, A. and S.-i. Teshima (1979). "Sedimentary Record of Faecal Pollution in Part of Lake Constance by Coprostanol Determination." Naturwissenschaften 66: 520-522.

Kaneda, T., A. Nakajima, et al. (1980). "Quantitative Analysis of Cholesterol in Foods by Gas-Liquid Chromatography." Journal Nutrional Science and Vitaminology 26: 497-505.

Katayama, H., A. Shimasaki, et al. (2002). "Development of a virus concentration method and its application to detection of enterovirus and norwalk virus from coastal seawater." Appl Environ Microbiol 68(3): 1033-9.

Kerr, R. G., C. Foss, et al. (1997). "Isolation and Structure Elucidation of Epipolasterol and 22,23-Dihydroepipolasterol from the Marine Sponge Epipolasis sp." Comparative Biochemistry & Physiology 117B(4): 561-563.

King, S., M. T. Childs, et al. (1990). "Shellfish: Proximate Composition, Minerals, Fatty Acids, and Sterols." Journal of the American Dietetic Association 90(5): 677-685.

Kingsley, D. H. and G. P. Richards (2001). "Rapid and efficient extraction method for reverse transcription-PCR detection of hepatitis A and Norwalk-like viruses in shellfish." Appl Environ Microbiol 67(9): 4152-7.

Knauer, J., S. M. Barrett, et al. (1999). "Assimilation of dietary phytosterols by Pacific oyster Crassostrea gigas spat." Aquaculture Nutrition 5: 257-266.

Knauer, J., R. G. Kerr, et al. (1998). "Sterol metabolism of Pacific oyster (Crassostrea gigas) spat." Comparative Biochemistry & Physiology 119B(1): 81-84.

Koonjul, P. K., W. F. Brandt, et al. (1999). "Inclusion of polyvinylpyrrolidone in the polymerase chain reaction reverses the inhibitory effects of polyphenolic contamination of RNA." Nucleic Acids Res 27(3): 915-6.

Koplin, D. W., E. T. Furlong, et al. (2002). "Pharmaceuticals, hormones, and other organic wastewater contaminants in U.S. streams, 1999-2000: A national reconnaissance." Environmental science and technology 36: 1202-1211.

Korpela, J. T. (1982). "Capillary gas-liquid chromatography of faecal free and esterfied neutral sterols." Scandinavian Journal of Laboratory Investigation 42: 529-534.

Krahn, M. M., C. A. Wigren, et al. (1989). "High-performance liquid chromatographic method for isolating coprostanol from sediment extracts." Journal of Chromatography 481: 263-273.

Kramer, J. K. G. and H. W. Hulan (1978). "A Comparison of Prcedures to Determoin Free Fatty Acids in Rat Tisse." Journal of Lipid Research 19: 103106.

Krzynowek, J., K. Wiggin, et al. (1983). "Sterol and Fatty Acid Content in Three Groups of Surf Clams (Sipisula solidissima): Wild Clams (60 and 120mm Size) and Cultured Clams (60mm Size)." Comparative Biochemistry and Physiology 74B(2): 289-293.

Laing, I. and N. H. Earl (1998). "The lipid content, spatfall and subsequent growth of early and late settling hatchery-reared Pacific Oyster, Crassostrea gigas Thunberg, larvae." Aquaculture Research 29: 19-25.

Le Guyader, F., V. Apaire-Marchais, et al. (1993). "Use of genomic probes to detect hepatitis A virus and enterovirus RNAs in wild shellfish and relationship of viral contamination to bacterial contamination." Appl Environ Microbiol 59(11): 3963-8.

Le Guyader, F., E. Dubois, et al. (1994). "Detection of hepatitis A virus, rotavirus, and enterovirus in naturally contaminated shellfish and sediment by reverse transcription-seminested PCR." Appl Environ Microbiol 60(10): 3665-71.

Le Guyader, F., L. Haugarreau, et al. (2000). "Three-year study to assess human enteric viruses in shellfish." Appl Environ Microbiol 66(8): 3241-8.

Lee, A., J. Wan, et al. (2001). "Use of Viraffiniy and Multiplex RT-PCR for the Detection of Food-Borne Viruses." Australian Society of Microbiology, 2001 Abstracts

Lee, C. M., B. Trevino, et al. (1996). "A Simple and Rapid Solvent Extraction Method for Determining Total Lipids in Fish Tissue." Journal of AOAC International 79(2): 487-492.

Leeming, R., A. Ball, et al. (1996). "Using faecal sterols from humans and animals to distinguish faecal pollution in receiving waters." Water Research 30(12): 2893-2900.

Leeming, R., N. Bate, et al. (1998). "Discriminating Faecal Pollution: A Case Study of Stormwater Entering Port Phillip Bay, Australia." Water Science and Technology 38(10): 15-22.

Lees, D. N., K. Henshilwood, et al. (1994). "Development of a method for detection of enteroviruses in shellfish by PCR with poliovirus as a model." Appl Environ Microbiol 60(8): 2999-3005.

Lees, D. N., K. Henshilwood, et al. (1995). "Detection of small round structured viruses in shellfish by reverse transcription-PCR." Appl Environ Microbiol 61(12): 4418-24.

Legeay, O., Y. Caudrelier, et al. (2000). "Simplified procedure for detection of enteric pathogenic viruses in shellfish by RT-PCR." J Virol Methods 90(1): 1-14.

Legeay, O., Y. Caudrelier, et al. (2001). "Detection of Enteric Pathogenic Viruses in Shellfish by RT-PCR." Promega Notes(78): 13-16.

Lew, J. F., A. Z. Kapikian, et al. (1994). "Molecular characterization of Hawaii virus and other Norwalk-like viruses: evidence for genetic polymorphism among human caliciviruses." J Infect Dis 170(3): 535-42.

Lewis, G. D., A. Hough, et al. (1996). "Modification of the polyethylene glycol 6000 precipitation mthod for recovering human indicator viruses from oysters and mussels." New Zealand Journal of Marine and Freshwater Research 30: 433-447.

Lewis, G. D., S. L. Molloy, et al. (2000). "Influence of environmental factors on virus detection by RT-PCR and cell culture." J Appl Microbiol 88(4): 633-40.

Lodder, W. J., J. Vinje, et al. (1999). "Molecular detection of Norwalk-like caliciviruses in sewage." Appl Environ Microbiol 65(12): 5624-7.

Mackensie, A. S., S. C. Brassell, et al. (1982). "Chemical fossils: The geological fate of steroids." Science 217: 491-504.

Marshall, J. A., L. K. Yuen, et al. (2001). "Multiple outbreaks of Norwalk-like virus gastro-enteritis associated with a Mediterranean-style restaurant." J Med Microbiol 50(2): 143-51.

Matsui, S. M. (1995). Astroviruses. Infections of the Gastrointestinal Tract. M. J. Blaser, P. D. Smith, J. I. Ravdin, H. B. Greenberg and R. L. Guerrant. New York, Raven Press, Ltd.: 1035-1045.

Maugeri, T. L., D. Caccamo, et al. (2000). "Potentially pathogenic vibrios in brackish waters and mussels." Journal of Applied Microbiology 89: 261-266.

McIver, C. J., E. A. Palombo, et al. (2000). "Detection of astrovirus gastroenteritis in children." J Virol Methods 84(1): 99-105.

Mitchell, D. K., D. O. Matson, et al. (1999). "Molecular epidemiology of childhood astrovirus infection in child care centers." J Infect Dis 180(2): 514-7.

Mitchell, D. K., S. S. Monroe, et al. (1995). "Virologic features of an astrovirus diarrhea outbreak in a day care center revealed by reverse transcriptase-polymerase chain reaction." J Infect Dis 172(6): 1437-44.

Moe, C. L., J. Gentsch, et al. (1994). "Application of PCR to detect Norwalk virus in fecal specimens from outbreaks of gastroenteritis." J Clin Microbiol 32(3): 642-8.

Monpoeho, S., A. Dehee, et al. (2000). "Quantification of enterovirus RNA in sludge samples using single tube real-time RT-PCR." Biotechniques 29(1): 88-93.

Monpoeho, S., A. Maul, et al. (2001). "Best viral elution method available for quantification of enteroviruses in sludge by both cell culture and reverse transcription-PCR." Appl Environ Microbiol 67(6): 2484-8.

Muniain-Mujika, I., R. Girones, et al. (2000). "Viral contamination of shellfish: evaluation of methods and analysis of bacteriophages and human viruses." J Virol Methods 89(1-2): 109-18.

Mustafa, H., E. A. Palombo, et al. (2000). "Epidemiology of astrovirus infection in young children hospitalized with acute gastroenteritis in Melbourne, Australia, over a period of four consecutive years, 1995 to 1998." J Clin Microbiol 38(3): 1058-62.

Ng, L.-K. and M. Hupé (1998). "Analysis of Sterols: a novel approach for detecting juices of pineapple, passionfruit, orange and grapefruit in compounded beverages." Journal of science food and agriculture 76: 617-627.

Nguyen, D.-K., A. Bruchet, et al. (1995). "Determination of sterols in sewage sludge by combined in situ

Trimethylsilyation/supercritical fluid extraction and GC/MS." Environmental Science &Technology 29: 1686-1690.

Noble, R. and J. A. Fuhrman (2001). "Enteroviruses detected by reverse transcriptase polymerase chain reaction from the coastal waters of Santa Monica Bay, California: low corrolation to bacterial indicator levels." Hydobiologia(460): 175-184.

Noel, J. S., T. W. Lee, et al. (1995). "Typing of human astroviruses from clinical isolates by enzyme immunoassay and nucleotide sequencing." J Clin Microbiol 33(4): 797-801.

Palombo, E. A. and R. F. Bishop (1996). "Annual incidence, serotype distribution, and genetic diversity of human astrovirus isolates from hospitalized children in Melbourne, Australia." J Clin Microbiol 34(7): 1750-3.

Paul, J. H., S. C. Jiang, et al. (1991). "Concentration of viruses and dissolved DNA from aquatic environments by vortex flow filtration." Appl Environ Microbiol 57(8): 2197-204.

Pina, S., M. Puig, et al. (1998). "Viral pollution in the environment and in shellfish: human adenovirus detection by PCR as an index of human viruses." Appl Environ Microbiol 64(9): 3376-82.

Pinto, R. M., F. X. Abad, et al. (1996). "Detection of Infectious Astroviruses in Water." Appl Environ Microbiol 62(5): 1811-1813.

Puig, M., J. Jofre, et al. (1994). "Detection of adenoviruses and enteroviruses in polluted waters by nested PCR amplification." Appl Environ Microbiol 60(8): 2963-70.

Ralph, J. P. Cromar, N. J. & Fallowfield H.J. (1997) Faecal sterol “profiles” as a tool for the assessment and management of water quality in urban catchments. Microbiology Australia, 18, A72 and

Ralph, J., Cromar, N.J., Cunliffe, D & Fallowfield, H.J (1999) Sourcing faecal pollution in the Torrens catchment by analysis of faecal steroids, pp 9 Australian Water and Wastewater Association 18th Federal Convention *Adelaide 11-14 April, 1999*. ISBN 0-0908255-47-0

Read, S. J. and J. B. Kurtz (1999). "Laboratory diagnosis of common viral infections of the central nervous system by using a single multiplex PCR screening assay." J Clin Microbiol 37(5): 1352-5.

Roser, D., N. Ashbolt, et al. (2002). "Application of physico-chemical parameters and particle-bound biomarkers to indicate microbial contamination of aquifers." Water Science and Technology 2(5): 419-426.

Ruan, B., J. Shey, et al. (1996). "Silver Ion High Pressure Liquid Chromatography Provides Unpresedented Separation of Sterols: Application to the Enzymatic Formation of Cholesta-5,8-dien-3ß-ol." Proceedings of the National Academy of Scinces of the United Staes of America 93: 11603-11608.

Sakamoto, T., H. Negishi, et al. (2000). "Molecular epidemiology of astroviruses in Japan from 1995 to 1998 by reverse transcription-polymerase chain reaction with serotype-specific primers (1 to 8)." J Med Virol 61(3): 326-31.

Sakon, N., K. Yamazaki, et al. (2000). "Genomic characterization of human astrovirus type 6 Katano virus and the establishment of a rapid and effective reverse transcription-polymerase chain reaction to detect all serotypes of human astrovirus." J Med Virol 61(1): 125-31.

Schreier, E., F. Doring, et al. (2000). "Molecular epidemiology of outbreaks of gastroenteritis associated with small round structured viruses in Germany in 1997/98." Arch Virol 145(3): 443-53.

Schvoerer, E., F. Bonnet, et al. (2000). "PCR detection of human enteric viruses in bathing areas, waste waters and human stools in Southwestern France." Res Microbiol 151(8): 693-701.

Schwab, K. J., R. De Leon, et al. (1995). "Concentration and purification of beef extract mock eluates from water samples for the detection of enteroviruses, hepatitis A virus, and Norwalk virus by reverse transcription-PCR." Appl Environ Microbiol 61(2): 531-7.

Schwab, K. J., F. H. Neill, et al. (1998). "Distribution of Norwalk virus within shellfish following bioaccumulation and subsequent depuration by detection using RT-PCR." J Food Prot 61(12): 1674-80.

Schwab, K. J., F. H. Neill, et al. (2000). "Development of methods to detect "Norwalk-like viruses" (NLVs) and hepatitis A virus in delicatessen foods: application to a food-borne NLV outbreak." Appl Environ Microbiol 66(1): 213-8.

Schwab, K. J., F. H. Neill, et al. (2001). "Development of a reverse transcription-PCR-DNA enzyme immunoassay for detection of "Norwalk-like" viruses and hepatitis A virus in stool and shellfish." Appl Environ Microbiol 67(2): 742-9.

Seah, E. L., I. C. Gunesekere, et al. (1999). "Variation in ORF3 of genogroup 2 Norwalk-like viruses." Arch Virol 144(5): 1007-14.

Seah, E. L., J. A. Marshall, et al. (1999). "Open reading frame 1 of the Norwalk-like virus Camberwell: completion of sequence and expression in mammalian cells." J Virol 73(12): 10531-5.

Shieh, Y., S. S. Monroe, et al. (2000). "Detection of norwalk-like virus in shellfish implicated in illness." J Infect Dis 181 Suppl 2: S360-6.

Shieh, Y. C., K. R. Calci, et al. (1999). "A method to detect low levels of enteric viruses in contaminated oysters." Appl Environ Microbiol 65(11): 4709-14.

Simkova, A. and J. Cervenka (1981). "Coliphages as ecological indicators of enteroviruses in various water systems." Bull World Health Organ 59(4): 611-8.

Small, A. C. and L. Lucas (2000). "Regulation and monitoring of aquaculture in the Netherlands." Journal of Applied Ichthyology 16: 187-191.

Smith, A. W., D. E. Skilling, et al. (1998). "Calicivirus emergence from ocean reservoirs: zoonotic and interspecies movements." Emerg Infect Dis 4(1): 13-20.

Stafford, R., D. Strain, et al. (1997). "An outbreak of Norwalk virus gastroenteritis following consumption of oysters." Commun Dis Intell 21(21): 317-20.

Sugieda, M., K. Nakajima, et al. (1996). "Outbreaks of Norwalk-like virus-associated gastroenteritis traced to shellfish: coexistence of two genotypes in one specimen." Epidemiol Infect 116(3): 339-46.

Sugiyama, A., K. Nishi, et al. (2002). "[Contamination of oyster sea farm with the Norwalk virus: mechanisms and control]." Nippon Rinsho 60(6): 1214-21.

Suprahatin, I. (2004) Ph.D. Thesis. Department of Environmental Health. Flinders University. Bedford Park.SA.

Takeda, N. (2002). "[Norwalk virus: recent findings]." Nippon Rinsho 60(6): 1138-42.

Teshima, S.-I., G. W. Patterson, et al. (1980). "Sterols of the Oyster, Crassostrea virginica." Lipids 15(12): 1004-1011.

Traore, O., C. Arnal, et al. (1998). "Reverse transcriptase PCR detection of astrovirus, hepatitis A virus, and poliovirus in experimentally contaminated mussels: comparison of several extraction and concentration methods." Appl Environ Microbiol 64(8): 3118-22.

Traore, O., G. Belliot, et al. (2000). "RT-PCR identification and typing of astroviruses and Norwalk-like viruses in hospitalized patients with gastroenteritis: evidence of nosocomial infections." J Clin Virol 17(3): 151-8.

Volkman, J. K. (1986). "A review of sterol markers or marine and terrigenous organic mater." Organic Geochemistry 9(2): 83-99.

Walker, R. W., C. K. Wun, et al. (1982). "Coprostanol as an indicator of fecal pollution." CRC Critical Reviews in Environmental Control.

Ward, J., A. Neill, et al. (2000). "Three nursing home outbreaks of Norwalk-like virus in Brisbane in 1999." Commun Dis Intell 24(8): 229-33.

Ward, R. D., L. J. English, et al. (2000). "Genetic improvement of the pacific oyster Crassostrea gigas (Thurnberg) in Australia." Aquaculture Research 31: 35-44.

Wilson, I. G. (1997). "Inhibition and facilitation of nucleic acid amplification." Appl Environ Microbiol 63(10): 3741-51.

Wright, P. J., I. C. Gunesekere, et al. (1998). "Small round-structured (Norwalk-like) viruses and classical human caliciviruses in southeastern Australia, 1980-1996." J Med Virol 55(4): 312-20.

Writer, J. H., J. A. Leenheer, et al. (1995). "Sewage contamination in the upper Mississippi river as measured by the fecal sterol, coprostanol." Water Research 29(6): 1427-1436.

Yuen, L. K., M. G. Catton, et al. (2001). "Heminested multiplex reverse transcription-PCR for detection and differentiation of Norwalk-like virus genogroups 1 and 2 in fecal samples." J Clin Microbiol 39(7): 2690-4.

Zerda, K. S., C. P. Gerba, et al. (1985). "Adsorption of viruses to charge-modified silica." Appl Environ Microbiol 49(1): 91-5.

Appendix 1. Intellectual Property.

**Appendix 2. Staff.**

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Assoc. Prof. John Edwards, Dept. of Environmental Health, Flinders University, Adelaide, South Australia.
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Peter Hallsworth, Flinders Medical Centre, Bedford Park, Adelaide, South Australia.
Mr. Ken Lee South Australian Shellfish Quality Assurance Program, Lincoln Marine Science centre, Port Lincoln. SA
Mr. Nick White, Dept. of Environmental Health, Flinders University, Adelaide, South Australia.

**Appendix 3. Virus PCR Methods**

**Detection of viruses by PCR**

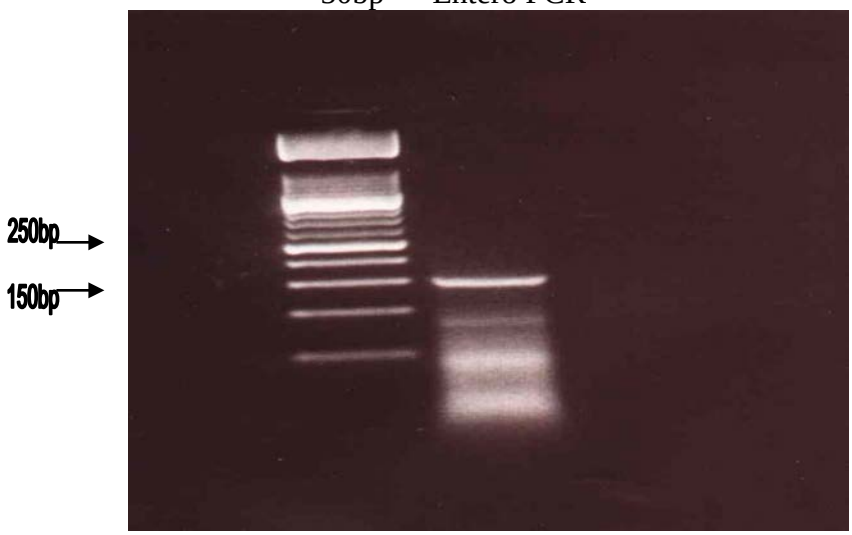
- PCR assays for detection of:
  - Enterovirus
  - Hepatitis A virus
  - Norovirus Genogroup 2
  - Astrovirus
  - Adenovirus
  - Coliphage phiX174.
- Assays have been optimized for the reagents of given source and for the given thermal cyclers. Alteration of these conditions may require the reoptimisation of the assay.
- RNA and DNA have been extracted using RNeasy and Dneasy Plant Minikits (Qiagen). Nucleic acids are eluted in DEPC treated water.
- Following extraction RNA must be stored on ice until use (up to about 1 hour) or at –20C or –70C for long term storage (up to 1 year).
- Following extraction DNA may be stored at room temperature for short-term storage (up to about 1 hour), at 4C for up to a week, or at –20C or –70C for long term storage (up to 1 year). This is also the case for cDNA or PCR products.

**1. Enterovirus single round PCR.**

Ref: Zoll et al (1992) J. Clin. Microbiol. 30(1):160-165
Used for diagnostics at SouthPath

Forward Primer "EP1" (20 mer): 5' -TCC TCC GGC CCC TGA ATG CG- 3'
Reverse Primer "EP2" (20 mer): 5' -ATT GTC ACC ATA AGC AGC CA- 3'

Product 154bp



**Reverse Transcription**

- 5X RT reaction buffer (Promega) 4µl
- DTT (0.1M) (Life Technologies) 2µl
- EP2 (10µM) 2µl
- dNTPs (5mM) (Promega) 1.75µl
- M-MLV (Promega) 0.25µl

TOTAL (10µl) + RNA (10µl)

**PCR Master Mix**

- DEPC treated water 14.6µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 1µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- EP1 (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (20µl) + cDNA (5µl)

Amplified at:
1 cycle – 95C, 5min
40 cycles – 95C, 30sec; 55C, 30sec; 72C, 30sec
1 cycle – 72C, 7min
4C

Can be amplified on either Perkin Elmer or Hybaid machines
Programmed in micro cyclers as entero or 3T55.

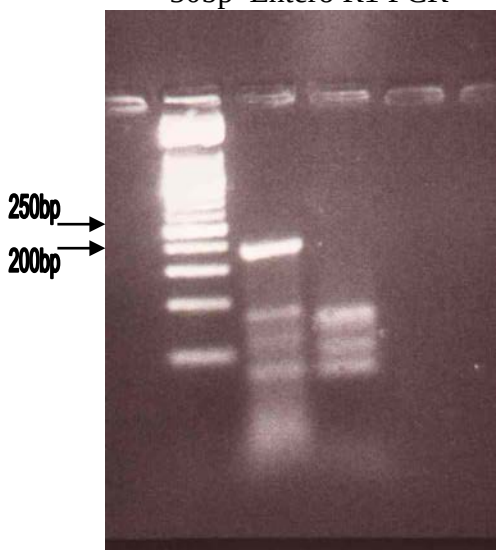
**2. Enterovirus nested PCR.**

Ref: Yuen et al (2001) J Clin Microbiol 39(7): 2690-2694
Used for diagnostics at SouthPath in a multiplex PCR
Nested PCR

**Round 1 Primers**
Forward Primer “EVOS” (17 mer): 5’ -CGG CCC CTG AAT GCG GC- 3’
Reverse Primer “EVOAS” (18 mer): 5’ -CAC CGC ATG GCC AAT CCA- 3’
Product 193bp

**Round 2 Primers**
Forward Primer “EVIS” (18 mer): 5’ -CCC CTG AAT GCG GCT AAT- 3’
Reverse Primer “EVIAS” - (20 mer): 5’ -AAT GTC ACC ATA AGC AGC CA- 3’
Product 146bp

50bp  Entero R1 PCR



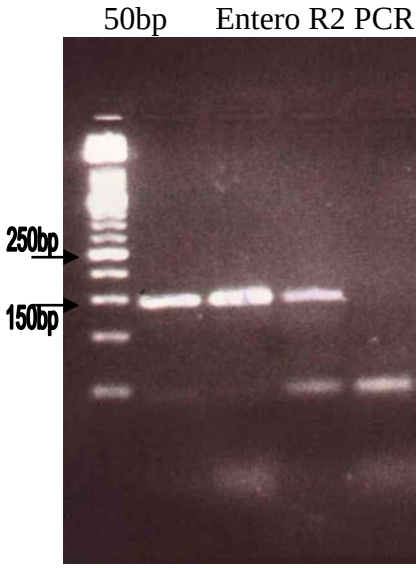

**Round 1 PCR Master Mix**

- DEPC treated water 12.55µl
- 5X RT reaction buffer (Promega) 10µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 4µl
- EVOS (10µM) 0.5µl
- EVOAS (10µM) 0.5µl
- M-MLV (Promega) 0.25µl
- Taq (5U/ml) (Promega) 0.2µl

TOTAL (30µl) + RNA (20µl)

Amplified at:
1 cycle – 37C, 15min; 94C, 40sec (reverse transcription stage)
33 cycles – 94C, 20sec; 60C, 20sec; 72C, 20sec
4C

Can be amplified on Hybaid machine. Programmed in micro cycler as multi 1.




**Round 2 PCR Master Mix**

- DEPC treated water 15.9µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 2µl
- EVOS (10µM) 0.25µl
- EVOAS (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.1µl

TOTAL (23µl) + R1 DNA (2µl)

Amplified at:
33 cycles – 94C, 20sec; 55C, 20sec; 72C, 20sec
4C

Can be amplified on Hybaid machine. Programmed in micro cycler as multi 2.

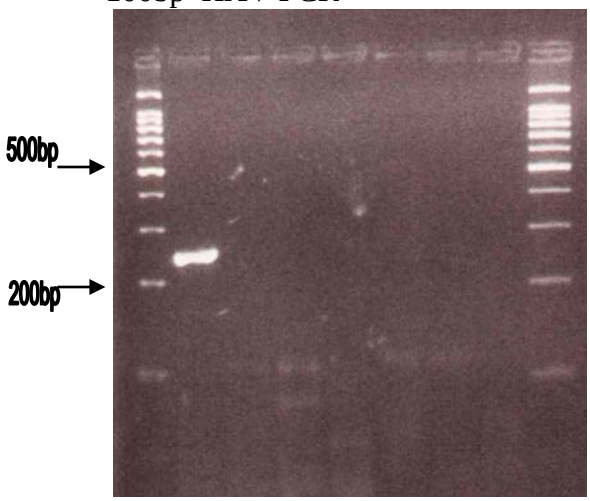
### 3. Hepatitis A

Ref: Cromeans et al (1997) Appl. Env. Microbiol. 63(6):2460-2463
Used for diagnostics at SouthPath

Forward Primer “HAVS” (26mer):5’ -GTT TTG CTC CTC TTT ATC ATG CTA TG- 3’
Reverse Primer “HAVAS” (25mer):5’ -GGA AAT GTC TCA GGT ACT TTC TTTG- 3’

Product 247bp

100bp HAV PCR




**Reverse Transcription**

- 5X RT reaction buffer (Promega) 4µl
- DTT (0.1M) (Life Technologies) 2µl
- HAVAS (10µM) 2µl
- dNTPs (5mM) (Promega) 1.75µl
- M-MLV (Promega) 0.25µl

TOTAL (10µl) + RNA (10µl)

**PCR Master Mix**

- DEPC treated water 14.6µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 1µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- HAVS (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (20µl) + cDNA (5µl)

Amplified at:
1 cycle – 95C, 5min
40 cycles – 95C, 30sec; 55C, 30sec; 72C, 30sec
1 cycle – 72C, 7min
4C

Can be amplified on either Perkin Elmer or Hybaid machines Programmed in micro cyclers as 3T55.

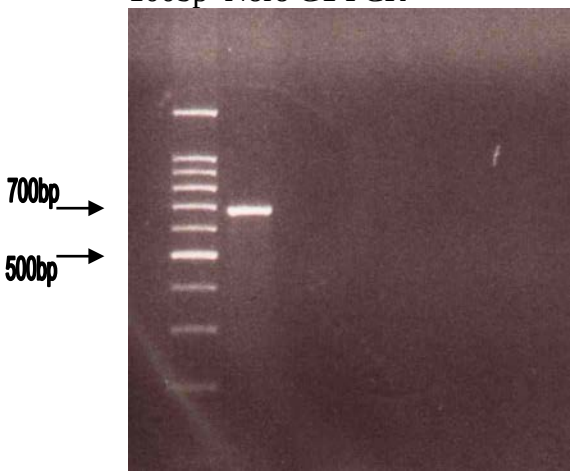
## 4. Norovirus Genogroup 2

Ref: Yuen et al (2001) J Clin Microbiol 39(7): 2690-2694
Semi-nested PCR

**Round 1 Primers**
Forward Primer "NV4611" (21 mer): 5' -C(A/T)G CAG C(A/C)C T(A/G/T)G AAA TCA TGG- 3'
Reverse Primer "NV5296" (21 mer): 5' -CCA (C/T)CT GAA CAT TG(A/G) CTC TTG- 3'
Product 685bp

**Round 2 Primers**
Forward Primer "NV4692" (23 mer): 5' -GTG TG(A/G) T(G/T)G ATG TGG GTG ACT TC- 3'
Reverse Primer "NV5296" - As above
Product 604bp

100bp Noro G1 PCR




**Reverse Transcription**

- 5X RT reaction buffer (Promega) 4µl
- DTT (0.1M) (Life Technologies) 2µl
- NV5296 (10µM) 2µl
- dNTPs (5mM) (Promega) 1.75µl
- M-MLV (Promega) 0.25µl

TOTAL (10µl) + RNA (10µl)

**Round 1 PCR Master Mix**

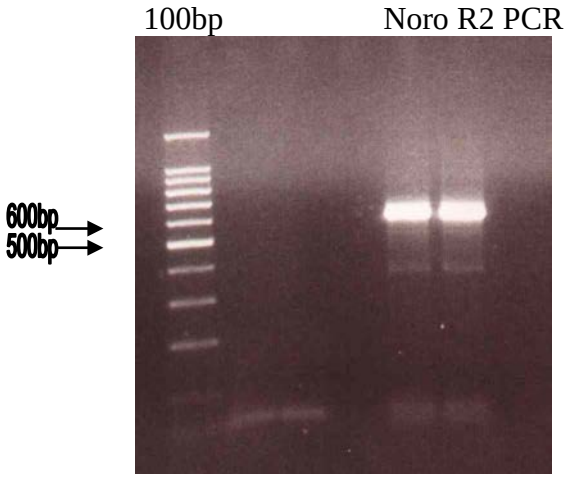
- DEPC treated water 14.4µl

- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 0.7µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- NV4611 (10µM) 0.5µl
- NV5296 (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (20µl) + cDNA (5µl)

Amplified at:
1 cycle – 94C, 2min
35 cycles – 94C, 30sec; 50C, 30sec; 72C, 30sec
1 cycle – 72C, 5min
4C

Can be amplified on either Perkin Elmer machine. Programmed in micro cycler as NV R1.




**Round 2 PCR Master Mix**

- DEPC treated water 18.4µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 0.7µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- NV4692 (10µM) 0.5µl
- NV5296 (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (24µl) + R1 DNA (1µl)
Amplified at:
1 cycle – 94C, 2min
25 cycles – 94C, 30sec; 50C, 30sec; 72C, 30sec

1 cycle – 72C, 5min
4C

Can be amplified on either Perkin Elmer machine. Programmed in micro cycler as NV R2.

**5. Norovirus Genogroup 1**

Ref: Yuen et al (2001) J Clin Microbiol 39(7): 2690-2694
Semi-nested PCR

**Round 1 Primers**
Forward Primer "NV4562" (22 mer): 5' -GAT GC(A/G/T) GAT TAC ACA GC(A/C/T) TGG G- 3'
Reverse Primer "NV5298" (22 mer): 5' –ATC CAG CGG AAC ATG GCC TGC C- 3'
Product 804bp

**Round 2 Primers**
Forward Primer "NV4562" - As above
Reverse Primer "NV5366" - (21 mer): 5' –CAT CAT CAT TTA C(A/G)A ATT CGG- 3'
Product 736bp

No PCR has been developed as a positive sample of Norovirus genogroup 1 could not be obtained. This group of viruses is rare and therefore difficult to source.

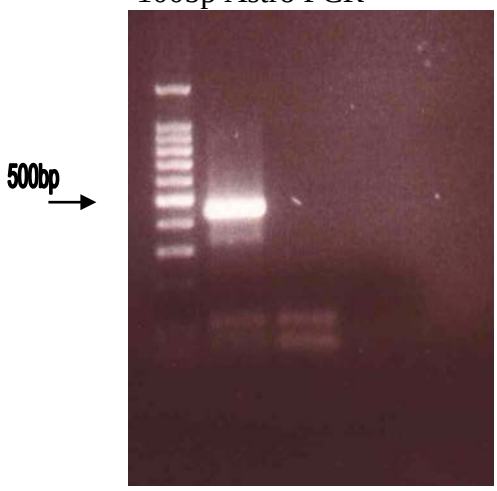
**5. Astrovirus**

Ref: Noel et al (1995) J. Clin. Microbiol. 33(4):797-801

Forward Primer "Mon269" (20 mer): 5' –CAA CTC AGG AAA CAG GGT GT- 3'
Reverse Primer "Mon270" (20 mer): 5' -TCA GAT GCA TTG TCA TTG GT - 3'

Product 449bp

100bp Astro PCR




**Reverse Transcription**

- 5X RT reaction buffer (Promega) 4µl
- DTT (0.1M) (Life Technologies) 2µl
- Mon270 (10µM) 2µl
- dNTPs (5mM) (Promega) 1.75µl
- M-MLV (Promega) 0.25µl

TOTAL (10µl) + RNA (10µl)

**PCR Master Mix**

- DEPC treated water 12.65µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 2µl
- Mon269 (10µM) 0.5µl
- Mon270 (10µM) 0.25µl
- Taq (5U/ml) (Promega) 0.1µl

TOTAL (20µl) + cDNA (5µl)

Amplified at:
40 cycles – 95C, 60sec; 55C, 60sec; 72C, 60sec
4C

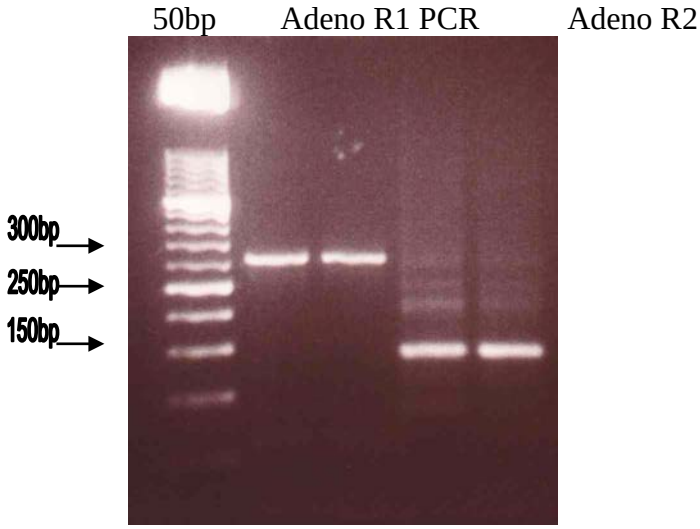
Can be amplified on either Perkin Elmer or Hybaid machines. Programmed in micro cyclers as Astro.

## 6. Adenovirus nested PCR.

Ref: Allard et al (1990) J Med Virol 37: 149-157
Nested PCR

**Round 1 Primers**
Forward Primer "hexAA1885" (25 mer): 5' –GCC GCA GTG GTC TTA CAT GCA CAT C- 3'
Reverse Primer "hexAA1913" (23 mer): 5' –CAG CAC GCC GCG GAT GTC AAA GT- 3'
Product 300bp

**Round 2 Primers**
Forward Primer "nehexAA1893" (24 mer): 5' -GCC ACC GAG ACG TAC TTC AGC CTG- 3'
Reverse Primer "nehexAA1905" (29 mer): 5' -TTG TAC GAG TAC GCG GTA TCC TCG CGG TC- 3'
Product 142bp




**Round 1 PCR Master Mix**

- DEPC treated water 13.7µl
- 10X PCR buffer (Roche) 5µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 3µl
- NV4611 (10µM) 0.5µl
- NV5296 (10µM) 0.5µl
- Taq (5U/ml) (Promega) 0.3µl

TOTAL (25µl) + DNA (25µl)

Amplified at:
1 cycle – 94C, 4min
30 cycles – 92C, 90sec; 55C, 90sec; 72C, 120sec
4C

**Round 2 PCR Master Mix**

- DEPC treated water 36.7µl
- 10X PCR buffer (Roche) 5µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 3µl
- NV4611 (10µM) 0.5µl
- NV5296 (10µM) 0.5µl
- Taq (5U/ml) (Promega) 0.3µl

TOTAL (48µl) + DNA (2µl)

Amplified as above.

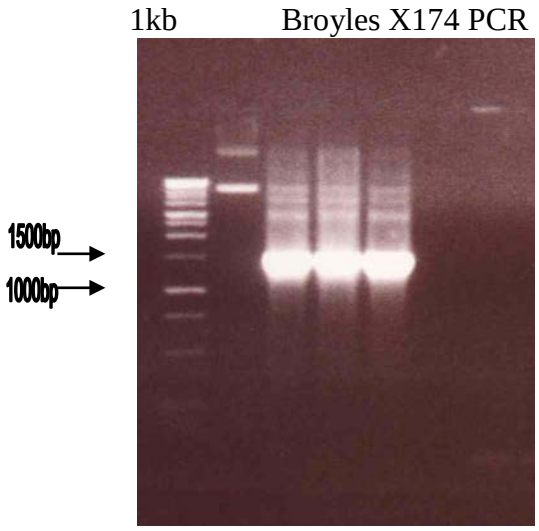
Can be amplified on Hybaid machine only. Programmed in micro cycler as Adeno.

## 7. PhiX174 single round PCR.

Ref: Broyles et al (2002) 40(8): 2725-2728

Forward Primer (18 mer): 5’ -GCT TGC GTT TAT GGT ACG- 3’
Reverse Primer (18 mer): 5’ -ATA CGA CGG CGC ATA ACG- 3’

Product size: 1.5kb



**PCR Master Mix**

- DEPC treated water 35.8µl
- 10X PCR buffer (Roche) 5µl
- dNTPs (5mM) (Promega) 2µl
- MgCl2 (25mM) (Roche or Promega) 3µl
- Broyles F (10µM) 1µl
- Broyles R (10µM) 1µl
- Taq (5U/ml) (Promega) 0.2µl

TOTAL (48µl) + DNA (2µl)

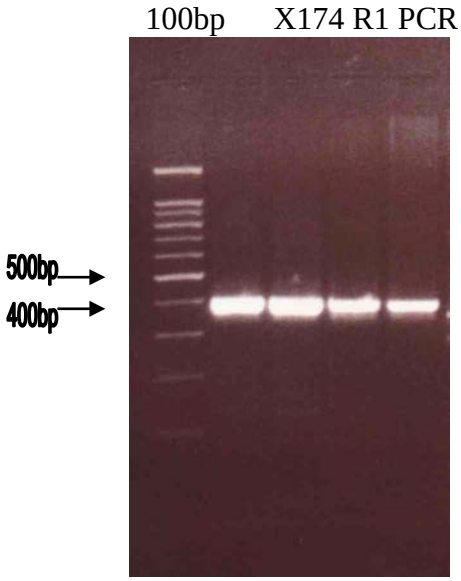
Amplified at:
1 cycle – 94C, 5min
35 cycles – 94C, 45sec; 54C, 60sec; 72C, 60sec
1 cycle – 72C, 10min
4C

Can be amplified on Perkin Elmer only. Programmed in micro cycler as Broyles.

**8. PhiX174 nested PCR.**

**Round 1 Primers**
Forward Primer "X174 forward" (20 mer): 5' -CAG CAA ACG CAG AAT CAG CG- 3'
Reverse Primer "X174 reverse" (21 mer): 5' -CTT ATG GAA GCC AAG CAT TGG- 3'
Product 396bp

**Round 2 Primers**
Forward Primer "X174 new forward" (20 mer): 5' -ATT GGC CCT ACT GCA AAG GA- 3'
Reverse Primer "X174 new reverse" (22 mer): 5' –AGA GCC TCG ATA CGC TCA AAG T- 3'
Product 293bp



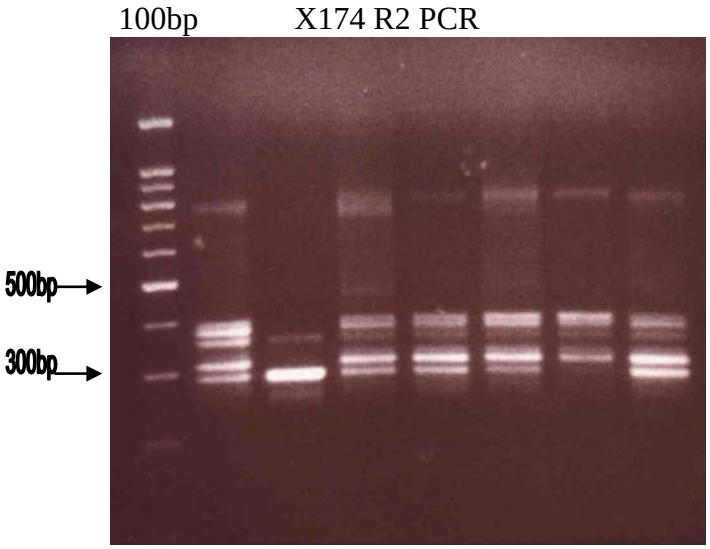

**Round 1 PCR Master Mix**

- DEPC treated water 13.85µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 1µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- X174 forward (10µM) 0.5µl
- X174 reverse (10µM) 0.5µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (20µl) + DNA (5µl)

Amplified at:
1 cycle – 95C, 5min
40 cycles – 95C, 30sec; 55C, 30sec; 72C, 30sec
1 cycle – 72C, 7min
4C

Can only be amplified on Perkin Elmer machine. Programmed in micro cycler as entero or 3T55.




**Round 2 PCR Master Mix**

- DEPC treated water 17.85µl
- 10X PCR buffer (Roche) 2.5µl
- dNTPs (5mM) (Promega) 1µl
- MgCl2 (25mM) (Roche or Promega) 1.5µl
- X174 new forward (10µM) 0.5µl
- X174 new reverse (10µM) 0.5µl
- Taq (5U/ml) (Promega) 0.15µl

TOTAL (24µl) + DNA (1µl of 1:100 diluted round 1 reaction)

Amplified at:
1 cycle – 94C, 5min
40 cycles – 94C, 30sec; 68C, 30sec; 72C, 30sec
1 cycle – 72C, 7min
4C

Can only be amplified on Perkin Elmer machine. Programmed in micro cycler as X174 R2.

**Appendix 4. Sensitivity and selectivity – the use of contingency table data in establishing predictive capacity of microbiological analyses.**

| | |
|---|---|
| *Sensitivity* - | The fraction of samples positive for virus by PCR that had a positive microbiological test |
| *Specificity* - | The fraction of samples negative for virus by PCR that had a negative microbiological test |
| *Positive - predictive value* | The fraction of samples that had a positive microbiological test that were positive for virus by PCR |
| *Negative - predictive value* | The fraction of samples that had a negative microbiological test that were negative for virus by PCR |
| *Likelihood - ratio* | If a positive microbiological test is seen how much more likely is it to be positive for viral contamination? If the LR is 6.0, then a sample with a positive microbiological test is six times more likely to be virus contaminated. |

**Appendix 5. Contingency table analyses for predictive capacity of microbiological analyses.**

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥7MPN** | 2 | 2 | *4* |
| **E. coli <7MPN** | 3 | 24 | *27* |
| *column totals* | *5* | *26* | *31* |

Fisher Exact Test, two-sided, p=0.112 – poor association

| | **Enteric virus (-adeno)** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥7MPN** | 1 | 3 | *4* |
| **E. coli <7MPN** | 2 | 25 | *27* |
| *column totals* | *3* | *28* | *31* |

Fisher Exact Test, two-sided, p=0.349 – poor association

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥1MPN** | 3 | 3 | *6* |
| **E. coli <1MPN** | 2 | 23 | *25* |
| *column totals* | *5* | *26* | *31* |

Fisher Exact Test, two-sided, p=0.038 – good association
sensitivity – 0.600
specificity – 0.885
positive predicitive value – 0.500
negative predictive value – 0.920
Likelihood ratio – 5.200

| | **Enteric virus (-adeno)** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **E. coli ≥1MPN** | 2 | 4 | *6* |
| **E. coli <1MPN** | 1 | 24 | *25* |
| *column totals* | *3* | *28* | *31* |

Fisher Exact Test, two-sided, p=0.088 – reasonable association
sensitivity – 0.667
specificity – 0.857
positive predicitive value – 0.333
negative predictive value – 0.960
Likelihood ratio – 4.667

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **phage ≥50 cfu** | 1 | 0 | *1* |
| **phage <50 cfu** | 4 | 24 | *28* |
| *column totals* | *5* | *24* | *29* |

Fisher Exact Test, two-sided, p=0.172 – poor association

| | **Enteric virus (-adeno)** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **phage ≥50 cfu** | 1 | 0 | *1* |
| **phage <50 cfu** | 2 | 26 | *28* |
| *column totals* | *3* | *26* | *29* |

Fisher Exact Test, two-sided, p=0.349 – poor association

| | **Enteric virus** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **faecal ≥1MPN** | 4 | 8 | *12* |
| **faecal <1MPN** | 1 | 18 | *19* |
| *column totals* | *5* | *26* | *31* |

Fisher Exact Test, two-sided, p=0.060 – good association
sensitivity – 0.800
specificity – 0.692
positive predicitive value – 0.333
negative predictive value – 0.947
Likelihood ratio – 2.600

| | **Enteric virus (-adeno)** | | *row totals* |
|---|---|---|---|
| | **present** | **absent** | |
| **faecal ≥1MPN** | 3 | 9 | *12* |
| **faecal <1MPN** | 0 | 19 | *19* |
| *column totals* | *3* | *28* | *31* |

Fisher Exact Test, two-sided, p=0.049 – good association
sensitivity – 1.000
specificity – 0.679
positive predicitive value – 0.250
negative predictive value – 1.000
Likelihood ratio – 3.111

## Appendix 6. Raw data from field sampling

| Sample name | Date | Shellfish species | Microbiology (PFU/g) | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | | | Total coliforms | Fecal coliforms | E. coli | Fecal Streptococci | Enterococci | Coliphage (PFU/g) | Virus |
| MR-1036 | 03.04.02 | Pacific Oyster | 1.1 | 1.1 | 0.7 | - | - | | 0 |
| MR-1044 | 08.04.02 | Pacific Oyster | 0.8 | 0.5 | 0.5 | - | - | | 0 |
| MR-1058 | 29.04.02 | Pacific Oyster | 3.3 | 0 | 0 | - | - | 0.8 | 0 |
| MR-1066 | 30.04.02 | Pacific Oyster | 2.2 | 1.1 | 1.1 | - | - | 0 | 1 |
| MR-1071 | 06.05.02 | Pacific Oyster | 13 | 0 | 0 | - | - | 1.86 | 0 |
| MR-1072 | 06.05.02 | Pacific Oyster | 0.7 | 0.7 | 0.7 | - | - | 0 | 0 |
| MR-1081 | 13.05.02 | Pacific Oyster | 0.5 | 0.5 | 0.5 | - | - | 0.53 | 0 |
| MR-1170 | 07.08.02 | Mussel | | | | | | 0 | 0 |
| MR-1173 | 12.08.02 | Mussel | | | | | | 0 | 0 |
| Outfall | 18.11.02 | Pacific Oyster | 7.9 | 0 | 0 | 2.3 | 2.3 | 0 | 0 |
| Noro 1 | 28.11.02 | Pearl Oyster | 2 | 0 | 0 | 2.3 | 2.3 | 0 | 0 |
| Noro 2 | 04.03.03 | Pearl Oyster | 2.2 | 0.68 | 0 | 4.9 | 2.2 | 0 | 0 |
| Coffin | 25.05.03 | Pacific Oyster | | | | | | 0.73 | 1 |
| PBS | 25.05.03 | Mussel | | | | | | 0 | 1 |
| PPS | 25.05.03 | Mussel | | | | | | 0.22 | 0 |
| BIS | 25.05.03 | Mussel | | | | | | 0.11 | 0 |
| Flow through | 18.06.03 | Pacific Oyster | 160 | 35 | 160 | 160 | 92 | 0 | 1 |
| SR | 10.10.03 | Sydney Rock | 160 | 17 | 0 | 0.78 | 0 | 0 | 0 |
| Noro 1 | 11.12.03 | Pearl Oyster | 160 | 0.2 | 0.2 | 7.9 | 0 | 0 | 0 |
| Noro 2 | 11.12.03 | Pearl Oyster | 160 | 0.18 | 0 | 6.9 | 0 | 0 | 0 |
| Noro 3 | 11.12.03 | Pearl Oyster | 160 | 0 | 0 | 3.6 | 0.36 | 0 | 0 |
| Noro 4 | 11.12.03 | Pearl Oyster | 160 | 0 | 0 | 92 | 0 | 0 | 0 |
| Noro 5 | 11.12.03 | Pearl Oyster | 160 | 0 | 0 | 17 | 0 | 0 | 0 |
| Noro 6 | 11.12.03 | Pearl Oyster | 160 | 0 | 0 | 7.9 | 0 | 0 | 0 |
| Retail A | 18.12.03 | Pacific Oyster | 160 | 0.54 | 0 | 54 | 0.56 | 0 | 0 |
| Retail B | 18.12.03 | Pacific Oyster | 2.3 | 1.3 | 1.3 | 1.3 | 0.2 | 0 | 0 |
| Retail C | 18.12.03 | Pacific Oyster | 1.7 | 0.68 | 0.45 | 2.3 | 0.78 | 0 | 0 |
| Retail 1 | 02.02.04 | Pacific Oyster | 160 | 35 | 0.18 | 44 | 54 | 44 | 0 |
| Retail 2 | 02.02.04 | Pacific Oyster | 44 | 17 | 0.45 | 160 | 160 | 44.4 | 0 |
| Noro 1 | 10.02.04 | Pacific Oyster | 4.8 | 4 | 0 | 4.9 | 0.79 | 10.18 | 0 |
| Noro 2 | 10.02.04 | Pacific Oyster | 8.1 | 6.9 | 0 | 7.9 | 2.3 | 244.31 | 1 |