

FINAL REPORT



**Aquatic Animal Health Subprogram:
Development of a disease zoning policy for
Marteilia sydneyi to support sustainable
production, health certification and trade in
the Sydney rock oyster**

Dr R.D. Adlard & Dr S.J. Wesche

Project No. 2001/214



Australian Government
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Queensland Government



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Development Corporation





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2001/214 Aquatic Animal Health Subprogram: Development of a disease zoning policy for *Marteilia sydneyi* to support sustainable production, health certification and trade in the Sydney rock oyster

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OBJECTIVES:

1. The primary objective is to implement and field-test the zoning policy framework developed under AQUAPLAN in a practical context and to facilitate the development of further zoning policies for other significant diseases of aquatic animals. This will be conducted using 'QX Disease'- aetiological agent *Marteilia sydneyi*, as a case study to develop an effective zoning policy that is consistent with internationally recognised (OIE) standards. The zoning policy will aim to:

- Reduce the risk of introducing this pathogen into the remaining disease-free production areas; and
- Facilitate domestic and international market access for the industry.

2. The sub-objectives necessary to achieve this are to:

- Identify through sampling and appropriate diagnosis 'QX Disease'-free and 'QX Disease'-endemic estuaries within oyster culture areas;
- Determine the specific identity of *Marteilia* sp. from positive samples through ultra-structural and molecular diagnostics;
- Develop a rational and effective program of surveillance for 'QX Disease' based on occurrence and an assessment of risk for each oyster producing estuary;
- In consultation with fisheries managers and industry, develop a coastal zoning plan for 'QX Disease'.

NON TECHNICAL SUMMARY:

OUTCOMES ACHIEVED

The identification of areas of risk to commercial culture of the Sydney Rock Oyster through the presence of the oyster pathogen *Marteilia sydneyi*, agent of QX disease, has been detailed through a comprehensive survey of estuaries in southeast Queensland and New South Wales (2001-2004).

The outcomes from this project have immediately effected changes in the domestic management of QX disease in oysters. The NSW and Queensland oyster industry and Department of Primary Industries of each state have adapted their disease management plans to recognize the wide geographic distribution of the disease. Furthermore, the presence of the QX pathogen in estuaries where disease has not been recorded has emphasised the likely role played by the dynamics of the parasite's lifecycle together with host immune defence and likely environmental factors as regulators of QX disease outbreaks in rock oysters.

Non-technical summary

The edible oyster industry in Australia is currently valued at around \$62.5 million annually of which rock oyster production accounts for approx 56%. For the industry to survive in the long-term requires the ability to service what may become a premium domestic market demanding a high quality product. The expansion of the industry is likely to be available only from international export, which in turn requires compliance with international regulations on oyster health with a transparent health audit trail. The rock oyster is potentially positioned for re-emerging export success, being a unique product with an extended shelf-life relative to other oyster species (e.g. the Pacific oyster, *Crassostrea gigas*) and this is an opportunity that should be exploited by the industry.

Within Australia, the Sydney Rock Oyster industry is subjected to periodic epizootics of disease induced by a range of parasitic organisms that produce significant mortality and morbidity of commercial oyster stocks. The most significant of these is the agent responsible for 'QX disease' (caused by the protistan parasite *Marteilia sydneyi*) affecting the Sydney rock oyster *Saccostrea glomerata*. Management of this disease has been based on quarantine of affected estuaries enforced through limitation on the movement of potentially infected stock. In this context, it was obvious that the oyster industry required a disease zoning policy based on scientifically defensible data to allow domestic best practice in oyster farming and to maximise market accessibility for the industry. This host/parasite system then formed the basis for a test of the zoning policy framework developed under the federal government's 'AQUAPLAN'.

A number of key issues related to zoning and surveillance for specific diseases were addressed through this project. Initially the design of field collection and the appropriate test to use for diagnosis were assessed to maximise, and allow quantification of, disease detection limits in the surveillance program.

1. The design of field sampling to identify disease infected oysters was critical in order to reach a statistically robust probability of disease detection. Global animal health standards (Office Internationale des Epizooties) recommend random sampling from a zone to detect a prevalence of 2% or greater disease in a population. This was fulfilled using a computer generated random selection of geographic co-ordinates under which individual oysters were sampled (Angus Cameron, AusVet).

2. The appropriate method for diagnosis of disease, another critical issue in disease surveillance programs, was assessed by comparing the sensitivity and specificity of: tissue imprints (cytology); or tissue sections (histology); or the presence of specific parasite DNA (by polymerase chain reaction - PCR). Our analysis showed clearly that PCR was the most sensitive diagnostic test followed by cytology then histology. PCR also detected the presence of sub-clinical infections which could not be unambiguously identified using either histology or cytology. Confirmatory diagnosis (following PCR) at sub-clinical levels was undertaken using DNA in situ hybridisation tests designed to stain the QX organism specifically in tissue section.

Combined surveillance results from 2001 (NSW estuaries only), 2002-03 (NSW and Queensland estuaries) and 2004 (Queensland estuaries only) demonstrated some significant departures from the geographic distribution expected for QX disease. In 2001 diagnosis was undertaken using cytology and no unexpected occurrences of the disease were observed, with positives recorded only from the Clarence River (1.5% of sample infected), Georges River (47% of sample infected). In 2002 the distribution of disease was significantly different to that expected. Initially using cytology for diagnosis there were no apparent unusual infections with Southern Moreton Bay (0.8% of sample infected), Richmond River (40.8% of sample infected), Clarence River (22% of sample infected) and Georges River (16% of sample infected) recording oysters positive for the disease. However, when PCR techniques were used for diagnosis in estuaries that had never recorded the presence of the disease agent it became obvious that the organism was more widespread than indicated by previous diagnostic testing or previous occurrences of disease outbreaks. In total 142 unexpected positives for *Marteilia sydneyi* were found in oysters scored as negative by cytological examination during surveillance in this project. Of these, 61 were identified in oysters sampled from estuaries with no prior record of *Marteilia sydneyi*. These represent oysters from Hastings River, Wallis Lake, Port Stephens, Bateman's Bay, Tuross Lake, Narooma and Merimbula.

Further testing of these infections confirmed the identity of the QX organism and found it to be present in the oyster tissues at a sub-clinical level i.e. prior to reaching the oyster's digestive gland where the parasite would normally produce spores. At this stage of development, pathology in the oyster is reduced and the condition factor of oysters is not seriously compromised.

In 2003 surveillance and diagnosis using PCR techniques showed a reduced impact of QX disease with Southern Moreton Bay (0.67% of sample infected), Brunswick River (1.3% of sample infected), Richmond River (13.3% of sample infected), Clarence River (6% of sample infected) and Georges River (0.67% of sample infected).

This project has had a significant impact on our understanding of QX disease in rock oysters as it applies to management. Rather than the disease agent being limited geographically to those estuaries that experience periodic outbreaks, the agent has been identified in most rock oyster growing areas on

the east coast of Australia. As such there is the potential for outbreaks of QX disease in all commercial growing areas (indeed such an outbreak occurred in 2004, with seasonal re-occurrence in 2005, in the Hawkesbury River) and that disease is likely to be regulated through a combination of the dynamics of the parasite lifecycle and the level of oyster fitness. Furthermore, in any aquatic system the environment will play an equally significant role in the outcomes of host/parasite interactions both through direct impact on stages (spores, infective stages) in the lifecycle of the parasite and indirectly through its impact on host fitness.

In the light of our new understanding of the distribution of the QX disease agent it could be argued that management through quarantine of identified QX-endemic estuaries is no longer appropriate. However, the biology of *Marteilia sydneyi* (dynamics of the life cycle of the parasite, interactions with alternate hosts) and its interaction with the host oyster's immune system are incompletely understood and the precautionary principle should be upheld especially in the case of such a serious disease.

While estuaries which undergo periodic outbreak should remain closed to export of oysters for relaying live in water elsewhere, local management will focus on disease seasonality and stock rotation to avoid the high risk periods in mid to late summer. These periods should be identified with accuracy to maximise available growth periods in disease endemic areas of estuaries. The ongoing projects to develop QX disease resistant oysters (NSW DPI and collaboration with Macquarie University) should run parallel with a program of incremental addition to the biological knowledge of this pathogen. Specifically, an absence of our ability to maintain a laboratory based infection model hampers research on identifying those factors (pathogen-specific, oyster-specific and environment-specific) which promote disease.

KEY WORDS: Sydney rock oyster, QX disease, *Marteilia sydneyi*, aquaculture, zoning policy, diagnostic method.

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BACKGROUND:

Recent trends in world trade have lead to increased globalization and the removal of both tariff and non-tariff trade barriers. Trade in both live animals and animal products have been liberalized and increased. This has lead to a greater threat of the spread of disease associated with this trade. The Office Internationale des Epizooties (OIE - World Organisation for Animal Health) and the World Trade Organisation have recognised and responded to this threat through the development of the Sanitary and Phytosanitary Agreement (SPS Agreement). The SPS agreement recognizes that differences in disease status between exporting and importing countries may be a legitimate reason to prevent trade in animals or animal products. In recent times, Australia has been a major player in the development of guidelines for the Responsible Movement of Live Aquatic Animals in Asia in conjunction with the Network of Aquaculture Centres in the Asia-Pacific.

Traditionally, the occurrence of a disease within a country has lead to the entire country being considered infected with that disease and its trading status affected commensurately. Recently however, the OIE has recognised the concept of zoning whereby recognised areas of disease freedom can be established within a country. Trade from these areas can continue unaffected by the presence of disease elsewhere. The establishment of zoning has also

proved useful in preventing the further spread of disease and in protecting unaffected production areas.

Australia recently recognised the value of zoning in its aquaculture industries with the adoption and endorsement of Zoning Policy Guidelines by Standing Committee on Fisheries and Aquaculture. These guidelines are a first step towards implementation of zoning policies in Australia. As yet the guidelines only:

"...explain the background and principles relevant to establishing a future zoning policy" (Zoning Policy Guidelines, Jan 2000 p.5)

Similarly, Australian States and Territories have recently endorsed (through Ministerial Council on Forestry, Fisheries and Agriculture - MCFFA) the National Policy for the Translocation of Live Aquatic Animals. This will provide:

"...a basis from which to develop translocation policies and guidelines specific to their jurisdictions" (Translocation Policy, Ministerial Foreword, Sep. 1999)

There is a need to begin implementing the guidelines in a practical context. The establishment of scientifically defensible zoning and translocation policies is critical to the future development of Australian aquaculture and the maintenance of international market access ultimately by supporting export health certification. These two policy areas are interlinked and are both critical components of AQUAPLAN - Australia's National Strategic Plan for Aquatic Animal Health 1998-2003. AQUAPLAN is the nationally agreed and endorsed strategy for policy development in the area of aquaculture health and program 3 of AQUAPLAN lists development of zoning policy as a priority.

Within Australia, the Sydney Rock Oyster industry is the industry most urgently in need of zoning policy development. Australia's oyster industry has for decades been subjected to periodic epizootics of disease induced by a range of protozoan parasites that produce significant mortality and morbidity of commercial oyster stocks. The most significant of these are the agents responsible for:

- * 'QX disease' (aetiological agent: *Marteilia sydneyi*) affecting the Sydney rock oyster *Saccostrea glomerata* [syn *S. commercialis*];
- * 'Winter Mortality' (aetiological agent: *Bonamia roughleyi* [syn *Mikrocytos roughleyi*]) affecting the Sydney rock oyster;
- * Bonamiasis (aetiological agent: *Bonamia* sp.) affecting the flat oyster, *Ostrea angasi*.

These pathogens are listed by the OIE as notifiable pathogens and are also included on the Australian National List of Reportable Diseases of Aquatic Animals. Without an internationally recognised disease zoning policy the presence of these pathogens compromises the ability of the industry to explore international export markets. These diseases have also had a severe impact on

domestic oyster productivity to the extent that some major traditional production areas have become non-viable and industry has been forced to abandon them. A scientifically-based zoning policy is necessary to prevent spread of these pathogens between states and production areas.

Growth of the oysters involves the translocation of live oysters between various rivers on the NSW and Queensland coasts at various stages of the life cycle. This regular translocation presents a potential threat to the industry because several diseases of rock oysters appear to have limited geographic distributions. In particular, QX disease was limited to the warmer waters of the Queensland and Northern NSW rivers until 1995 when QX appeared in the central regional growing area of the Georges River where stocks were subsequently decimated. Anecdotal evidence strongly suggests that QX disease has been spread to previously unaffected areas through the movement of affected stock. The Rock Oyster Industry thus offers a classic opportunity to serve as a case study in the development and application of both zoning and translocation policies for Australian aquaculture.

The development and implementation of a zoning policy is contingent upon establishment of surveillance and monitoring programs to define the disease status of the various geographic regions and ensure freedom from disease. This proposal will:

- * Serve as a first case study for the implementation of the National Zoning Policy Guidelines;
- * Collect the baseline surveillance and monitoring data necessary to identify QX disease-free and affected areas;
- * Recommend a scientifically defensible zoning policy for QX disease that is consistent with internationally recognised standards by the Office International des Epizooties and with the National Policy for the Translocation of Live Aquatic Animals and national Zoning Policy Guidelines. The zoning policy will propose appropriate stock movement restrictions that will protect QX disease-free areas and facilitate domestic and international trade/market access.

NEED:

The edible oyster industry in Australia is currently valued at around \$62.5 million annually of which rock oyster production accounts for approx 56%. For the industry to survive in the long-term requires the ability to service what may become a premium domestic market demanding a high quality product. The expansion of the industry is likely to be available only from international export, which in turn requires compliance with international regulations on oyster health with a transparent health audit trail. The rock oyster is potentially positioned for re-emerging export success, being a unique product with an extended shelf-life

relative to other oyster species (e.g. the Pacific oyster, *Crassostrea gigas*) and this is an opportunity that should be exploited by the industry.

The techniques of surveillance and diagnosis for molluscan pathogens required by the OIE for imported oyster products are not only stringent and accepted as the worldwide standard, but are also applicable to domestic requirements within Australia. In essence, the regulations state that appropriate diagnostic tests are applied for detecting the presence of pathogens of molluscs (microscopic identification techniques with the potential for specific molecular identification using monoclonal antibodies or DNA probes) which have been collected as part of a surveillance program within delimited coastal zones. The sample size, period and frequency are determined with reference to the cycle of infection of the particular pathogen and its prepatent period. There is an initial 2 year period of surveillance before a zone can be granted a disease-free status, with ongoing surveillance required for this status to be maintained.

The development of a zoning policy framework for QX disease will provide a valuable opportunity to implement and field-test Australia's zoning policy guidelines in a practical context to assist with the development of further zoning policies for diseases of aquatic animals. Considerable interest has already been expressed in the case study by State authorities and it was discussed at an Aquatic Animal Disease Zoning Workshop in Canberra on 23 January 2001, hosted by the National Offices of Animal and Plant Health. Furthermore, the development of the zoning policy will be of direct benefit to the oyster industry by facilitating domestic and international market access, and through identifying and protecting the remaining disease-free production areas

OBJECTIVES ACHIEVED:

1. The primary objective is to implement and field-test the zoning policy framework developed under AQUAPLAN in a practical context and to facilitate the development of further zoning policies for other significant diseases of aquatic animals. This will be conducted using 'QX Disease'- aetiological agent *Marteilia sydneyi*, as a case study to develop an effective zoning policy that is consistent with internationally recognised (OIE) standards. The zoning policy will aim to:

- * Reduce the risk of introducing this pathogen into the remaining disease-free production areas; and

- * Facilitate domestic and international market access for the industry.

This objective has been achieved.

2. The sub-objectives necessary to achieve this are to:

- * Identify through sampling and appropriate diagnosis 'QX Disease'-free and 'QX Disease'-endemic estuaries within oyster culture areas;

- * Determine the specific identity of *Marteilia* sp. from positive samples through ultra-structural and molecular diagnostics;
- * Develop a rational and effective program of surveillance for 'QX Disease' based on occurrence and an assessment of risk for each oyster producing estuary;
- * In consultation with fisheries managers and industry, develop a coastal zoning plan for 'QX Disease'.

This objective has been achieved.

METHODS:

Sampling frequency and timing:

The seasonal nature of QX disease suggests that sampling once a year is an appropriate frequency, conforming with OIE guidelines for surveillance of such pathogens, that sampling should occur 'at least once a year'. It is the timing of samples that is critical. Throughout this surveillance project, sampling was carried out during autumn (March to May). This period for sampling offers maximum detection potential by allowing time for the parasite to develop through to sporulation, in the digestive gland. At this stage of development the parasite is most abundant in the digestive gland and easily identified. It was apparent that for defining infection status for the purpose of zoning, diagnosis of early infections was not a critical issue.

Specimen sampling in NSW estuaries:

250 oysters were sampled individually at random from commercial culture areas in 18 estuaries (ie. Tweed, Brunswick, Richmond, Clarence, Wooli, Bellingen, Macleay, Hastings, Manning, Wallis Lake, Port Stephens, Hawkesbury, Georges, Shoalhaven, Bateman's, Tuross, Narooma and Merimbula) along the NSW coast (see fig 1). Random GPS points within each estuary's culture area were generated and loaded into hand-held GPS units. Oysters were collected by relocating GPS points on site and selecting an oyster at that position. Oysters were collected in NSW by NSW Fisheries officers during 2001 to 2003 resulting in 3 consecutive years of sampling. The collection and processing of samples during 2001 was funded through NSW Fisheries and the NSW Oyster Research Advisory Committee (NSW ORAC).

Specimen sampling in south-east Queensland estuaries:

250 oysters were sampled individually at random from commercial culture areas in 3 putative zones (southern Moreton Bay-SMB, central Moreton Bay-CMB, and northern Moreton Bay-NMB) in south-east Queensland (see fig 1). Random points in distance along the axis of oyster leases were generated. Oysters were then collected at or nearest to these random points. Oysters were collected in south-east Queensland by Drs Rob Adlard (PI), Stephen Wesche (RO) and Mal Bryant (Queensland Museum funded Research Assistant), for the period 2002 to 2004 resulting in 3 consecutive years of sampling.



Figure 1: Estuaries in which oysters were collected as part of the sampling regime for the surveillance program along the NSW (2001–2003) and south-east Queensland (2002–2004) coastline. **NB** Cudgen Lake, Bonville Ck, Sydney Harbour and Sussex Inlet were added to the sampling program for NSW in response to survey results obtained in 2002 from a related project (FRDC 2001/630). See text ‘Responsive variations to sampling and processing’ for details.

Sample processing:

Oyster samples were received at the Queensland Museum. For each oyster sample received the collection date, date of delivery, estuary and location within the estuary (when applicable) was recorded. Prior to opening, each oyster was allocated a unique sample number against which all data was recorded. Field numbers (see 'Definitions A' below) were also recorded as supplied.

Oysters were opened by inserting an oyster knife into the hinge of the oyster and twisting. When the oyster shells were released, the knife was pushed into the shell cavity and manoeuvred to sever the adductor muscle. The top shell was then removed without damaging the oyster tissue.

Oysters were removed from the shell and placed into a sterile Petrie dish. The size (see 'Definitions A' below) of each oyster shell was measured and recorded.

Definitions A:

Sample No. - The number allocated to each oyster after it has been received and prepared for processing. Numbers are sequential and chronological (i.e. unique to each oyster) according to receipt of oyster samples and assigned irrespective of the origin of the oyster samples.

Field No. - The number allocated to oysters while in the field. Field numbers were allocated to a proportion of collected oysters to allow precise identification of exact collection site within an estuary.

Size: maximum diameter across shell margins.

Dissection:

A transverse incision was made with a sterile scalpel blade through the oyster dissecting the gonad. At this stage a semi-quantitative assessment of condition factor and digestive gland colour was recorded (see Definitions B below).

Representative tissues from the palps, gills, mantle, gonad and digestive gland were removed for fixation in 10% formalin in seawater. Each preserved specimen contains a label recording Sample No., Estuary and collection date. Preserved specimens were removed from formalin after 24-48 hours and placed in 70% alcohol for storage (Howard and Smith 1983).

Imprinting:

A small piece of digestive gland (approx. 2-4 mm³) was removed from the remaining portion of fresh oyster with sterile forceps and scalpel, and blotted dry on a clean piece of paper towel for imprinting. The blotted tissue was then imprinted on a frosted end slide. Each oyster was imprinted multiple times on a slide, and a new slide was used for each oyster. Slides are labelled with the Estuary name and Sample No. After imprinting, the remaining tissue was stored in 90% ethanol, for molecular analysis. Tissue preserved in ethanol, as for formalin, contains a label recording Sample No., Estuary and collection date.

Imprinted slides were air dried before staining with Hemacolor (Merck) a commercial rapid blood stain kit. The slides are allowed to air-dry before having coverslips applied using mounting medium (ie. DEPEX, Merck).

Definitions B:

Condition factor: is qualitative and expressed on a 5-point scale from 1-5. 1 indicates the oyster is thin, lacking gonad; 2 indicates that gonad is present but in small amounts and the digestive gland is clearly visible; 3 indicates some gonad surrounding the digestive gland; 4 indicates developed gonad; 5 indicates the oyster is ripe with gonad and ready to spawn.

Digestive gland colour: is qualitative and expressed on a 3-point scale from 1-3. 1 indicates the colour of the digestive gland is pale (yellow-brown to white); 2 indicates the digestive gland is light-medium brown in colour; 3 indicates a dark brown digestive gland.

Sterilization: Oyster knives are washed under running water after each oyster in a sample batch and sterilised between batches. Dissection implements and oyster knives are sterilised by rinsing in alcohol and flaming over a Bunsen burner. Commercially sterilised Petrie dishes were single use only.

Microscopic examination:

Slides were scanned for the presence of *Marteilia sydneyi*, using x20 and x40 objectives. The 100x objective and oil was required for definitive identification of some cells, (i.e. primary cell stages). Viewing time for each slide was a minimum of 5 min, or until all imprints on a slide had been viewed. When *M. sydneyi* was present on a slide the intensity of infection was recorded using a 5-point scale. 1 represents a heavy infection (>100 cells/ 200x field). 2 represents a moderate/heavy infection (20-100 cells/200x field). 3 represents a moderate infection (5-20 cells/200x field). 4 represents a light/moderate infection (1-5 cells/200x field). 5 represents a light infection (<1 cell/200x field). The stages of *M. sydneyi* present on each slide were recorded using three terms: 1° cells (any single or binucleate cell stages), 2° cells (any cells showing advanced replication i.e. plasmodia) and sporonts.

Histology:

Tissue was preserved in 10% formalin in seawater for histology if required for confirmatory diagnosis. The samples are subsequently handled in accordance with classical histological methods, i.e. embedded in paraffin, sectioned at 5-7µm, stained with haematoxylin/eosin (see Chapter I.2. of OIE Diagnostic Manual for Aquatic Animal Disease, 2000).

Molecular diagnostics:

DNA extraction

Total genomic DNA was extracted from a small sub-sample of tissue excised from the digestive gland of each oyster. Extractions were performed using commercially available standard DNA tissue extraction kits (DNeasy Tissue Kit, Qiagen).

Polymerase Chain Reaction (PCR)

Protocols for the detection of *Marteilia sydneyi* in oyster tissue using PCR used in this project, were optimised and validated during the formation of the Australian and New Zealand Standard Diagnostic Procedure (ANZSDP) (Adlard and Worthington Wilmer, 2003). Double stranded PCR reactions were performed on all DNA extracts using *Marteilia sydneyi* specific primers which target an ~200 base pair (bp) section of ITS1 (internal transcribed spacer) (see Table 1). For each batch of PCR reactions performed, a negative control i.e. a PCR reaction containing no DNA template, and a positive control i.e. a PCR reaction containing DNA from a known *Marteilia sydneyi* infected sample, were included. The former is run to ensure that the presence of an amplified product was not due to contamination of the PCR reagents, and the latter to ensure that the absence of any amplified product was not a result of a failed reaction.

Table 1: *Marteilia sydneyi* ITS1 PCR Primers

Primer Name	Sequence 5'-3'	Source/Ref
LEG1 (forward)	CGA TCT GTG TAG TCG GAT TCC GA	Kleeman and Adlard, 2000
PRO2 (reverse)	TCA AGG GAC ATC CAA CGG TC	Kleeman and Adlard, 2000

All PCR reactions, including the controls, are carried out in 25µl volume under the reaction conditions described in Table 2.

Table 2: PCR reagents used for the diagnosis of *Marteilia sydneyi*

PCR reagents/sample	Volume	Final conc./sample
Water (molecular biology grade)	14.35µl	
10x <i>Taq</i> polymerase buffer	2.5µl	1x
10mM dNTPS	2.0µl	0.8mM
10µM Primer LEG1	1.0µl	0.4µM
10µM Primer PRO2	1.0µl	0.4µM
25mM MgCl ₂	2.0µl	2.0mM
<i>Taq</i> polymerase	0.15µl	0.75 Units
Genomic DNA template	2.0µl	20-50ng

The *Taq* polymerase used in the reaction protocol is a hot-start polymerase (e.g. Applied Biosystems AmpliTaq Gold) that requires a 5-10 minute initial denaturation cycle prior to the commencement of the remaining cycle parameters.

- a) Initial denaturation 95°C for 10 min.
- b) Denaturation..... 95°C for 30 sec
 Annealing 55°C for 30 sec
 Extension..... 72°C for 30 sec } x 35 cycles
- c) Final Extension 72°C for 5 min
 22°C for 30 sec

Gel electrophoresis

The examination for the presence or absence of an amplified product (presumptive diagnosis of *Marteilia sydneyi*) was conducted by the addition of 1 µl of loading dye to 9 µl of PCR product followed by electrophoresis through submarine 1.4% (w/v) agarose gels incorporating ethidium bromide stain, and then photographed on a ultra-violet transilluminator. A molecular weight standard (100bp DNA ladder, MBI Fermentas) was used to estimate the size of the products.

Labelling of *in situ* hybridisation DNA probe:

A specific DNA probe (Kleeman and Adlard, 2000) was employed to unambiguously identify *M. sydneyi* cellular stages *in situ*. The DNA probe was synthesised by the incorporation of dioxygenin-11-dUTP during PCR using the *Marteilia sydneyi* specific primers PRO2 and LEG1, genomic DNA extracted from the digestive gland of QX infected oysters and the PCR DIG Probe synthesis Kit (Roche Diagnostics) according to the protocol suggested by the manufacturer. Incorporation of digoxigenin was indicated by an increase in molecular mass as analysed on ethidium bromide-stained agarose gels.

***In situ* hybridisation:**

Tissue was preserved in 10% formalin in seawater for DNA probe *in situ* hybridisation (ISH) if required for confirmatory diagnosis. Initial embedding of tissue in paraffin was identical to standard histological procedures. Sections were then mounted onto silanized slides (PROSCITEC) and baked for 45 min at 60°C. Sections were deparaffinized, rehydrated in ethanol and air dried. Sections were permeabilized with 100 µg ml⁻¹ Proteinase K (PK) in 1x TE (10 mM Tris-HCl, 1 mM EDTA.2H₂O, pH 8.0) for approx 30 min at 37°C in a humid chamber. PK was spread evenly for digestion by the application of glass coverslips. Sections were dehydrated then air dried. Sections were prehybridised in hybridisation buffer (500 µl/slide) for 30 min at 42°C. Buffer solution was replaced with 55 µl of hybridization buffer containing 5 µl of DIG-labelled DNA probe. Sections were covered with coverslips to help prevent drying of slides and to allow for even coverage. Slides were heated on a hot plate at 95°C for 5 min to denature the target DNA then immediately cooled on ice for 5 min and allowed to hybridise overnight in a humid chamber at 42°C. Post hybridization washes in pre-warmed buffers included 2xSSC twice for 5 min at room temp (RT) and 0.4xSSC once for 10 min 42°C, followed by equilibrium in maleic acid buffer (1 min, RT). Dig-

labelled probe detection included blocking sections with 200ul blocking buffer for 30min at RT followed by incubation for 1hr at 37°C with 200ul of dilute anti-digoxigenin-alkaline phosphatase conjugate (1:500 in blocking buffer) in humid chamber. Unbound antibody was removed with two 1 min washes in Maleic acid buffer followed by equilibrium in detection buffer with one 5 min wash. BCIP/NBT was diluted in detection buffer (20ul in 1 ml) and 200ul was added to the tissue and incubated in the dark at RT for approx 2 hr. The reaction was stopped by washing slides in TE buffer for 15min at RT. The slides were rinsed in ddH₂O and counterstained in Bismark brown Y, followed by ethanol dehydration and mounted in DEPEX via xylene (see protocols in Appendix 3a).

Transmission Electron Microscopy:

Oyster tissue from samples in estuaries that scanned positive for *M. sydneyi* in cytology and fixed in 10% formalin in seawater were re-fixed in 3% glutaraldehyde in cacodylate buffer for 24hr. Samples were post-fixed in 1% OsO₄ for 1hr dehydrated in an acetone series then embedded in Epon resin blocks. Ultrathin sections were cut from the block faces using a glass knife, stained in uranyl acetate and lead citrate and viewed at 80 kV in a JEOL 1010 transmission electron microscope.

Responsive variations to sampling and processing:

Under the related project (FRDC 2001/630) 1837 oysters were screened for the presence of *Marteilia sydneyi* using PCR, while developing an Australian and New Zealand Standard Diagnostic Procedure (ANZSDP). 142 unexpected positives for *M. sydneyi* were found in oysters scored as negative by cytological examination. Of these, 74 were identified in oysters sampled from estuaries with no prior record of *M. sydneyi*. These represent oysters from NSW: Hastings R, Wallis Lake, Port Stephens, Bateman's Bay, Tuross Heads, Narooma, Merimbula; and QLD: Northern Moreton Bay and Central Moreton Bay. Examination of archived tissues from these estuaries using histological and DNA probe *in situ* hybridisation techniques confirmed the presence of the pathogen in an atypical, (with respect to seasonality) apparent early stage of development. No proliferation through to sporulation (development of spores) was identified in the samples examined. Note that Crookhaven/Shoalhaven and Hawkesbury R samples were the only estuaries tested by PCR techniques which returned negative PCR results for 100 oysters screened per estuary during this period (2002).

Because of these research findings a special meeting was called by NSW Fisheries (Mercure Hotel, St Leonards, Sydney: 19th February 2003). Stakeholders felt that applying PCR diagnostics to 2003 samples was of immediate significance to strategic management of QX disease. Furthermore, stakeholders also felt it a priority need to identify the presence/absence of naturally occurring infections of QX disease. Therefore the following changes to methodology were adopted:

1) Presumptive diagnosis changed from cytological examination of tissue imprints to PCR screening with a reduced sample size of 150 for the 2003 sampling period onwards.

Rationale: A combination of the results from this project and FRDC 2001/630 clearly demonstrate that the diagnostic resolution power of PCR exceeds that of all microscopic examination, thus sample sizes can be reduced to 150 without compromising detection levels ($\geq 2\%$ prevalence). Stakeholders felt that applying PCR diagnostics to 2003 samples was of immediate significance to strategic management of QX disease, particularly given that the pathogen would not have been discovered in estuaries where it had previously been unrecorded using any other available diagnostic technique.

2) Expansion of sampling regime to include 4 uncultivated estuaries during the final year of sampling (2003) in NSW.

Rationale: NSW Fisheries has recorded a high level of movement of commercial oyster stocks between estuaries, and as such it is statistically probable that the QX organism has been translocated unknowingly to many current areas of culture. Stakeholders felt it a priority need to examine estuaries with only natural populations of rock oysters (i.e. those without any history of cultivation or translocation of stock from other estuaries) to identify the presence/absence of naturally occurring infections of QX disease. Estuaries selected were Cudgen Lake, Bonville Creek, Sydney Harbour and Sussex Inlet.

Source of funding for project variation:

The variation in diagnostic method and extension of 4 estuaries in NSW were funded through an FRDC project grant awarded to NSW Fisheries with Dr Adlard contracted to undertake the work as a consultant. This source provided funds for molecular consumables, while this project (FRDC 2001/214) provided salary for the RO to physically undertake the PCR testing.

Sub-sampling tissue

To determine any influence that sub-sampling tissue may have on diagnostic protocols, 50 oysters known to be infected with QX disease (by cytology) were fixed in 10% formalin in seawater and embedded in wax using standard histological processing methods. Six sections were cut at equal intervals along the length of the oyster digestive gland so that the entire digestive gland had been sectioned. The sections were then stained with hematoxylin and eosin (H&E). Each section was viewed with light microscope and the number of infected digestive tubules was recorded. Sections cut from each oyster were compared to establish whether QX infections were homogenous throughout the digestive gland.

Comparison of diagnostic methods

The true infection status of a zone can only be determined using the best available diagnostic test and must take into account the specificity and sensitivity of all available methods. As such, it is of paramount importance that the

limitations of all available diagnostic methods for pathogen identification be well-defined.

Cytology compared with Histology

A sample of 80 rock oysters used in this comparison, were collected from Limekiln Bar, Georges River, New South Wales, in April 2002. The timing of collection and the sample site were selected to maximize the potential of selecting oysters infected with *Marteilia sydneyi*. Each oyster was opened and a portion of the digestive gland was removed (1mm³) for imprinting. The remaining oyster tissue was preserved in 10% formalin in seawater before being embedded in wax, processed for histology and stained with hematoxylin and eosin (H&E).

Imprints of the digestive gland were made on a microscope slide, air dried, then fixed and stained with Hemacolor™. The imprinted slides were examined under the light microscope (mag x200) for the presence or absence of *Marteilia sydneyi*. Each infected oyster was classified into one of 5 grades of intensity of infection. Grade 1 – heavy; > 100 cells/field. Grade 2 – moderate to heavy; 20-100 cells/field. Grade 3 – moderate; 5-20 cells/field. Grade 4 – light to moderate; 1-5 cells/field. Grade 5 – light; < 1 cell/field.

The histological slides were arbitrarily labelled with numbers to enable blind testing, with an electronic record of their original collection number retained to allow for comparison of results. Histological sections were then screened for the presence or absence of *M. sydneyi*. Each infected oyster was classified into one of 5 grades of intensity of infection: Grade 1 - Light; a few (<5) parasites present, only observed after extensive searching of the section. Grade 2 - light to moderate; a few parasites observed in many digestive tubules. Grade 3 - moderate; parasites readily observed, but not often in great numbers, in most digestive tubules. Grade 4 - moderate to heavy; parasites abundant in nearly all digestive tubules, sometimes extending into the lumen of the tubule. Grade - 5 heavy; parasites abundant in all tubules, tubules congested with breakdown of tubule epithelia.

Cytology compared with PCR

A sample of 1839 rock oysters used in this comparison was collected from 17 estuaries in southeast Queensland and New South Wales (see Table 3).

Each oyster was opened and imprints of the digestive gland were made on a microscope slide, air dried, then fixed and stained with Hemacolor™ before examination by light microscopy. A tissue sample from the digestive gland of each oyster was preserved in 90% ethanol for DNA extraction, and tested for the presence of *M. sydneyi* using PCR screening in accordance with the ANZSDP (Adlard and Worthington Wilmer, 2003). The results from cytology and PCR were compared.

Table 3: List of estuaries sampled and their corresponding sample sizes, used in the comparison of cytology and PCR diagnostic methods.

Estuary	No. of oysters sampled
<i>Queensland</i>	
Northern Moreton Bay	100
Central Moreton Bay	100
Southern Moreton Bay	102
<i>New South Wales</i>	
Brunswick River	100
Richmond River	102
Clarence River	155
Macleay River	100
Hastings River	100
Wallis Lakes	100
Port Stephens	100
Hawkesbury River	100
Georges River	140
Shoalhaven/Crookhaven	100
Bateman's Bay	100
Tuross Lake	100
Narooma	100
Merimbula	140

RESULTS/DISCUSSION:

Estuary Surveillance 2001:

During the 2001 sampling period a total of 5206 oysters were received and processed from 18 NSW estuaries. *Marteilia sydneyi* was diagnosed in samples from 2 estuaries: Clarence River (5/330, 1.52%) and the George's River (123/260, 47.3%) (see Table 4). Both the Clarence and the Georges River are known endemic estuaries for QX disease therefore these results were not surprising. One result that was unexpected was the absence of a detectable prevalence in the Richmond River given that QX disease is known to be endemic in this estuary.

Table 4: Surveillance results for samples collected from NSW during March-May 2001. Cytology was the diagnostic method used to detect for the presence of *Marteilia sydneyi*

2001 Survey Results

Estuary	N	N infected	%
Tweed River	316	0	0
Brunswick River	320	0	0
Richmond River	248	0	0
Clarence River	330	5	1.52
Wooli River	294	0	0
Kalang /Bellinger Rivers	295	0	0
Macleay River	261	0	0
Hastings River	330	0	0
Manning River	286	0	0
Wallis Lakes	271	0	0
Port Stephens	263	0	0
Hawkesbury River	323	0	0
Georges River	260	123	47.31
Shoalhaven/Crookhaven	255	0	0
Bateman's Bay	300	0	0
Tuross Lake	304	0	0
Narooma	300	0	0
Merimbula	250	0	0

Estuary Surveillance 2002:

During the 2002 sampling period a total of 5250 oysters were received and processed from 18 NSW estuaries and 3 Queensland zones using cytological methods (see Table 5). *Marteilia sydneyi* was diagnosed in samples from 4 estuaries, Southern Moreton Bay (2/250, 0.8%), Richmond River (102/250,

40.8%), Clarence River (55/250, 22%) and Georges River (40/250, 16%) of the 21 estuaries surveyed.

Table 5: Surveillance results for samples collected during March-May 2002. Cytology was the diagnostic method used to detect for the presence of *Marteilia sydneyi*

2002 Survey results

Estuary	N	N infected	%
Northern Moreton Bay	250	0	0
Central Moreton Bay	250	0	0
Southern Moreton Bay	250	2	0.8
Tweed River	250	0	0
Brunswick River	250	0	0
Richmond River	250	102	40.8
Clarence River	250	55	22
Wooli River	250	0	0
Kalang /Bellingen Rivers	250	0	0
Macleay River	250	0	0
Hastings River	250	0	0
Manning River	250	0	0
Wallis Lakes	250	0	0
Port Stephens	250	0	0
Hawkesbury River	250	0	0
Georges River	250	40	16
Shoalhaven/Crookhaven	250	0	0
Bateman's Bay	250	0	0
Tuross Lake	250	0	0
Narooma	250	0	0
Merimbula	250	0	0

Under the related project (FRDC 2001/630) 142 unexpected positives for *Marteilia sydneyi* were found in oysters scored as negative by cytological examination (Table 6) during surveillance in this project. Of these, 61 were identified in oysters sampled from estuaries with no prior record of *Marteilia sydneyi*. These represent oysters from Hastings R, Wallis Lake, Port Stephens, Bateman's Bay, Tuross Lake, Narooma and Merimbula. Note that Crookhaven/Shoalhaven and Hawkesbury R samples returned negative PCR results for 100 oysters screened from each estuary.

Table 6: PCR results from the related project FRDC 2001/630 on oysters which tested negative to QX using cytology in this project (FRDC 2001/214).

Estuary	Number sampled	PCR +ve's
Northern Moreton Bay	100	7
Central Moreton Bay	100	6
Southern Moreton Bay	100	16
Brunswick River	100	8
Clarence River	100	9*
Macleay River	100	35
Hastings River	100	3
Wallis Lakes	100	7
Port Stephens	100	2
Hawkesbury River	100	0
Georges River	100	0
Shoalhaven/Crookhaven	100	0
Bateman's Bay	100	4
Tuross Lake	100	19
Narooma	100	25
Merimbula	140	1

Once the results from PCR analysis were obtained, cytology slides for each of the oysters (+ve PCR) were scanned a second time to confirm the infection. Only 7/9 oysters from the Clarence R showed early developmental cell stages of *Marteilia sydneyi* however *M. sydneyi* cell stages were unsighted on any other slides. To confirm the presence of *M. sydneyi* from these other estuaries, archived tissues were processed for histological and DNA *in situ* hybridisation techniques.

Parasite stages were identified in oyster tissue from these samples using the *in situ* hybridisation process confirming the presence of the pathogen in an atypical, (with respect to seasonality) apparent early stage of development (Table 7). No proliferation through sporulation (development of spores) was identified in these samples. The results from *in situ* hybridisation then aided the location and identification of parasite stages in H&E stained histological sections. Note that Crookhaven/Shoalhaven and Hawkesbury R samples returned negative PCR results for 100 oysters screened from each estuary.

Table 7: Histology and *in situ* hybridisation results for oyster samples found positive using PCR from estuaries with no prior record of *Marteilia sydneyi*. Indicative pathology is defined as the presence of either focal or general haemocytosis – a non-specific indicator of the presence of a pathogen.

Estuary	PCR sample size	# PCR +ve	Histopathology	DNA probe <i>In situ</i> hybrid.
Hastings R	100	2	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised
Wallis Lake	100	6	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised
Port Stephens	100	2	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised
Bateman's Bay	100	7	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised
Narooma	100	22	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised
Merimbula	140	1	Yes, indicative pathology	<i>M.sydneyi</i> cells hybridised

Estuary Surveillance 2003:

During the 2003 sampling period a total of 4450 oysters were received and processed from 22 NSW estuaries and 3 Queensland zones using PCR methods. Of the 25 estuaries sampled in 2003, 6 estuaries (Cudgen Lake, Brunswick River, Richmond River, Clarence River, Georges River and southern Moreton Bay) tested positive for QX (see Table 8).

Table 8: Surveillance results for samples collected during March-May 2003. PCR was the diagnostic method used to detect for the presence of *Marteilia sydneyi* in accordance with the ANZSDP protocols (Adlard and Worthington Wilmer, 2003).

Survey Results

Estuary	N	N infected	%
Northern Moreton Bay	150	0	0
Central Moreton Bay	150	0	0
Southern Moreton Bay	150	1	0.67
Tweed River	150	0	0
Cudgen Lake (P2)	150	3	2
Brunswick River	150	2	1.3
Richmond River	150	20	13.3
Clarence River	150	9	6
Wooli River	150	0	0
Bonville Creek (P1)	150	0	0
Kalang /Bellinger Rivers	150	0	0
Macleay River	150	0	0
Hastings River	150	0	0
Manning River	150	0	0
Wallis Lakes	150	0	0
Port Stephens	150	0	0
Hawkesbury River	150	0	0
Sydney Harbour (P4)	150	0	0
Georges River	150	1	0.67
Shoalhaven/Crookhaven	150	0	0
Sussex Inlet (P3)	150	0	0
Bateman's Bay	150	0	0
Tuross Lake	150	0	0
Narooma	150	0	0
Merimbula	150	0	0

The prevalence of QX in sampled estuaries during 2003 was less than has been recorded during this project in previous years. The presence of QX in oysters sampled from Cudgen Lake, while previously unrecorded from this estuary is not surprising given that Cudgen is located in the northern rivers area of northern NSW, an area known historically for its QX endemicity and one in which positive estuaries have been identified during this project.

Further investigation of infected oysters from the Brunswick River and Cudgen Lake revealed the presence of the pathogen in an atypical early stage of development (with respect to seasonality) with no proliferation through sporulation recorded. We examined cytology slides for all infected oysters from

Cudgen Lake and Brunswick River and confirmed that these oysters would be diagnosed as false negatives using microscopic examination.

Estuary Surveillance 2004:

During the 2004 sampling period a total of 450 oysters were received and processed from 3 Queensland zones using PCR methods. In the Southern Moreton Bay zone (SMB) 21 oysters returned positive results using PCR testing. This represents the highest prevalence of QX disease in Southern Moreton Bay (SMB) for the 3 consecutive years of surveillance in this project. In southern leases within this zone the disease was at an advanced stage with oyster mortality and sporulation obvious in samples collected in mid-March of 2004. Further north in this zone, *M. sydneyi* was detected by PCR only, indicating a less-advanced stage of development. Differences in the development of infection are likely to be due to differences in a combination of parameters e.g. oyster fitness and infective dose and how these interact with environment. In both the Central and Northern Moreton Bay zones, *Marteilia sydneyi* was not detected by PCR or cytological examination (Table 9).

Table 9: Surveillance results for samples collected during March-May 2004. PCR was the diagnostic method used to detect for the presence of *Marteilia sydneyi* in accordance with the ANZSDP protocols (Adlard and Worthington Wilmer, 2003).

2004 Survey results

Estuary	N	N infected	%
Northern Moreton Bay	150	0	0
Central Moreton Bay	150	0	0
Southern Moreton Bay	150	21	14

The 3 years of surveillance in Moreton Bay zones confirm results for 2001-2003 testing in NSW in which the pathogen presented in populations with marked temporal (=seasonal) and spatial variability. Nonetheless, there are patterns of infection in those estuaries where *M. sydneyi* is endemic, with more estuarine localities having higher risk of disease.

The most significant complicating factor discovered through this project to date is the presence of the pathogen in estuaries where it had been previously unrecorded. Its presence obviously increases the risk of development of patent disease in those areas and given its sub-clinical presentation also increases the risk of translocation of 'apparently healthy' stock. Such estuaries should be ranked accordingly for management of stock translocation to other areas for relaying live in water.

Transmission Electron Microscopy:

The positive identification to species of *Marteilia sydneyi* was made from samples collected in 2002 in accordance with OIE guidelines. All electron microscopy analysis was undertaken with formalin fixed material which allowed species determination (as *Marteilia sydneyi* defined as possessing 2 tri-cellular spores in a sporont) (see Figures 2a&b).

Figure 2a: Electron micrograph of a formalin-fixed plasmodium of *Marteilia sydneyi* containing developing sporonts. Each sporont contains 2 developing spores (SP1 & SP2). PM - Plasmodium membrane; RG - refringent granules; arrow heads - sporont membranes.

Bar = 1µm

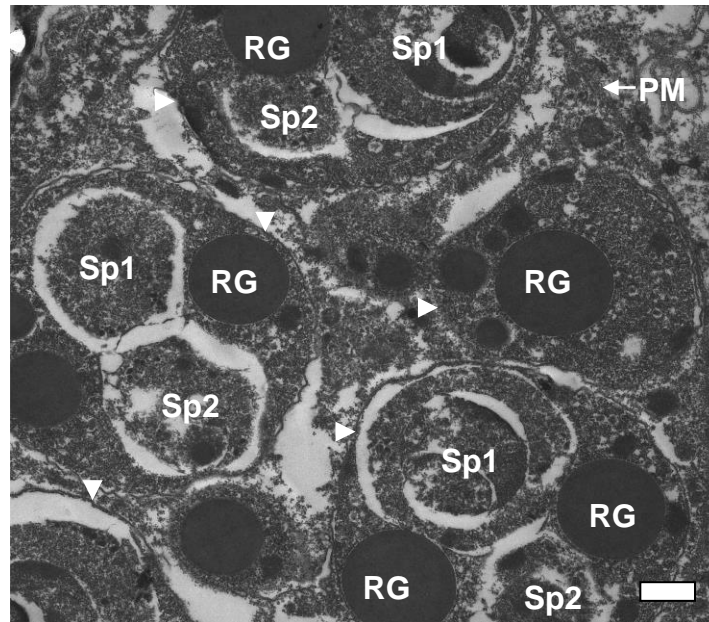
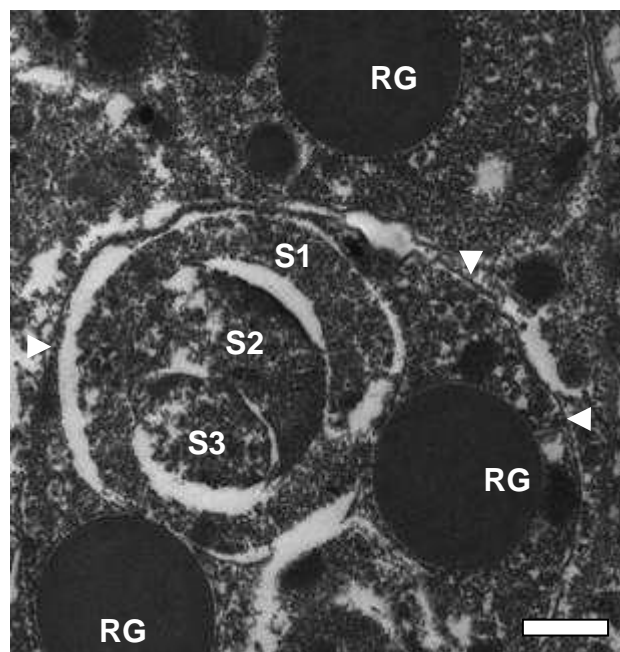


Figure 2b: Electron micrograph of formalin-fixed material showing 1 of 2 spores within a sporont. Spore contains 3 sporal cells (S1, S2, and S3). Refringent granules (RG) are visible in the extraspore cytoplasm. Arrows - sporont membranes. Bar = 1µm



Sub-sampling tissue

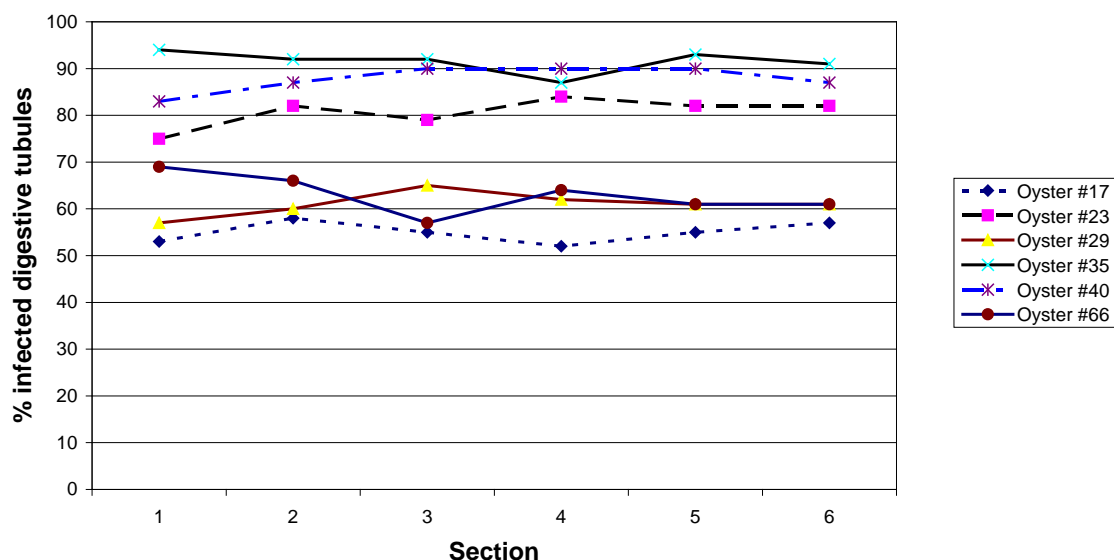
Sub-sampling of tissues for diagnostic testing is an issue to be considered regardless of the type of diagnostic technique then applied. It is of the utmost importance to ensure that tissues samples used for diagnostic purposes in a surveillance regime are accurate representatives of the whole.

In the case of QX disease, previous studies (Kleeman et al., 2002) on the progression of infection in individual oysters from gill/palp through connective tissue to digestive epithelium (in which sporulation occurs) allows diagnostic sampling to focus on the most likely target tissue (=digestive gland). Sub-sampling of this tissue is required for any diagnostic test thus the issue of homogeneity of infection within the tissue is critical.

In this study 44/50 (88%) oysters examined contained sporulating stages of QX. When the 'percentage of infected digestive tubules' is compared between sections from each of these oysters it is obvious that the infection is homogenous throughout and that sub-sampling does not compromise the ability to detect an infection because once sporulation commences almost all digestive tubules (99.88%) have been invaded by the parasite (see Appendix 4).

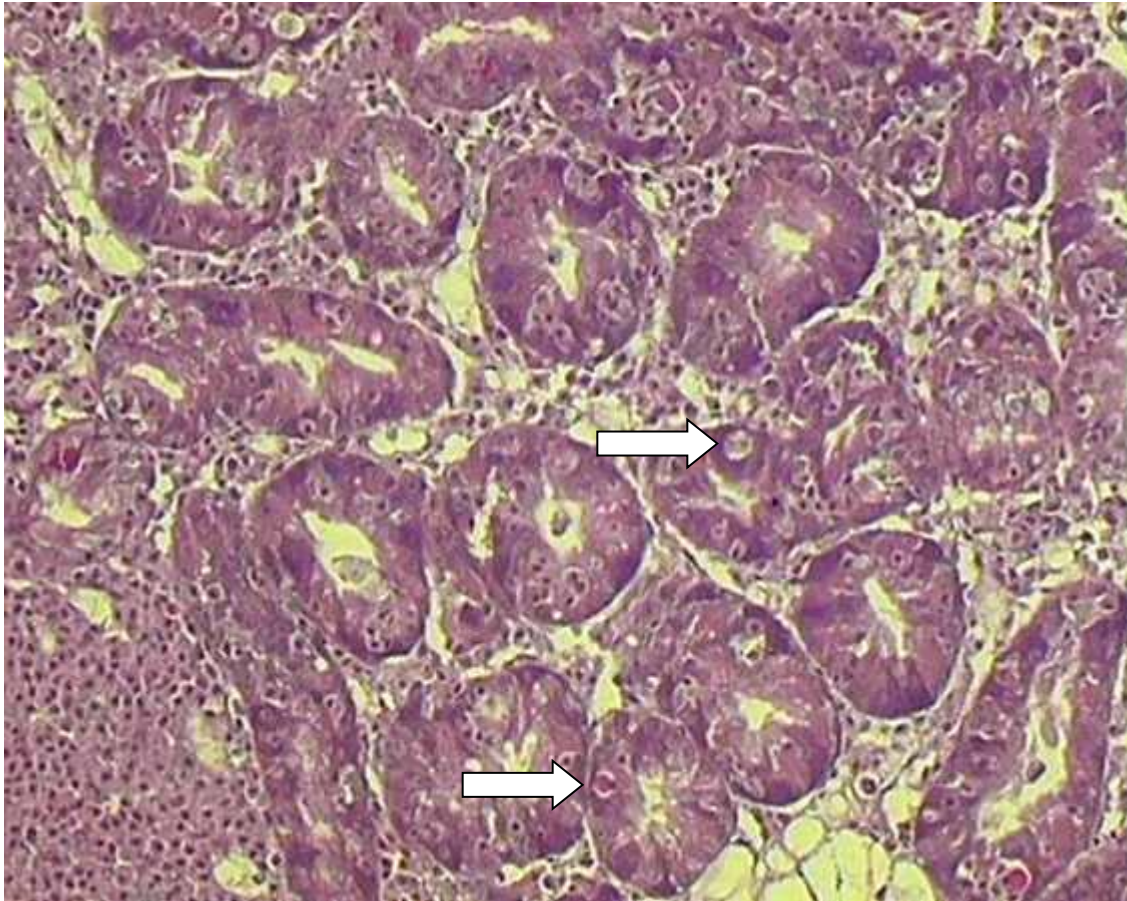
The remaining 6/50 (12%) oysters contained pre-sporulating stages of QX. When the 'percentage of infected digestive tubules' is compared between sections from each of these oysters (Figure 3) results show only minor variation. Oyster #66 had the greatest variation between sections of 6% from the mean (mean=63%, range=57-69%).

Figure 3: Comparison of '% infected digestive tubules' between sections cut from the digestive gland of QX infected oysters



Because not all digestive tubules have been invaded during early phases of QX development it is important to consider the distribution of QX cells within each section. As can be seen in Figure 4, although not all digestive tubules have been parasitized QX shows no preference for a specific region within the digestive gland with cells scattered evenly throughout.

Figure 4: Digestive gland of a rock oyster with pre-sporulating stages (arrows) of *M. sydneyi*. Note haemocyte infiltration throughout the tissue, common in pre-sporulating infections.



Therefore in all 50 oysters examined, infections were identified as homogenous throughout the digestive gland. Differences in '% digestive tubules infected' were observed between oysters however these differences were related to the stage of development of *M. sydneyi* in each oyster emphasizing the fact that the seasonality of QX disease and the development cycle in individual oysters remain significant issues in diagnosis and should be carefully considered when designing sampling protocols.

Comparison of Diagnostic Methods

Cytology compared with Histology

For this comparison 80 oysters were examined independently using both histology and cytology to diagnose the presence or absence of QX. The apparent prevalence of infection as recorded by histology and cytology were 80% (64 of 80) and 90% (72 of 80), respectively. By comparing the 2 diagnostic methods in a 2x2 contingency table (Table 10), making the assumption that both tests have a 100% specificity, because the 2 diagnostic procedures yield pathognomonic findings, the results show that cytology (96.88%) had a greater sensitivity than histology (86.11%) explaining the greater prevalence recorded by cytology. Calculations were made according to the unbiased method described in Staquet et al. (1981).

Table 10: A 2x2 contingency table summarising diagnostic results for *Marteilia sydneyi* from histology and cytology, for 80 oysters examined.

		Histology		
		Positive	Negative	
Cytology	Positive	62	10	72
	Negative	2	6	8
		64	16	80

Cytology compared with PCR

For this comparison 1839 oysters were examined independently using both PCR and cytology to diagnose the presence or absence of QX. The apparent prevalence of infection as recorded by PCR and cytology were 18.43% (339 of 1839) and 10.82% (199 of 1839), respectively. By comparing the 2 diagnostic methods in a 2x2 contingency table (Table 11), making the assumption that both tests have 100% specificity, the results show PCR (98.99%) had a greater sensitivity than cytology (58.11%). Calculations were made according to the unbiased method described in Staquet et al. (1981). The significant difference in sensitivity is not unexpected given that this study has shown that PCR is able to detect *M. sydneyi* infections at a much earlier stage of development than was previously possible by cytology or histology alone. PCR allows for detection long

before pre-sporulating and sporulating stages are easily identifiable through microscopy in the oyster digestive gland. These results have been confirmed by unambiguous identification of cells in the connective tissue, palps and gills using a combination of *in situ* hybridisation to locate the cells and histology to determine cell morphology.

Table 11: A 2x2 contingency table summarising diagnostic results from PCR and cytology, for 1839 oysters examined.

		PCR		
		Positive	Negative	
Cytology	Positive	197	2	199
	Negative	142	1498	1640
		339	1500	1839

Zoning Policy – Case Study

This project has collected the baseline surveillance and monitoring data necessary to identify QX disease-free and QX disease-affected areas. It was designed to be consistent with internationally recognised standards by the Office International des Epizooties and with the National Policy for the Translocation of Live Aquatic Animals and national Zoning Policy Guidelines. As such the results not only provide a scientifically defensible basis for implementing zoning policy but also highlight issues which are likely to be faced by other aquaculture industries in dealing with disease.

The most significant outcome on the implementation of zoning policy has been the discovery of the QX organism, *Marteilia sydneyi*, (a listed and reportable pathogen) throughout the whole geographic range of rock oyster commercial culture on the east coast of Australia. Furthermore, it is unlikely that unexpected infections in estuaries formerly thought disease free would have been detected without using the high sensitivity of diagnosis afforded by PCR.

Prior to the results of this project QX disease had been reported in epizootic condition from SE Queensland, the northern rivers of NSW and from Georges River, Sydney. The only recently confirmed report of the presence of the disease

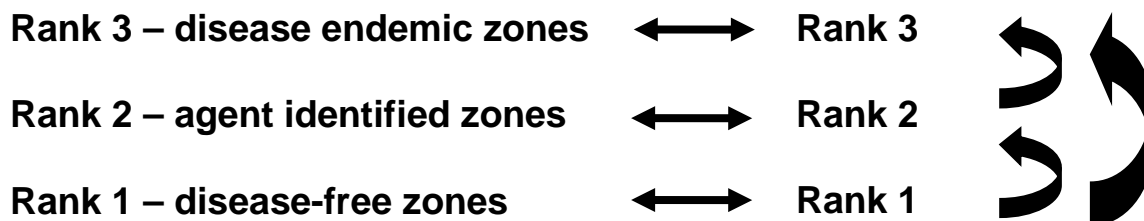
at low prevalence was from the Brunswick River, an estuary within the northern rivers district of NSW. All other estuaries were thought to be free of *Marteilia sydneyi* largely due to an absence of noticeable evidence of disease. This distribution had suggested that the pathogen was restricted geographically with management consequently predicated on the restriction of movement of potentially infected stock from disease endemic areas. However, huge numbers of oysters have been translocated between estuaries in NSW during the last few decades and given the volume of stock translocated it is statistically probable that the disease agent would have been transferred in that process. Indeed, recently published evidence (Kleeman, Adlard, Zhu & Gasser, 2004) lends support to past disease translocation events. *M. sydneyi* isolates from non-neighbouring estuaries (e.g. Great Sandy Strait in Queensland, Richmond & Georges Rivers in NSW) showed similar genetic sequence profiles while isolates from neighbouring estuaries showed genetic differences (e.g. Richmond & Clarence Rivers in NSW).

Given those considerations, it is more surprising that outbreaks of QX disease have not occurred in estuaries other than those in which it is considered to be endemic. A closer examination of oysters positive for *M. sydneyi* recorded in this project in formally 'QX-free' estuaries showed that the disease development appeared to be at an earlier stage than that seen typically in infected oysters during epizootics of disease. Both the lack of outbreaks and this aberrant development of disease indicate the presence of factors which regulate QX disease. There are now data implicating oyster immuno-competence as a determining factor in the success of disease establishment with changes in such immuno-competence correlated with environmental stressors (Peters & Raftos, 2003; Newton, Peters & Raftos, 2004). Equally, the dynamics of parasite transmission through an as yet undetermined alternate host (see Kleeman, Adlard, Zhu & Gasser, 2004 for discussion) and the dose-dependent nature of infection are likely to regulate development of clinical disease. The third effector in disease triangles, i.e. the environment, cannot be ignored particularly since the impact of environmental parameters (e.g. water salinity, pH, temperature, chemical composition, tidal flow) on *Marteilia sydneyi* transmission and establishment in oysters has been little studied. Indeed, environmental parameters have been shown to impact on the ability of oysters to defend themselves from disease (Peters & Raftos, 2004), while differences in the pH of estuarine water showed no causal link to QX disease outbreaks in SE Queensland (Anderson, Wesche & Lester, 1994; Wesche, 1995). Understanding the dynamic interactions between the host/s (oyster and unknown intermediate) the pathogen (*M. sydneyi*) and the environment are key to developing a full suite of management tools for QX disease and currently hampered by our inability to provide a laboratory model for testing such parameters.

What impact does this have on zoning policy and implementation? In determining disease status within zones, the OIE makes no distinction between either the presence of the pathogen or the presence of clinical disease. A zone

is considered to be free of disease if ‘no disease *agent* ... has been detected in any mollusc tested during operations of an official mollusc health surveillance scheme....’ (OIE, 2004). As such, for exports controlled by global animal health guidelines, all estuaries in which the agent of QX disease has been identified would be classed as infected and exports would not be allowed until those areas had proven to be disease-free for 2 consecutive years. Industry and management should consider that if export access for the rock oyster industry is to be developed, key estuaries in which *Marteilia sydneyi* has not yet been recorded (e.g. Crookhaven/Shoalhaven) are subject to a high level of protection against translocation of the disease agent.

In terms of domestic management there are clearly differences between estuaries: 1) those in which recurrent epizootics of QX disease occur, 2) those in which the agent has been identified but no epizootic disease has been recorded, 3) those that have been tested but no disease or agent identified and 4) those that have not been tested (these classified as infected until proven otherwise). It is recommended that these 4 distinctions are retained when assessing disease management plans and that a risk ranking be applied such that stock from low risk estuaries can be moved to estuaries of like or increased ranking but not the reverse i.e.:



BENEFITS:

The Sydney rock oyster industries in Queensland and New South Wales have suffered from a lack of basic information about QX disease for many years. Recently, concern has been increased by the first recorded outbreak of disease in the Georges River in 1994 which demonstrated clearly that this disease was not limited to subtropical latitudes of SE Queensland and northern New South Wales; and increased again by the outbreak of disease in the Hawkesbury River in 2004. Knowledge of the true geographic range of the parasite through the systematic sampling undertaken in this project has provided critical baseline information on which industry and management can assess the risk to commercial culture of oysters. Furthermore, through the information provided by this project, emphasis has moved towards local management of areas now known to harbour the pathogen. These include management of rock oyster stock movement to avoid periods of risk, consideration of alternative species for culture (e.g. *Crassostrea gigas*), adoption of hatchery-produced disease resistant lines of rock oysters.

Industry and management have already adopted the results of this project and have considered their response in the context of current farming practices.

FURTHER DEVELOPMENT:

We are currently in the process of preparing scientific manuscripts which will be aimed at reputable peer-reviewed journals as a means of disseminating further the information gained through this project. All draft manuscripts will be referred to the FRDC via the Aquatic Animal Health Subprogram for their information and approval prior to submission for publication.

PLANNED OUTCOMES:

The major outcome achieved through this project was the implementation and field-testing of the zoning policy framework developed under AQUAPLAN using QX disease of oysters as the practical context. Outputs from this project in the form of advice to industry groups and fisheries management, and publications in the scientific literature will provide the basis for the development of further zoning policies for other significant diseases of aquatic animals.

CONCLUSION:

The pathway that this project has taken reflects responses to all the issues of major concern in determining the distribution of an aquatic disease in the framework of management through regulating movement of stock.

The development of a statistically robust, theoretical sampling regime was coupled with field-truthing through the use of geographic co-ordinates placed within a 'stratified random' system within each estuary examined. Confidence (99%) of detecting the presence of QX disease at a prevalence of 2% or greater in the population (OIE recommended level) was satisfied through this sampling regime. Strict seasonality of QX disease has previously been recognised and documented, allowing sampling to be undertaken at a single time during each yearly cycle and further allowing sampling to be undertaken at that time when the probability of detection of infection was maximised (March-May) while the parasite is undergoing sporulation in the digestive tubules of the oyster.

After satisfying the requirements for field sampling, the diagnostic method for the presence of the disease was investigated. Regardless whether the method employed is histology, cytology or molecular, issues of tissue sub-sampling and homogeneity of infection can have a significant impact on the sensitivity of detection of disease. This issue was investigated by undertaking serial sectioning of the digestive gland of oysters in early-, mid- and late-stage infections. Results confirmed that homogeneity of infection in all but very early stages of disease agent incursion into the digestive gland provided sufficient homogeneity that sub-sampling of tissue for diagnosis did not have a significant negative impact on the ability of diagnostic tests to detect the disease.

Traditional diagnostic methods (histology, cytology derived from stained impression smears of digestive gland) were compared for their relative ability to detect QX disease. Eighty oysters were used in this trial with results indicating that cytology (96.88%) had a greater sensitivity of detecting QX disease than

histology (86.11%). Furthermore, cytology is more a cost-effective option for surveillance for QX disease. However, these diagnostic methods should be taken in the context of their use. Histology provides cues to the presence of pathogens through observation of pathological changes (e.g. inflammatory reactions) but these changes are often non-specific and cannot be used as diagnostic indicators. Equally, histological examination may detect unknown pathogens which would escape detection using cytology or molecular methods.

After development and optimisation of a PCR (polymerase chain reaction) based diagnostic test through a separate project (FRDC2001/630) this method was compared to cytology and found to have a greater sensitivity of detection (98.99%) than cytology (58.11%) in trials conducted on 1,839 oysters. As such, the diagnostic method used in surveillance in years 1 & 2 of this project was changed from cytology to PCR to allow more sensitive detection of QX disease in field surveys of 22 localities on the east coast of Queensland and New South Wales.

The sampling and diagnostic methods described above were applied to field samples collected from 18 estuaries in New South Wales in 2001, 2002 and 2003, and to samples collected from 3 zones in SE Queensland in 2003, 2003, and 2004. The most significant outcome from this study was the identification of the QX disease agent, *Marteilia sydneyi*, throughout the whole extent of commercial rock oyster farming, from Merimbula on the south coast of New South Wales, to Koorringal at Moreton Island, the most northerly zone examined during the surveillance program. This outcome alone has major implications to the management of such a pathogenic aquatic animal disease. The available information on distribution of QX disease prior to this project was patchy both temporally and spatially, but indicated that the disease and agent was limited to estuaries in northern NSW and SE Queensland with its most recent range extension being into the Georges River in Sydney in 1994. Management had recognised this apparent geographic limitation and responded by applying quarantine (for relaying of oysters live elsewhere) to those estuaries deemed to be endemic. The evidence provided by this project now indicates that the disease is regulated, not by the absence of the pathogen, but by either factors that regulate the intensity of infection (i.e. dose-dependence) and/or by estuary-specific factors that impact on the susceptibility of host oyster to disease.

The impact of host susceptibility on the progression of disease is now being investigated through a study funded by ARC Linkage projects (Raftos, Nell & Adlard).

A final point of consideration is the outbreak of QX disease in the upper reaches of the Hawkesbury River in 2004, the first recorded incidence in that estuary. Coincidentally, this outbreak immediately followed 3 consecutive years of surveillance in which QX disease was not detected in the Hawkesbury River (at a detection limit prevalence of 2% or greater). This outbreak indicates that QX disease has the ability to become established at a significant level (with concomitant mortality) in an oyster population within a single season. The dynamics underlying such a phenomenon are worthy of biological investigation,

particularly the identification and population dynamics of the as yet unidentified intermediate host of this disease.

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APPENDIX 1

INTELLECTUAL PROPERTY:

No intellectual property issues have been identified as arising from this project.

APPENDIX 2.

Staff List

Dr Robert D. Adlard - Principle Investigator
(Senior Curator, Biodiversity Program, Queensland Museum)

Dr Stephen Wesche - Research Officer
(FRDC funded, Research Officer, Biodiversity Program, Queensland Museum)

Dr Malcolm Bryant - Queensland Museum Research Assistant
(Senior Technician, Biodiversity Program, Queensland Museum)

Tony Charles - casual Research Assistant
(FRDC funded, casual appointment)

Matthew Mackay - casual Research Assistant
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Jodi Salmond - casual Research Assistant
(FRDC funded, casual appointment)

Paula Weatherhog - casual Research Assistant
(FRDC funded, casual appointment)

APPENDIX 3

IN SITU HYBRIDISATION ASSAY METHOD – QUEENSLAND MUSEUM

1. Fix tissue in 10% Formalin in seawater for up to 1 week
2. Embed tissue in paraffin and cut sections 6µm in thickness. Float sections onto silanized slides
3. Heat slides at 62°C => 45min to over night
4. Deparaffinize:

xylene	2x	10min each
Absolute alcohol	2x	10min each
5. Air dry
6. Prepare proteinase K (PK) fresh at 100µg/ml in 1xTE. Pipette 200µl of PK soln/slide and incubate for 30min at 37°C in a humid chamber. (coverslipping slides helps spread ProK evenly. The time for this step is variable, dependant on required digestion. This step may be repeated before moving to prehyb/ hyb buffers)
7. Dehydrate:

95% alc	1x	1min
100% alc	1x	1min
8. Air dry
9. Prehybridise 500µl/slide, 60min at 42°C with hybridization buffer
10. Dilute probe 5µl in 50µl of hybridization buffer (this may be reduced to 1µl if needed, but must be tested first)
11. Pipette 55µl of probe onto section and coverslip (coverslipping helps prevent drying of slides and allows even coverage)
12. Heat slides on hot plate 95°C for 5min. Quench on ice => 5min
13. Incubate in humid chamber, 42°C => overnight
14. Wash slides in prewarmed buffers

2xSSC	2x	5min	room temp (RT)
0.4xSSC	1x	10min	42°C
Maleic Acid Buffer	1x	1min	RT
15. Block sections with 200µl Blocking buffer for 30min at RT
16. Dilute anti-DIG-alkaline phosphatase (AP) conjugated antibody in Blocking buffer at a dilution of 1:500
17. Flood tissue with 200µl of diluted conjugate and incubate in humid chamber for 1hr at 37°C
18. Wash slides in Maleic Acid buffer

2x	1min
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19. Equilibrate slides in Detection buffer for 5min
20. Dilute 20µl BCIP/NBT in 1ml Detection buffer and pipette 200µl per slide and incubate in the dark for 10min to overnight (3-5hr has been the best so far)
21. Stop the reaction by washing slides in TE buffer for 15min RT. Rinse the slides in distilled water
22. Counter stain sections in Bismark Brown Y for 1min at RT
23. Dehydrate slides:

95% alc	2x	1min
100%alc	2x	1min
xylene	3x	1min
24. Mount with Depex

Appendix 4a: Oyster tissue sub-sampling - raw data

Oyster #	Infection intensity (cytology)	Parasite Stages Present	% Digestive Tubules Infected with <i>M. sydneyi</i> (Histology)						Average %
			Section 1	Section 2	Section 3	Section 4	Section 5	Section 6	
2	3	sporulating	100	100	99	100	99	100	99.67
3	1	sporulating	100	100	100	100	100	100	100.00
4	2	sporulating	100	100	100	100	100	100	100.00
5	1	sporulating	99	100	100	100	100	100	99.83
102	1	sporulating	99	100	100	100	100	100	99.83
7	1	sporulating	100	100	100	100	100	100	100.00
8	1	sporulating	100	100	100	100	100	100	100.00
9	1	sporulating	100	100	100	100	100	100	100.00
10	1	sporulating	100	100	100	100	100	100	100.00
11	1	sporulating	100	100	100	100	100	100	100.00
16	3	sporulating	100	100	100	100	100	100	100.00
17	5	pre-sporulating	53	58	55	52	55	57	55.00
20	3	sporulating	100	100	100	100	100	100	100.00
21	2	sporulating	100	100	100	100	100	100	100.00
23	5	pre-sporulating	75	82	79	84	82	82	80.67
27	2	sporulating	100	100	100	100	100	100	100.00
29	5	pre-sporulating	57	60	65	62	61	61	61.00
35	4	pre-sporulating	94	92	92	87	93	91	91.50
40	5	pre-sporulating	83	87	90	90	90	87	87.83
41	1	sporulating	100	100	100	100	100	100	100.00
43	1	sporulating	99	100	98	99	96	96	98.00
44	1	sporulating	100	100	100	100	100	100	100.00
47	1	sporulating	100	100	100	100	100	100	100.00
49	1	sporulating	100	100	100	100	100	100	100.00
50	1	sporulating	100	100	100	100	100	100	100.00
54	1	sporulating	100	100	100	100	100	100	100.00
58	1	sporulating	100	100	100	100	100	100	100.00
60	1	sporulating	100	99	100	100	100	100	99.83
61	1	sporulating	100	100	100	100	100	100	100.00
62	1	sporulating	100	99	100	100	100	100	99.83
64	1	sporulating	100	100	100	100	100	100	100.00
66	4	pre-sporulating	69	66	57	64	61	61	63.00
69	1	sporulating	100	100	100	100	100	100	100.00
70	1	sporulating	99	99	100	100	100	99	99.50
77	1	sporulating	100	100	99	100	100	100	99.83
79	1	sporulating	100	100	100	100	100	100	100.00
80	1	sporulating	100	100	100	100	100	100	100.00
81	1	sporulating	100	100	100	100	100	100	100.00
84	1	sporulating	100	100	99	100	100	100	99.83
87	2	sporulating	100	100	100	99	100	100	99.83
89	1	sporulating	100	100	100	100	100	100	100.00
90	1	sporulating	100	100	100	100	100	100	100.00
92	1	sporulating	100	100	100	100	100	100	100.00
93	1	sporulating	100	100	100	100	100	100	100.00
94	1	sporulating	100	100	100	99	99	99	99.50
95	1	sporulating	100	100	100	100	100	100	100.00
96	1	sporulating	100	100	100	100	100	100	100.00
98	1	sporulating	100	100	100	100	100	100	100.00
100	1	sporulating	98	100	100	100	99	100	99.50
101	1	sporulating	100	100	100	100	100	100	100.00

Appendix 4b: Master data from surveillance study - raw data is available in MS Excel format from Dr Robert Adlard contact R.Adlard@qm.qld.gov.au

Sample data (complete list details 15,357 individually identified oyster samples):

Oyster No.	Estuary	Location	Date Collect	Size (mm)	Cond. Factor (1-5)	DigGld Colour (1-3)	QX Intensity (1-5)	DNA Extract	PCR	PCR Result	Archived Tissue		
											Formalin	Ethanol	Frozen -80°C
14860	Sydney Harbour (P4)	Zone A	1/06/2003	72	3	3		1x	1x	neg	Yes	Yes	No
14861	Sydney Harbour (P4)	Zone A	1/06/2003	56	3	3		1x	1x	neg	Yes	Yes	No
14862	Sydney Harbour (P4)	Zone A	1/06/2003	54	4	3		1x	1x	neg	Yes	Yes	No
14863	Sydney Harbour (P4)	Zone A	1/06/2003	73	3	3		1x	1x	neg	Yes	Yes	No
14864	Sydney Harbour (P4)	Zone A	1/06/2003	68	3	3		1x	1x	neg	Yes	Yes	No
14865	Sydney Harbour (P4)	Zone A	1/06/2003	67	4	3		1x	1x	neg	Yes	Yes	No
14866	Sydney Harbour (P4)	Zone A	1/06/2003	71	3	3		1x	1x	neg	Yes	Yes	No
14867	Sydney Harbour (P4)	Zone A	1/06/2003	89	3	3		1x	1x	neg	Yes	Yes	No
14868	Sydney Harbour (P4)	Zone A	1/06/2003	63	3	3		1x	1x	neg	Yes	Yes	No
14869	Sydney Harbour (P4)	Zone A	1/06/2003	74	5	3		1x	1x	neg	Yes	Yes	No
14870	Sydney Harbour (P4)	Zone A	1/06/2003	48	4	3		1x	1x	neg	Yes	Yes	No
14871	Sydney Harbour (P4)	Zone A	1/06/2003	65	4	3		1x	1x	neg	Yes	Yes	No
14872	Sydney Harbour (P4)	Zone A	1/06/2003	75	4	3		1x	1x	neg	Yes	Yes	No
14873	Sydney Harbour (P4)	Zone B	1/06/2003	83	3	3		1x	1x	neg	Yes	Yes	No
14874	Sydney Harbour (P4)	Zone B	1/06/2003	60	3	3		1x	1x	neg	Yes	Yes	No
14875	Sydney Harbour (P4)	Zone B	1/06/2003	74	4	3		1x	1x	neg	Yes	Yes	No
14876	Sydney Harbour (P4)	Zone B	1/06/2003	58	4	3		1x	1x	neg	Yes	Yes	No
14877	Sydney Harbour (P4)	Zone B	1/06/2003	65	3	3		1x	1x	neg	Yes	Yes	No