FINAL REPORT

aquatic animal health subprogram

Aquatic Animal Health Subprogram: Identification of host interactions in the life-cycle of QX disease

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2006/062	**Aquatic Animal Health Subprogram: Identification of host interactions in the life cycle of QX disease**

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OBJECTIVES:

- To determine what members of the benthic macrofauna contribute as intermediate hosts in the life cycle of *Marteilia sydneyi*, agent of QX disease.
- To identify and characterise previously unknown stages of *Marteilia sydneyi* through in-situ DNA probe hybridisation and histological examination.

NON TECHNICAL SUMMARY:

For decades QX disease has caused increased mortality of Sydney rock oysters and presented an impediment to commercial oyster culture particularly in the estuaries of southern Queensland and northern New South Wales. In 1994, an outbreak of QX disease in the Georges River, Sydney raised concerns for the whole oyster industry, while 10 years later, an outbreak in the Hawkesbury River confirmed QX as a clear industry-wide threat to production.

Quarantine of estuaries has been used to prevent the spread of QX disease by restricting movement of known infected stock. In the absence of data on transmission and on the causative elements that combined to create disease outbreaks, this was the most conservative course of action to protect the industry and the only management tool available at that time. It has since become known that the organism that causes QX disease, the protistan parasite, *Marteilia sydneyi*, exists in most estuaries in which major rock oyster culture is undertaken even though many have not yet suffered from significant disease events. As such, while quarantine of heavily infected stock still remains a sensible precaution, research has now turned to the production of QX disease-resistant oysters and to the discovery of factors that may contribute to triggering epizootics of disease.

All diseases are regulated by a web of variables that interact to produce a varying severity of effect. Such variables will include the fitness of the animals subject to infection, which itself is impacted by environmental conditions and nutritional status at any one time. Equally, parasitic infection is regulated by the defensive response of the animal it is invading, and by environmental conditions that act upon stages that are vulnerable to it, typically it is those stages just prior to infection that are most impacted. Furthermore, the abundance of definitive hosts (in this case oysters) and alternate hosts in the life cycle of any pathogen will determine the number of infective stages cycling through the system and becoming available for infection at the start of the next cycle of infection, i.e. the 'dose rate' will vary temporally.

The first suggestion that *M. sydneyi* in Australia required more than one host in its life cycle came over two decades ago when attempts to cross-infect rock oysters by either transplanting infected tissue or by association of uninfected stock with infected stock, failed. Since then the discovery that spores of this parasite could not remain viable in a marine environment for longer than 35 days at optimal temperature and salinity provided the final piece of evidence pointing undeniably to the presence of an alternate host in the life cycle. *Marteilia sydneyi* could not 'overwinter' in the environment but required a host other than rock oysters in which to develop and produce infective stages for the next seasonal cycle of QX infection.

What had further constrained research on the life cycle of both *Marteilia* spp was the lack of molecular tools with which to unambiguously link parasite stages in different hosts, assuming of course, that such hosts could be found. Now DNA-based tools are available and can be used not only for diagnosis of infection in oysters but they also provided an opportunity to investigate the presence of the pathogen in other hosts. Thus, the objective of this study was clear, to identify alternate hosts in the life cycle of QX disease and to characterise them.

The study site was restricted to 2 lease areas in the Hawkesbury River, Cobar and Kimmerikong, where QX disease was first reported in 2004 and prevalence of disease has been extreme in the 2 years following. A stratified random sample of 114 bottom sediment grabs were collected in November 2006 and covered areas within and adjacent to existing oyster culture leases. All samples were sieved to retain benthos (bottom dwelling invertebrates) >0.5mm (standard macro-benthic sampling). Segmented marine worms (polychaetes) were the dominant fauna recovered and these were sorted while alive into

21 putative species after which representatives of each species were preserved both in ethanol (for initial DNA screening for the presence of parasite DNA) and in formalin (for DNA probe staining and confirmation of parasite development in alternate host tissues). Of a total 1,247 individual worms tested using the DNA assay for *M. sydneyi*, 75 representatives from 8 different species were positive for the presence of parasite DNA. Of these species, 2 specimens showed confirmatory DNA probe staining that unambiguously indicated the presence of developing stages of *M. sydneyi*.

Both confirmed positive specimens came from the same species of marine bristle worm, *Nephtys australiensis*, a member of the polychaete family Nephtyidae. These worms are common in Australia's east coast estuaries and show some preference for muddier areas within them. They burrow and swim strongly and prey on bivalves and other polychaetes. Their reproduction is thought to be seasonal but is largely unstudied.

The developing stages of *M. sydneyi* revealed by DNA probe staining occurred in the epithelium of the gut of the worms and were clearly not the product of accidental ingestion of spores taken up from the environment and passing through the gut. The stained bodies varied in structure but were consistent with parasites at different stages of development.

The major aims of this project have thus been achieved and considering that the risk of failure was high it is a major breakthrough in our understanding of the dynamics associated with QX disease.

While this information alone does not provide a solution to mitigating the effects of QX disease on commercial Sydney rock oyster culture it does provide an opportunity to augment research on both QX disease resistance and on identifying factors that lead to outbreaks of disease. We now have enabling information to develop a laboratory model of infection and expedite industry outcomes.

KEYWORDS: QX disease, aquaculture, Sydney rock oysters, life cycle, polychaetes.

OUTCOMES ACHIEVED TO DATE

The most significant outcome of the results from this project lies directly in the confirmation that the life cycle of *Marteilia sydneyi*, the causative organism of QX disease in rock oysters, requires 2 hosts for its completion. Additionally is the identification of marine bristle worms (polychaetes) as an alternate host for *M. sydneyi* and one in which the parasite develops to produce stages infective to rock oysters. The impact of these results on the oyster industry is profound not only from a social perspective (i.e. providing industry members with an understanding of how the disease cycles) but also because these results provide information to enable the development of experimental models for QX infection. Development of such models will in turn allow assessment of parameters that predispose outbreaks of disease (e.g. genetic inheritance of disease resistance in oysters; parasite dose-related factors; physico-chemical properties of estuaries). It thus removes a major limitation to research on QX disease and in so doing provides novel pathways to explore reduction of disease impact for the industry and assist in ensuring consistent market supply of quality product.

ACKNOWLEDGEMENTS:

The authors wish to acknowledge with thanks the ongoing support of this project from members of the oyster industry. Particular appreciation goes to Robert Moxham and John Stubbs, oyster farmers from the Hawkesbury River, whose assistance both in discussions of the QX disease problem in their estuary and in their logistical support while collecting benthos on-site was unstintingly generous. Thanks are also due to members of the NSW Department of Primary Industries, particularly Jane Frances, for their support and their interest and assistance with disseminating the results. The Fisheries Research and Development Corporation (FRDC) funded this project with in-kind support from the Queensland Museum. Dr Peter Dundas-Smith led an FRDC-funded strategic workshop on QX disease in oysters from which the impetus to proceed with this project was agreed by industry, federal and state government participants, thanks to you all.

BACKGROUND:

Rock oysters and QX disease:

The strategic challenge for the rock oyster industry in Australia is to maximise production from current aquaculture lease areas, particularly in an environment where increased lease space is unlikely to be made available due to competition for use of estuarine resources from conservation, development and tourism.

QX disease has had a major impact on the oyster industry's ability to utilise its resources fully, not only from the direct effect of the disease on significantly increasing oyster mortality, but also from fallowing of lease areas in locations where, and at those times when, susceptibility to QX disease is at its highest.

A recently completed project (Adlard & Wesche, 2005; FRDC project 2001/214) demonstrated the widespread geographic distribution of the aetiological agent (*Marteilia sydneyi*) of QX disease suggesting that control of the disease through estuary quarantine is unlikely, by itself, to prevent future outbreaks of disease.

There are currently 2 approaches to reducing the impact of QX disease, a project being undertaken through New South Wales DPI is attempting to produce by selective breeding a QX disease resistant oyster, while a recently completed ARC Linkage project (Raftos, Nell & Adlard) investigated the contribution of oyster immuno-competence and

environmental parameters on disease initiation in rock oysters. The project described in this report was complementary to both approaches and a logical parallel project through addressing one of the major deficiencies in our knowledge of the biology of QX disease, the identity of the intermediate host/s required for the life cycle of QX disease to be viable.

Parasite life cycles: the case for an alternate host

Parasites have developed a number of different transmission strategies which can be broadly differentiated into direct and in-direct. Direct life cycles involve an infection pathway that does not require other host species in the life cycle, there is direct host to host transmission often after a short period in the environment. This category includes some significant pathogens of bivalves e.g. *Bonamia* spp.

Indirect life cycles require two (or more) different host species to cycle through a biological system, with developmental stages occurring in each of the required hosts. The increased complexity of in-direct transmission has substantial impacts on the dynamics of parasitic disease including, but not limited to, a built-in time lag for peaks of transmission to cycle through the system. Equally, environmental effectors in such a system act on multiple hosts with potentially varying responses often resulting in high variability in spatial and temporal distribution of disease.

Life cycle of Marteilia *spp*

The most well known element of the life cycle of the two most significant paramyxean parasites of oysters, *Marteilia sydneyi* and *M. refringens* from Europe is the development within their respective hosts, *Saccostrea glomerata* and *Ostrea edulis*. In many papers published on these parasites the existence of a complex life cycle had been postulated but not yet proven (Berthe et al. 1998; Kleeman and Adlard 2000, Audemard et al. 2001, Audemard et al. 2002, see review in Berthe et al. 2004).

The first suggestion that *M. sydneyi* in Australia required more than one host in its life cycle came over two decades ago when attempts to cross-infect rock oysters by either transplanting infected tissue or by association of uninfected stock with infected stock, failed (Lester, 1986). In that paper the author suggested the presence of '… an intermediate host, the discovery of which would facilitate research…'. Since then the discovery *in vitro* that spores of this parasite could not remain viable in a marine environment for longer than 35 days at optimal temperature and salinity (Wesche et al.

1999) provided the final piece of evidence pointing undeniably to the presence of an alternate host in the life cycle.

What had further constrained research on the life cycle of both *Marteilia* spp was the lack of molecular tools with which to unambiguously link parasite stages in different alternate hosts, assuming of course, that such hosts could be found. Now tools such as species-specific PCR assays and DNA probes for *in-situ* hybridisation (ISH) are available for both *Marteilia* species, (see Anderson, Adlard & Lester, 1995; Le Roux et al., 1999; Kleeman & Adlard, 2000; Kleeman, Adlard & Lester, 2002; Kleeman et al., 2002) with those developed and optimised for *M. sydneyi* having been partly funded through previous FRDC projects (Adlard & Worthington Wilmer 2003 (FRDC 2001/630), Adlard & Wesche 2005 (FRDC 2001/214)). These tools were directed primarily towards diagnosis of infection in oysters, allowing sensitive and specific detection with a rapid through-put of samples, while ISH provided confirmatory diagnosis of unexpected PCR assay positives. Nonetheless, these tools also provided an opportunity to investigate life cycle interactions in these pathogens.

Audemard et al. (2002) working in the claire ponds of western France, applied PCR and *in-situ* DNA probe hybridisation to screen potential alternate hosts for the presence of stages of *Marteilia refringens*. In their favour was the relatively depauperate fauna of these systems with a total of less than 100 species that could serve potentially as alternate hosts for *M. refringens*. In open systems it is estimated that there is an order of magnitude higher diversity than that found in claire ponds, while in the lower-latitude estuaries of eastern Australia the diversity is likely to be higher again. In that publication, Audemard et al. (2002) implicated the copepod *Paracartia grani* in the life cycle of *M. refringens*. An initial PCR screening which returned positive results was confirmed through DNA probe staining. The authors conclude that while the copepod can now be recognised as one of the hosts of *M. refringens* it may not be the only other host in the life cycle since they could not infect *Ostrea edulis* from infected copepods. Equally, the conditions for oyster infection may not have been optimal in their experimental system. Recently, Carrasco et al. (2007) reported the identification using PCR of *Marteilia* sp. in two new copepod hosts sampled from a natural enzootic area. While Carrasco et al. (2008) showed differences in development of *Marteilia maurini* and *M. refringens* after experimental infection from oysters into the copepod *Paracartia grani* the non-molluscan host first reported by Audemard et al. (2002).

In Australia, data from the initial outbreak of QX disease in the Georges River, Sydney in 1994-95 provided some clues to the life cycle of *M. sydneyi*. During the course of investigation into the outbreak funded by the FRDC (Adlard & Lester, 1996, FRDC 94/156) a disjunct distribution of QX disease was identified with high prevalence (approaching 100%) on upriver leases, medium prevalence (20-30%) on mid-river leases and no infection identified from oysters on down-river leases near the mouth of Botany Bay. In an attempt to capture ecological data on this phenomenon, benthic sampling was undertaken at that time which suggested an inverse correlation between sediment particle size and prevalence of *M. sydneyi* infection. In turn, the abundance of the major component of the benthos, the polychaete worms, appeared directly correlated with prevalence of QX disease. These results were intriguing but could not be verified absolutely nor could it be suggested without other data that there existed any causal link between benthic fauna presence/abundance and QX disease infection in oysters.

Since then, attempts at using PCR assays and in-situ DNA probe staining (ISH) for detection of *M. sydneyi,* revealed polychaetes collected from QX endemic localities positive in PCR but none of these were confirmed through ISH (Kleeman & Adlard, unpublished). At that time it was hypothesised that the PCR positives indicated either the presence of *M. sydneyi* spores that had been ingested and lay within the intestinal lumen (i.e. 'false positives', those not indicating development in an alternate host), or that *M. sydneyi* was actually developing in the tissues of the polychaetes but was localised and/or at low intensity. If the latter were true, and taking into account that tissue is sub-sampled during processing for ISH, then these factors combined may well have contributed to the lack of detection.

Prior to the start of the current project we knew how *Marteilia sydneyi* developed from initial infective stages through to sporulation in the oyster host, that spores emanating from end-stage infection of oysters could not 'overwinter' in the environment, that infection of oysters occurred in a reduced temporal window typically during mid- to late summer and that leases in more marine, sandy areas were less at risk. Nonetheless, the life cycle remained incompletely known (Figure 1).

NEED:

There have been some significant advances in our knowledge of QX disease of Sydney rock oysters in recent years. The pathogen has been isolated from many farming areas without being accompanied by patent disease and the influence of host fitness

together with environmental effectors are now being implicated as disease precursors. Nonetheless, the devastating oyster mortalities in the Hawkesbury River which started in 2004 highlight our problems in devising intelligent management strategies to minimise the impact of this disease.

A key obstacle to developing knowledge on parameters that control whether a disease outbreak will occur or whether the pathogen remains in estuaries at or beneath detectable levels is the lack of an experimental model of infection. In turn, the development of such a model is predicated on identifying the alternate (i.e. intermediate) host in the life cycle of QX disease, a stage which is required for the pathogen to viably cycle repeatedly through an estuary. Furthermore, if an experimental model could be identified and later developed, obvious benefit would flow to strategic programs of selective breeding for disease resistant oysters. For example, an experimental model of infection would then provide a consistent and quantifiable challenge to assess the level of resistance in selected stock. Equally, the interactions of oyster immuno-competence and environment could then be assessed in a controlled system without the risk of spatial and temporal variation in QX disease prevalence and intensity that occurs in natural estuarine systems.

A successful outcome of this research would have major benefit to our understanding of the biology of QX disease and have direct application to parallel projects aimed at benefiting the industry in eastern Australia.

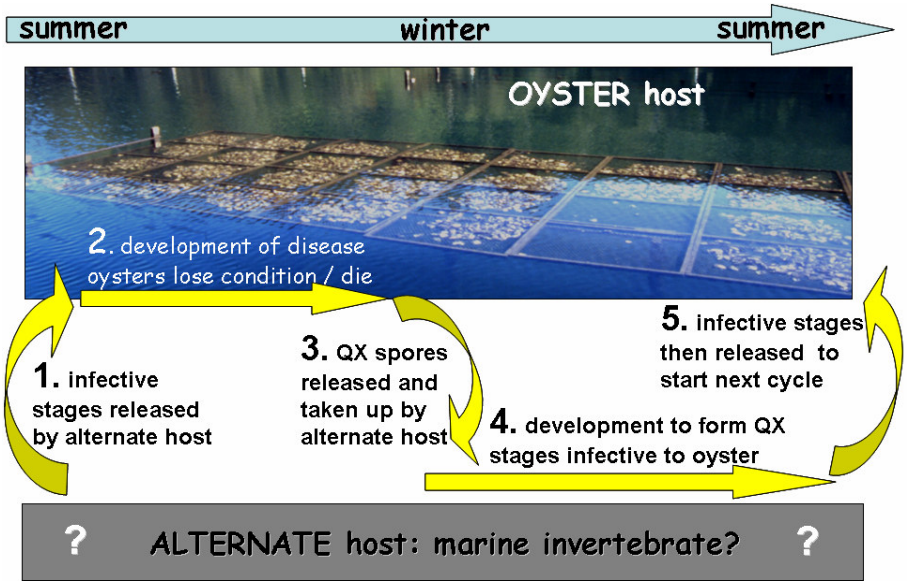


Figure 1. Hypothetical seasonal cycle of QX disease with development within marine invertebrate (at least) required for the disease to cycle every 12 months. Note that at Stage 3, released spores of *Marteilia sydneyi* can only survive in the environment for a maximum of 1 month at optimal temperature and salinity.

OBJECTIVES:

1. To determine what members of the macrofauna contribute as intermediate hosts in the life cycle of *Marteilia sydneyi*, agent of QX disease.

2. To identify and characterise previously unknown stages of *Marteilia sydneyi* through *in-situ* DNA probe staining and histological examination.

MATERIALS AND METHODS:

Sample Timing

The current study used an optimised PCR protocol (Adlard & Worthington Wilmer, 2003, FRDC Project 2001/630) to detect the presence of *M. sydneyi* in potential benthic invertebrate alternate hosts. As such it was anticipated that PCR positives would fall into two categories; 'false positives' that are the product of incidental ingestion of spores which lie in the digestive tract, particularly of filter-feeding and deposit feeding benthos (probably the majority of positives would fall here), and; 'real positives' which relate to uptake and development within true alternate hosts.

Given the seasonal nature of QX disease (Bower et al. 1994) it was anticipated that sampling alternate hosts in November would minimise the presence of false positives since most infected oysters would have already shed spores and died (and spores do not remain viable in the environment for more than ca. 35 days). Furthermore, development within an alternate host was anticipated to be well advanced to allow infection of oysters during the following (mid-summer) infection period (Bower, et al., 1994). As such, the probability of detecting developing *M. sydneyi* stages in alternate hosts was maximised.

Sampling the Hawkesbury River

Sample sites in each study area were generated using randomised GPS points; however, rather than undertaking a quantitative study of macrobenthos, the aims of this project require comprehensive coverage of lease and surrounding areas, resulting in actual sampling at a subset of those pre-selected random sites to ensure coverage.

Maps of the Hawkesbury River region, NSW, were provided by NSW Department of Primary Industries. Two areas, Cobar and Kimmerikong, both associated with current and former oyster leases and unfarmed neighbouring areas were selected for sampling and are shown in Figure 2. Both areas were overlaid with a numbered grid; 150 computer

generated random numbers were plotted for each area and the latitude and longitude determined for each (see Appendix 3).

GPS points for each sampled site were located on site using a hand-held GPS unit; 50 sites were sampled at Cobar (Figure 3) and 64 at Kimmerikong (Figure 4). Benthic samples were collected using a van Veen grab sampler between the 7th and 20th of November 2006 (see Appendix 4). One sample was taken at each site with only five samples collected at one time prior to sieving and sorting to prevent live benthos deteriorating while held in containers. Each sample was placed in a five litre container for transport and labelled with the site number (1-150 for Cobar and 151-300 for Kimmerikong samples).

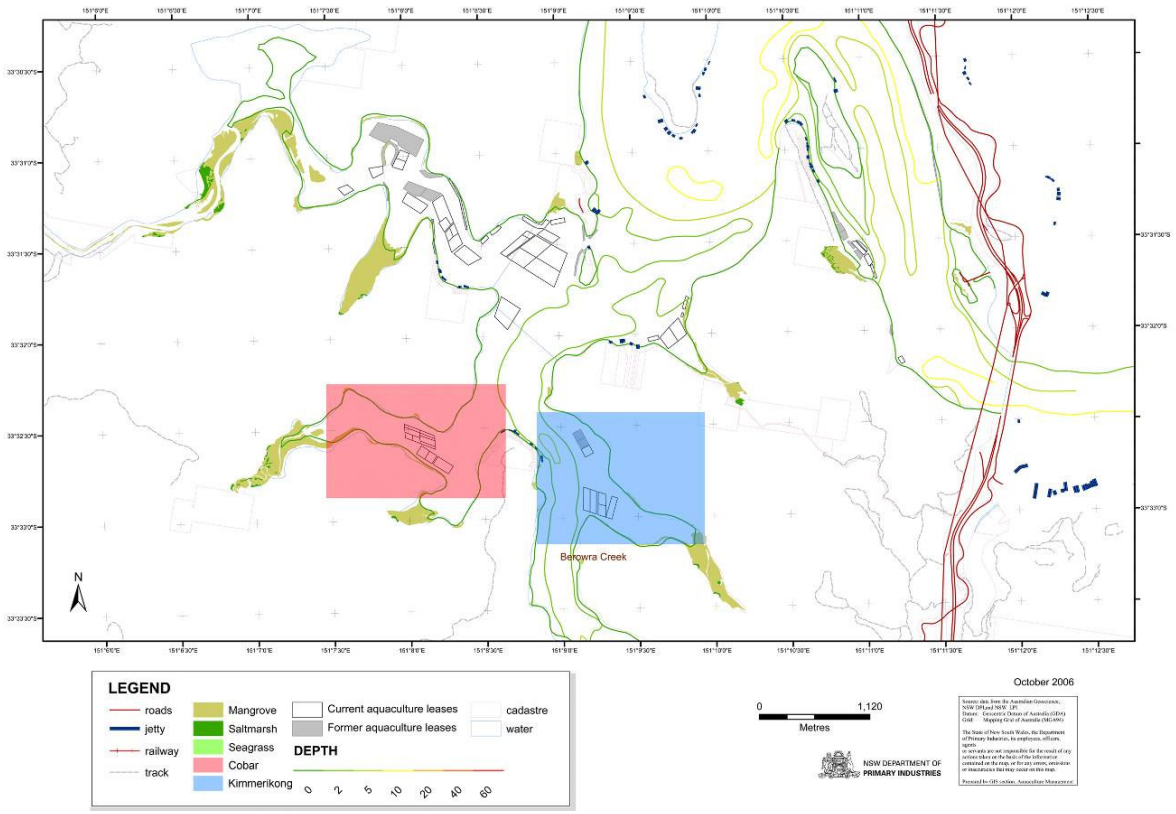


Figure 2. Location of benthic sampling areas in the upper Hawkesbury River.

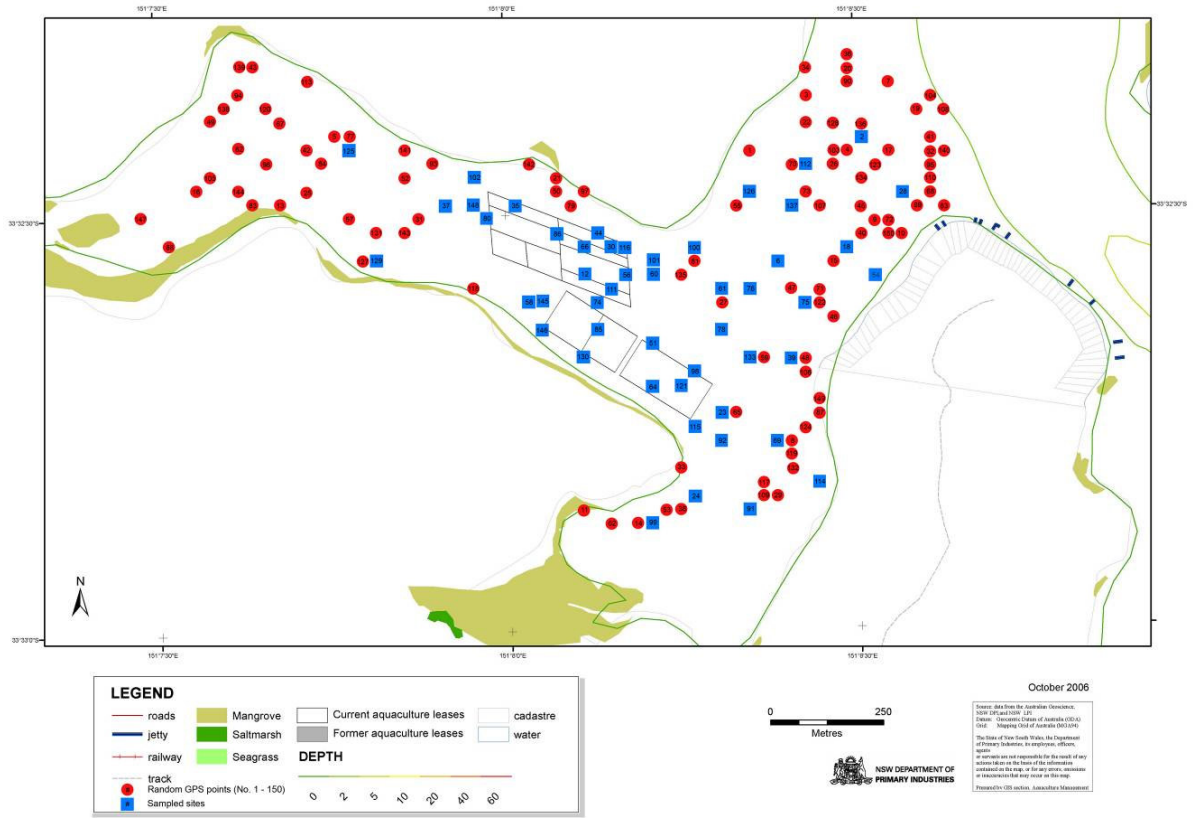


Figure 3. Random GPS points (red circles) plotted for Cobar; 50 sites (blue squares) were sampled.

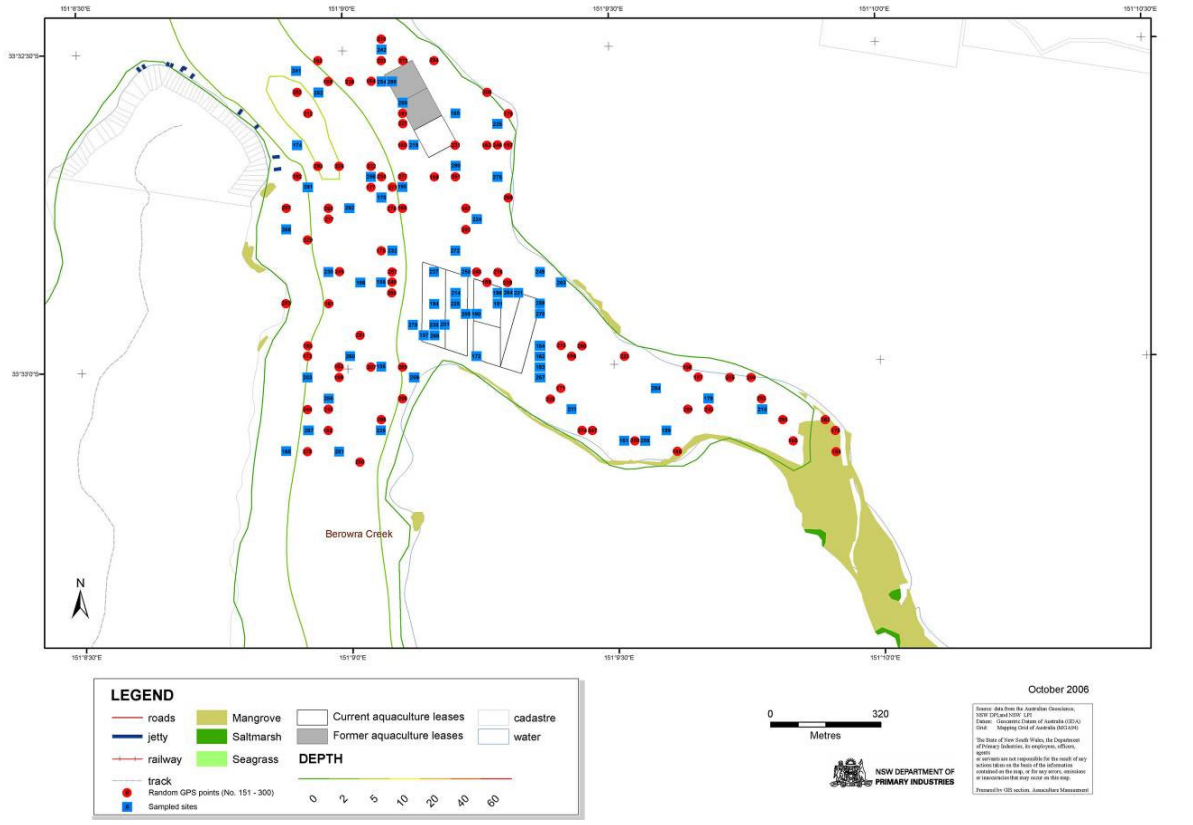


Figure 4. Random GPS points (red circles) plotted for Kimmerikong; 64 sites (blue squares) were sampled.

Sample Processing

Benthic samples were reduced in volume by washing each individually through a series of two stacked sieves (1 mm, 500 µm) which sat in a bracket attached to the side of an oyster punt (see Appendix 4). Estuary water was pumped over samples using a 12V 500 GPH bilge pump. Once 'clean' samples were placed in separate 1 litre containers (labelled with the corresponding site number) and left in river water for 45 minutes. Samples were then rewashed through the 500 µm sieve to remove sediment loosened during this soaking process.

Macrobenthos collection and preservation

Clean samples were poured into Petri dishes and allowed to settle for 10-15 minutes before being scanned using a dissecting microscope. Polychaetes were removed from the sample using feather-weight forceps and stored in hemagglutination trays in river water (see Appendix 4). Polychaetes were classified into operational taxonomic units (OTUs) (family; putative species) for each site. Numbers for each OTU for each site were recorded (see Appendix 5) before individual site OTUs were combined and half the specimens fixed in ethanol (for DNA extraction) and half in formalin (to represent an OTU or for *in situ* hybridisation (ISH)). Specimens for ISH were changed from formalin to ethanol after 1-2 weeks in accordance with the requirements for completing ISH. Formalin fixed OTU specimens were photographed using a Nikon Digital Sight camera (DS-5M) attached to a compound and a dissecting microscope at the Queensland Museum.

DNA Extraction

Total genomic DNA (gDNA) was extracted from ethanol preserved polychaetes using a QIAGEN DNeasy® Blood and Tissue Kit as per manufacture's instructions. DNA was extracted from single polychaete specimens only (samples not pooled during extraction); however, on occasions large polychaete individuals were divided into two to three sections so host DNA would not overwhelm parasite DNA during the PCR process.

Polymerase Chain Reaction (PCR)

Protocols used in this project were originally taken from Kleeman & Adlard (2000) and optimised in FRDC Project No. 2001/630; (Adlard & Worthington Wilmer, 2003). The optimised protocol was then modified slightly, in accordance with instructions for newly

available reagents (for example, HotMaster™ Taq DNA polymerase; Eppendorf) and the use of dimethyl sulfoxide (DMSO), as required.

A 195 nucleotide base fragment from the first internal transcribed spacer (ITS1) of ribosomal DNA (rDNA) was amplified using the forward primer 'LEG1' (5' – CGATCTGTGTA GTCGGATTCCGA – 3') (positioned 48 nucleotide bases downstream from the 5' end of ITS1) (Kleeman & Adlard, 2000) and the reversve primer 'PRO2' (5' – TCAAGGGACATCCAACGGTC – 3') (positioned approximately 240 nucleotide bases from the 5' end of ITS1) (Kleeman & Adlard, 2000). PCR cycling reactions were performed in 25 µl volumes containing; 2.5 µl of 10x HotMaster™ Taq buffer with 25 mM Mg^{2+}, 2.0 µl of deoxyribonucleotide triphosphate (dNTP) (10 mM), 1.0 µl of each primer (10 µM), 1.25 µl of dimethyl sulfoxide (DMSO) (5.0% final concentration) and 0.15 µl of HotMaster™ Taq DNA polymerase (5 U/µl), 2 µl of DNA template (20–50 ng) and made up to 25 µl with ultra pure water. Cycling reactions were run on a Gradient PalmCycler (supplied by Corbett Research) with the following protocol, initial denaturation at 95°C for 10 mins; 35 cycles of 95°C for 30 s, 55°C for 30 s and 65°C for 30 s, and; final extension at 65°C for 5 mins and 22°C for 30 s.

For each set 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 oyster from the Pimpama River collected in 2004, were included. The former was run to ensure the presence of an amplified product was not the result of contaminated PCR reagents and the latter to ensure the absence of any amplified product was not the result of a failed reaction.

Gel Electrophoresis

To visualise for the presence or absence of an amplified PCR product for *M. sydneyi* from each extracted DNA sample 25 µl of PCR product together with 2 µl of loading dye were run on a 1.4% submarine agarose check gel (in Tris-borate EDTA (TBE) buffer) stained with ethidium bromide. A molecular weight standard (TrackIt™ 100 bp DNA ladder, Invitrogen™) was used to estimate the size of products. Agarose gels were electrophoresed at 80 V for 45 minutes. Amplified DNA was visualised on a ultra-violet Alpha Digi Transilluminator (Alpha Innotech Corporation) and photographed using a Kodak DC290 zoom digital camera with an AlphaDigiDoc™ (Alpha Innotech Corp.) attachment.

Purification and Confirmatory Sequencing of PCR Products

Four of six operational taxonomic units which scored a positive result for *M. sydneyi* DNA (Lumbrineridae sp. 1, Magelonidae sp., Nephtyidae sp. 1 and Trichobranchidae sp.) were sequenced from Cobar and Kimmerikong. The sequences were then compared to *M. sydneyi* sequence data available on the GenBank database to confirm the identity of the amplified product.

Purification of PCR products prior to sequencing was completed using a QIAGEN QIAquick® PCR Purification Kit according to manufacturer's instructions. Purified DNA was quantified by running 2 µl of each sample on a 1.4% submarine agarose gel stained with ethidium bromide against 2 µl of Low DNA Mass Ladder (Invitrogen™).

DNA was sequenced using the same primers that were used during PCR amplification and an ABI Prism BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (version 3.1). Sequencing reactions were performed in 10 µl volumes containing; 0.3 µl of BigDye Terminator (BDT) ready reaction mix, 2.0 µl of 5x BDT dilution buffer, 0.5 µl of primer (separate reactions were performed for each sample using either the forward or reverse primer) (10.0 uM), 1-3 ng of purified PCR product and ultra pure water, in a Corbett Research PalmCycler with a protocol of 1 cycle of 96℃ for 1min, 25 cycles of 96℃ for 10 s, 50℃ for 5 s, 60℃ for 4 mins (each temperature reached using rapid thermal ramp, 1℃/s) and 1 cycle at a final holding temperature of 4℃.

Sequenced products were precipitated in 2.0 µl of 125 mM ethylene diamine tetracetic acid (EDTA), 2.0 µl of 3 M sodium acetate (pH 4.6) and 50 µl of 100% EtOH (room temperature) (added in that order) to remove dye terminators and the pellets dried at 37℃ for a period of 30 mins. Samples were sent to the Australian Genome Research Facility (AGRF) at the University of Queensland for visualisation on an ABI 3730x1 automated sequencer.

Raw sequence data was checked for accuracy by comparison to electrophoretograms (using Sequence Scanner v1.0 (AppliedBiosystems, 2005)) and consensus sequences were constructed in BioEdit Sequence Alignment Editor v7.0.5 (Hall, 1999) and CAP3 Sequence Assembly Program (Huang & Madan, 1999). The taxonomic accuracy and affinity of sequences was checked by BLAST searching (GenBank) and sequence homology compared with previously published *M. sydneyi* sequence data (Gen Bank accession numbers AY504628-AY504632, AF159248).

Sequence alignments were performed using CLUSTALW Version 1.83 (Thompson et al. 1994) and the following settings (pairwise alignment parameters of gap opening penalty 10.0, gap extension penalty 0.1, DNA weight matrix International Union of Biochemistry (IUB); multiple alignment parameters of gap opening penalty 10.0, gap extension penalty 0.2, delay divergent sequences 30%, DNA weight matrix IUB) (Thompson et al., 1994). Alignments were checked by eye for accuracy using BioEdit (Hall, 1999) and the final alignment exported with sequence identities identical to the first sequence replaced with a dot.

Labelling of ISH DNA Probe

The primer set used in the synthesis of the DNA probe for ISH in this study was CS2 (5' – GCAAGTCTGGTGCCAGCAGC – 3') (positioned 776-796 nucleotide bases downstream of the 5' end of 18S) and SAS1 (5' – TTCGGGTGGTCTTGAAAGGC – 3') (positioned 1117-1137 nucleotide based downstream of the 5' end of 18S) (Le Roux et al., 1999). This primer pair incorporates the region reported as 'Smart 2', the most specific probe used to detect *M. refringens* 18S transcript in infected European flat oysters (*Ostrea edulis*) and naturally infected mussels (*Mytilus edulis* and *Mytilus galloprovincialis*) (Le Roux et al., 1999). Despite its specificity to *M. refringens,* Kleeman et al. (2002) found this probe provided a stronger signal in detection of all stages of *M. sydneyi* compared to the species specific ITS1 probe (Kleeman & Adlard, 2000). We did not use the 'Smart 2' in isolation because one primer, SS2 (Le Roux, et al., 1999), required for its construction shows no homology with the corresponding region of the *M. sydneyi* sequence.

The DNA probe was synthesised by incorporation of digoxigenin-11-dUTP during PCR and using a PCR DIG Probe Synthesis Kit (Roche Diagnostics Australia Pty Limited) according to the manufacturer's instructions. Incorporation of digoxigenin (DIG) was signalled by an increase in molecular mass as indicated on an ethidium bromide stained agarose TBE check-gel. Labelled PCR products were purified using a High Pure PCR Product Purification Kit (Roche Diagnostics Australia) as per the provided instructions.

In-Situ Hybridisation

Formalin-fixed polychaetes were embedded in paraffin, longitudinal histological sections were cut 6 µm thick, floated onto silane slides (2% (3-aminopropyl)triethoxysilane in acetone) and baked at 62℃ overnight (O/N). Sections were dewaxed by immersion in

Histo-Clear II for 10 minutes (two times in 100% solution). The solvent was eliminated by immersion in ethanol for 10 minutes (two times in 100% solution) and sections air dried.

Each slide was treated with 200 µl of Proteinase K (100 µg/ml) in 1X TE buffer (10X solution; 100 mM Tris-Cl (pH 8.0), 10 mM EDTA (pH 8.0)) (*CSH Protocols*; 2006; doi:10.1101/pdb.rec 8018), at 37°C for 30 mins. Sections were dehydrated in a single 1 min wash of each of 95% and 100% ethanol and air dried. Sections were prehybridised with 500 µl of hybridisation buffer (3X SSC, 50% formamide, 1X Denhardt's solution, 0.5 mg ml^{-1} heat denatured herring sperm DNA and 5% dextran sulphate) per slide at 42°C for 60 mins (20X SSC; 3 M NaCl, 0.3 M Na-citrate, pH 7.0). Sections were covered with 55 µl of diluted DIG-labelled probe in hybridisation buffer (5 µl in 50 µl, respectively), cover-slipped and placed on a heating block at 95°C for 5 mins. Slides were cooled on ice for 5 mins before O/N hybridisation at 42°C in a humid chamber. Post hybridisation washes included two washes for 5 mins each in 2X SSC at room temperature and one wash for 10 mins in 0.4X SSC at 42°C followed by equilibration in Maleic acid buffer (100 mM Maleic acid, 150 mM NaCl, pH 7.5) once for 1 min at room temperature. DIG-labelled probe detection included blocking sections with 200 µl of blocking buffer (Maleic acid buffer, 1% blocking reagent) at room temperature for 30 mins followed by incubation in a humid chamber for 60 mins at 37°C with dilute anti-digoxigenin-alkaline phosphatase (AP) conjugated antibody (1:500 in blocking buffer). Unbound antibody was removed with two 1 min washes in Maleic acid buffer and slides were equilibrated in detection buffer (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 5 mins. BCIP/NBT (5-Bromo-4-chloro-3-indolyl phosphate / Nitro Blue Tetrazolium) was diluted in detection buffer (20 µl in 1 ml, respectively) and 200 µl of the solution pipetted onto sections which were incubated in the dark at room temperature for 4 hrs. The reaction was stopped by washing slides in 1X TE buffer for 15 mins at room temperature. Slides were washed with MilliQ water, counter stained in Bismark Brown Y (9% solution) for 1 min at room temperature, dehydrated in two washes of each of 95% and 100% ethanol for 1 min, washed three times in Histo-Clear II (1 min each) and mounted in Depex (Adlard & Wesche, 2005).

Table 1. Total number of each operational taxonomic unit (OTU) at each of Cobar and Kimmerikong, the numbers preserved in formalin (for reference and ISH) and ethanol (for PCR) from each site and the number of PCR positives for *M. sydneyi* for each OTU.

		Site									
		Cobar					Kimmerikong				
Polychaeta OTU.	Putative sp.	Total	No. formalin fixed (for reference & ISH)	No. ethanol fixed (for PCR)	No. PCR positives	% PCR positives	Total	No. formalin fixed (for reference & ISH)	No. ethanol fixed (for PCR)	No. PCR positives	% PCR positives
Capitellidae sp. 1							1	1			
Capitellidae sp. 2		2	2				3	3			
Cirratulidae sp.		10	3	7	2	28.6	26	8	18	3	16.7
Lumbrineridae sp. 1	*Augeneria verdis*	108	72	36	4	11.1	244	165	79	9	11.4
Lumbrineridae sp. 2		1	0	1	0	0.0	6	2	4	0	0.0
Lumbrineridae sp. 3		14	7	7	0	0.0	20	13	7	0	0.0
Magelonidae	*Magelona* sp.	6	2	4	3	75.0	76	44	32	9	28.1
Nephtyidae sp. 1	*Nephtys australiensis*	86	46	40	10	25.0	85	47	38	5	13.2
Nephtyidae sp. 2	*Nephtys inornata*						1	0	1	0	0.0
Opheliidae sp. 1	*Armandia intermedia*	11	5	6	0	0.0	26	14	12	0	0.0
Phyllodocidae	*Paranaitis* (*Compsanaitis*) *inflata*						2	1	1	0	0.0
Polynoidae		1	1								
Sabellidae sp. 1	*Jasminiera* sp.	13	6	7	3	42.9	40	19	21	2	9.5
Sabellidae sp. 2	*Laonome triangularis*						1	1			
Scalibregmatidae	*Scalibregma inflatum*	1	1				1	1			
Spionidae sp. 1		17	10	7	0	0.0	9	6	3	1	33.3
Spionidae sp. 2		14	8	6	0	0.0	12	5	7	4	57.1
Spionidae sp. 3		14	14				3	3			
Spionidae sp. 4							1	1			
Terebelidae sp.	*Polycirrus rosea*	3	2	1	0	0.0	19	9	10	0	0.0
Trichobranchidae	*Terebellides stroemii*	885	462	423	12	2.8	990	521	469	8	1.7
		1186	641	545	34		1566	864	702	41	

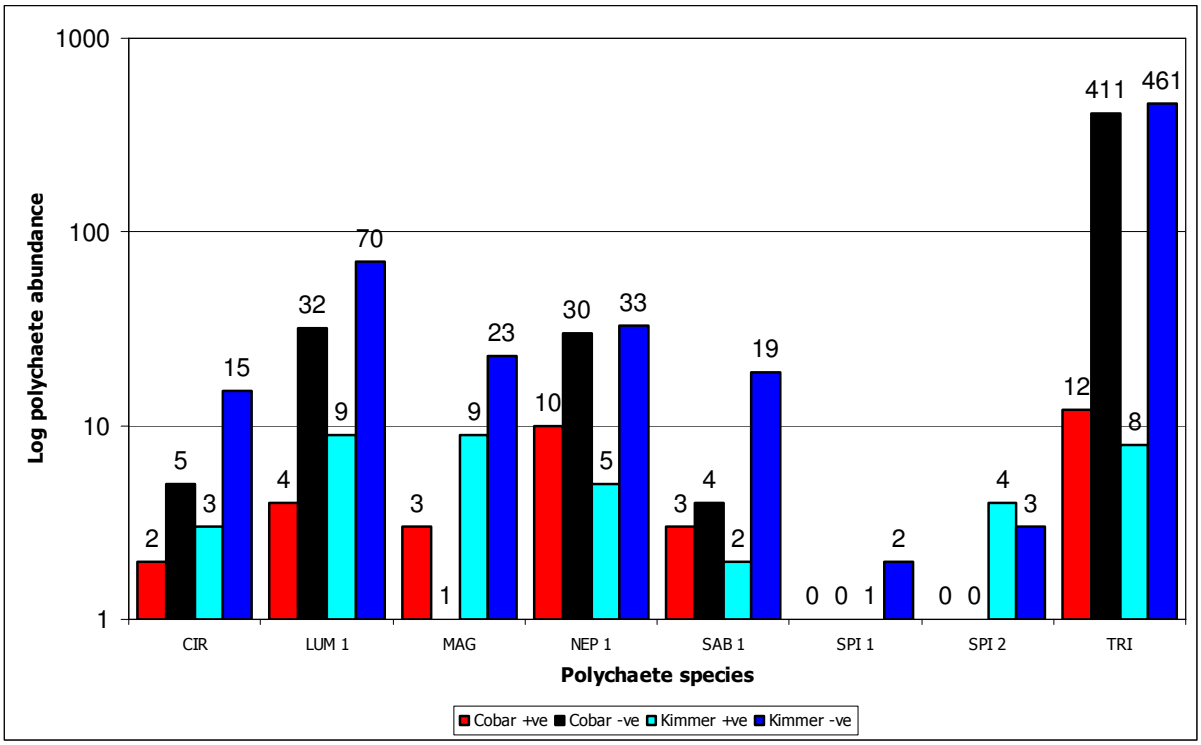


Figure 5. Polychaete taxa of which a proportion were positive for *M. sydneyi* in PCR. Figures plotted above each bar represent the actual number of positive and negative examined individuals for each OTU at each collection area.

RESULTS

Polychaeta at Cobar and Kimmerikong

Provisional identification of polychaetes in the Hawkesbury River found a combined total of 21 putative species from 13 families at Cobar (16 OTUs in 12 families) and Kimmerikong (20 OTUs in 12 families) (see Table 1, Figure 5 and Appendix 5). Combined totals for individual OTUs from both sample areas ranged from one specimen of Capitellidae sp. 1, Nephtyidae sp. 2, Polynoidae sp., Sabellidae sp. 2 and Spionidae sp. 4 to 1875 specimens of Trichobranchidae sp. At Cobar 12 OTUs numbered between 0 and10 specimens, six numbered between 11 and 20 specimens, one numbered between 21 and 100 specimens while Lumbrineridae sp.1 and Trichobranchidae sp. numbered 108 and 885 specimens, respectively. At Kimmerikong 11 OTUs numbered between 0 and 10 specimens, three numbered between 11 and 20 specimens, five numbered between 21 and 100 specimens while Lumbrineridae sp.1 and Trichobranchidae sp. numbered 244 and 990 specimens, respectively. For both collection localities, over 95% of the total polychaete abundance was provided by members of only 6 polychaete families (Figure 6 A-F).

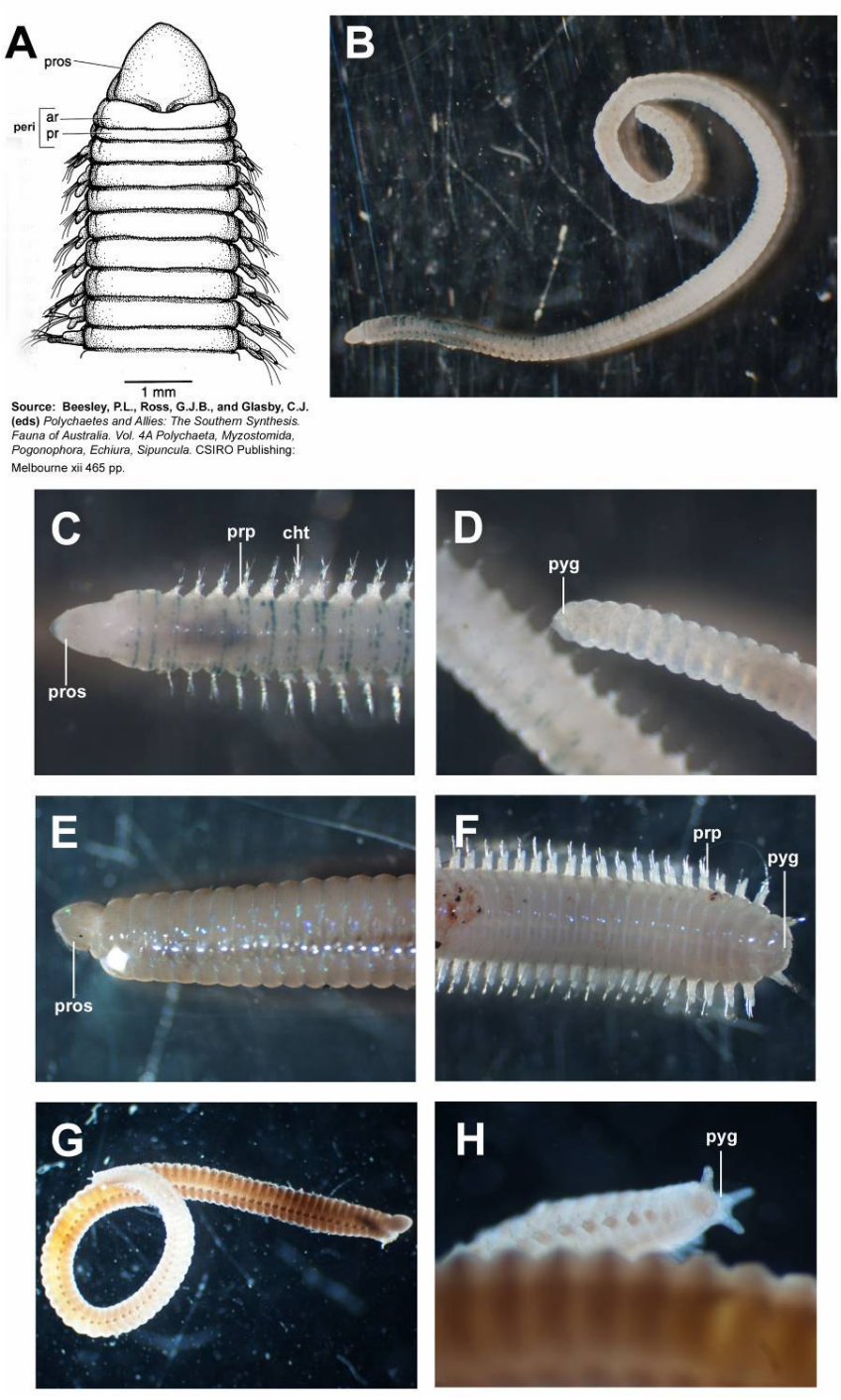


Figure 6A. **Lumbrineridae** Schmarda, 1861. A. Line drawing of representative of Lumbrineridae. B-D. Digital images of Lumbrineridae sp. 1 from benthic samples collected in the Hawkesbury River, NSW, November 2006. B. Whole specimen, lateral view. C. Anterior end, dorso-ventral view. D. Posterior end. E-F. Digital images of Lumbrineridae sp. 2 from the Hawkesbury River. E. Anterior end, lateral view. D. Posterior end, dorso-ventral view. G-H. Digital images of Lumbrineridae sp. 3 from the Hawkesbury River. G. Whole specimen, lateral view. H. Pygidium. *Abbreviations:* AR, anterior ring; CHT, chaetae; PERI, peristomium; PR, posterior ring; PROS, prostomium; PRP, parapodia; PYG, pygidium.

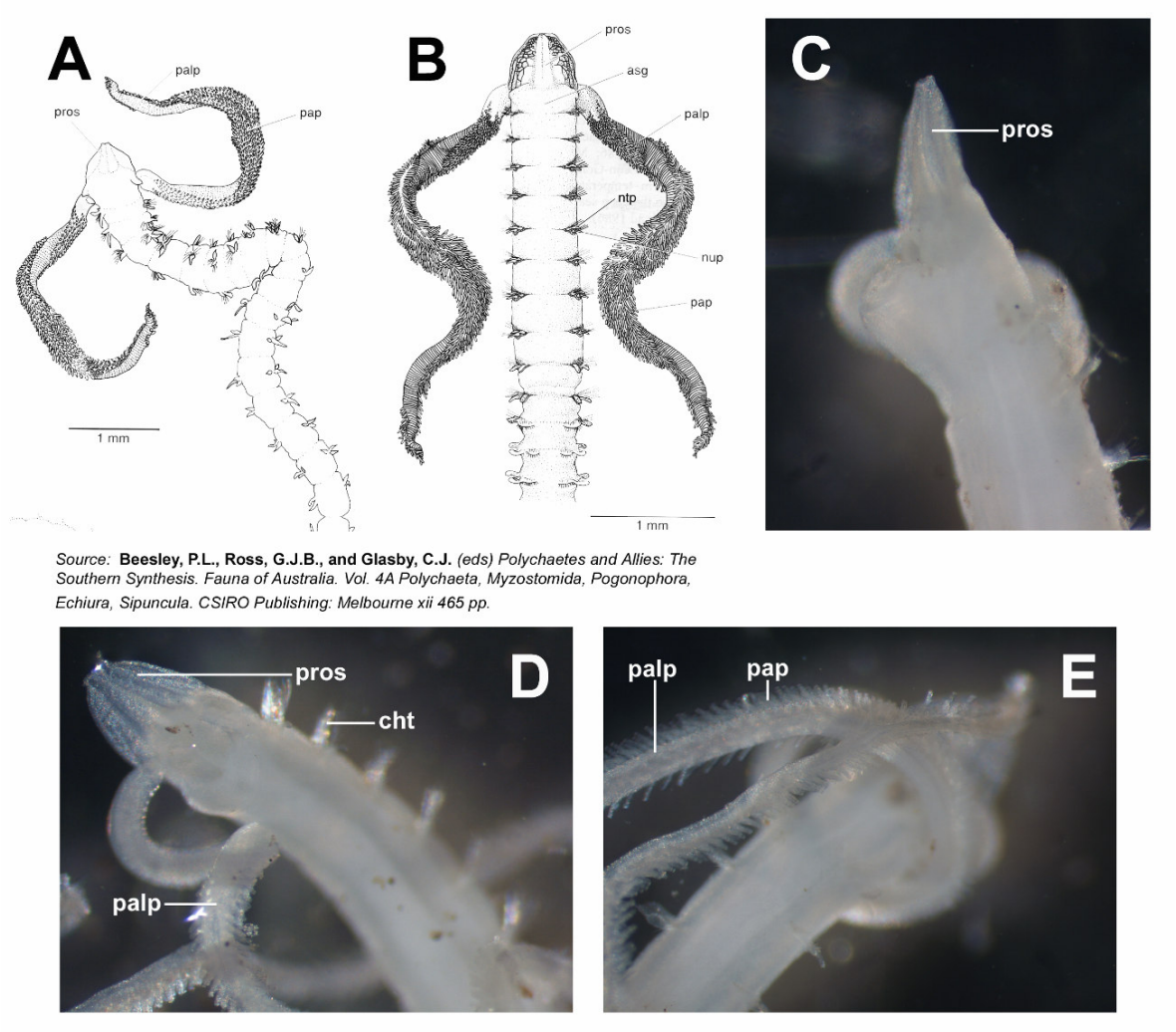


Figure 6B. **Magelonidae** Cunningham & Ramage, 1888. A-B. Line drawings of representative of Magelonidae. C-E. Digital images of Magelonidae sp. from benthic samples collected in the Hawkesbury River, NSW, November 2006. C. Prostomium, lateral view. D. Prostomium and palp (the second has broken off), dorso-ventral view. E. Palps and palpal papillae. *Abbreviations:* ASG, achaetous segment; CHT, chaetae; PALP, palp; PAP, palpal papillae; PROS, prostomium; NTP, notopodium; NUP, neuropodium.

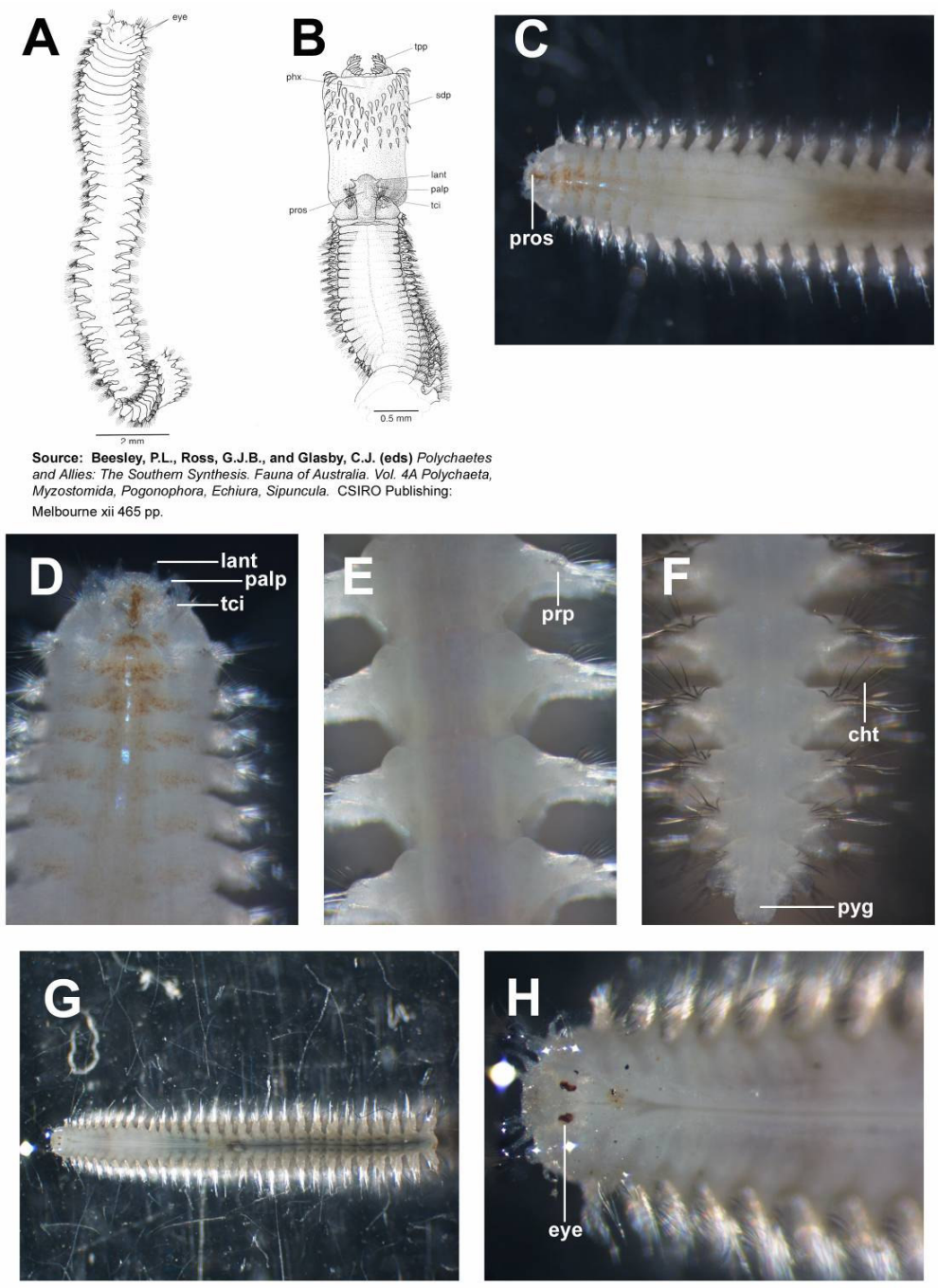


Figure 6C. **Nephtyidae** Grube, 1850. A-B. Line drawings of representative of Nephtyidae. C-F. Digital images of Nephtyidae sp.1 from benthic samples collected in the Hawkesbury River, NSW, November 2006. C. Anterior end, dorsal view. D. Prostomium which bears lateral antennae, palps and tentacular cirrus. E. Mid-section, dorsal view. F. Pygidium, dorsal view. G-H. Digital images of Nephtyidae sp. 2 from the Hawkesbury River. G. Partial specimen, dorso-ventral view. H. Prostomium bearing two eye spots. *Abbreviations:* CHT, chaetae; EYE, eye; LANT, lateral antenna; PALP, palp; PHX, pharynx, everted; PROS, prostomium; PRP, parapodia; PYG, pygidium; SDP, subterminal pharyngeal papillae; TCI, tentacular cirrus; TPP, terminal pharyngeal papillae.

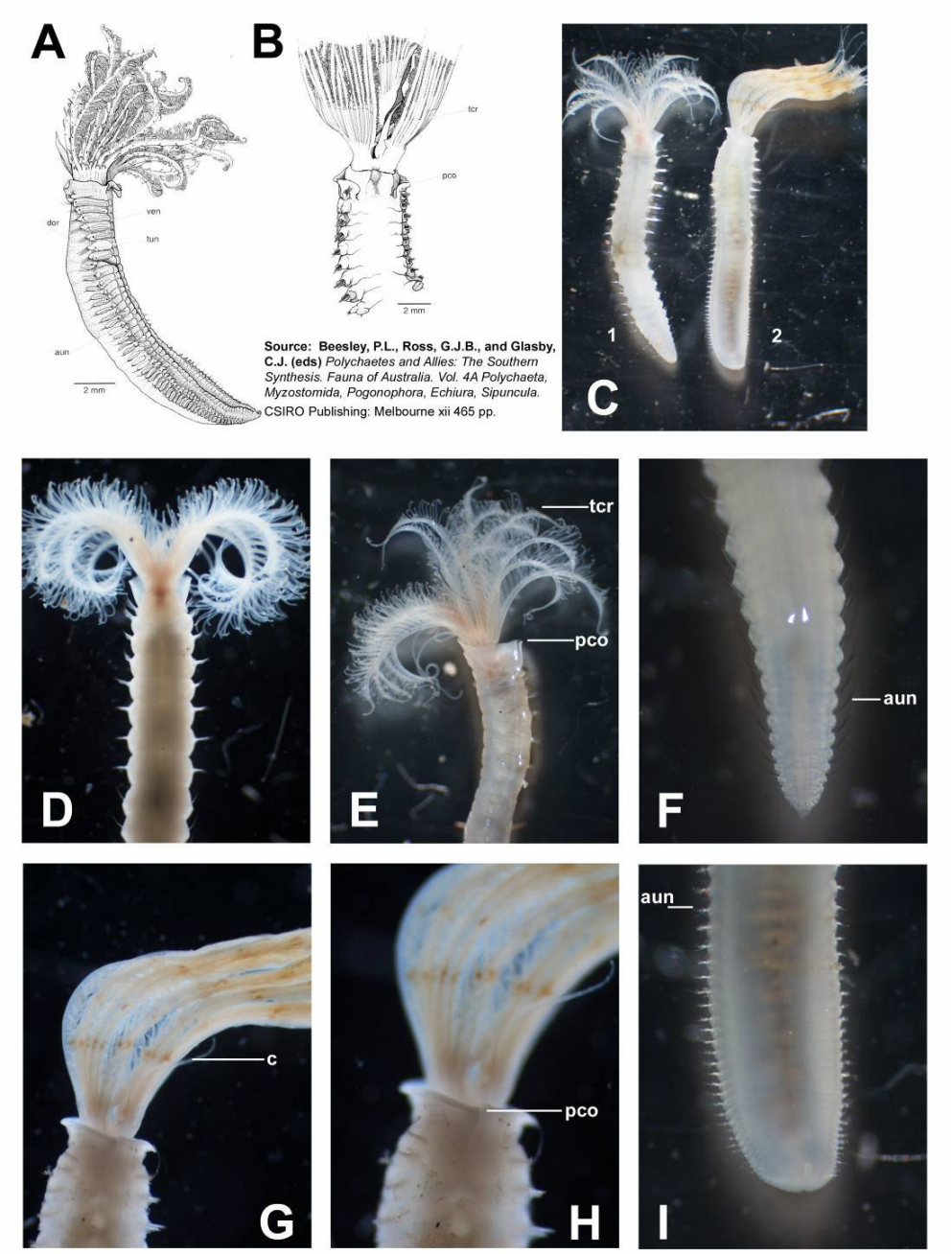
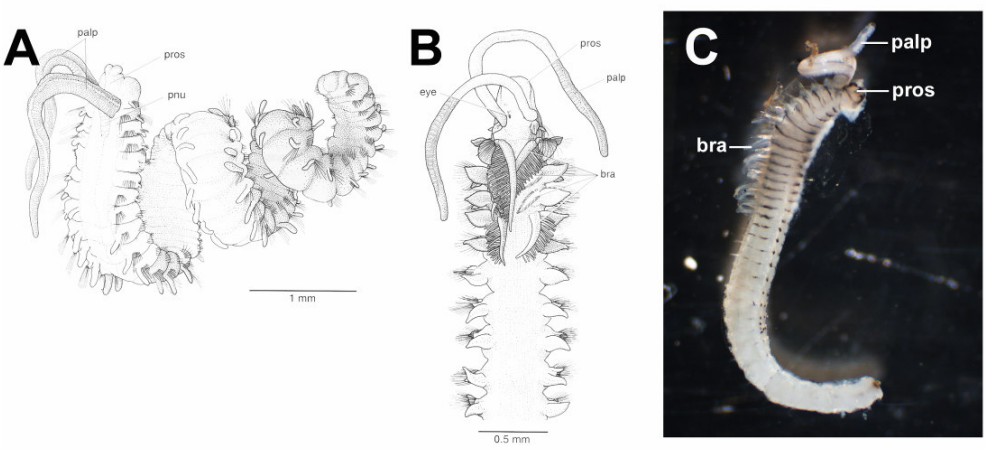


Figure 6D. **Sabellidae** Malmgren, 1867. A-B. Line drawings of representative of Sabellidae. C-I. Digital images of Sabellidae sp. 1 and 2 from benthic samples collected in the Hawkesbury River, NSW, November 2006. C. Sabellidae sp. 1 and 2, whole specimens. D-F. Sabellidae sp. 1. G-I Sabellidae sp. 2. *Note:* Differences between species apparent in presence/absence of coloration on tentacular crown and length of abdominal chaetae. *Abbreviations:* 1, species one; 2, species 2; AUN, abdominal uncini (notopodial); C, coloration on tentacular crown; DOR, dorsum; PCO, peristomal collar; TCR, tentacular crown; TUN, thoracic uncini (neuropodial); VEN, ventral surface.



Source: **Beesley, P.L., Ross, G.J.B., and Glasby, C.J. (eds)** *Polychaetes and Allies: The Southern Synthesis. Fauna of Australia. Vol. 4A Polychaeta, Myzostomida, Pogonophora, Echiura, Sipuncula.* CSIRO Publishing: Melbourne xii 465 pp.

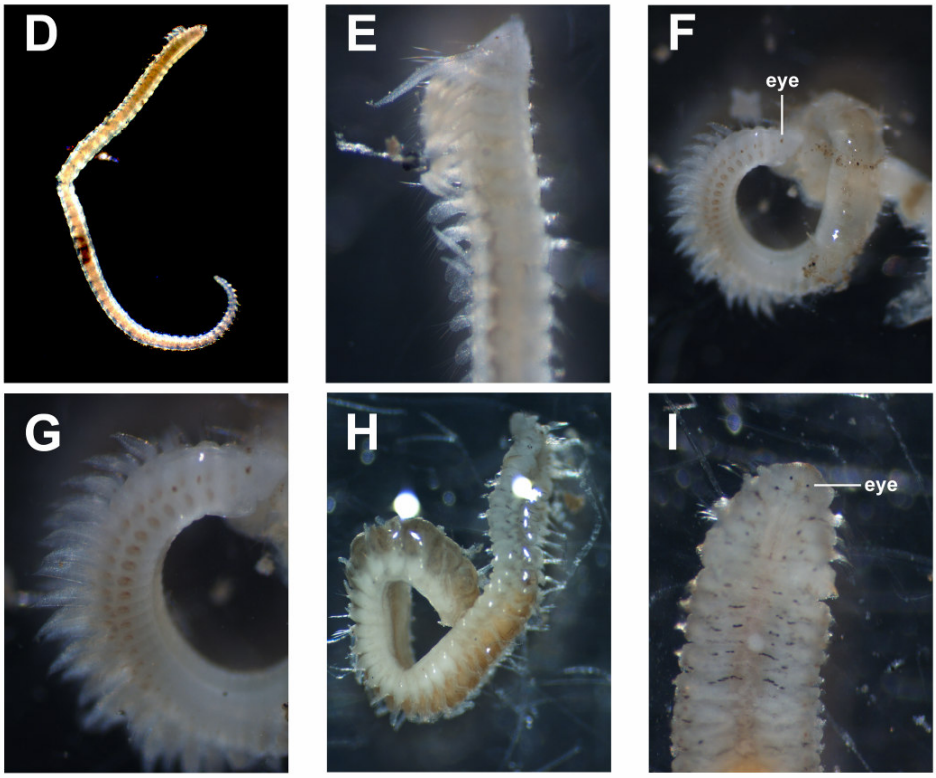
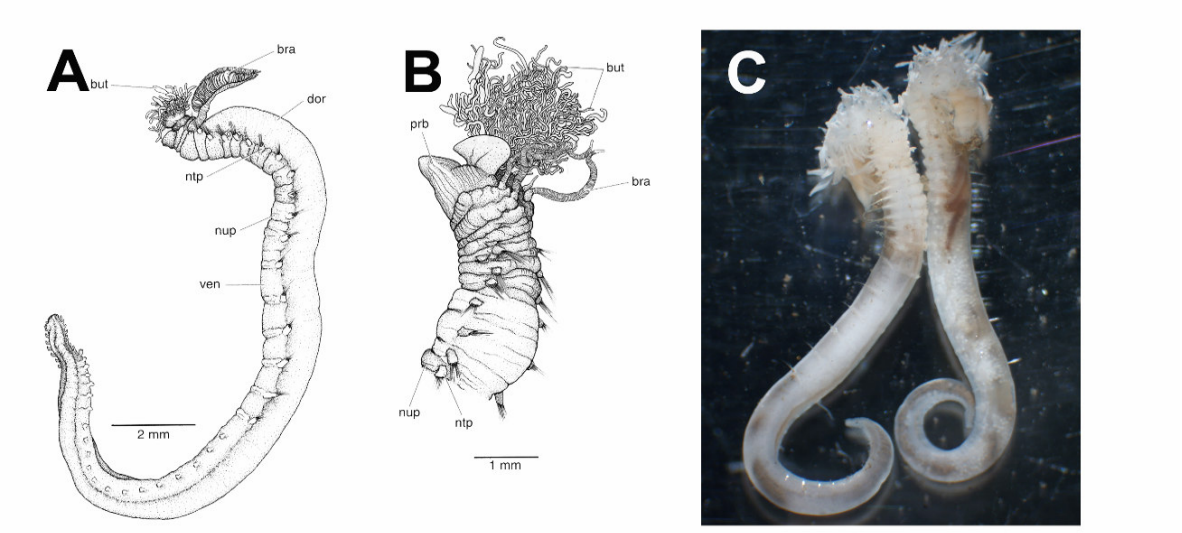


Figure 6E. **Spionidae** Grube, 1850. A-B. Line drawings of representative of Spionidae. C. Digital image of Spionidae sp. 1 from benthic samples collected in the Hawkesbury River, NSW, November 2006. Partial specimen, lateral mount. D-E. Spionidae sp. 2 from the Hawkesbury River. D. Whole specimen, lateral view. E. Anterior end, lateral view. F-G. Spionidae sp. 3 from the Hawkesbury River. Anterior end, lateral view. H-I. Spionidae sp. 4 from the Hawkesbury River. H. Partial specimen, lateral view. I. Anterior end, dorso-ventral view. *Note* differences in pigmentation patterns on body, shape of the body, size and shape of the prostomium, palps and branchiae. *Abbreviations:* BRA, branchiae; EYE, eye; PALP, palp; PNU, posterior projection of prostomium carrying the nuccal organs; PROS, prostomium.



Source: **Beesley, P.L., Ross, G.J.B., and Glasby, C.J. (eds)** *Polychaetes and Allies: The Southern Synthesis. Fauna of Australia. Vol. 4A Polychaeta, Myzostomida, Pogonophora, Echiura, Sipuncula.* CSIRO Publishing: Melbourne xii 465 pp.

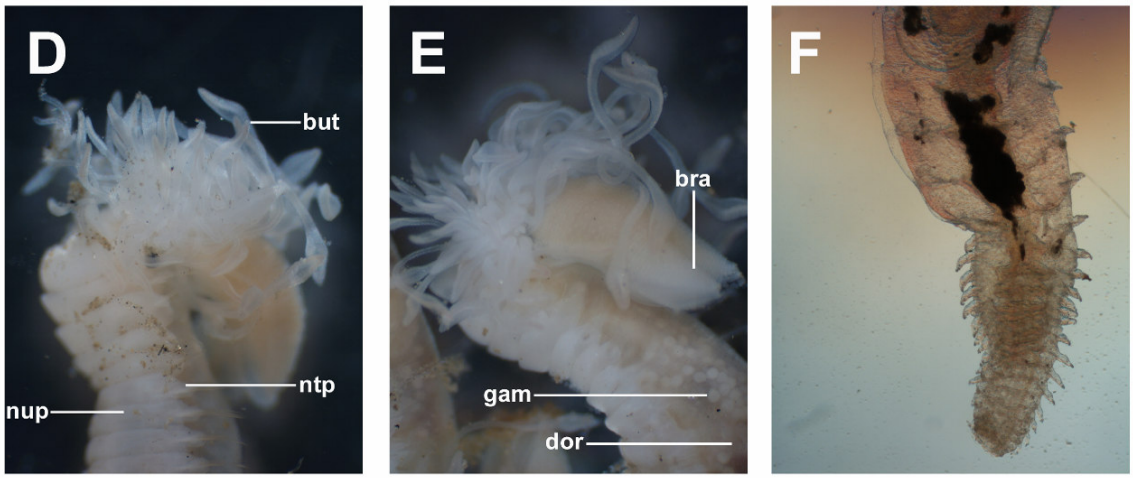


Figure 6F. **Trichobranchidae** Malmgren, 1866. A-B. Line drawings of representative of Trichobranchidae. C-F. Digital images of Trichobranchidae sp. from benthic samples collected in the Hawkesbury River, NSW, November 2006. C. Whole specimen, lateral view. D-E. Anterior region of trichobranchid sp., lateral view, bears distinct oral filaments around mouth and branchiae. *Note:* Female gametes present within coelom (white spheres) in Figure E. F. Posterior end trichobranchid sp. taken on compound microscope. *Abbreviations:* BRA, branchia; BUT, buccal tentacles; DOR, dorsum; GAM, gamete; NTP, notopodium; NUP, neuropodium; PRB, proboscis; VEN, ventral surface.

Table 2. Host/ location combinations sequenced for the internal transcribed spacer 1 (ITS1) of *Marteilia sydneyi*, accession numbers and source.

Species	Host (Family)	Locality	Wild/ Farmed oysters	Accession numbers	Source
Phylum Paramyxea					
Class Marteiliidea					
Genus *Marteilia*					
Species *M. sydneyi*	*Saccostrea glomerata* (Ostreidae)	Great Sandy Strait (QLD)	Farmed	AY504628	Kleeman *et al*. 2004
	Saccostrea glomerata (Ostreidae)	Richmond River (NSW)	Farmed	AY504629	Kleeman *et al*. 2004
	Saccostrea glomerata (Ostreidae)	Clarence River (NSW)	Wild	AY504630	Kleeman *et al*. 2004
	Saccostrea glomerata (Ostreidae)	Georges River (NSW)	Farmed	AY504631	Kleeman *et al*. 2004
	Saccostrea glomerata (Ostreidae)	Pimpama River (QLD)	Wild	AY504632	Kleeman *et al*. 2004
	Saccostrea glomerata (Ostreidae)	Pimpama River (QLD)		AF159248	Kleeman & Adlard, 2000
	Saccostrea glomerata (Ostreidae)	Pimpama River (QLD)			This study
	Augeneria verdis (Lumbrineridae)	Kimmerikong, Hawkesbury River (NSW)			This study
	Magelona sp. (Magelonidae)	Cobar, Hawkesbury River (NSW)			This study
	Nephtys australiensis (Nephtyidae)	Kimmerikong, Hawkesbury River (NSW)			This study
	Terebellides stroemii (Trichobranchidae)	Kimmerikong, Hawkesbury River (NSW)			This study

Molecular data – PCR positives for M. sydneyi

From the 21 polychaete OTUs collected from the Hawkesbury River 545 polychaete individuals from Cobar and 702 from Kimmerikong were examined for the presence of *M. sydneyi* by PCR (seeTable 1). A proportion of Cirratulidae sp., Lumbrineridae sp. 1, Magelonidae sp., Nephtyidae sp. 1, Sabellidae sp. 1 and Trichobranchidae tested positive in PCR for the presence of *M. sydneyi* at both Cobar and Kimmerikong while Spionidae sp. 1 and 2 tested positive from Kimmerikong only (see Table 1 and Figure 5). With one exception, large differences between the proportion of PCR positives for the six shared taxa may be attributed to differences in sample sizes between the two areas; however, Nephtyidae sp. 1 had comparable numbers of individuals preserved from both areas (40 from Cobar, 38 from Kimmerikong) but PCR positives from Cobar (25.0%) were almost double those from Kimmerikong (13.2%).

Sequencing of the amplified fragment from four polychaete taxa from both areas (see Table 2) and comparison to sequence data available on GenBank and our positive control (QX positive oyster, Pimpama River, 2004) confirmed the presence of *M. sydneyi* (see Figure 7) DNA.

```
                5          15         25         35         45         55         65
AY504628     CGATCTGTGT AGTCGGATTC CGATTTGGTC CTCGTCGTCG AAAWACGGAT CTTCGACCAT ARCGGTCGCG
AY504631     .......... .......... .......... .......... .......... .......... ..........
AY504629     .......... .......... .......... .......... .......... .......... ..........
AY504630     .......... .......... .......... .......... ...T...... .......... .G........
AY504632     .......... .......... .......... .......... ...T...... .......... .G........
AF159248     .......... .......... .......... .......... ...T...... .......... .G........
PIMP.OYST.   .......... .......... .......... .......... ...T...... .......... .G........
LUM.SP.1     .......... .......... .......... .......... ...T...... .......... .G........
MAG.SP.      .......... .......... .......... .......... ...T...... .......... .G........
NEP.SP.1     .......... .......... .......... .......... ...T...... .......... .G........
TRI.SP.      .......... .......... .......... .......... ...T...... .......... .G........

                75         85         95        105        115        125        135
AY504628     TCCATGTTCG TACTTGGGAG GATCGGTCAC TCATGTYCGT CATCCGTGAC GTCTTTCCGA TCGGTCCTTT
AY504631     .......... .......... .......... .......... .......... .......... ..........
AY504629     ..S....... .......... .......... .......... .......... .......... ..........
AY504630     ..G....... .......... .......... ......C... .......... .......... ..........
AY504632     ..G....... .......... .......... .......... .......... .......... ..........
AF159248     ..G....... .......... .......... ......T... .......... .......... ..........
PIMP.OYST.   ..G....... .......... .......... ......T... .......... .......... ..........
LUM.SP.1     ..G....... .......... .......... ......T... .......... .......... ..........
MAG.SP.      ..G....... .......... .......... ......T... .......... .......... ..........
NEP.SP.1     ..G....... .......... .......... ......T... .......... .......... ..........
TRI.SP.      ..G....... .......... .......... ......T... .......... .......... ..........

               145        155        165        175        185        195
AY504628     CCATGGGACG CCATCCTATC GTATAGTCGA TGTACGACCG TTGGATGTCC CTTGA
AY504631     .......... .......... .......... .......... .......... .....
AY504629     .......... .......... .......... .......... .......... .....
AY504630     .......... .......... .......... .......... .......... .....
AY504632     .......... .......... .......... .......... .......... .....
AF159248     .......... .......... .......... .......... .......... .....
PIMP.OYST.   .......... .......... .......... .......... .......... .....
LUM.SP.1     .......... .......... .......... .......... .......... .....
MAG.SP.      .......... .......... .......... .......... .......... .....
NEP.SP.1     .......... .......... .......... .......... .......... .....
TRI.SP.      .......... .......... .......... .......... .......... .....
```

Figure 7: Sequence alignment of 195 base ITS1 DNA probe of *Marteilia sydneyi* (isolated from *Saccostrea glomerata* and polychaete taxa) designed by Kleeman & Adlard (2000). *Taxon legend:* those with prefix 'AY' and 'AF' were sourced from GenBank; PIMP.OYST., *M. sydneyi* from QX positive oyster used as outgroup; LUM.SP.1, *M. sydneyi* from *Augeneria verdis* (Lumbrineridae); MAG.SP., *M. sydneyi* from *Magelona* sp. (Magelonidae); NEP.SP.1, *M. sydneyi* from *Nephtys australiensis* (Nephtyidae); TRI.SP., *M. sydneyi* from *Terebellides stroemii* (Trichobranchidae).

There were no nucleotide differences between *M. sydneyi* ITS1 sequences from different polychaete OTUs or different collection areas.

Location of infections from PCR positives

Individual site's OTUs were combined each day; this practice prevented us from assigning PCR positive samples to a specific numbered site. However, we plotted every sampled site from which a PCR positive OTU (all sites including positive and negative ones) was collected during field work at Cobar and Kimmerikong and isolated only those sites collected on days from which positive samples were detected by PCR (Figures 8 and 9). It was hoped that a comparison of days from which PCR positive samples for *M. sydneyi* were detected to those from which they were not would allow the determination of a pattern in the distribution of *M. sydneyi* in polychaetes. For Cobar PCR positive samples

were collected across the entire sample area; similarly, positive samples collected from Kimmerikong appear to have had a broad coverage of the sampled area.

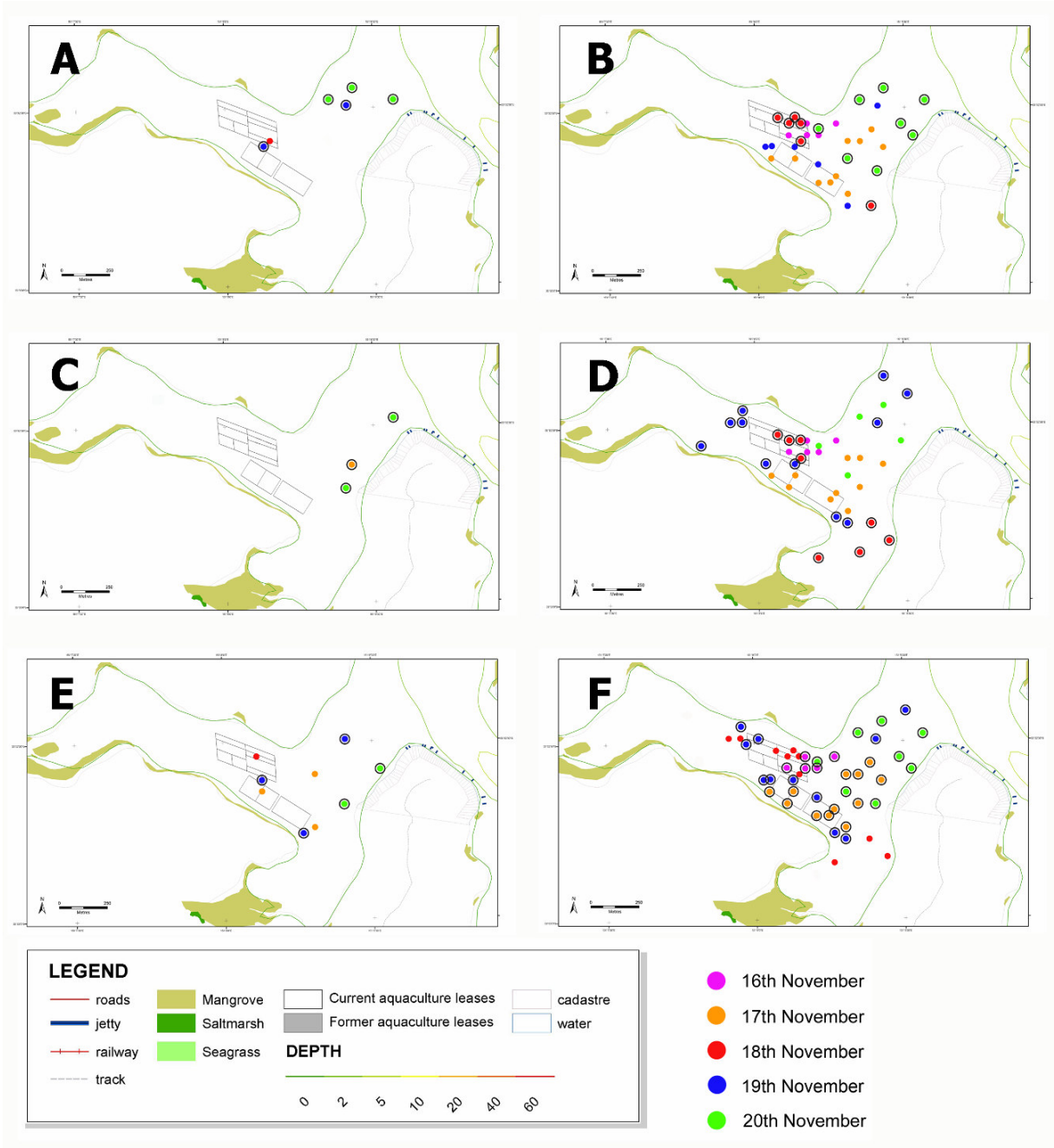


Figure 8. Cobar sites, by date, from which PCR positives for *M. sydneyi* were detected (circled) and those that were negative (un-circled). A, Cirratulidae sp. B, Lumbrineridae sp. 1. C, Magelonidae sp. D, Nephtyidae sp. 1. E, Sabellidae sp. 1. F, Trichobranchidae sp.

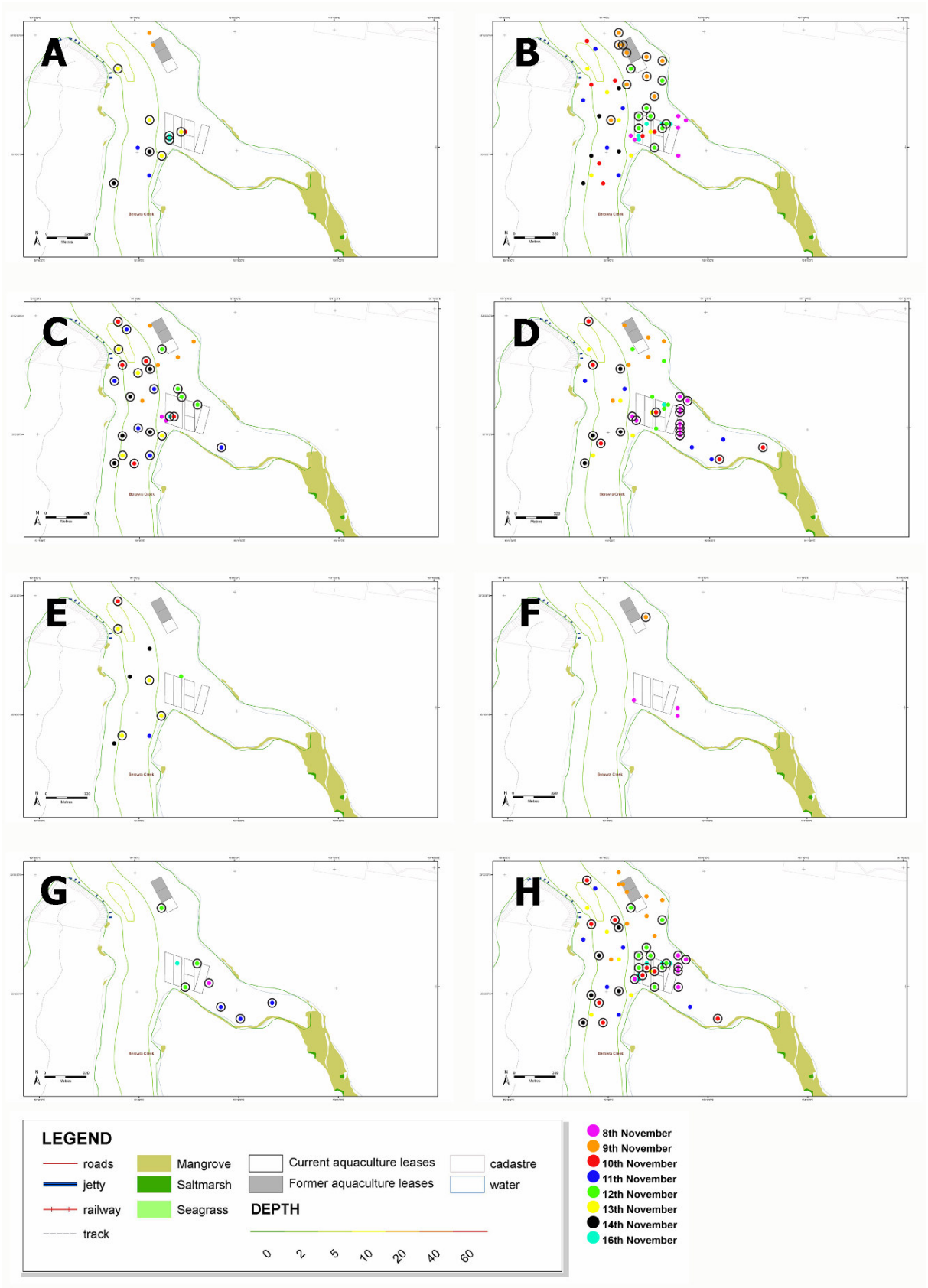


Figure 9. Kimmerikong sites, by date, from which PCR positives for *M. sydneyi* were detected (circled) and those that were negative (un-circled). A, Cirratulidae sp. B, Lumbrineridae sp. 1. C, Magelonidae sp. D, Nephtyidae sp. 1. E, Sabellidae sp. 1. F, Spionidae sp.1. G, Spionidae sp. 2. H, Trichobranchidae sp.

Table 3. Number of *in-situ* hybridization positive worms from each OTU from each of Cobar and Kimmerikong

		Site								
		Cobar					Kimmerikong			
Polychaeta OTU.	Putative sp.	Total	No. formalin fixed	No. examined ISH	No. ISH positives	% ISH positives	Total	No. formalin fixed	No. examined ISH	No. ISH positives	% ISH positives
Cirratulidae sp.		10	3	3			26	8	4		
Lumbrineridae sp1	*Augeneria verdis*	108	72	9			244	165	13		
Magelonidae sp.	*Magelona* sp.	6	2	2			76	44	18		
Nephtyidae sp1	*Nephtys australiensis*	86	46	24	2	8.3	85	47	26		
Sabellidae sp1	*Jasmineira* sp.	13	6	4			40	19	13		
		223	129	42	2		471	283	74		

In-Situ Hybridisation – positive polychaete OTUs

Of the six polychaete OTUs which tested positive in PCR for *M. sydneyi* DNA at both Cobar and Kimmerikong five were embedded for ISH analysis. Of these only two Nephtyidae sp. 1 specimens from Cobar were confirmed as positive for developing stages of *M. sydneyi* DNA (see Table 3 and Figure 11 A-F).

The polychaete was identified as *Nephtys australiensis* Fauchauld, 1965 and is a pale brown worm that ranges in length from 7mm to 85mm usually possessing dorsal pigmentation (Hutchings & Rainer, 1979). The recorded distribution of *N. australiensis* is given as Australia-wide with specific collections made from southern Queensland, through New South Wales, Victoria, Tasmania, South Australia and in south-western Western Australia (Rainer & Hutchings, 1977). This polychaete is a common inhabitant of coastal lagoons, estuaries or sheltered bays and in sea-grass beds, but appears more common in muddy rather than sandy sediments (Rainer & Hutchings, 1977). It is a muscular species that swims and burrows strongly using rapid lateral sinusoidal movements. Records suggest this species preys on small molluscs, crustaceans and other polychaetes (Glasby, 2000).

For comparison with infected material, histological sections showing the internal structure of a normal (i.e. uninfected) nephtyid were examined (Figure 10).

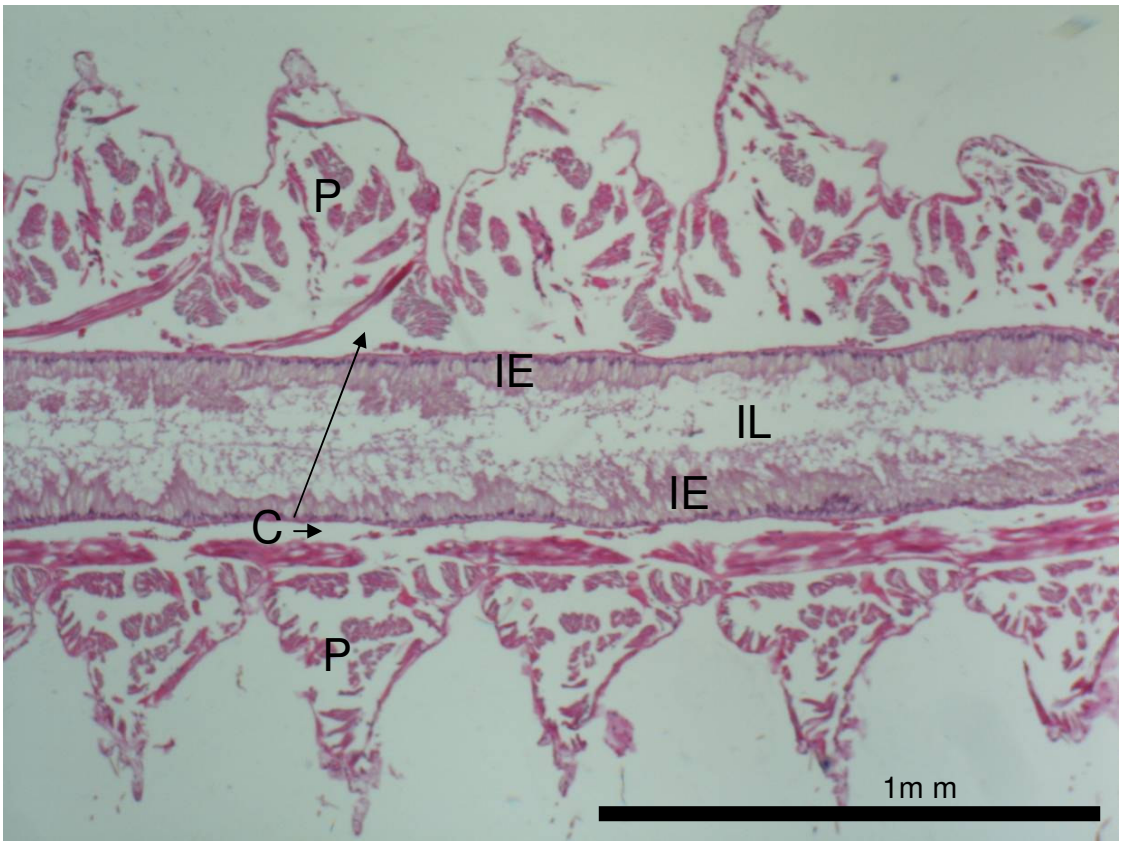


Figure 10. Haematoxylin and eosin stained histological section through a normal (uninfected) individual of *Nephtys australiensis*. P = parapodium, C = coelomic cavity, IE = intestinal epithelium, IL = intestinal lumen.

Marteilia sydneyi DNA was identified through in-situ DNA probe staining and located in the epithelium of the intestine of *N. australiensis* (Figure 11). Two major morphological forms were identified: a 'primordial' cell which contained a well-defined nucleus but had little differentiation in the cytoplasm; and a 'plasmodial' cell which showed an apparent syncytial structure. These 2 morphotypes measured 30 x 10 µm in dimension and were intimately associated with or adhered to the membrane of polychaete intestinal epithelial cells.

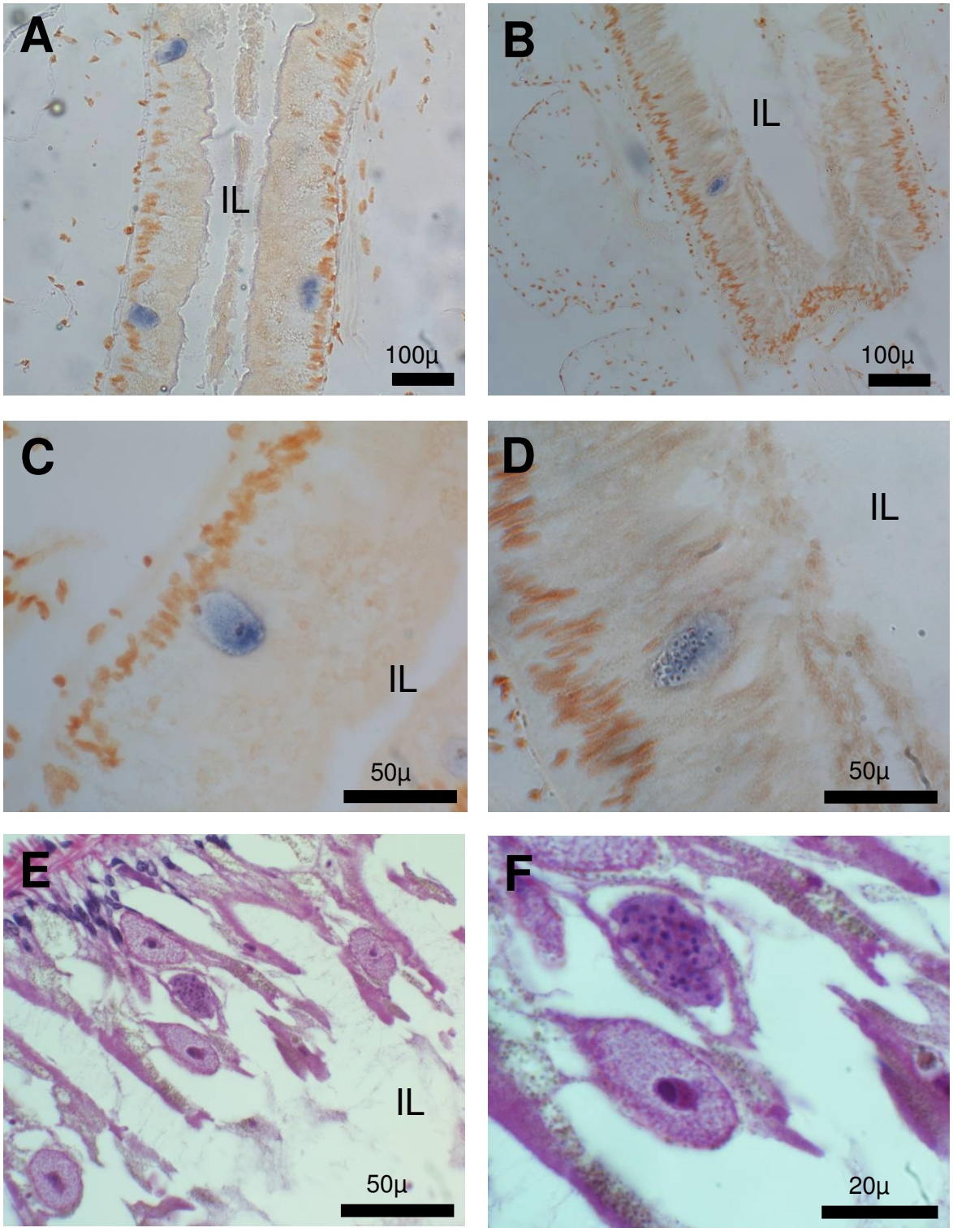


Figure 11. Photomicrographs of tissue sections from the polychaete worm, *Nephtys australiensis*. A-D Stages of development stained with ISH DNA probe specific for *Marteilia* spp, E-F haemotoxylin and eosin stained sections from the same histological preparation. A-B ISH staining of bodies in the intestinal epithelium of the polychaete; C-D ISH staining of 2 distinct morphologies, E both morphologies with H&E staining; F high magnification of 'primary' and 'plasmodial' morphologies. IL = intestinal lumen.

DISCUSSION

The results reported here represent the first record of the identification of *Marteilia sydneyi* being parasitic in an organism other than an oyster and only the second record of any species of *Marteilia* identified from non-molluscan hosts collected from open coastal systems. The presence of this member of the Phylum Paramyxea in a polychaete worm is in hindsight, perhaps unsurprising. The first member of the phylum described by Chatton (1911), *Paramyxa paradoxa*, was found in polychaete larvae (*Poecilochaetus serpens*, Poecilochaetidae), while more recently Larsson & Koie (2005) described a new species of parasite, *Paramyxoides nephtys*, from a polychaete in Scandinavia. However, neither of these species has been connected through their life cycles to a bivalve mollusc. Nonetheless, the presence of these two species in polychaetes only serves to strengthen the proposal that polychaetes can act as hosts for paramyxean parasites. It remains intriguing that a copepod has been implicated in the life cycle of *Marteilia refringens* (see Audemard et al., 2002) while we have established that a polychaete is implicated in the life cycle of *M. sydneyi*. These results may initially appear to be conflicting but rather may represent different evolutionary strategies for transmission developed by different, geographically-isolated species. Equally, in the broader context of parasitology there are many examples of hosts which become infected with stages of parasites but those hosts are 'dead ends' in terms of the life cycle, for example the infection of humans by ingestion of L3 infective larvae of anisakid nematodes in fish (normal definitive hosts would be cetaceans and pinnipeds).

The work undertaken by our French colleagues (Audemard et al. 2002; Carrasco et al. 2007; Carrasco et al. 2008) over the last few years has been parallel in approach but focussed on the identification of stages of *Marteilia refringens* developing within the copepod, *Paracartia grani*. They have been successful in transmitting *M. refringens* from infected oysters (*Ostrea edulis*) into *P. grani* where they have detected development of the parasite but have as yet failed in their attempts to infect oysters from infected copepods (Carrasco et al., 2008). Audemard et al. (2001) hypothesised that either a period of maturation in the environment is required or that a second intermediate host is necessary. However, Carrasco et al. (2008) reinforced that the difficulty in either collecting sufficient infected hosts or producing them through laboratory infections severely curtails experimental capacity to establish the true nature of that infection.

This latter issue is reinforced by the low number of ISH-confirmed developing infections we discovered during the current study. While positive polychaetes were relatively common from our PCR screening, with Cobar and Kimmerikong returning prevalences of 6.2% and 5.8%, respectively, it is difficult to quantify what proportion of those positives was indicative of actual development of *M. sydneyi*. Equally, it is unlikely that the two worms confirmed with developing infections through ISH in our study were the only hosts in which such development occurs.

Our aim was to unambiguously identify *M. sydneyi* developing in an alternate host which required following a methodology that reduced sample sizes and detection sensitivity with each step. First, collected worms had to be identified to 'operational taxonomic units' (OTU) while still alive to allow fixation of equivalent OTU both in ethanol (for PCR) and in formalin (for ISH). This immediately reduced the effective sample size for each OTU by 50%. PCR amplification using sensitive, specific and optimised protocols offers the least loss of detection possible. Nonetheless, amplification of parasite DNA can be swamped by the presence of overwhelming amounts of host DNA (see Kleeman & Adlard, 2000) leading to a lack of detection. Conversely, the presence of parasite DNA in the intestinal lumen of worms as a result of incidental ingestion of *M. sydneyi* spores while feeding, potentially overestimates the prevalence in alternate hosts (what we refer to as 'false positives'). The last level of reduced detection occurs during processing for ISH. Detection was maximised methodologically by optimising the duration of formalin fixation and by using DNA probes designed in the small subunit region of rDNA (rather than the ITS region) to maximise ISH staining signal (see Kleeman et al., 2002). However, this technique relies on histological sectioning which, of necessity, sub-samples the target tissue. Where the distribution (and intensity of infection) of a parasite in the tissues of its host is unknown, sub-sampling may or may not impact on detection levels.

Given the issues detailed above it is perhaps more surprising that this study returned <u>any</u> confirmed positives in alternate hosts at all, rather than the low number that were actually identified. Furthermore, approval for this project to proceed was predicated on a modest financial and temporal budget and was rightly ranked as a high-risk venture with a relatively low probability of any successful outcome.

Implications of this study

There are at least 3 possible scenarios that must be considered before assessing the implications of this study:

1. That *Nephtys australiensis* is the only alternate host required for completion of the life cycle of *Marteilia sydneyi*.
2. That *Nephtys australiensis* is one of a number of species that can act as an alternate host for the completion of a 2-host life cycle of *Marteilia sydneyi*.
3. That *Nephtys australiensis* and *Saccostrea glomerata* are two of a 3-or-more host, life cycle of *Marteilia sydneyi*.

If scenario 3 is correct it is unlikely that the benefit of continuing research on developing a laboratory model of infection would outweigh the cost of doing so. However, if scenarios 1 or 2 prove to be correct then the development of an experimental model of infection is enabled by the outcome of this project. The first phase would involve the growth of a parasite-free culture of *Nephtys australiensis* to allow *in vivo* confirmation of infection by the introduction of mature spores of *M. sydneyi* into controlled experiments. This would then parallel results reported by Audemard et al. (2002) from flat oysters in France.

The second phase would involve the infection of parasite-free oysters with parasite stages derived from *M. sydneyi* infected polychaetes. It is this phase which has been unsuccessful so far in the attempted experimental infections of *Ostrea edulis* from infected copepods in France (see Audemard et al., 2002, Carrasco et al., 2007; Carrasco et al., 2008) and has led them to hypothesise that a third host (or period of maturation) is required in the life cycle of *M. refringens*.

However, we would expect that the host oysters themselves used for any experimental infection, in terms of their genetics and level of immuno-competence, would play a significant role in determining whether infective stages of *M. sydneyi* originating from polychaete alternate hosts would be able to establish infections.

Studies on the mechanism of resistance developed through the Sydney rock oyster selective breeding program conducted by the NSW Department of Primary Industries (see Nell & Perkins, 2006) implicates one form of the defensive enzyme, phenyloxidase, as being negatively selected in resistant lines (Bezemer et al., 2006). Furthermore, resistant

lines have now been shown to have higher phagocytic activity and other forms of phenyloxidase activity than wild-type oysters (Butt & Raftos, 2008). As such there is compelling evidence that oyster genetics directly impacts on their susceptibility to infection with *M. sydneyi*. Another confounding issue is the link between environmental stressors and immuno-supression. Butt and Raftos (2007) suggested that the presence of a transient environmental stressor in the Hawkesbury River in 2004-05 may have affected phenyloxidase activity and, in turn, increased the susceptibility of oysters to *M. sydneyi* infection. Such a scenario would explain the appearance of severe mortalities in an estuary that had previously been unaffected by QX disease.

As such, any attempts to either confirm or further develop *in vitro* life cycle studies of *M. sydneyi* should involve experimental oysters of known genetic susceptibility and be undertaken with sufficient replicates to allow for experimental assessment of environmental stressors.

BENEFITS

The flow of benefits is directly linked with the commercial oyster industry and management sectors of NSW (90%) and Queensland (10%) with proportional benefit notionally based on the contribution of industry production from these states. The objectives achieved will clarify industry member's understanding of disease interactions in the aquatic environment and will be of broader interest to the global aquatic animal health community through the novel identification of alternate hosts required for disease transmission.

In production terms the actual and notional losses that can be attributed to the presence or potential presence of QX disease are a significant proportion of industry production but are difficult to quantify. For those estuaries in which significant outbreaks have occurred at sometime in the past, e.g. Georges River first outbreak in 1995, Hawkesbury River first outbreak in 2004, production has been reduced dramatically. Furthermore, assessment of QX disease risk has now become a central and limiting issue for commercial growers, particularly those considering disease translocation issues when translocating stock as part of the normal production process.

We have estimated the impact of QX disease on Sydney rock oyster cultivation based on a drop in the production figures in the Hawkesbury River estuary from the year that QX disease was first recorded there. In the absence of data on specific losses (partly

due to confidentiality issues) we have made a conservative estimate that 50% of the loss in production is attributable to either the presence or risk of presence of QX disease or changes in the dynamics of oyster cultivation brought about following the QX disease outbreak.

Production figures for the Hawkesbury River (NSW Department of Primary Industries 2004, 2008) for 2003/04 were 846,261 dozens of oysters. QX disease was identified in the estuary in June of 2004 and production figures for the following period (2004/05) dropped to 54% of the previous year at 456,030 dozens. Since then production has dropped more dramatically to 5,450 dozen in 2005/06 and 10,500 dozen in 2006/07. If the average production for the 5 year period to 2003/04 is 945,238 dozens and the average production for the 05/06 and 06/07 years after QX disease had become established (2005/06 and 2006/07) is 7,975 dozens, production is currently at only 8% of that prior to QX disease. Even allowing a conservative estimate of 50% of those losses attributable to QX disease, Hawkesbury River has lost an average production of 468,631 dozens of oysters per year due to QX disease. In current farm gate prices assuming an equal proportion of plate, bistro and bottle oysters are produced in that estuary, it amounts to a loss of production of $2.6 million/year for that estuary alone.

FURTHER DEVELOPMENT

This project was the first planned phase of research which, depending on the outcome, was classified as an enabling phase to determine whether the development of an experimental model of infection for QX disease was warranted. Clearly, if this project had not been successful further research on establishing such a model of infection would have been either limited or curtailed. Strategic priorities for research on QX disease now need to be re-assessed to determine whether such an approach would derive real benefit to the industry.

If such an approach was considered it is recommended that investigations not only include the annelid host but also take into account the level of immuno-competence of oysters and the environmental conditions that predispose them to infection. No proposal for extending this work will be submitted by the authors of the current study, due largely to lack of infrastructure at the Queensland Museum for such a study. We suggest that if this study is deemed beneficial to the industry, that it be a collaborative project but based and driven at the NSW DPI research facility at Port Stephens.

The first phase would require establishment of a QX-free polychaete population to allow controlled infection from infected oysters. Second phase would be confirmation of transmission from infected polychaetes to QX-free oysters. Third phase would be the collection and identification of environmental stages of QX released by polychaetes and infective to oysters. Once these phases have been undertaken, a laboratory model of infection exists which can then be applied to investigate environmental precursors to epizootics and furthermore, provides a controlled infection protocol against which to assess disease resistant lines.

PLANNED OUTCOMES

This project was specifically designed to be of short duration and of low cost since there was an identified risk that the primary outcome may not be achieved. As such, the major planned outcome was restricted to the unambiguous identification of intermediate host/s required for QX disease to cycle within an estuary. Now that this outcome has been achieved it may lead to the development of collaborative research applications (see Further Development above) to develop a laboratory model of infection (infection cycling through oysters and intermediate hosts) for direct uptake through:

1. the provision of a quantifiable and controlled challenge in the selective breeding for disease resistance program to enhance and expedite this program; and

2. the investigation of the role of environmental parameters and oyster immune defence using laboratory systems that enable identification of the principal effectors and the presence of synergistic effects on outbreak and severity of QX disease.

CONCLUSION

The benthic fauna associated with sediment near oyster leases in the upper Hawkesbury River is dominated by polychaete worms with 21 species identified at various levels of abundance. Of 1,247 individual polychaetes tested in a PCR designed to detect the presence of *Marteilia sydneyi* DNA specifically, 75 individuals representing 8 different species of polychaete returned positive results. A single species, *Nephtys australiensis* (Polychaeta: Nephtyidae), was confirmed to harbour developing stages of *Marteilia sydneyi*. Morphotypes of developing stages of *M. sydneyi* were identified in the epithelium

of the intestine of these 2 individuals. This represents a breakthrough in research into the biology of the agent of QX disease and may correspond to the identification of the only two hosts required in the life cycle of the parasite. Such a contention would require confirmation through experimental infection of *N. australiensis* from infected oysters then back-infection from infected polychaetes to known uninfected oysters.

If the life cycle described above is viable there is an opportunity to develop an *in vivo* laboratory model of infection which will enable the enhancement of selected QX disease resistant stock and the identification of parameters that predispose disease to outbreak levels in estuaries. The value would be significant of developing a laboratory system in which a quantified number of pathogens can be produced on demand to test selected resistance in stock.

EXTENSION OUTPUTS

This project drew significant attention from the media and public:

2007: Television interview Network 10 *National News* – breakthrough in oyster disease research.
2007: Public Lecture, for Biotechnology Week – Dusting off DNA at the Queensland Museum - tracking aquatic disease in open aquaculture.
2007: Television interview Network 10 *Totally Wild* – oyster disease research.
2007: Radio interviews – ABC National '*A.M.*', ABC Newcastle talk show – oyster disease breakthrough.
2007: Newspaper interviews – *Brisbane Courier Mail* and *Sydney Morning Herald* – oyster disease breakthrough.
2007: Feature article in *FISH* magazine (FRDC News).
2007: Television interview ABC National Science Show *Catalyst* – QX disease of commercial oysters updated – Museum research using DNA diagnostics.
2006: Television interview ABC National *LandLine* – Chasing the lifecycle of QX disease using DNA tools.

Results have been delivered formally at scientific conferences and as an invited lecture:

Nolan, M.J. & Adlard R.D. 2008. Identification of host interactions in the life cycle of *Marteilia sydneyi*. *Australian Society for Parasitology & ARC/NHMRC Research Network for Parasitology Annual Conference*, Glenelg, South Australia, July. (Abstract).

Adlard, R.D. 2007. Tracking aquatic disease in open aquaculture: QX disease in rock oysters in Australia. Oregon State University, sponsored by the Centre for Fish Disease Research. November.

Adlard, R.D. & Nolan, M.J. 2007. Identification of host interactions in the life-cycle of QX disease. *FRDC Aquatic Animal Health Subprogram Scientific Conference*, Cairns, Queensland, July (Abstract).

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APPENDIX 1: Intellectual Property

No intellectual property issues have been identified as arising from this project. The outcomes of this project have been/will be published, widely disseminated and promoted. Outputs will be available in the public domain.

APPENDIX 2: Staff List.

Dr Robert D. Adlard - Principal Investigator
Head of Marine Zoology & Senior Curator of Parasitology, Biodiversity Program
Queensland Museum (funded by the Queensland Government).

Dr Matthew Nolan – Research Officer
Biodiversity Program, Queensland Museum (funded by the FRDC research project).

APPENDIX 3A: Stratified random GPS points calculated for benthic sampling at **Cobar** locality, Hawkesbury River, NSW.

SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE
1	151°8.333 E	33°32.416 S	**51**	151°8.200 E	33°32.650 S	**101**	151°8.200 E	33°32.550 S
2	151°8.500 E	33°32.400 S	**52**	151°7.841 E	33°32.450 S	**102**	151°7.941 E	33°32.450 S
3	151°8.416 E	33°32.350 S	**53**	151°8.216 E	33°32.850 S	**103**	151°8.450 E	33°32.416 S
4	151°8.475 E	33°32.416 S	**54**	151°8.516 E	33°32.566 S	**104**	151°8.591 E	33°32.350 S
5	151°7.741 E	33°32.400 S	**55**	151°8.316 E	33°32.483 S	**105**	151°7.566 E	33°32.450 S
6	151°8.375 E	33°32.550 S	**56**	151°8.158 E	33°32.566 S	**106**	151°8.416 E	33°32.683 S
7	151°8.533 E	33°32.333 S	**57**	151°7.766 E	33°32.500 S	**107**	151°8.433 E	33°32.483 S
8	151°8.391 E	33°32.766 S	**58**	151°8.025 E	33°32.600 S	**108**	151°8.616 E	33°32.366 S
9	151°8.516 E	33°32.500 S	**59**	151°8.358 E	33°32.666 S	**109**	151°8.358 E	33°32.833 S
10	151°8.558 E	33°32.516 S	**60**	151°8.200 E	33°32.566 S	**110**	151°8.591 E	33°32.450 S
11	151°8.100 E	33°32.850 S	**61**	151°8.300 E	33°32.583 S	**111**	151°8.141 E	33°32.583 S
12	151°7.958 E	33°32.566 S	**62**	151°8.141 E	33°32.866 S	**112**	151°8.416 E	33°32.433 S
13	151°7.666 E	33°32.483 S	**63**	151°8.616 E	33°32.483 S	**113**	151°7.708 E	33°32.333 S
14	151°8.175 E	33°32.866 S	**64**	151°8.200 E	33°32.700 S	**114**	151°8.433 E	33°32.816 S
15	151°8.450 E	33°32.550 S	**65**	151°8.316 E	33°32.733 S	**115**	151°8.258 E	33°32.750 S
16	151°7.541 E	33°32.466 S	**66**	151°8.100 E	33°32.533 S	**116**	151°8.158 E	33°32.533 S
17	151°8.533 E	33°32.416 S	**67**	151°7.666 E	33°32.383 S	**117**	151°8.358 E	33°32.816 S
18	151°8.475 E	33°32.533 S	**68**	151°8.591 E	33°32.466 S	**118**	151°7.941 E	33°32.583 S
19	151°8.575 E	33°32.366 S	**69**	151°8.375 E	33°32.766 S	**119**	151°8.391 E	33°32.783 S
20	151°8.475 E	33°32.316 S	**70**	151°8.391 E	33°32.433 S	**120**	151°7.641 E	33°32.366 S
21	151°8.058 E	33°32.450 S	**71**	151°8.433 E	33°32.583 S	**121**	151°8.241 E	33°32.700 S
22	151°8.416 E	33°32.383 S	**72**	151°8.533 E	33°32.500 S	**122**	151°8.433 E	33°32.600 S
23	151°8.300 E	33°32.733 S	**73**	151°8.416 E	33°32.466 S	**123**	151°8.516 E	33°32.433 S
24	151°8.258 E	33°32.833 S	**74**	151°8.116 E	33°32.600 S	**124**	151°8.416 E	33°32.750 S
25	151°7.708 E	33°32.466 S	**75**	151°8.416 E	33°32.600 S	**125**	151°7.766 E	33°32.416 S
26	151°8.450 E	33°32.433 S	**76**	151°8.333 E	33°32.583 S	**126**	151°8.333 E	33°32.466 S
27	151°8.300 E	33°32.600 S	**77**	151°7.766 E	33°32.400 S	**127**	151°7.783 E	33°32.550 S
28	151°8.558 E	33°32.466 S	**78**	151°8.300 E	33°32.633 S	**128**	151°8.450 E	33°32.383 S
29	151°8.375 E	33°32.833 S	**79**	151°8.075 E	33°32.483 S	**129**	151°7.808 E	33°32.550 S

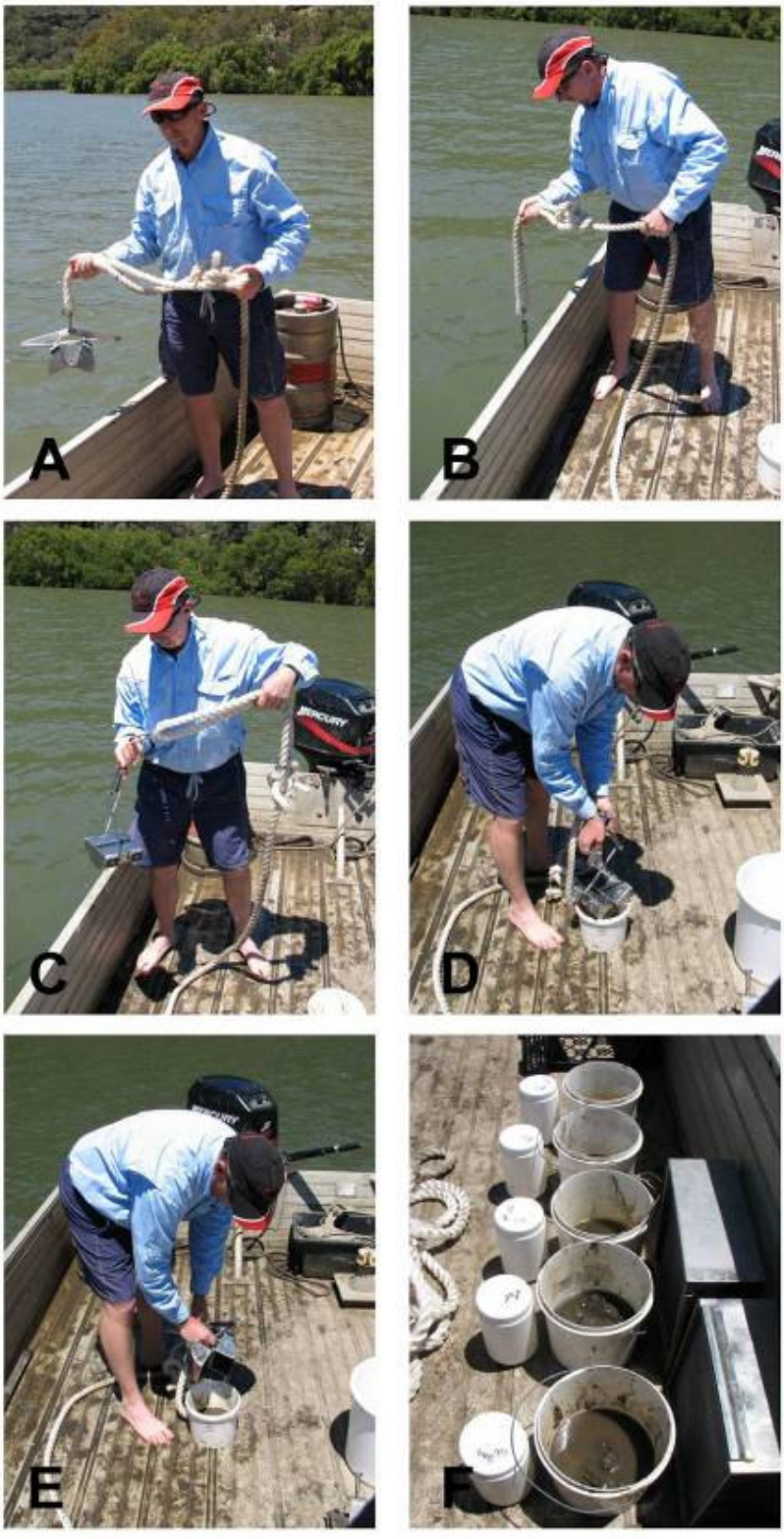
SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE
30	151°8.141 E	33°32.533 S	**80**	151°7.958 E	33°32.500 S	**130**	151°8.100 E	33°32.666 S
31	151°7.858 E	33°32.500 S	**81**	151°8.258 E	33°32.550 S	**131**	151°7.808 E	33°32.516 S
32	151°8.591 E	33°32.416 S	**82**	151°7.608 E	33°32.416 S	**132**	151°8.391 E	33°32.800 S
33	151°8.241 E	33°32.800 S	**83**	151°7.625 E	33°32.483 S	**133**	151°8.333 E	33°32.666 S
34	151°8.416 E	33°32.316 S	**84**	151°7.725 E	33°32.433 S	**134**	151°8.500 E	33°32.450 S
35	151°8.000 E	33°32.483 S	**85**	151°8.116 E	33°32.633 S	**135**	151°8.241 E	33°32.566 S
36	151°8.475 E	33°32.300 S	**86**	151°8.058 E	33°32.516 S	**136**	151°8.500 E	33°32.383 S
37	151°7.900 E	33°32.483 S	**87**	151°8.433 E	33°32.733 S	**137**	151°8.391 E	33°32.483 S
38	151°8.241 E	33°32.850 S	**88**	151°7.508 E	33°32.533 S	**138**	151°7.583 E	33°32.366 S
39	151°8.391 E	33°32.666 S	**89**	151°8.575 E	33°32.483 S	**139**	151°7.608 E	33°32.316 S
40	151°8.500 E	33°32.516 S	**90**	151°8.475 E	33°32.333 S	**140**	151°8.616 E	33°32.416 S
41	151°8.591 E	33°32.400 S	**91**	151°8.333 E	33°32.850 S	**141**	151°7.841 E	33°32.416 S
42	151°7.708 E	33°32.416 S	**92**	151°8.300 E	33°32.766 S	**142**	151°8.025 E	33°32.433 S
43	151°7.625 E	33°32.316 S	**93**	151°7.883 E	33°32.433 S	**143**	151°7.841 E	33°32.516 S
44	151°8.116 E	33°32.516 S	**94**	151°7.608 E	33°32.350 S	**144**	151°7.608 E	33°32.466 S
45	151°8.500 E	33°32.483 S	**95**	151°8.591 E	33°32.433 S	**145**	151°8.041 E	33°32.600 S
46	151°8.450 E	33°32.616 S	**96**	151°7.641 E	33°32.433 S	**146**	151°8.041 E	33°32.633 S
47	151°8.391 E	33°32.583 S	**97**	151°8.100 E	33°32.466 S	**147**	151°7.466 E	33°32.500 S
48	151°8.416 E	33°32.666 S	**98**	151°8.258 E	33°32.683 S	**148**	151°7.941 E	33°32.483 S
49	151°7.566 E	33°32.383 S	**99**	151°8.200 E	33°32.866 S	**149**	151°8.433 E	33°32.716 S
50	151°8.058 E	33°32.466 S	**100**	151°8.258 E	33°32.533 S	**150**	151°8.533 E	33°32.516 S

APPENDIX 3B: Stratified random calculated GPS points for benthic sampling at **Kimmerikong** locality, Hawkesbury River, NSW.

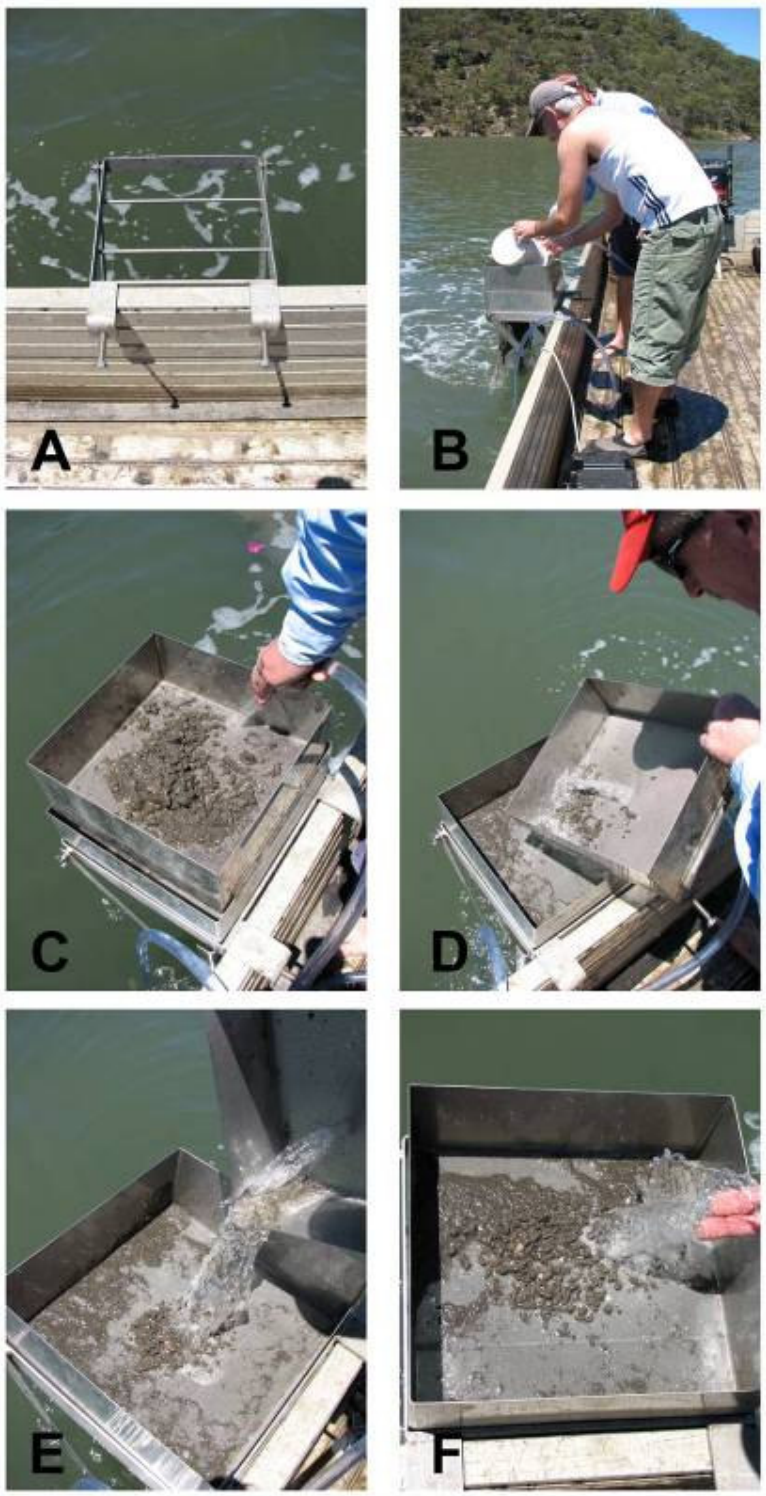
SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE
151	151°9.508 E	33°33.116 S	**201**	151°9.158 E	33°32.933 S	**251**	151°9.183 E	33°32.700 S
152	151°8.950 E	33°33.100 S	**202**	151°9.200 E	33°32.783 S	**252**	151°8.925 E	33°32.566 S
153	151°9.341 E	33°33.000 S	**203**	151°8.908 E	33°33.016 S	**253**	151°9.758 E	33°33.050 S
154	151°9.900 E	33°33.133 S	**204**	151°9.733 E	33°33.016 S	**254**	151°9.050 E	33°32.550 S
155	151°9.050 E	33°33.000 S	**205**	151°9.200 E	33°32.916 S	**255**	151°9.700 E	33°33.016 S
156	151°9.616 E	33°33.000 S	**206**	151°9.083 E	33°33.050 S	**256**	151°9.025 E	33°32.700 S
157	151°9.125 E	33°32.950 S	**207**	151°9.025 E	33°33.000 S	**257**	151°9.066 E	33°32.850 S
158	151°9.050 E	33°32.866 S	**208**	151°9.083 E	33°32.583 S	**258**	151°9.341 E	33°32.900 S
159	151°9.583 E	33°33.100 S	**209**	151°9.616 E	33°33.066 S	**259**	151°9.108 E	33°33.016 S
160	151°8.866 E	33°33.133 S	**210**	151°9.050 E	33°32.483 S	**260**	151°8.991 E	33°32.983 S
161	151°8.950 E	33°32.900 S	**211**	151°9.400 E	33°33.066 S	**261**	151°9.008 E	33°32.950 S
162	151°9.341 E	33°32.983 S	**212**	151°8.908 E	33°32.600 S	**262**	151°9.875 E	33°33.083 S
163	151°9.241 E	33°32.650 S	**213**	151°9.383 E	33°32.966 S	**263**	151°9.383 E	33°32.866 S
164	151°9.025 E	33°32.550 S	**214**	151°9.183 E	33°32.883 S	**264**	151°9.275 E	33°32.883 S
165	151°9.816 E	33°33.116 S	**215**	151°9.758 E	33°33.066 S	**265**	151°8.950 E	33°32.750 S
166	151°9.008 E	33°32.866 S	**216**	151°9.258 E	33°32.850 S	**266**	151°9.275 E	33°32.733 S
167	151°9.200 E	33°32.750 S	**217**	151°8.950 E	33°32.766 S	**267**	151°9.341 E	33°33.016 S
168	151°9.083 E	33°32.750 S	**218**	151°8.950 E	33°33.066 S	**268**	151°8.866 E	33°32.783 S
169	151°8.975 E	33°33.016 S	**219**	151°9.108 E	33°32.650 S	**269**	151°9.141 E	33°32.950 S
170	151°9.050 E	33°32.733 S	**220**	151°9.050 E	33°33.100 S	**270**	151°9.341 E	33°32.916 S
171	151°9.383 E	33°33.033 S	**221**	151°9.183 E	33°32.650 S	**271**	151°9.066 E	33°32.716 S
172	151°9.225 E	33°32.983 S	**222**	151°9.025 E	33°32.683 S	**272**	151°9.183 E	33°32.816 S
173	151°8.908 E	33°32.983 S	**223**	151°9.508 E	33°32.983 S	**273**	151°9.083 E	33°32.516 S
174	151°8.891 E	33°32.650 S	**224**	151°9.225 E	33°32.766 S	**274**	151°9.425 E	33°33.100 S
175	151°9.050 E	33°32.816 S	**225**	151°9.183 E	33°32.900 S	**275**	151°9.258 E	33°32.700 S
176	151°9.900 E	33°33.100 S	**226**	151°8.975 E	33°32.683 S	**276**	151°9.066 E	33°32.750 S
177	151°9.025 E	33°32.716 S	**227**	151°9.083 E	33°32.616 S	**277**	151°9.083 E	33°32.700 S
178	151°9.275 E	33°32.600 S	**228**	151°9.366 E	33°33.050 S	**278**	151°9.108 E	33°32.933 S
179	151°9.658 E	33°33.050 S	**229**	151°8.908 E	33°32.800 S	**279**	151°8.908 E	33°33.133 S
180	151°9.083 E	33°32.650 S	**230**	151°8.950 E	33°32.850 S	**280**	151°9.008 E	33°33.150 S

SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE	SITE NUMBER	LONGITUDE	LATITUDE
181	151°9.083 E	33°32.600 S	**231**	151°9.300 E	33°32.883 S	**281**	151°8.975 E	33°33.133 S
182	151°8.925 E	33°32.516 S	**232**	151°9.066 E	33°32.816 S	**282**	151°8.891 E	33°32.566 S
183	151°8.975 E	33°33.000 S	**233**	151°9.050 E	33°32.516 S	**283**	151°8.866 E	33°32.900 S
184	151°9.341 E	33°32.966 S	**234**	151°9.050 E	33°32.700 S	**284**	151°9.558 E	33°33.033 S
185	151°9.141 E	33°32.700 S	**235**	151°9.258 E	33°32.616 S	**285**	151°9.066 E	33°32.883 S
186	151°9.600 E	33°33.133 S	**236**	151°8.991 E	33°32.550 S	**286**	151°8.950 E	33°33.050 S
187	151°9.641 E	33°33.016 S	**237**	151°9.141 E	33°32.850 S	**287**	151°8.908 E	33°33.100 S
188	151°9.183 E	33°32.600 S	**238**	151°9.141 E	33°32.933 S	**288**	151°9.541 E	33°33.116 S
189	151°9.241 E	33°32.866 S	**239**	151°9.275 E	33°32.866 S	**289**	151°9.083 E	33°33.000 S
190	151°9.225 E	33°32.916 S	**240**	151°9.066 E	33°32.866 S	**290**	151°9.183 E	33°32.683 S
191	151°9.258 E	33°32.900 S	**241**	151°8.891 E	33°32.533 S	**291**	151°8.908 E	33°32.716 S
192	151°8.891 E	33°32.700 S	**242**	151°9.050 E	33°32.500 S	**292**	151°8.991 E	33°32.750 S
193	151°8.908 E	33°32.966 S	**243**	151°9.658 E	33°33.066 S	**293**	151°8.925 E	33°32.683 S
194	151°9.141 E	33°32.900 S	**244**	151°8.908 E	33°33.066 S	**294**	151°9.141 E	33°32.516 S
195	151°9.083 E	33°32.716 S	**245**	151°8.975 E	33°32.850 S	**295**	151°9.800 E	33°33.083 S
196	151°9.400 E	33°32.983 S	**246**	151°9.258 E	33°32.650 S	**296**	151°9.050 E	33°33.083 S
197	151°9.275 E	33°32.650 S	**247**	151°9.441 E	33°33.100 S	**297**	151°8.866 E	33°32.750 S
198	151°9.258 E	33°32.883 S	**248**	151°9.225 E	33°32.850 S	**298**	151°9.425 E	33°32.966 S
199	151°8.950 E	33°32.550 S	**249**	151°9.341 E	33°32.850 S	**299**	151°9.066 E	33°32.550 S
200	151°9.241 E	33°32.566 S	**250**	151°9.200 E	33°32.850 S	**300**	151°9.525 E	33°33.116 S

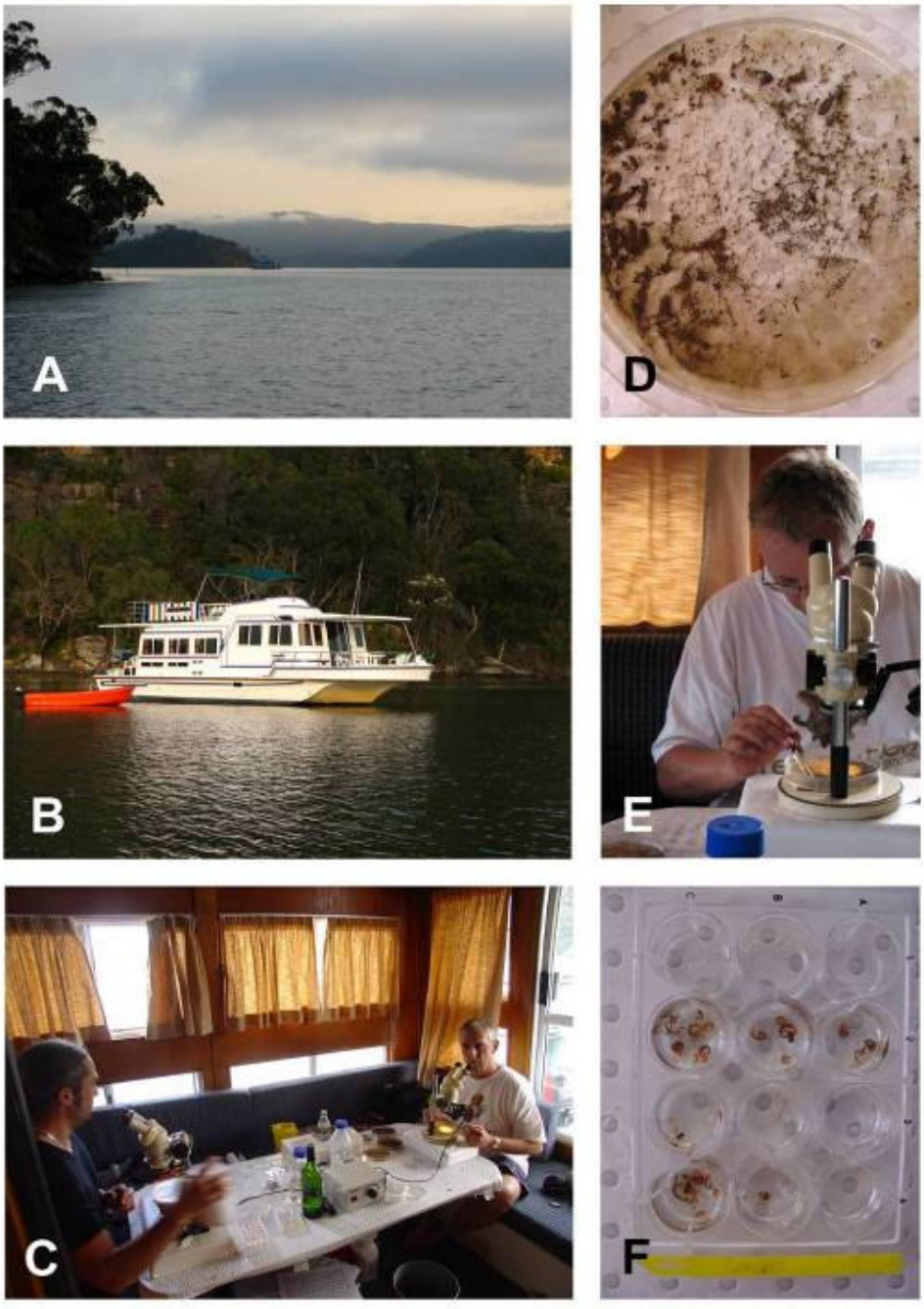
APPENDIX 4A: Benthic samples collection from randomised sites in Cobar and Kimmerikong, Hawkesbury River using a ¼ size van Veen grab sampler.



APPENDIX 4B: Benthic sample initial processing through two stacked sieves (mesh size 1mm, 500µm) to remove fine sediment.



APPENDIX 4C: Hawkesbury River, New South Wales. A, Hawkesbury River from Cobar oyster leases. B-C, Houseboat used as a laboratory for the collection of polychaetes. D, Sieved benthic samples in Petrie dishes were scanned for the presence of benthos using a dissecting microscope. E-F, Polychaetes were removed from the sample using feather-weight forceps and sorted to 'operational taxonomic unit' in a hemagglutination tray prior to preservation.



APPENDIX 5A: Numbers of each polychaete operational taxonomic unit (OTU) collected at sample sites from **Kimmerikong** oyster leases.

		Polychaeta operational taxonomic units																			
Date	Site	Capitellidae sp. 1	Cirratulidae sp. 1	Glyceridae	Lumbrineridae sp. 1	Lumbrineridae sp. 2	Magelonidae	Nephtyidae sp. 1	Nephtyidae sp. 2	Opheliidae sp. 1	Opheliidae sp. 2	Phyllodocidae	Polynoidae	Sabellidae	Scalibregmatidae	Spionidae sp. 1	Spionidae sp. 2	Spionidae sp. 3	Spionidae sp. 4	Terebelidae	Trichobranchidae	Total
8/11/2006	258				2			1													20	23
	249				3	1		2	1												13	20
	270	1						1													10	12
	157				7		4	1								1					9	22
	162							2		1						2					2	7
	267		1		2			3								1						7
	278				1		1	1														3
	153							1														1
	184							2									3					5
	263				2			1		1											18	22
9/11/2006	254				9		1			1	1										6	18
	224			1	4																17	22
	195				1		7	1													10	19
	290				9		2	1													25	37
	242		1		9																25	35
	166			1	5		3	3													31	43
	188				8			2		1						2					34	47
	208				3						1										20	24
	235				10		1	2													19	32
	299		2		6			2		1	1										16	28
10/11/2006	225					2										1					22	25
	201				3		1			5	1										37	47
	288							2													1	3
	215							1									1					2
	190		1		4			7													15	27

		Polychaeta operational taxonomic units																				
Date	Site	Capitellidae sp. 1	Cirratulidae sp. 1	Glyceridae	Lumbrineridae sp. 1	Lumbrineridae sp. 2	Magelonidae	Nephtyidae sp. 1	Nephtyidae sp. 2	Opheliidae sp. 1	Opheliidae sp. 2	Phyllodocidae	Polynoidae	Sabellidae	Scalibregmatidae	Spionidae sp. 1	Spionidae sp. 2	Spionidae sp. 3	Spionidae sp. 4	Terebelidae	Trichobranchidae	Total
	291			1	3		7	2													25	38
	286				4			2									1				1	8
	256				7		2														18	27
	241				4		2	2						2							24	34
	281				8		6														28	42
11/11/2006	159																					0
	232				8		2	2													8	20
	252				4		1														20	25
	260		5		7		4														5	21
	211						1	5									1				1	8
	220		1		2		1			1				19							31	55
	268				8		1	2		1											8	20
	284							1														1
	179																1					1
	151							1									3					4
12/11/2006	194			1	3					1											20	25
	272				4		1														42	47
	172				2			2									1			1	12	18
	275				2			1													27	30
	250			1	4		1	2		4				1						1	17	31
	237			5	4																26	35
	264			2	1		2	2									1				14	22
	191				2			2													9	13
	219			2	5		2	1		1							1				12	24
13/11/2006	287				3		1	1						3						2	18	28
	158		3	1	7			1						2	1			1		1	30	47
	259		1		7		6	1						2							10	27
	174		2	2	1		5	1						1				1			22	35
	205	1	1		5			3								1					25	36
	292			1	4		1												1		15	22

		Polychaeta operational taxonomic units																				
Date	Site	Capitellidae sp. 1	Cirratulidae sp. 1	Glyceridae	Lumbrineridae sp. 1	Lumbrineridae sp. 2	Magelonidae	Nephtyidae sp. 1	Nephtyidae sp. 2	Opheliidae sp. 1	Opheliidae sp. 2	Phyllodocidae	Polynoidae	Sabellidae	Scalibregmatidae	Spionidae sp. 1	Spionidae sp. 2	Spionidae sp. 3	Spionidae sp. 4	Terebelidae	Trichobranchidae	Total
14/11/2006	160		2		10		1	3		2				5						1	16	40
	203				10		2	3													25	40
	170				4		1	1						1				1		1	15	24
	230			1	3		3					1		4		1					6	19
	155		1	1	1		1	2				1								2	43	52
16/11/2006	231				1			5													18	24
	238	1	2	1	9		2			1											22	38
	214	1			4												1				11	17
	198				2			1													14	17
	269		1		7					1											12	21
Total	64	4	24	21	248	3	76	85	1	22	4	2	0	40	1	9	14	3	1	9	1000	1567

APPENDIX 5B: Numbers of each polychaete operational taxonomic unit (OTU) collected at sample sites in the area of the **Cobar** oyster leases.

		Polychaeta operational taxonomic units																			
Date	Site	Capitellidae sp. 1	Cirratulidae sp. 1	Glyceridae	Lumbrineridae sp. 1	Lumbrineridae sp. 2	Magelonidae	Nephtyidae sp. 1	Nephtyidae sp. 2	Opheliidae sp. 1	Opheliidae sp. 2	Phyllodocidae	Polynoidae	Sabellidae	Scalibregmatidae	Spionidae sp. 1	Spionidae sp. 2	Spionidae sp. 3	Spionidae sp. 4	Terebelidae	Trichobranchidae	Total
16/11/2006	56				1			2													8	11
	100				2			1													19	22
	60				2			4													16	22
	12				2			2													13	17
	116				3			1													37	41
17/11/2006	75				6			1		1											39	47
	61				1			5						2		1					34	43
	85				2			1		1				1		2					18	25
	146				2			2													4	8
	64				1																8	9
	23			2	2			5						1							39	49
	133			1				2		1								1			29	34
	130		1					2													17	20
	76		1	1	2			2		1											38	45
	121				2			2													14	18
	98			1	3			1												1	38	44
	6			2	2															1	27	32
18/11/2006	69				11			5								1		1			16	34
	30	1			1		1	2													18	23
	111		1		1			3										1			14	20
	66				1		1	2						1		1					16	22
	99							5														5
	114							3													2	5
	91			1				6														7
	86				1			1								1		11			14	28

		Polychaeta operational taxonomic units																				
Date	Site	Capitellidae sp. 1	Cirratulidae sp. 1	Glyceridae	Lumbrineridae sp. 1	Lumbrineridae sp. 2	Magelonidae	Nephtyidae sp. 1	Nephtyidae sp. 2	Opheliidae sp. 1	Opheliidae sp. 2	Phyllodocidae	Polynoidae	Sabellidae	Scalibregmatidae	Spionidae sp. 1	Spionidae sp. 2	Spionidae sp. 3	Spionidae sp. 4	Terebelidae	Trichobranchidae	Total
	24															2		1			1	4
	44				1											1		1			19	22
19/11/2006	2			1				1		1						1					16	20
	92				1			2										2			21	26
	51				1			3		3						1		1			25	34
	35																				3	3
	145				1											2					2	5
	80																				30	30
	125						1															1
	129							1														1
	102							1													1	2
	37							2								1		3				6
	148							1										1				2
	74		1		1			2		2				2				1			11	20
	137		1	3	6			2						1							41	54
	115							2						1				1			6	10
	58				2			2								2		2			6	14
20/11/2006	28		1		8		2														21	32
	112		3	1	2			1												1	34	42
	78				4			2		1			1								36	44
	39				7		1							1							38	47
	101				3			2													32	37
	18				14			1													10	25
	126	1	1		1			1													34	38
	54			4	9									2	1	1		2			21	40
Total	50	2	10	17	109	0	6	86	0	11	0	0	1	12	1	17	0	29	0	3	886	1190