Control of Winter Mortality and QX Disease in Sydney rock oysters

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Report prepared by R.J.G. Lester and R.D. Adlard

(ii) **Summary**:

Growout culture of Sydney rock oysters in New South Wales and Queensland is hampered by two diseases, 'winter mortality' and 'QX' disease. These are caused by the protistans *Mikrocytos roughleyi* and *Marteilia sydneyi* respectively. Our survey results, based on stained imprints and PCR assay, demonstrated that *Mi. roughleyi* is in oysters throughout the year in areas known to be susceptible to winter mortality. To ameliorate the disease oysters should be obtained from disease free areas and kept in growout areas susceptible to winter mortality for a limited time prior to selling for consumption. The most significant finding is that oysters may become infected throughout the whole year and not only in early winter and just prior to obvious impact of the disease (characterised by the presence of focal lesions on oyster tissues and significant mortality of oysters).

Our epidemiological studies on QX disease indicate a strong correlation between the disease, fine mud and benthic fauna. The PCR assay, perfected late in the project, suggests that a component of the benthic fauna may play an integral part in the development of the *Ma. sydneyi*. Further research on this is necessary to confirm the link and to develop control measures.

Our research shows clearly that outbreaks of QX disease are not correlated with significant changes of pH in the water of culture areas.

(iii) **Background to the research project**

For several decades two diseases have been known to hamper production in the culture of Sydney rock oysters *Saccostrea commercialis*. They are 'Winter Mortality' and 'QX' disease, both caused by protozoans. Management practices have developed to keep losses within manageable limits, however, in the last 8 years losses have escalated.

Winter mortality

From Crookhaven to Eden on the south coast of NSW consistently high losses of oysters on trays as a result of winter mortality have occurred in recent years. Losses averaged 35% per annum, equivalent to an annual loss on the south coast of NSW of about 2 million dollars.

In 1992, 70% of the oysters being grown on trays in the Georges River were destroyed by winter mortality, a loss of 3.5 million dollars. Some oyster farmers lost 100% of tray stock, with individual losses as high as $150,000. Significant losses also occurred in Port Stephens. Control measures usually applied are to raise the oyster trays or to move the oysters to low salinity areas. Both these measures were ineffective in the Georges River in 1992.

Winter mortality is caused by the protozoan, *Mikrocytos roughleyi*. The project aimed to determine where the parasite was between outbreaks of winter mortality (Adlard, 1994a).

QX Disease

QX Disease kills oysters on leases in estuaries and bays in southeast Queensland and northern NSW, usually during the summer. To avoid the disease leases are left empty from

January to March each year. This reduces the growing season for oyster farmers in QX endemic areas by 25%.

During the project a devastating outbreak of QX disease was diagnosed by us in the Georges River in 1994 (Adlard, 1994b) and again in 1995. This epizootic apparently differed in its timing from outbreaks in northern NSW, and was 400km further south than any previous record of QX. A detailed investigation of these outbreaks became the subject of a separate project (FRDC94/156 Adlard & Lester) and is reported elsewhere.

QX disease is caused by the protozoan *Marteilia sydneyi*. Its life cycle, method of transmission and reservoir host are unknown.

FIRTA, the forerunner of FRDC, funded an earlier project at the University of Queensland (FIRTA 81/20) to investigate the epizootiology and life cycle of the QX organism. Many aspects of the disease were clarified. Some farmers had noticed that disease often accompanied the 'first fresh', i.e. the first heavy rain of the summer. They concluded that stress associated with low salinity caused the disease latent in the oysters to become overt. I confirmed that on at least one lease, the oysters became infected immediately after heavy rain. These were new infections, however, as oysters put out just before the rain became as heavily infected as those already on the lease. In other words the parasite arrived at the time of the fresh and was not lying latent in the oysters (Lester, 1986). Some heavily infected creeks drained cane farms or other agricultural land; others, such as those in Pummicestone Passage, ran from pristine forest. This indicated that agricultural practices were not necessarily linked to outbreaks. I found that in Moreton Bay and the Tweed/Terranora Lakes new infections occurred only in the summer. The timing varied but the last infections for the season occurred before the second week of April. Oysters died within 6 weeks (as reported by Wolf, 1979) at the height of the summer; those infected late in the summer gradually died off during the winter with some surviving until the following spring. There was no relationship between the presence of overwintering infections and the likelihood or severity of an outbreak the following summer suggesting that the parasite normally overwintered away from the oyster. Oysters from areas where the disease was endemic had no more resistance to infection than oysters from outside the QX area (Lester, 1986).

Growers believed that the parasite was not transmitted directly from oyster to oyster. My data supported this as I was unable to transmit the parasite experimentally. The spore of *M. sydneyi* is thin walled and apparently dies within a few days in sea water. Most spores are released into the water before the oysters die (Roubal, Masel & Lester, 1989). In ultrastructure, the spore resembles that of the PKX organism found in the kidney of salmonids. This suggested that a fish might be the unknown alternate host in the life cycle of the parasite. Two species of mullet and one of bream were fed spores, with encouraging results at first (Lester & Healey, 1986) but the sporoplasms did not penetrate the fish and little further development took place. We turned our attention to possible infections in invertebrates around oyster leases and developed an indirect fluorescent antibody test (IFAT) to detect the parasite in environments away from the oyster (Roubal & Lester, 1987). The antibody reacted strongly with the refringent granules and the spore wall but only weakly to the contents of the spore itself (Roubal *et al.*, 1989). As only the contents of the spore are likely to be present in the alternate host, the IFAT was considered to be a unreliable guide to infection. Therefore, in the absence of a reliable method to detect the parasite in the environment, this line of research had to be temporarily discontinued.

In 1992 Dr Joan Vickers, then a postdoctoral fellow with me in the Department of Parasitology, developed a DNA probe for monodon baculovirus (MBV), a virus of prawns (Vickers *et al.*, 1992). With this new molecular capability, Dr Mialhe and I successfully applied for a grant from the Australian Research Council (ARC) to develop DNA probes for *Mikrocytos roughleyi* and *Marteilia sydney*. The first probes for these organisms were made by Dr R. Adlard, postdoctoral fellow on the project, and Tim Anderson, Ph.D. student (Adlard & Lester, 1995; Anderson, Adlard & Lester, 1995). We were then in a position to apply to FRDC for funds to develop the probes for routine use and apply them to clarify the epidemiology and the life cycles of the parasites.

The details of the biology of the parasites are essential to formulate management practices specifically targetted to a particular stage of the parasite. For example, if the QX organism was found to develop in a fish, a borer, a mudworm, a flatworm or a crab, all of which have been suggested to be potential reservoirs, control of the the alternate host may be possible, perhaps by modifying the environment, and screening of them for QX parasites would be a useful strategy for risk assessment. In addition, knowledge of the life cycle permits the development of experimental infections in oysters so that effectiveness of treatments can be assessed and genetically resistant stocks selected. This project aimed to perfect the pilot DNA based tests developed under the ARC grant and to apply them to meet specific objectives important to the industry.

(iv) **Objectives of the research project**

1. To find where the disease-causing parasites are when there is no disease apparent in the oysters
2. To evaluate the epidemiology of the two diseases through investigations of reservoir hosts, resting stages and prepatent infections
3. To evaluate the possible relationship between infection and low pH in QX disease
4. To develop strategies to reduce the impact of QX disease and winter mortality on oysters.

The milestones specified were:

Dec 1993 Set up sampling regime for winter mortality in two estuaries in NSW and evaluate prevalence of the parasite.

July, 1994 Probe to detect *Mikrocytos roughleyi* now functional

Dec. 1994 Survey for *M. roughleyi* in Georges River complete

July 1995 Survey of one NSW and one Qld estuary for QX disease and its association with pH, salinity and temperature complete

Dec. 1995 Probe for *Marteilia sydneyi* now functional

July 1996 Survey for *Mikrocytos roughleyi* in second estuary complete. Search and survey for *Ma. sydneyi* in alternate hosts complete. Recommendations to growers to avoid disease.

(v) **Introductory technical information concerning the problem or research need**

The two organisms *Mikrocytos roughleyi* and *Marteilia sydneyi* are difficult to diagnose in lightly infected oysters. Indeed, *Mikrocytos roughleyi* was only recognised to be the cause of winter mortality in 1988 (Farley, Wolf & Elston, 1988) though the disease had been described and studied since the 1920s (Roughley, 1926). Both organisms are virtually impossible to detect in the water column or in another organism on the basis of their morphology.

Molecular techniques, particularly using DNA probes, are very sensitive and can detect small amounts of particular DNA. We used the polymerase chain reaction (PCR) to amplify part of the DNA of the parasite. If the parasite is present in a piece of oyster tissue, the piece of parasite DNA is in such a high concentration after amplification by PCR that it can be visualised on a gel or sequenced. Thus the technique, when properly developed, can detect very small numbers of parasites not detectable by normal microscopy.

(vi) **Research methodology**

Winter mortality

Oysters from 5 sites were regularly sampled for the survey: Merimbula (lease holder Hugh Wheeler), Narooma (lease holder John Ritchie), and in the Georges River at Wooloware Bay, Oyster Bay and Double Bay (various lease holders co-ordinated by Andrew Derwent) (Fig. 1a, 1b). Samples of 30 oysters were bagged and bags fixed to culture trays at each site. Bags were collected approximately monthly and checked for infection. Extraordinary samples were taken elsewhere as reports of sick or dying oysters came to us either directly or through officers of NSW State Fisheries.

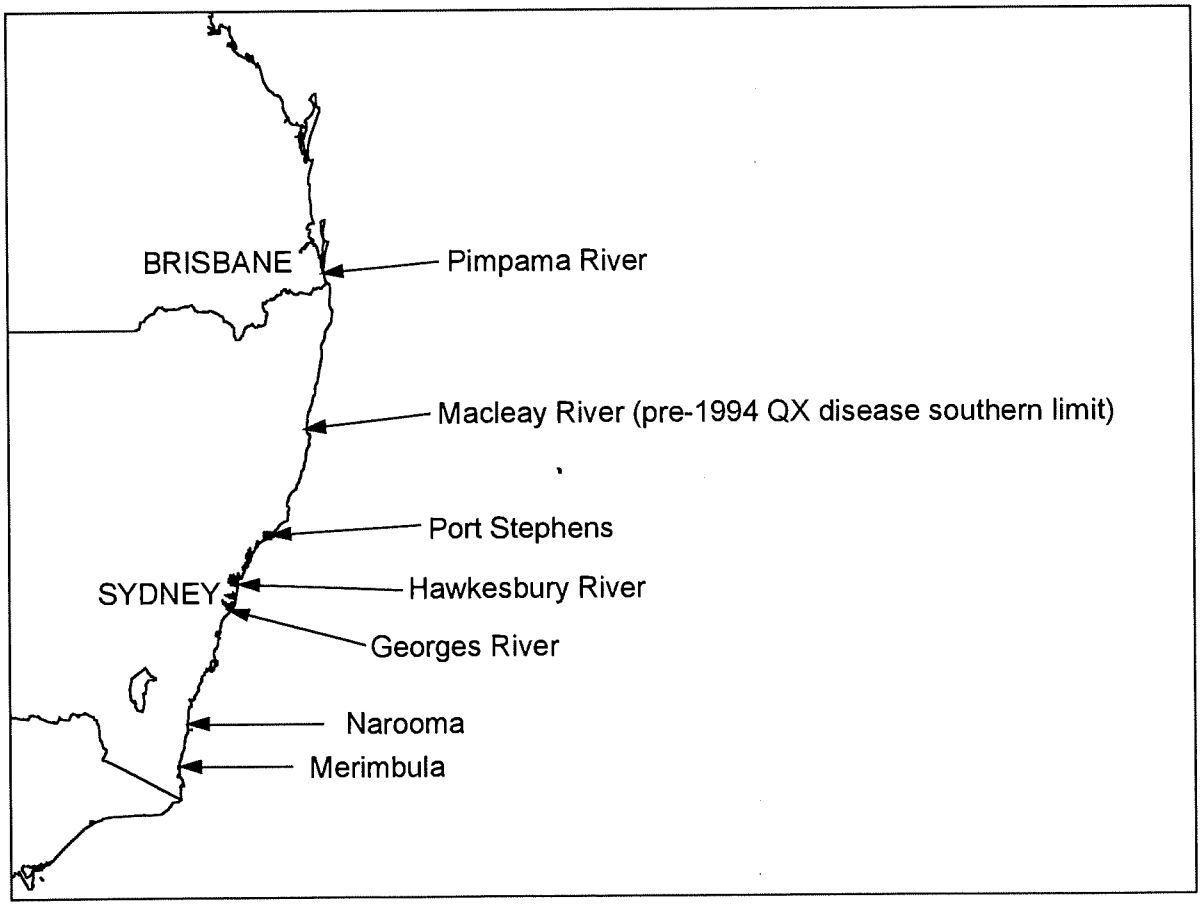

Figure 1a: Locality map of oyster sampling sites.

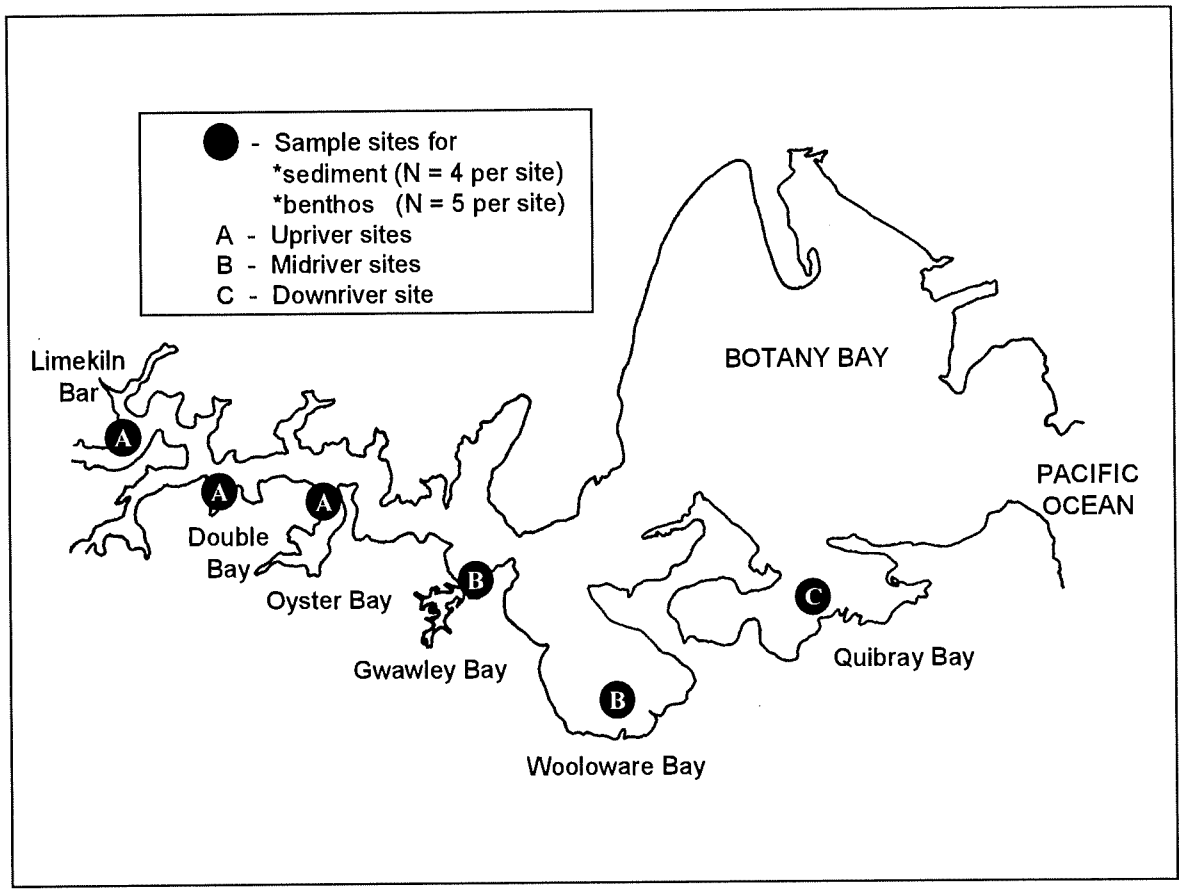

Figure 1b: Locality map of oyster, sediment and benthic fauna sampling sites within the Georges River, Sydney.

Routine diagnosis of winter mortality was based on seeing microcells in tissue imprints of one of the heart, mantle, gill, palp, or adductor muscle. The imprints were made on glass slides and stained with Hemacolor (Merck). Intensity of infection was recorded as the average number of parasites per 400 host cells. The presence of focal lesions on the gills, palps and mantle which are indicative of winter mortality (Roughley, 1926) was also recorded.

In the PCR based test a pair of primers were used to amplify a piece of parasite rDNA 680 base pairs long. The DNA was visualised on an agarose gel as described by Adlard & Lester (1995). DNA was extracted from known infected and known uninfected tissue using standard proteinase K digestion, phenol/chloroform purification and cold ethanol precipitation and stored in ultrapure water at -20°C. Amplification of the internal transcribed spacer region (ITS) within the ribosomal gene cluster (rRNA) was undertaken using two PCR primers (Table 1). Total reaction volume for PCR was 50µl, with the following reaction parameters (final concentrations): $MgCl_2$ - 2mM, buffer - 67mM Tris-HCl, 16.6mM $(NH_4)SO_4$, 0.45% (v/v) Triton X-100, dNTP's - 200mM, primers - 10pmol, Taq polymerase (Bresatec) - 3 Units, DNA - 4µl of 50µl (extracted from 1mm cube of oyster tissue or homogenised pellet), ultrapure water to 50µl total. Thermal cycling parameters were as follows: denaturation at 95°C for 60s, primer annealing at 55°C for 30s, chain extension at 72°C for 60s.

DNA from oysters from Moreton Bay that had been infected experimentally was used in PCR to test for the presence of *M. roughleyi*-specific amplified products (i.e. as positive controls). The experimental infections were produced by inoculating into the naive oysters a filtered but unpurified homogenate from oysters infected naturally from southern NSW collection sites determined by microscopic examination. The disease was allowed to develop in inoculated oysters that were maintained in laboratory aquaria for 5 months at 10°C. The

presence of *M. roughleyi* in experimentally infected oysters was confirmed from microscopic examination of stained tissue imprints. DNA from uninfected oysters (diagnosed from imprints) from the three sites in NSW and from uninfected oysters from Moreton Bay were used as a series of negative controls.

Amplified products (amplicons) were electrophoresed on a submarine agarose gel (1.2% (w/v) agarose, 1.2µl ethidium bromide (10mg/ml w/v) in 20ml total, for 30mins at 100V/20mA) and visualised under ultraviolet light. A molecular weight standard (100bp ladder, Gibco BRL) was used to estimate the size of amplicons.

The PCR based assay was applied to oyster stock that had been placed on leases in Merimbula. The oysters had been *in situ* for 12 months and were sampled in May, at least a month before winter mortality normally appeared.

Table 1. Oligonucleotide primers used in PCR amplification of DNA extracted from oysters that were infected with *Mikrocytos roughleyi*, *Marteilia sydneyi* or were uninfected oysters. Primer designation, sequence and approximate position relative to the rDNA gene cluster (SSU - small subunit, LSU - large subunit). Primers marked with an asterisk (*) were used to produce a *Mi. roughleyi* specific amplicon, those with ([a]) were used for developing the *Ma. sydneyi* primers.

PRIMER DESIGNATION	PRIMER SEQUENCE	PRIMER POSITION AND DIRECTION
SB2[a]	5'-GTTCCCCTTGAACGAGGAATTC-3'	3' end SSU, forward
RA2*	5'-GTCCCTGCCCTTTGTACACA-3'	3' end SSU, forward
RA58[a]	5'-CGCATTTCGCTGCGTTCTTC-3'	5.8S, reverse
ITS2.2*	5'-CCTGGTTAGTTTCTTTTCCTCCGC-3'	5' end LSU, reverse

QX disease

The PCR based test for *Marteilia sydneyi* developed by Anderson *et al.* (1995) had been shown previously to be specific for the pathogen. However, its sensitivity had not been quantified, and it was likely that a high sensitivity would be required to detect the presence of the pathogen in an intermediate host. To assess its sensitivity, sporonts *of M. sydneyi* were collected from infected oysters, purified and counted using a Nebauer haemocytometer. Serial dilutions of the sporonts (between 1 x 10^6 sporonts and 1 x 10^3 sporonts) were made and DNA extracted from each diluent. PCR amplification was performed (as detailed in Anderson *et al.*, 1995) and results assessed on ethidium bromide stained agarose gels. The sensitivity proved to be relatively low (consistent positive amplification from 1 x 10^4 sporonts and not less) regardless of attempts to optimise the reaction environment. It was decided that, in its current form, the PCR test was inappropriate to apply in the detection of *M. sydneyi* stages in alternative hosts.

While this work was underway, we took samples of sediment and invertebrates from the Georges River following a recent epizootic of QX disease. Sediment was collected and benthic fauna sampled from the Georges River (N=25 benthic samples - 5 sites with 5 samples at each site) using a 0.25 sq.m. Van Veen grab. Particle size of sediments in areas of high, medium and low QX prevalence was analysed (Bowler Geotechnical Pty. Ltd., Brisbane; sieve method). Benthos was collected from the same sites as the sediment, sieved through 400µm mesh and the fauna characterised. PCR was then used to test part of the benthic fauna for the presence of DNA of *M. sydneyi*, using samples from Port Stephens as negative controls.

To redevelop the PCR bioassay, DNA was extracted from sporonts of *Marteilia sydneyi* taken from the hepatopancreas of infected oysters from Moreton Bay, Queensland and from the Georges River, NSW. Extraction methods and PCR amplification followed the protocol as given above.

The relationship of QX infection and the pH of the water was determined at two sites, the mouth of the Brisbane River and the mouth of the Pimpama River, both in southeast Queensland. A third site on the Clarence River was abandoned because of theft of the oyster bags. Bags were placed in the water every 2 weeks and retrieved after 6 weeks to establish the date of any new infection to within 2 weeks. pH was monitored adjacent to the bags by a Hydrolab Datasonde 3 (CHK Industries) on the Brisbane River and by a TPS 90FL unit with a programmable logger on the Pimpama River. Readings were recorded 60 cm below the surface every 30 minutes (Anderson *et al.*, 1994; Wesche, 1995).

(vii) **Detailed results**

Winter mortality

Objective 1. *To find where the disease-causing parasites are when there is no disease apparent in the oysters.*
Objective 2. *To evaluate the epidemiology of the disease through investigations of reservoir hosts, resting stages and prepatent infections.*

These objectives were met together as part of our survey for winter mortality. We used gross signs and tissue imprints stained with Hemacolor for our routine tests and the PCR test for specific cases. Assessment of the sensitivity of the PCR assay for *M. roughleyi* showed that the lowest intensity that was tested (1 microcell per 800 host cells) gave negative results. At 1 microcell per 400 host cells the diagnostic band at 680bp was present. We estimate the test is at least one order of magnitude more sensitive than previous diagnostic techniques.

Gross signs of the disease were prevalent in late winter, the recognised danger period for winter mortality, and occasionally at other times of the year. *Mikrocytos roughleyi* was detected in imprints from oysters with lesions during the late winter but was also found in oysters during the summer. Of the total of 40 oysters positive by this test, 33 (82.5%) were sampled between June and October, and the remaining 7 (17.5% of total infections detected by imprint) were oysters sampled in December, February, March and April. (Table 2). Designation gives the range of the oysters numbered in the sample. 'Young stock' refers to oysters 1 year old; 'old stock' refers to oysters 2 to 3 years old. Though most infections were in old stock two infections were detected in 1 year-old oysters (GRY3 and GRY111). The oyster that returned the highest intensity of infection of all oysters sampled was ME66 which was sampled from Merimbula in December, 1994.

Table 2. Prevalence of gross lesions characteristic of winter mortality in oysters from NSW in 1993 and 1994 and the occurrence of *Mikrocytos roughleyi* 'microcells' as detected by the stained imprint method.

SAMPLE LOCATION	DESIGNATION	DATE COLLECTED	GROSS LESIONS	POSITIVE FOR *M. roughleyi*
Georges River (Oyster Bay)	GRA1-30	30/7/93	7 of 30	2 of 30
"	GRA31-68	6/9/93	15 of 38	12 of 38
"	GRA69-98	5/10/93	20 of 30	11 of 30
"	GRA100-134	27/10/93	18 of 35	2 of 35
"	GRA135-154	20/1/94	1 of 19	0 of 19
"	GRA155-191	8/4/94	8 of 37	1 of 37
"	GRA192-209	20/5/94	1 of 17	0 of 17
"	GRA210-228	9/6/94	2 of 18	0 of 18
"	GRA229-247	15/7/94	4 of 18	2 of 18
Georges River (Double Bay)	GRB1-34	31/7/93	4 of 34	0 of 34
"	GRB35-54	10/3/94	2 of 19	1 of 19
"	GRB55-93	8/4/94	0 of 38	0 of 38
"	GRB94-133	1/6/94	1 of 39	0 of 39
"	GRB134-147	15/7/94	0 of 13	0 of 13
Georges River (Wooloware Bay) young stock	GRY1-20	1/8/93	0 of 20	1 of 20
"	GRY21-40	22/1/94	0 of 20	0 of 20
"	GRY41-71	8/4/94	5 of 30	0 of 30
"	GRY72-91	20/5/94	1 of 19	0 of 19
"	GRY92-111	9/6/94	2 of 19	1 of 30
"	GRY112-128	15/7/94	1 of 16	0 of 16
Narooma	NA1-20	23/1/94	0 of 20	0 of 20
"	NA21-40	7/3/94	0 of 20	2 of 20
"	NA41-56	18/3/94	0 of 16	0 of 16
"	NA57-74	28/4/94	0 of 18	0 of 18
"	NA75-92	7/6/94	0 of 18	0 of 18
"	NA93-125	17/8/94	5 of 33	2 of 33
Merimbula	ME1-40	2/9/93	1 of 30	0 of 30
"	ME41-60	20/10/93	1 of 20	0 of 20
"	ME61-100	20/12/93	7 of 40	1 of 40
"	ME101-120	2/2/94	5 of 20	0 of 20
"	ME121-150	15/2/94	1 of 9	1 of 9
"	ME151-169	8/3/94	0 of 19	1 of 19
"	ME170-188	6/4/94	1 of 19	0 of 19
"	ME189-207	28/4/94	0 of 19	0 of 19
"	ME208-227	8/6/94	1 of 20	0 of 20
"	ME228-266	17/8/94	1 of 39	0 of 39

Oysters for the PCR assay were taken in May prior to any sign of winter mortality. They were first assessed using the imprint method. All were negative (2 minute scan per

tissue, N= 48 oysters). The PCR assay gave positive results for 30 of the 48 oysters (63%), indicating that *M. roughleyi* was indeed present. Imprints of the oysters that tested positive from PCR were re-examined microscopically (unlimited scan time per tissue) and microcells were eventually found in 4 of the 30 oysters, albeit at very low intensities (< 1 microcell per 1,000 host cells). It is clear from these results that *M. roughleyi* was present in a high proportion of oysters during a period when no disease was apparent in the oysters.

The PCR test for winter mortality is currently being used to diagnose the presence of *M. roughleyi* in triploid and diploid oysters in a collaborative study with Dr John Nell of NSW Fisheries Port Stephens Research Centre.

(**Objective 3.** Relates to QX only.)

Objective 4: *To develop strategies to reduce the impact of winter mortality on oysters.*

Our results on the epidemiology of winter mortality indicate that the organism can be detected in oysters during a whole year's cycle rather than just prior to and during the period of significant mortality in winter. This implies that current control methods (which involve translocating oysters into the upper reaches of estuaries), are successful **not** because infections are avoided by such translocation, but rather because environmental conditions in those areas preclude disease development in already infected oysters.

Anecdotal evidence suggests that winter mortality occasionally occurs in areas thought 'safe' from the disease, our hypothesis then explains such occurrences if they are linked to shifts in environmental parameters at those sites e.g. increased salinity due to periods of low rainfall.

Given that 1 year old oysters do not suffer the same mortality as 2-3 year old oysters and that we have identified *M. roughleyi* in the young stock, it follows that the development of the disease is slow in the oyster. In the laboratory we found that infections of oysters inoculated with thousands of parasites took several months to develop. Even after that time the intensity of infection was relatively low. Corroborating evidence was provided after collaboration with Dr Dominique Hervio from the Pacific Biological Station (Nanaimo, Canada) who was studying *Mikrocytos mackini* (Denman Island disease) in the Pacific oyster. The intensity of eperimental infections with *M. mackini* were found to be directly proportional to the original dose given. These facts suggest that with *M. roughleyi*, overt disease may develop more rapidly if oysters are continually acquiring infections. Though it is not known how the parasite is transmitted in the field, the ease with which we were able to transmit infection by inoculation suggests that transmission is directly from oyster to oyster. Oysters in areas of 'light' winter mortality are repeatedly exposed at a low level and mortalities are not likely until the oysters have reached a potential lethal intensity of the organism. Whether mortalities then occur may depend on environmental variables such as temperature and salinity as healthy unstressed oysters may be able to control the multiplication of the parasite. Oysters in areas of 'heavy' winter mortality apparently develop lethal intensities of the organism more quickly and mortalities then occur rapidly given the correct conditions.

It follows that any effective long-term control of winter mortality must be related to the place of origin of the oyster stock and the length of time that it has been exposed to infection by association with infected stock. This will require a co-ordinated effort by members of the oyster industry. To optimise production in areas of known winter mortality, if there is a choice

between stock caught in areas of known winter mortality and stock from disease free areas, then stock from disease free areas should, in preference, be used for growout. We hypothesise that immediately this stock is placed in a known winter mortality area it will be subjected to repeated infection (rather than only subjected to infection during the start of winter). Therefore, growout duration in areas of known winter mortality should be kept to a minimum.

QX Disease

Objective 1. *To find where the disease-causing parasites are when there is no disease apparent in the oysters.*
Objective 2. *To evaluate the epidemiology of the disease through investigations of reservoir hosts, resting stages and prepatent infections.*

While perfecting the DNA probe, sediment samples were taken from the Georges River for correlations between QX infection and particle size. The results show that fine mud (particle size less than 0.075mm) is strongly correlated with QX prevalence (Fig. 2). Details of the prevalence of QX in the Georges River are given in the final report for project FRDC94/156.

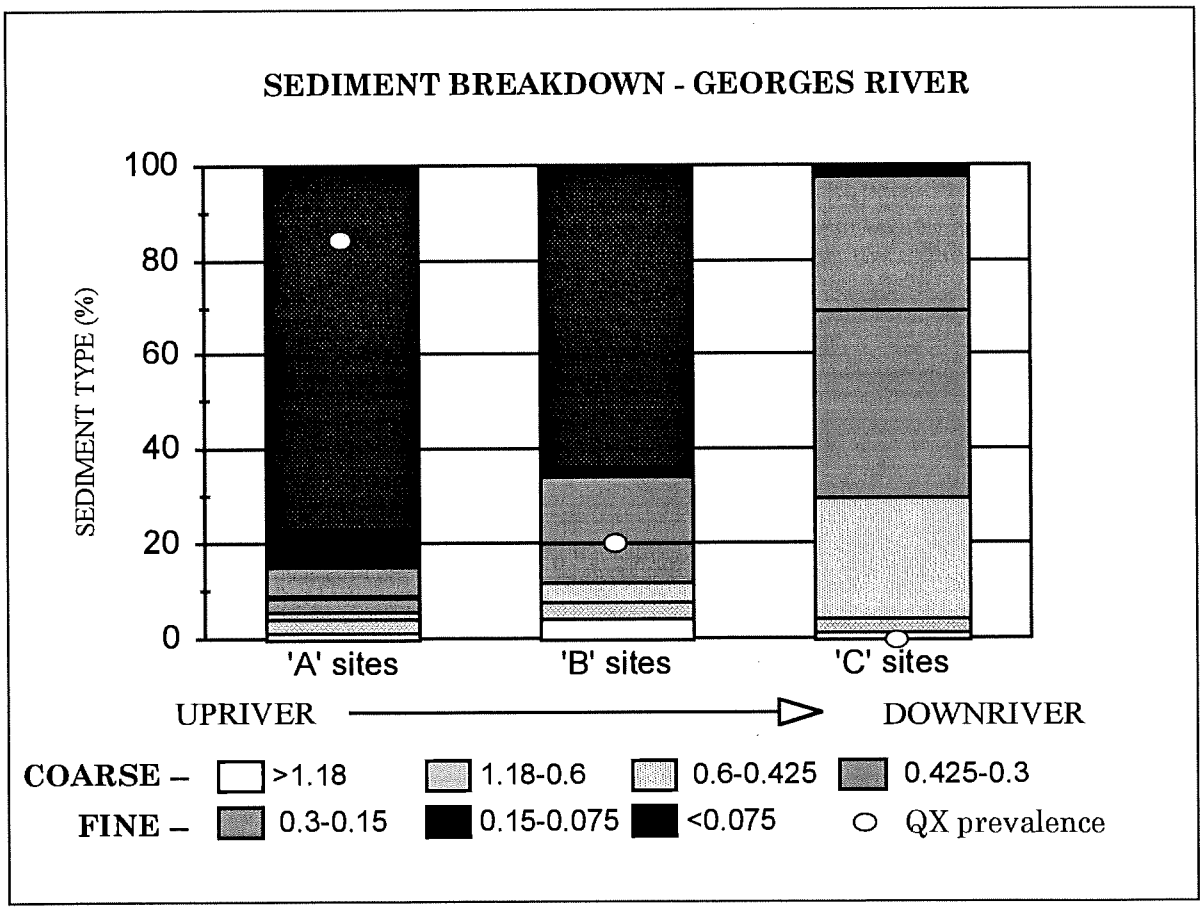

Figure 2. Particle size of sediments from three localities in the Georges River and the prevalence of QX in those localities in July.

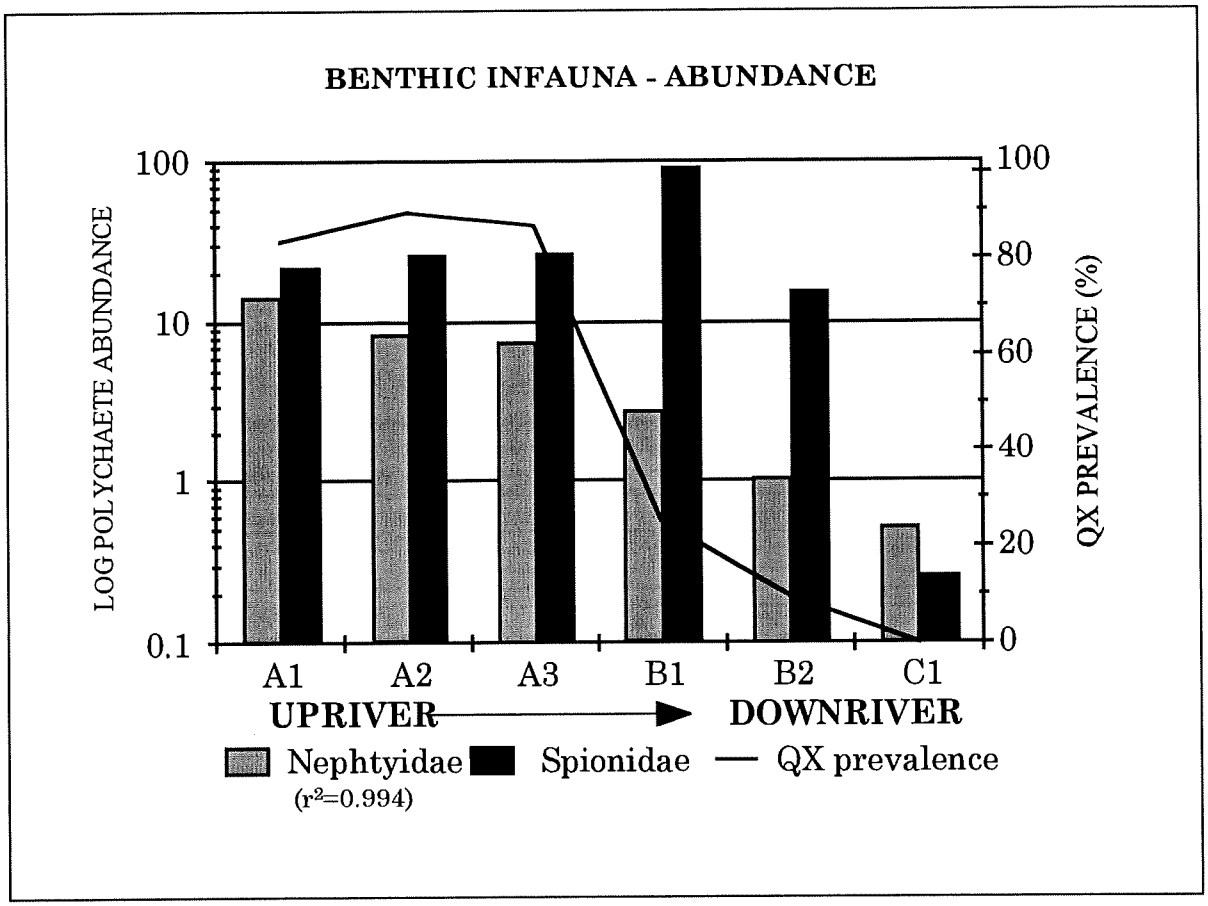

Figure 3. Abundance of polychaetes at 6 sites in the Georges River. The correlation coefficient refers to the correlation between QX prevalence and nephtyid abundance.

Of the invertebrates collected in the samples, polychaete worms were by far the most abundant. Most of the polychaetes belonged to the families Spionidae and Nephtyidae. Both families were less abundant at the mouth of the River (C sites, Figures 1 and 3). QX disease was not diagnosed at any time from these sites (Fig. 2).

The PCR test was developed to improve its sensitivity. Primers designated ([a]) in Table 1 amplified the rDNA of *M. sydneyi* and the DNA sequence was determined for the Internal Transcribed Spacer region (ITS1) and flanking region of the Small Subunit (SSU) of rDNA. The results showed that the original PCR test was unreliable because of a mismatch in the sequence of the forward primer. A second generation of PCR primers (LEG1 forward primer 5' ITS1 region and PRO2 reverse primer 3' ITS1 region) were designed directly from the sequence (details in manuscript in preparation). As the primers were designed directly from the spacer region of *M. sydneyi* it was likely that they were specific for this parasite. The sensitivity of these primers was measured using DNA extracted from known numbers of sporonts and gave positive results with at least 500 sporonts. This level of sensitivity was now appropriate to detect the presence *of M. sydneyi* DNA in alternative hosts and the test now suitable to apply in a assay of likely candidates.

Because polychaetes were abundant and were closely correlated with the distribution of QX disease in oysters, they were examined for *M. sydneyi* using the PCR test. Samples of nephtyids (N=10) and spionids (N=10) collected from the Georges River in January, from

areas that had high QX prevalence the previous winter, were tested for the presence of *Marteilia sydneyi* DNA using the PCR bioassay. Of these, 7 nephtyids and 5 spionids gave a positive result. Polychaetes (nephtyids N=3, spionids N=3) collected from Port Stephens were used as negative controls. January is thought to be when oysters in the Georges River are not releasing QX sporonts and is just prior to appearance of new infections (see final report from FRDC94/156 Adlard & Lester). This preliminary finding should be treated with caution because there remains a possibility that a positive result could arise from a cross reaction with either another organism in the sediment or contamination with QX spores from oysters still holding infections. Confirmation requires further evidence.

Objective 3. *To evaluate the possible relationship between infection and low pH in QX disease.*

Data collected on the Brisbane River in 1992/3 showed that there was a pH change of less than 1 unit, yet heavy infections of QX occurred (Anderson et al., 1994). Similarly in 1993/94 heavy infections occurred on the Brisbane and Pimpama Rivers with little change in pH (Wesche, 1995).

Objective 4. *To develop strategies to reduce the impact of QX disease on oysters.*

Our results implicate polychaetes as hosts in the life cycle of *Marteilia sydneyi*. This in turn explains previous anecdotal evidence and evidence gathered during this study that QX is more prevalent in muddy areas than sand. Polychaete species and abundance is probably directly related to sediment type, and QX disease in oysters could possibly be related indirectly to sediment type through a polychaete in the life cycle. This finding, if confirmed, offers a new direction for potential control of the disease. It may also explain why, after 4 years of low rainfall and high siltation in the Georges River prior to 1994, that the disease outbreak occurred when and where it did.

We feel that it is highly likely that *M. sydneyi* has been introduced repeatedly into many estuaries on the NSW coast during the movement of oyster stock/spat. The collection of benthos from Port Stephens, while primarily meant to provide polychaetes as negative controls and not designed to assess abundance, did indicate a markedly lower abundance of nephtyids and spionids when compared with benthic samples from QX endemic areas within the Georges River. This alone may explain why QX disease has not been reported from Port Stephens even though the pathogen may have been introduced into that and other estuaries.

The link between *Marteilia sydneyi,* polychaetes and sediment type if confirmed, offers a new opportunity to manage QX disease. Previously, oysters could be monitored for signs of the disease, but once diagnosed little could be done to reduce the impact. Now, monitoring of the abundance of polychaetes in high production areas may provide some hard data on risk assessment. Furthermore, polychaetes can be tested for the presence of the pathogen prior to its effect being transmitted to oysters thus knowledge of the possible life cycle could provide industry with some warning of impending epizootics.

(viii) Discussion of results including an analysis of research outcomes compared with the objectives.

The first objective, to find where the disease causing organisms are when there is no disease apparent in the oyster, was reached for winter mortality. The parasite remains in older oysters for most of the year and these oysters evidently infect newly introduced oysters. The disruption to the project caused by the major QX associated mortalities in our chief study site, the Georges River, did not prevent us from reaching the objective.

Perfection of the QX probe took longer than expected. Only at the end of the project were we able to start our search for the QX organism outside the oyster. However, the preliminary results are very promising.

The second objective, to evaluate the epidemiology of the two diseases, was completed for winter mortality. The QX component requires further work.

Objective 3, to evaluate the relationship between infection and low pH in QX disease, was completed. There was no relationship between QX infection and low pH in the two estuaries we studies. Heavy QX infections occurred without any appreciable change in pH. However, though pH changes are not required for an outbreak, if oysters are immunologically or metabolically compromised by sudden changes in physical parameters such as pH, it is possible that the severity of a QX outbreak may be increased although there is no evidence for this. However, the pH did not drop in our estuaries during the 2 years of monitoring and several waves of QX infection occurred.

The last objective, to develop strategies to reduce the impact of the two diseases, is the overall purpose of the project. New strategies are proposed in Section (vii) 'Detailed results' above and in (ix) below.

(ix) Implications and recommendations - costs and benefits to the Australian industry. Future research needs

The survey for winter mortality showed that some oysters become infected in their first year and that many older oysters in areas known for winter mortality are infected throughout the year. It would be advisable to use young stock without any previous contact with winter mortality for growout in susceptible areas as the progress of the disease is generally slow. Oyster growers are aware of which areas are susceptible to winter mortality in general. Imprint and probe diagnostic methods could be used to specifically identify which sources of stock are disease free.

The QX outbreak in the Georges River provided an opportunity to evaluate environmental and biological parameters in relation to the disease. There was clearly a relationship with fine mud. Preliminary DNA tests suggest that polychaetes may play an important role. If this is confirmed in future studies, growers will be in a better position to predict areas and times most at risk to major QX outbreaks and may be able to modify the lease environment to reduce the availability of infective stages and hence the severity of the disease.

(x) Intellectual property arising from the research

The PCR probe for QX is potentially of commercial significance. However, once the life cycle is confirmed, it is likely to have little demand apart from as a research tool.

(xi) Technical summary of all information developed.

The technical data is given above and in the publications referred to below. In developing the PCR tests, it is most important to control for false positives by ensuring use of negative and positive controls, and preferably by using experimental infections as positive controls.

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Those in bold type are papers arising from the project.

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WESCHE, S.J. 1995. Outbreaks of *Marteilia sydneyi* and their relationship with environmental pH. *Bulletin of the European Association of Fish Pathologists* 15:23-27.

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Communication of results to industry during the project

August 19, 1994: Overview of QX disease and winter mortality presented by Dr Adlard at the OFA Executive Council Meeting in Sydney (invited by Richard Roberts - President, NSW Oyster Farmers Association).

September 24, 1994: Dr Adlard presented a seminar on oyster pathogen research at the Annual General Meeting of the QOGC in Brisbane (invited by Justin Bender - President, Queensland Oyster Growers Corporation).

October 27, 1994: An award was presented to Dr Adlard by the Hon Wayne Goss (Premier of Queensland) from the Queensland Oyster Growers Association at their Inaugural Industry Awards, for 'Fostering outstanding advances in the genetics of oysters and their diseases.'

Plus personal visits by Dr Adlard to oyster farmers and NSW Fisheries personnel from Port Macquarie to Merimbula (Aug. '93, 14 contacts; Jan. '94, 8 contacts; then jointly with project FRDC94/156, Oct '94, 13 contacts; Jan. '95 6 contacts; May '95 16 contacts, July, '95, 11 contacts; Oct. '95, 22 contacts; Feb. '96, 7 contacts; Nov. '96, 4 contacts). On each occasion progress on the project was discussed.

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Distribution

Copies of this report were distributed directly to the following beneficiaries, relevant libraries and relevant authorities:

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