Paving the way for continued rapid development of the flat (angasi) oyster (*Ostrea angasi*) farming industry in New South Wales

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Final Report to the Department of Transport & Regional Services
Project No. NT002/0195

June 2004
NSW Fisheries Final Report Series No. 66
ISSN 1440-3544

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Published By:	NSW Fisheries
Postal Address:	Private Bag 1, Nelson Bay NSW 2315
Internet:	www.fisheries.nsw.gov.au

ISSN 1440-3544

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ACKNOWLEDGMENTS

The authors acknowledge the generous hospitality and practical assistance provided by south coast oyster farmers, Steve Feletti (Batemans Bay), David Maidment (Narooma), John Smith (Bermagui), Chris and Dominic Boyton (Merimbula) and Mike "Banjo" Young (Pambula), during field survey and collection operations in southern NSW. We are also most grateful of the assistance of interstate colleagues Neil Hickman and Richard Gasior (Primary Industries Research Victoria), Nick Savva (Springbay Seafoods P/L, Tasmania), Dr Xiaoxu Li (South Australian Aquaculture Research and Development Institute), Matthew Muggleton (Yorke Regional Development Board, South Australia), Phil Button, (Ocean Foods International P/L, Western Australia), for their collection and supply of additional wild flat oysters for genetic assay. We thank NSW Fisheries technical staff Ian Diemar and Ben Finn for their after hours assistance in the preparing and dispatching of samples for histopathology and genetic investigations. We also thank colleagues Dr Angus Cameron (Ausvet P/L), Dr Robert Adlard (Qld Museum) and Dr Judith Handlinger (DPI Fisheries, Tasmania) for their valued technical advice on sampling protocols. Finally, we thank Helena Heasman for assistance in the preparation of this report.

NON-TECHNICAL SUMMARY

A diving survey and systematic sampling of live populations of flat oysters was done at 5 locations on the south coast of NSW. These were Merimbula Lake, Pambula Lake, the Bermagui River estuary, Wagonga Inlet at Narooma and the Clyde River estuary at Batemans Bay. At all locations live flat oysters were found on hard substrates projecting above sand, silt or mud sediments or on other vertical substrates especially oyster lease posts and rail structures, jetty piles, natural rocky outcrops and boulders of breakwaters near the mouths of these estuaries. The great majority of live flat oysters collected occurred within a sub-tidal depth band spanning 1 to 4 m below the low water mark.

A total of 474 oysters ranging from 80 to 120 mm were collected from the 5 locations. Samples from each oyster were examined to determine their gender, breeding condition and for the presence of disease agents, especially *Bonamia* that has decimated most wild populations of flat oysters elsewhere in southern Australia and overseas. These and additional flat oysters collected from wild populations in Port Phillip Bay, Victoria, Bicheno, Tasmania, Streaky Bay in South Australia, and Albany, Western Australia, were examined to determine genetic relationships throughout southern Australian distribution of this species.

A microscopic parasite, thought to be *Bonamia,* was detected in wild flat oysters from all 5 southern NSW sites. It occurred in 13% of oysters sampled from Pambula Lake, up to 44% for oysters sampled from Merimbula Lake, with an overall average of 26%. Several other parasites and microbial disease agents were also detected at varying levels. These included a possible viral infection in up to 10% of sampled oysters.

Results of the genetics research showed there are little or no differences between populations of flat oysters throughout southern NSW, nor throughout its distribution across southern Australia. Preliminary assessment suggests that the Australian flat oyster is also highly akin to its famous European cousin, the belon oyster. Further investigations into whether Australian and European flat oysters are the same species are underway. The extremely low level of genetic variation detected in this study suggests that the range of the Australian flat oyster has expanded to its current distribution relatively recently in evolutionary terms, perhaps following European settlement. Based on these data, there may be no genetic reason to limit movement of small seed oysters, commonly known as “spat”, for culture among estuaries in NSW or to source breeding stock from particular areas to meet local spat requirements. As long as the genetic diversity of broodstock used in hatchery production is high, there should be little or no danger of transporting hatchery produced spat among estuaries in NSW and beyond.

KEYWORDS: *Ostrea angas*, flat oyster, Australia, *Bonamia*, disease survey, population genetics.

1. BACKGROUND AND NEED

Sydney rock oyster farming is the state's dominant aquaculture industry directly employing around 2000 people in regional NSW and generating about $31 million in farm sales. This represents two-thirds of the state's total aquaculture earnings. However, the industry sustained a production decline of about 40% between 1975 and 1995 and has not recovered since. Principal causes of this decline have been high and rising costs (especially labour), of producing good quality, safe-to-eat oysters. Also standing in the way of profitable farming of Sydney rock oysters are a slow growth rate (3 to 4 years to market), a susceptibility to two major protozoan diseases (Winter mortality and QX) and environmental pollution, especially sewerage and acid sulphate soil run-off in the north of the state. These problems have been further compounded by increasing market competition from Pacific oysters grown in Tasmania, South Australia and New Zealand.

One aspect of research and development undertaken by NSW Fisheries in support of the ailing oyster industry has been to provide hatchery-produced seed of other high value but faster growing estuarine bivalves. During the period 1997 to 2004, more than one million seed flat oysters have been supplied each year to a small group of south coast oyster farmers for trial farming. From 2000/2001, NSW Fisheries has assisted oyster growers to set up their own pump-ashore field nurseries to on-grow smaller, but much cheaper, hatchery spat. Experience gained during the 3 initial years (1997 to 1999) of trial farming and marketing of flat oysters on the south coast was reviewed in industry workshops hosted by NSW Fisheries in 2000 and 2001. Results showed that when flat oysters are grown on existing intertidal "rack and tray" oyster farming systems, market size is achieved in 18 to 24 months and even sooner with suspended culture on deeper sub-tidal leases. This is half the period required to farm Sydney rock oysters. Flat oysters also proved more tolerant of reduced salinities and more resistant to bio-fouling and mudworm infestation than anticipated. Average local and interstate farm-gate market prices have increased from about $6/dozen in 2000/01 to $9/dozen in 2002/3. This is much higher than that the equivalent prices of from $4 to $6/dozen for plate grade Sydney rock oysters achieved over the same period.

The NSW flat oyster farming workshop hosted by NSW Fisheries in March 2000 (Heasman & Lyall 2000), identified the following high priority research and development issues to help ensure continued expansion of flat oyster farming:

(1) Health risks posed by the disease, *Bonamia*. Although this disease had not been detected in flat oysters in NSW or South Australia to that time, it had caused major losses to natural and farmed stocks in Victoria, Tasmania and Western Australia (Hine & Jones 1994) as well as to most overseas fisheries for this and other species of flat oyster (*Ostrea* and *Tiostrea* spp.). The importance of *Bonamia* also extends to export marketing being a notifiable disease under the Office International des Epizootics (OIE) regulations that govern food seafood imports into major potential markets within the European Economic Community.

(2) The potential to seriously damage or narrow the gene pool of wild flat oyster stocks through centralised hatchery production and distribution of seed oysters.

2. OBJECTIVES

(1) To develop and apply flat oyster survey and sampling techniques.

(2) To identify the location, density, age/size and sex composition of natural populations of flat oysters at 5 south coast locations where flat oysters have been routinely farmed over the past 5 years. The locations, from south to north, are: Pambula Lake, Merimbula Lake, the Bermagui River estuary, Wagonga Inlet (Narooma) and the Clyde River estuary (Batemans Bay).

Use the results of (1) and (2):

(3) To map the distribution, density and age/size structure of natural populations of flat oysters at each location.

(4) To determine if and how these separate populations differ genetically.

(5) To determine the status of the disease *Bonamia* in these populations.

3. METHODS

3.1. Field survey and sampling of wild *O. angasi* in southern NSW

An initial diving survey of live flat oysters in Merimbula and Pambula Lakes was done by NSW Fisheries staff in August 2002. Because of hypothermia experienced by divers after protracted exposure seawater temperatures of 12 to 13°C, surveys of the remaining 3 estuaries at Bermagui, Wagonga Inlet and the Clyde River were postponed until the following autumn (March/April 2003). This rescheduled date was identified by veterinary pathologists from the Queensland Museum and the Tasmanian Department of Primary Industries, as the time of highest probable incidence of Bonamia infection.

In consultation with an epidemiologist, (Dr Angus Cameron, AusVet P/L, pers. comm.), preliminary randomised collection of oysters was set at 50-100 oysters per estuary to be sampled from oyster leases, jetties, rock outcrops and breakwaters. In the event that no Bonamia was detected, sampling would be expanded to 250 to 300 oysters per estuary thereby ensuring a 95% probability of detecting disease if present in as little as 2% of stock. This degree of sampling exceeds disease-free certification requirements set by the relevant international body, namely the Office International des Epizootics (OIE).

The distributions of live populations of flat oysters within each of the five locations on the south coast of NSW were surveyed by divers, mapped and sampled by NSW Fisheries staff in April 2003. Flat oysters at all five estuaries were found on hard substrates projecting above sand, silt or mud sediments or other vertical substrates especially oyster farm structures, jetties and natural rocky outcrops, walls and breakwaters. The oysters were also largely collected from a sub-tidal band spanning 1 to 4 m below the of mean low water spring tide level (MLWS). No live oysters were found on extensive shell beds located in deeper more central parts of these particular bays and estuaries. Possible factors influencing these observed patterns of size and spatial distribution are higher levels of predation and smothering by fine sediments experience by small oysters especially after settling in deeper lower current areas.

A total of 474 flat oysters were collected (Table 1); 88 from 7 sites at Bermagui; 102 from 6 sites at Merimbula; 98 from 6 sites at Narooma; 94 from 6 sites at Pambula and 92 from 3 sites at Batemans Bay. Almost all oysters fell within a relatively narrow size range of 80 to120 mm without discontinuities needed to distinguish annual cohorts.

Tissue samples of all flat oysters collected from the five locations comprised longitudinal sections, including gut and gonad tissues, together with gill and adductor muscle tissue. Duplicate sets of samples were fixed in 10% formalin in seawater for histopatholgical examination and in 70% ethanol for genetic assay. During histological examination, the gender and breeding status of all oysters was determined with each individual classified as either male, female, recently spent (female) or immature.

Additional adductor muscle tissue samples (Table 1) fixed in 70% ethanol, were sourced by colleagues from four additional wild *O. angasi* populations located elsewhere throughout southern Australia. These included 28 from Port Phillip Bay, Victoria, 21 from Triabunna, Tasmania, 30 from streaky Bay South Australia and 28 from Albany, Western Australia that were dispatched for genetic assay between October and December 2003.

Table 1. Sample locations.

Sample Location	Sites	Sample Size
Bateman's Bay (NSW)	1	59
	A	22
	B	11
	Sub total	**92**
Narooma (NSW)	A	30
	1	11
	2	13
	3	10
	4	14
	5(i)	15
	5(ii)	5
	Sub total	**98**
Bermagui (NSW)	1&4	3
	2	30
	3	14
	5	15
	6	17
	7	9
	Sub total	**88**
Merimbula (NSW)	1&2	47
	3	23
	4	18
	5	8
	6	6
	Sub total	**102**
Pambula (NSW)	1(i)	19
	1(ii)	11
	2	19
	3(i)	18
	3(ii)	13
	4	14
	Sub total	**94**
Port Phillip Bay (Vic)	1	28
Triabunna (Tas)	1	21
Streaky Bay (SA)	1	30
Albany (WA)	1	28

Figure 1. Location map.

3.2. Disease survey of wild *O. angasi* in southern NSW

This component of research was subcontracted by competitive tender to Dr Ben Diggles on behalf of the New Zealand Institute of Water and Atmospheric Sciences (NIWA).

Formalin-fixed samples sent from Australia were placed into histopathology cassettes. The tissues were embedded in paraffin wax, and sections 5 µm thick were cut at one level in the block. The section was then deparaffinized, hydrated, stained with hematoxylin and eosin, then dehydrated, cleared and mounted on microscope slides using standard techniques (Howard & Smith 1983).

The one section from each oyster was then examined with a compound microscope at both low and high magnification for *Bonamia* spp., *Haplosporidium* spp., *Marteilia* spp., *Mikrocytos* spp., and *Perkinsus* spp., all notifiable disease agents of molluscs listed by the OIE (OIE 2002). Any other disease agents or pathological abnormalities observed were also recorded. A semi-quantitative scoring method (light = 1, moderate = 2, heavy = 3) was used to describe the intensity of parasitic infections, metabolic processes such as diapedesis and some lesions such as digestive tubule atrophy.

It should be noted that the level of diagnosis achieved by histological techniques was generally presumptive. Any requirements for definitive diagnosis past genus level for any of the putatively identified disease agents require more detailed analysis for validation.

3.3. Genetics survey of wild *O. angasi*

A collaboration was formed with Professor Peter Mather and Dr David Hurwood of the School of Natural Resource Sciences, Queensland University of Technology (QUT), to address genetics aspects of this project.

As described above, samples of wild *O. angasi* were sourced from populations from five farming locations on the south coast of NSW and from an additional four locations spanning most of the southern Australian distribution of *O. angasi* extending from Moreton Bay, Queensland (Robert Adlard, Qld. Museum, personal communication), around the southern seaboard including Tasmania, through to the Swan River, Western Australia (Thompson 1954). The four interstate locations were the Geelong arm of Port Phillip Bay, Victoria, Triabunna on the east coast of Tasmania, Streaky Bay in South Australia and from Albany in Western Australia.

Total genomic DNA was extracted from adductor muscle tissue from wild caught oysters. A 700bp fragment of the mitochondrial Cytochrome Oxidase subunit I (COI) was amplified using polymerase chain reaction (PCR).

The initial aim of this part of the study was to determine the genetic structure within and among estuaries. However, preliminary sequence analysis indicated little differentiation between the most geographically distant sites within NSW. It was therefore considered more appropriate to directly sequence several samples from across the range of the species in Australia. Individuals were sequenced from all four additional interstate locations cited above. The fragment was directly sequenced in one direction only.

4. RESULTS AND DISCUSSION

4.1. Disease survey of wild *O. angasi* in southern NSW

A microcell parasite closely resembling *Bonamia* spp. was detected in oysters from all sites examined at an overall prevalence of 26% (Table 2). Prevalence of the microcells ranged between 12.8% for oysters sampled from Pambula, up to 44.1% for oysters sampled from Merimbula (Table 2). Most oysters had light infections (Table 2) characterised by low numbers of microcells in focal areas of haemocytosis in the vesicular connective tissues and gills (Appendix 9.2, Plate 1). Occasionally, higher numbers of microcells were present in focal areas of intense haemocytosis in the gills (Appendix 9.2, Plate 2) and gonad. Microcells were observed both intracellularly inside haemocytes, and extracellularly (Appendix 9.2, Plates 3, 4). The microcells ranged between 2 and 3 μm in diameter with a nucleus around 1 μm diameter.

A *Marteilia*-like paramyxean was detected in the epithelium of the digestive gland of two oysters (overall prevalence 0.4%, Table 2). One oyster from Bermagui had a moderate infection while another from Narooma had a heavy infection (Table 3) with all tubules in section infected. Prevalence of the parasite at both sites was around 1% (Table 2). Both grossly affected oysters had very pale digestive glands. Infected nurse cells were large (mean diameter 14.3 μm, range 9.6-19.2 μm, n = 15) and contained 1 to at least 10 parasite daughter cells (mean 3.8 x 3.5 μm, range 3.2-5.6 x 2.4-4 μm, n = 10) in the plane of section (Appendix 9.2, Plates 5, 6). Sporogenesis was observed in both infected oysters, but was particularly apparent in the heavily infected oyster from Narooma. Up to 14 spore-like bodies (mean 4.5 μm diameter, range 4-4.8μm, n = 10) were observed in sections through sporangiosaurus-like structures within the tubule epithelium (Appendix 9.2, Plate 7). These spore-like structures stained acid fast using Zeihl Neelsen stain (Appendix 9.2, Plate 8).

Densely basophillic inclusions (Appendix 9.2, Plate 9) containing chlamydiales-like organisms (C-LOs) (Hine *et al*. in prep) were present in the digestive gland epithelium of oysters from all sites at an overall prevalence of 29.8% (Table 2). Prevalence at each site ranged from 19.4% at Narooma to 38.2% at Merimbula. Most oysters were lightly infected, however one or two heavily infected oysters were recorded from each site (Table 3). Many of the C-LO inclusions contained striations which were probably due to bacteriophage infection (Hine *et al.* in prep.).

Focal lysis and necrosis of the epithelium of the digestive gland (Appendix 9.2, Plate 10) was observed in oysters from all sites and was associated with the presence of epithelial cells with abnormal hypertrophied nuclei with marginated chromatin (Appendix 9.2, Plate 11). The affected nuclei appeared to contain inclusion bodies (Appendix 9.2, Plate 11), possibly due to infection by a virus. The overall prevalence of lesions associated with the putative virus was 10.3%, with highest prevalence being at Pambula (16%), and lowest at Narooma (6.1%) (Table 2).

Table 2. Prevalence of parasites and lesions from histopathological examination of 474 *O. angasi* from all sites in southern NSW.

Site and sample data	Batemans Bay	Bermagui	Merimbula	Narooma	Pambula	All sites combined
No. oysters examined	92	88	102	98	94	474
Males	34.9%	35.2%	28.4%	25.5%	23.4%	29.3%
Females	10.8%	36.4%	28.4%	29.6%	16%	24.3%
Spent	13%	7.9%	21.6%	21.4%	23.4%	17.7%
Hermaprodite	41.3%	20.5%	21.6%	23.5%	37.2%	28.7%
Bonamia sp.*	18.5%	19.3%	44.1%	32.7%	12.8%	26%
Marteilia sp.*	0%	1.1%	0%	1%	0%	0.4%
Pseudomyicola sp.	1.1%	0%	0%	0%	0%	0.2%
Digestive tubule atrophy	5.4%	4.6%	7.8%	8.2%	9.6%	7.2%
Diapedesis	12%	12.5%	25.5%	21.4%	7.5%	16%
Haemocytosis	41.3%	38.6%	49%	43.9%	38.3%	42.4%
Ancistrocoma -like ciliates	0%	1.1%	1%	0%	0%	0.4%
Chlamydiales-like organisms	31.5%	21.6%	38.2%	19.4%	37.2%	29.8%
Putative Virus*	12%	11.4%	6.9%	6.1%	16%	10.3%
Necrotic Foci	12%	11.4%	7.8%	6.1%	16%	10.6%
Bacterial Infection	1.1%	0%	0%	0%	1.1%	0.4%
Digenean infection	23.9%	14.8%	5.9%	22.5%	30.9%	19.4%

* Confirmation of the identity of the *Bonamia* sp., *Marteilia* sp. and putative virus requires TEM or molecular analysis, and is beyond the scope of this study.

Table 3. Mean intensity of parasites and lesions from histopathological examination of 474 *O. angasi* from all sites in southern NSW.

Site and sample data	Intensity Score	Batemans Bay	Bermagui	Merimbula	Narooma	Pambula	All sites combined
Bonamia sp.	Mean*	1.06	1.18	1.18	1.25	1	1.16
	1*	16	15	37	24	12	104
	2*	1	1	8	8	0	18
	3*	0	1	0	0	0	1
Marteilia sp.	Mean*	-	2	-	3	-	2.5
	1*		0		0		0
	2*		1		0		1
	3*		0		3		1
Digestive tubule atrophy	Mean*	1.20	1	1.38	1.25	1.22	1.24
	1*	4	4	6	7	7	28
	2*	1	0	1	0	2	4
	3*	0	0	1	1	0	2
Diapedesis	Mean*	1.27	1.36	1.35	1.14	1	1.25
	1*	9	9	18	18	7	61
	2*	1	0	7	3	0	11
	3*	1	2	1	0	0	4
Chlamydiales-like organisms	Mean*	1.34	1.32	1.28	1.26	1.14	1.26
	1*	20	15	30	15	32	112
	2*	8	2	7	3	1	21
	3*	1	2	2	1	2	8
Location of haemocytosis	No. affected	38	34	50	43	36	201
	Connect. Tiss.	22	20	21	23	18	104
	Gut	6	3	9	9	2	29
	Gills	0	1	6	2	3	12
	Digestive Gl.	6	6	3	2	6	23
	Mantle	0	0	1	0	0	1
	Gonad	4	4	10	7	7	32

* Infection intensity determined by semiquantitative methods. The numbers below the value for mean intensity are raw counts of affected oysters classified using the following semi-quantitative scoring method (1 = light, 2 = moderate, 3 = heavy).

Sporocysts of a digenean (Appendix 9.2, Plate 13) were prominent in the gills and connective tissues of oysters from all sites (overall prevalence 19.4%, range 5.9% (Merimbula) to 30.9% (Pambula) (Table 2). Bacterial infections were observed in two oysters, one each from Batemans Bay and Pambula. The affected oyster from Pambula presented with a large abscess which was characterised by peripheral haemocytosis, inside of which large areas of connective tissue and digestive tubule epithelium had been infiltrated by bacteria. The centre of the abscess was characterised by widespread necrosis of connective tissue and digestive tubule epithelium (Appendix 9.2, Plates 14, 15).

Symbionts found included *Pseudomyicola*-like copepods in the digestive tubule of one oyster from Batemans Bay, and *Ancistrocoma*-like ciliates in the lumen of the digestive tubules of one oyster each from Bermagui and Narooma (Table 2).

The detection of the *Bonamia*-like cells associated with areas of haemocytosis in oysters from all sites sampled is significant as all microcell infections in molluscs are notifiable to the OIE Molluscan Reference Laboratory (OIE 2002). There are two described microcell genera, *Bonamia* and *Mikrocytos*. *Bonamia* spp. are primarily parasites of flat oysters, but have also been recorded in crassostreid oysters (Cochennec *et al.* 1998, Cochennec-Laureau *et al.* 2003). *Mikrocytos* spp. are parasites of crassostreid oysters and have been recorded from Pacific oysters (*M. mackini*, Farley *et al.* 1988) and Sydney Rock Oysters (*Saccostrea glomerulata*), (as *M. roughleyi*, Farley *et al.* 1988, but recently reclassified as *Bonamia roughleyi* (Cochennec-Laureau *et al.* 2003). *M. mackini* is commonly found in vesicular connective tissue immediately adjacent to abscesses (foci of haemocytosis), while *B. roughleyi* and *Bonamia* spp. are most commonly intracellular within haemocytes (Hine & Wesney 1994; OIE 2000, B. Diggles, personal observation). The microcells found in the *O. angasi* from these samples thus appear most likely to be *Bonamia* sp.

Given that a *Bonamia* spp. has already been recorded in *O. angasi* in Victoria, Tasmania and Western Australia (Hine & Jones 1994), this suggests that the microcells found during this study are not *B. roughleyi* as previously recorded from NSW *S. glomerata*. However, due to the very small size of these cells, clearly any attempt at description to species level using the light microscope is meaningless. Definitive diagnosis for *Bonamia* spp. to satisfy OIE requirements is currently based on transmission electron microscopy (TEM) examination of the microcells. Molecular probes are available (Adlard & Lester 1995; Cochennec *et al*. 2000; Carnegie *et al.* 2003, Diggles *et al.* 2003), but these have not been validated for southern hemisphere microcells and hence their use for diagnostic purposes is currently limited. This suggests that additional sampling is required to collect more material, preferably from Merimbula or Narooma, specifically for TEM for initial attempts at a definitive diagnosis. At the same time, it would be advisable to collect samples in ethanol for molecular analysis so these could be analysed at a later date by Dr Serge Corbeil (CSIRO) as part of his FRDC funded study into development of the molecular diagnostic techniques for Australian *Bonamia* spp.

One potential problem with collecting additional samples for TEM is the generally low intensity and focal nature of the microcell infections in the oysters examined. This is not surprising as these sites were surveyed in the absence of clinical disease. It is considered unlikely that microcells would be successfully visualised by TEM in lightly infected oysters. This is because microcells are usually detected reliably by TEM only in diseased, heavily infected oysters (M. Hine, personal communication). This suggests that before additional samples are taken for TEM, the oysters should be stressed to try and increase infection intensity, perhaps by overcrowding and/or increase in water temperature (which can promote the course of disease in the case of *Bonamia exitiosus* (see Hine *et al.* 2002), and/or a decrease in water temperature, which promotes disease in the case of *B. roughleyi* (see Farley *et al*. 1988). Alternatively, some published techniques for isolating and purifying microcells (Mialhe *et al.* 1988; Hervio *et al.* 1996; Joly *et al.* 2001) could be utilised to attempt to obtain pure material for ultrastructural and/or molecular analysis.

The other internationally significant finding from these oysters was the presence of a *Marteilia*-like paramyxean in the digestive gland of two oysters, 1 each from Bermagui and Narooma. Comparison of the various stages present in these oysters with reference slides of *Marteilia sydneyi* from NSW *S. glomerata* suggests that the paramyxean in *O. angasi* is not *M. sydneyi* based on differences in spores and sporogenesis (Diggles & Hine, personal observation). The spores and sporogenesis of the parasite in *O. angasi* also appeared dissimilar to those of *M. refringens* from *O. edulis* in France, and *M. lengehi* from *Saccostrea cucullata* from Western Australia (Diggles & Hine, personal observation). At this early stage it appears that the paramyxean in *O. edulis* may be a new species of *Marteilia*, however further work will be required using TEM and molecular probes to confirm this, and assess whether cross reaction occurs with molecular diagnostic tools developed for *M. sydneyi* (Kleeman *et al*. 2002a,b).

Focal areas of necrotic and sloughing digestive gland epithelium in oysters from all sites were associated with the presence in sloughed cells of hypertrophied nuclei with marginated chromatin and apparent viral inclusions. Additional work would be required using TEM and/or molecular techniques to determine whether these lesions were associated with viral infection. Digestive epithelial virosis has been previously reported in shellfish from New Zealand (Jones *et al.* 1996; Hine & Wesney 1997) and are thought to be due to the presence of small, unenveloped RNA viruses. The viruses appear to be associated with sloughing of the digestive tubule epithelium but their actual role in any disease process remains unclear. In New Zealand, for example, these viruses occur in all scallops examined, but at particularly high levels in dying or lethargic scallops. These viruses may therefore play a role in the natural process of renewal of the gut lining, but if they reach high levels, they may cause disease (Hine & Wesney 1997).

There may be a relationship between digestive tubule atrophy and the presence of the putative viral infection, as oysters from Pambula, which had the highest prevalence of digestive tubule atrophy, also had the highest prevalence of the putative viral infection. However, the severity of tubule atrophy (mean intensity 1.38 out of 3) was greatest at Merimbula, which had the second lowest prevalence of putative virus, hence a more detailed study of both lesions would be required to determine if a relationship truly exists between the two. Other possible causes for atrophy of digestive gland tubules include bacterial infections, metazoan infection, toxic insults and starvation caused by fluctuations in food supply.

Chlamydiales-like organisms (C-LOs) have been previously recorded from Australian *O. angasi* (see Hine *et a*l. in prep.). In the present study the C-LOs were relatively common (19.4-38.2% prevalence) in oysters from all sites. C-LOs, RLOs and other related intracytoplasmic bacteria are probably ubiquitous in marine bivalves (Hine & Diggles 2002). Usually they occur at low intensities, and are not associated with disease. However, if the host becomes stressed, due to factors which may include unfavourable environmental conditions or metabolic imbalances post-spawning, the C-LOs and RLOs can proliferate and may cause disease (Hine & Diggles 2002).

Diapedesis (migration of haemocytes across epithelia), was occasionally observed in the gut epithelium. Diapedesis is a metabolic process in oysters, used to remove harmful elements or metabolic by products, as well as parasites such as *Bonamia* (see McGladdery *et al*. 1993). As diapedesis also occurs in healthy oysters as well as in diseased ones, the presence or absence of diapedesis may not necessarily be related to the presence of disease agents or pollutants.

In very rare cases bacterial infections were found to be associated with areas of necrosis and haemocytosis. In the oyster from Pambula, the bacterial infection was associated with an abscess-like lesion which, due to its large size, appeared to present a significant threat to the health of that oyster. The copepod and ciliate parasites found are common symbionts of healthy oysters (McGladdery *et al.* 1993) and are of little pathological significance.

4.2. Genetics survey of wild *O. angasi*

The sequence analysis detected 10 unique haplotypes (Figure 2) separated by a single or two base pair differences only. It is clear from Figure 2 that there is no geographic structure of the populations. Under coalescent principles, internal haplotypes (Haplotype 1) should be older and therefore more numerous and widespread, which is the case here. Also, tip haplotypes (Haplotypes 2-10) are more recently derived and should be less common with restricted distributions.

Although the tip haplotypes in this study appear to be fewer in abundance, several of them (Haplotypes 2, 3 & 5) are found in more than one sampling site. Although this is not an uncommon pattern, the fact that Haplotype 5 is found in both Western Australia and Tasmania suggests that contemporary gene flow is occurring, and on a large geographic scale. Similarly, Haplotype 3 is found in New South Wales and South Australia. These results show that across the total range in Australia, there is very little divergence among sites, which was not expected given the life history traits of *O. angasi*. It is therefore reasonable to assume that there is little or no population structure in this species.

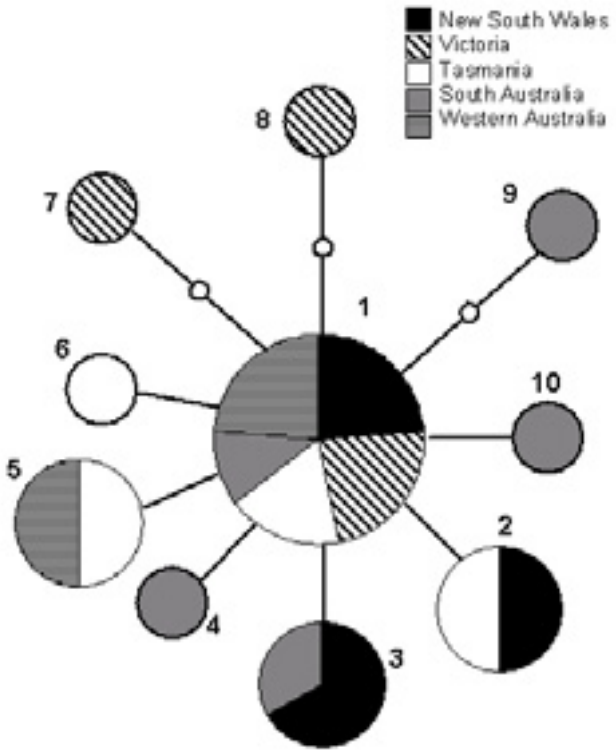

Figure 2. A network displaying the relationship between haplotypes. Lines represent single base pair differences and the size of the circles represents the relative abundance of each haplotype in the sample (largest circle >10; medium circle >1; small circle =1). The smallest open circles represent haplotypes that were not detected in the sample. The relative abundance by sampling location is given.

Although there is significant evidence for high levels of contemporary gene flow around the southern coast of Australia, these data cannot determine whether dispersal was anthropomorphically mediated or the result of natural dispersal capabilities of *O. angasi*. If stocks have been translocated for farming purposes, it still may be expected that divergent individuals would be detected from the natural populations, which is not the case.

Taxonomic considerations

The results of this study have raised some interesting and potentially important taxonomic inconsistencies. Firstly, two of the samples from South Australia appear to be *O. aupouria*, an endemic species confined to New Zealand (see Figure 3). Whether this species is found naturally in Australian waters or is the result of active translocation from New Zealand is unknown.

Figure 3. Neighbouring joining tree showing relationship among *O. angasi* COI data from this study and other congenerics. (Sequences obtained from GenBank with accession numbers given).

Secondly, the relationship between *O. angasi* and *O. edulis* is puzzling. *O. angasi* should be more closely related to other southern hemisphere species (e.g. *O. aupouria* from New Zealand and *O. chilensis* from Chile and New Zealand) and be highly divergent from northern hemisphere species such as *O. edulis*. Figures 3 & 4 show the level of genetic divergence between *O. angasi* and *O. edulis* to be an order of magnitude lower than that between *O. angasi* and its nearest neighbours. In fact the reconstructed phylogeny in Figure 4 shows the relationship between *O. angasi* and *O. edulis* to be unresolved.

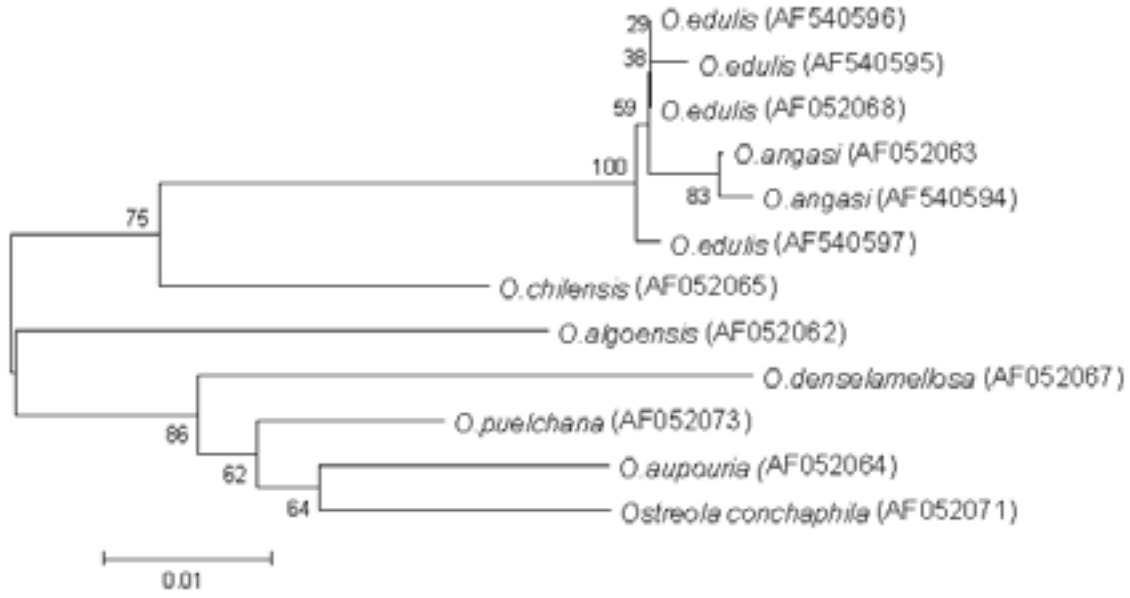

Figure 4. Neighbouring joining tree showing relationship among *O. angasi* 16s data and other congenerics. (All sequences obtained from GenBank with Accession numbers given).

O. edulis has been documented in Australia and it is assumed that this species has arrived with Europeans in the last 200 years. Yet these data suggest that *O. edulis* is the ancestral type to *O. angasi* and therefore gene flow has occurred between the northern hemisphere and Australia relatively recently.

Another scenario consistent with these data is that *O. angasi* and *O. edulis* are in fact the same species with both lineages independently arriving from Europe in the last 200 years. The strongest support for this argument is that the level of divergence within *O. angasi* is equal to the divergence level between *O. angasi* and *O. edulis*. Although Figure 2 indicates that *O. angasi* and *O. edulis* are reciprocally monophyletic (i.e. individuals within species are more genetically similar to each other), this may simply be the result of separate introductions from geographically disparate locations within the natural range of *O. edulis* in the northern hemisphere. The easiest way to resolve this dilemma is to investigate the genetic structure of sympatric populations of both species using nuclear markers (e.g. microsatellites) in order to determine whether random mating occurs between lineages.

5. KEY FINDINGS, CONCLUSIONS & RECOMMENDATIONS

5.1. Disease survey of wild *O. Angasi* in southern NSW

A microcell parasite, probably *Bonamia* spp., was detected in oysters from all sites examined at an overall prevalence of 26%. Prevalence of *Bonamia* spp. ranged between 13% for oysters sampled from Pambula, up to 44% for oysters sampled from Merimbula. A *Marteilia*-like paramyxean was detected in the epithelium of the digestive gland of two oysters (overall prevalence 0.4%). One oyster from Bermagui had a moderate infection while another from Narooma had a heavy infection with all tubules in section infected. Prevalence of the paramyxean parasite at both sites was around 1%.

Densely basophillic inclusions containing chlamydiales-like organisms (C-LOs) were present in the digestive gland epithelium of oysters from all sites at an overall prevalence of 30%. Most oysters were lightly infected, however, one or two heavily infected oysters were recorded from each site. Focal lysis and necrosis of the epithelium of the digestive gland observed in oysters from all sites was associated with the presence of intranuclear inclusion bodies in affected cells, possibly due to infection by a virus. The overall prevalence of the putative viral lesions was 10%. Other parasites and symbionts found included digenean sporocysts in oysters from all sites (overall prevalence 19%); *Pseudomyicola*-like copepods in the digestive tubule of one oyster from Batemans Bay, and *Ancistrocoma*-like ciliates in the lumen of the digestive tubules of one oyster each from Bermagui and Narooma. Bacterial infections were observed in two oysters, one each from Batemans Bay and Pambula.

The microcell-like cells and the paramyxean should be followed up as parasites from these groups are known to cause diseases in molluscs which are notifiable to the OIE Molluscan Reference Laboratory. Additional work would be required using TEM and/or molecular techniques to determine whether the microcell-like cells observed here are aligned with *Bonamia* spp. or *Mikrocytos* spp., and also to determine the specific identity of the *Marteilia*-like paramyxean. The low intensity and focal nature of the microcell infections in these oysters may hinder TEM diagnosis and some possible methods for obtaining heavily infected material for TEM are discussed. Similarly the low prevalence of the *Marteilia*-like paramyxean will most probably result in the need for focussed research to obtain the information required to determine its affinities.

Ongoing research initiatives and collaborations stemming from this project include research by Dr Serge Corbeil (AAHL/CSIRO) to develop a rapid diagnostic test for *Bonamia.* Another initiative is a project led by Dr Alex Hyatt (AAHL/CSIRO) to confirm the identity of a possible virus infection and of a paramyxean parasite as a species of *Marteilia.* An additional possible development is to extend the *Bonamia* survey to include estuaries and bays of the central and northern coasts of NSW and of southern Qld (Moreton and Hervey Bays). The latter will in any case be undertaken by Qld Department of Primary Industries and Fisheries in collaboration with a Moreton Bay based commercial partner (David Williams, Aquafarms, Qld. P/L; personal communication).

5.2. Genetics survey of wild *O. angasi*

Results of this study suggest there is little or no population structure for *O. angasi* among estuaries in southern New South Wales. In fact, there is strong evidence that *O. angasi* exist as a single panmictic population across the total range sampled (i.e. from east to west coast). The extremely low level of genetic divergence among haplotypes detected in this study suggests that the range of this species has expanded to its current distribution relatively recently in evolutionary terms.

Therefore, the translocation of spat from one estuary to another, and the potential hybridisation with wild stocks, is unlikely to erode the genetic integrity of the wild local populations.

It should be noted however, that this study only investigated structure based on mitochondrial DNA which is considered to be a neutral genetic marker. Therefore, any differential selection pressure, creating genetic differences at the local scale, will not have been detected.

It is planned that the genetic relationship between *O. edulis* and *O. angasi* be pursued to resolve whether they are the same species by investigating the genetic structures of sympathetic populations of both species using nuclear markers (e.g. microsatellites).

5.3. Implications of findings for industry

The ubiquitous presence of *Bonamia* detected at moderate to high levels in wild *O. angasi* in all five surveyed estuaries in southern NSW, together with the finding that none of these oysters were exhibiting acute levels of disease, implies that future stock movements between estuaries south from Batemans Bay should not be restricted on the basis of this particular disease agent. These findings also mean that any further initiatives by industry to have estuaries declared *Bonamia* free as a means of accessing potential live export markets into Europe would be futile. Instead, future export marketing initiatives by industry can be more fruitfully focussed on chilled half shell, frozen and other processed products and away from live oysters.

The original scope of this study to survey wild flat oyster populations in Southern NSW for the presence of *Bonamia* was expanded to include an array of other potentially important, internationally notifiable diseases and also to assess the general health and condition of stocks. A common array of additional pathogens were found to infect flat oysters collected from all five locations. These findings further negate arguments to restrict an expansion of flat oyster production into new sites or to restrict production flexibility associated with translocation on the NSW coast south from Batemans Bay.

Evidence that *O. angasi* throughout southern Australia constitute a common genetic stock means that future translocation of stock between estuaries throughout Australia may not be influenced by a need to conserve genetic diversity or genes unique to particular regional populations. This of course assumes that the genetic diversity of hatchery produced seed oysters is kept high. The finding that *O. angasi* cannot be distinguished from its European counterpart *O. edulis* could have positive export marketing implications such as a right to be marketed under common use names such as “belon”.

The number of jobs that will be created directly or indirectly as an outcome of this project will depend on the extent to which NSW oyster farmers shift away from Sydney rock oysters in favour of flat oysters. At the inception of this project the shift was expected to be large. However, in the intervening two years, major advances have also been made by NSW Fisheries in overcoming hatchery production difficulties with faster growing, selected breeding lines of Sydney rock oysters. These genetically improved Sydney rock oysters will rapidly promote expanded production of premium grade, single seed plate oysters. During the current 2003/4 breeding season, more than 12 million of these genetically superior Sydney rock oyster seed have been produced by NSW Fisheries at Port Stephens and distributed to nine farmer operated field nurseries on the southern and central coasts. Ironically, these field nurseries are the same or an extension of those initiated by NSW Fisheries over the past four years to promote expanded farming of flat oysters.

Accordingly, the revised role of the field nurseries for intermediate production of genetically improved Sydney rock oyster spat will effectively be at the cost of slowing the anticipated expansion of the industry into flat oysters.

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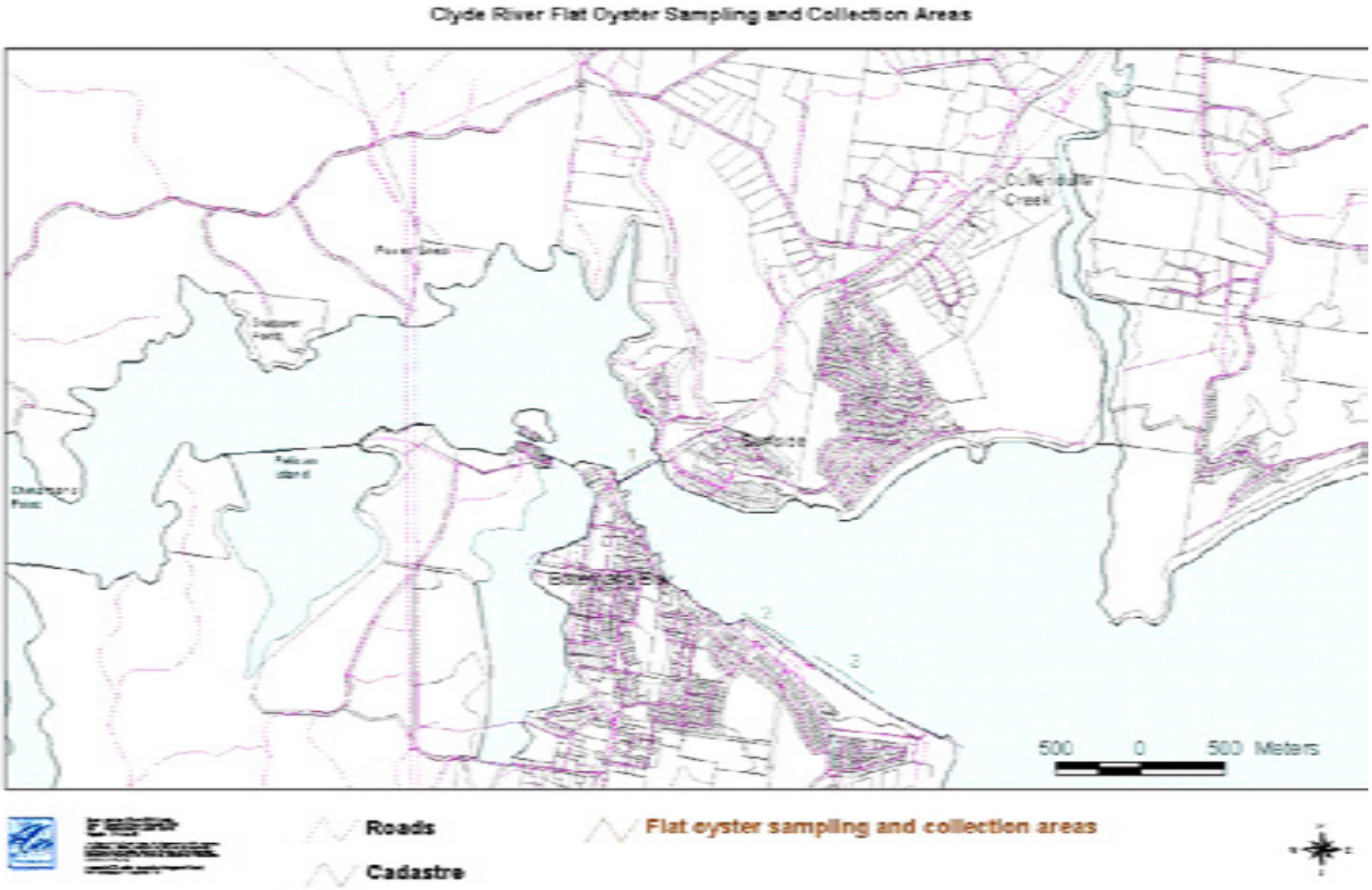
7. APPENDICES

7.1. Five surveyed collection sites on the south coast of NSW

(Pages 23-28)

7.2. Parasites and lesions observed in *O. angasi* from NSW

(Pages 29-36)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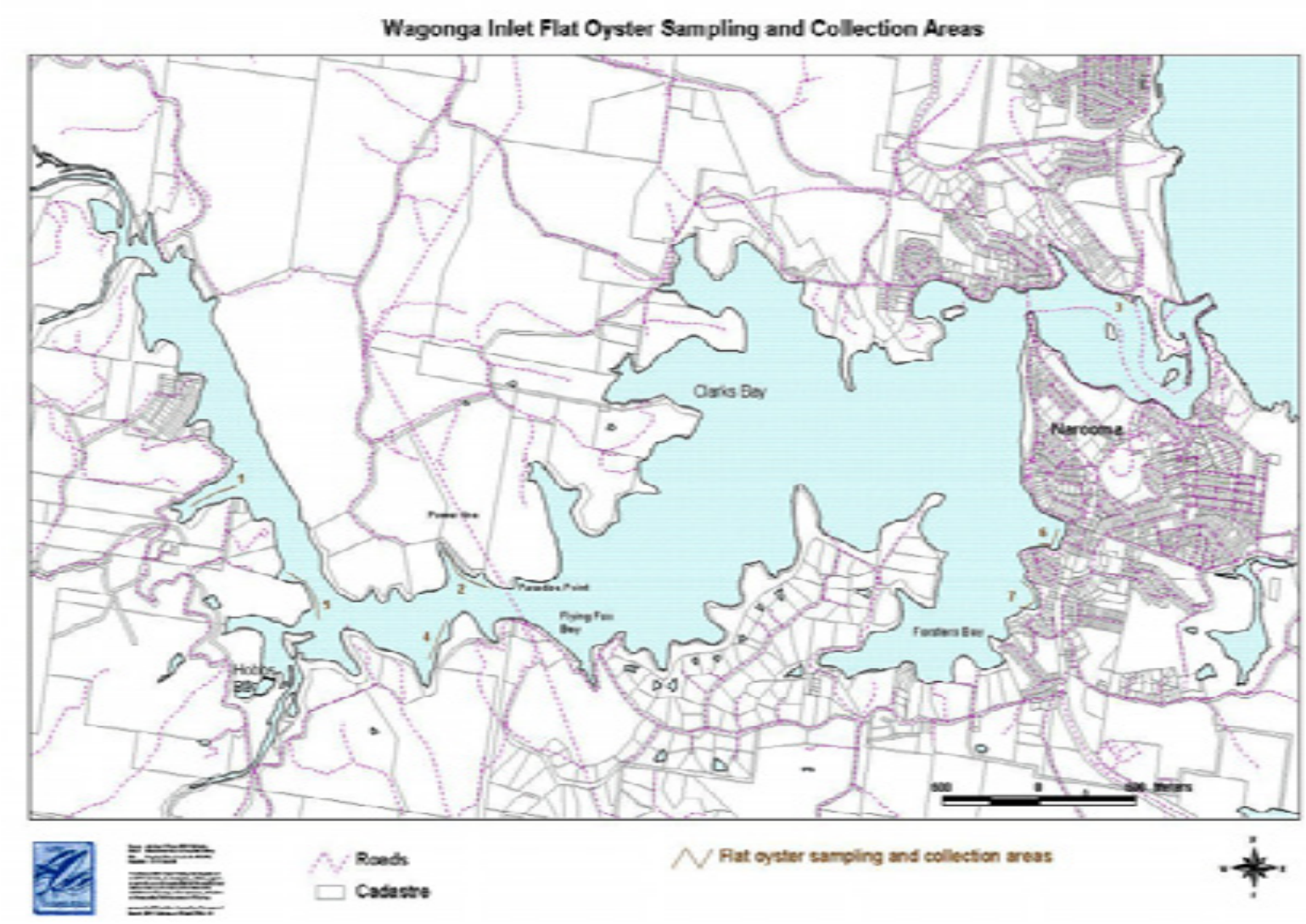
Flat oyster sampling and collection areas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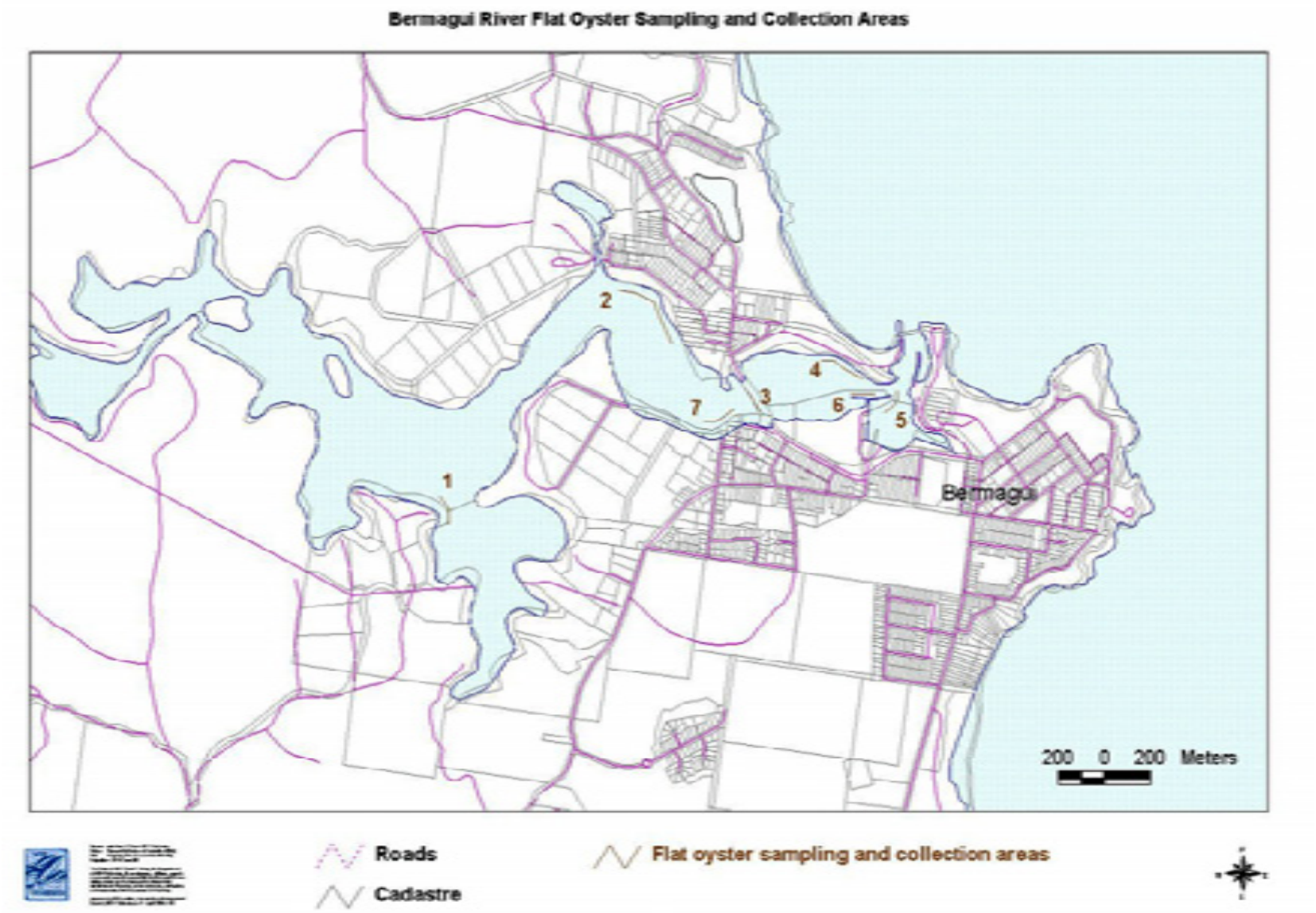
Clarks Bay
Narooma
Hobbs Bay
Roads
Cadastre
Flat oyster sampling and collection areas


Bermagui River Flat Oyster Sampling and Collection Areas
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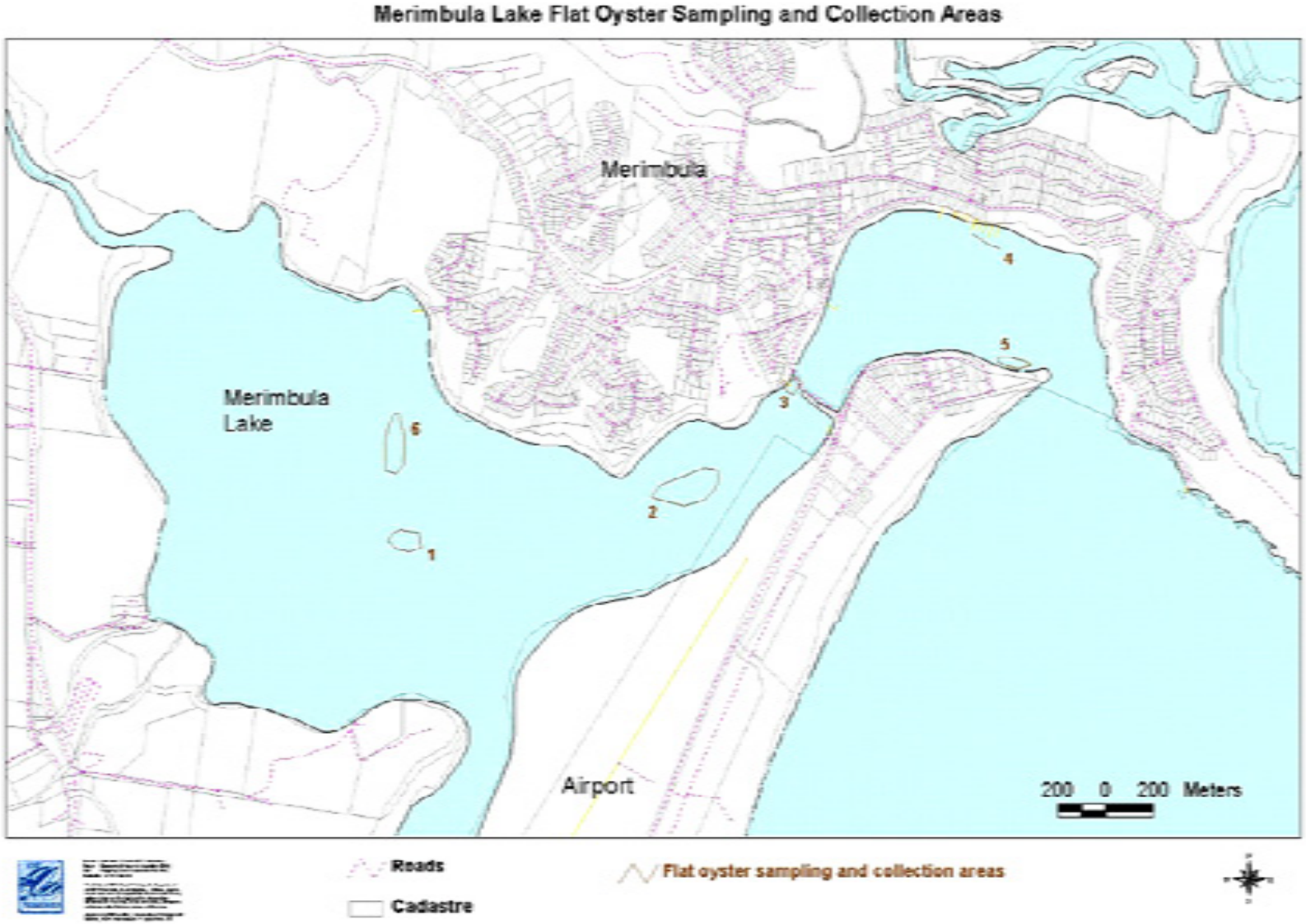
Flat oyster sampling and collection areas


Merimbula Lake Flat Oyster Sampling and Collection Areas
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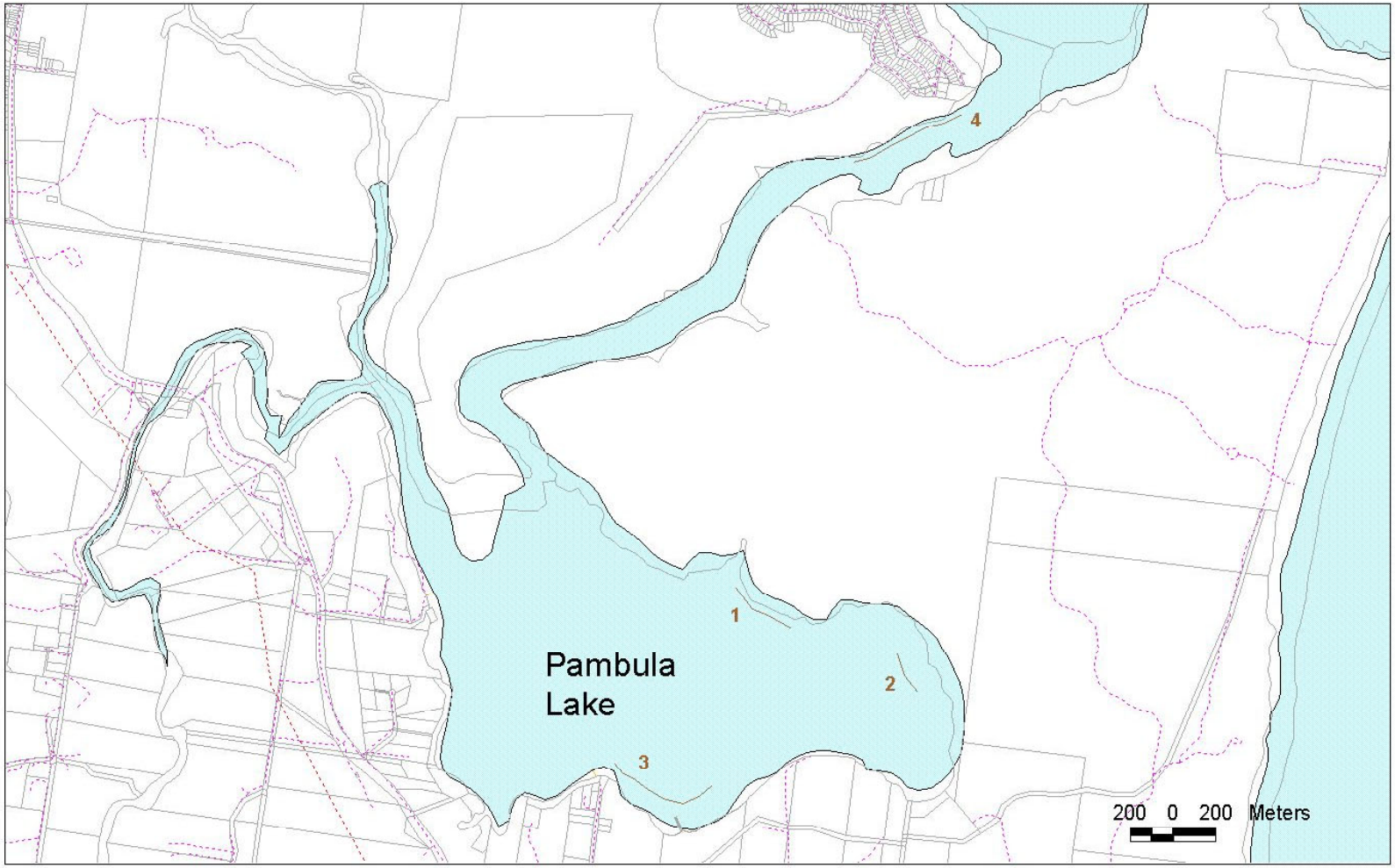
Flat oyster sampling and collection areas

Pambula Lake Flat Oyster Sampling and Collection Areas



7.3. Parasites and lesions observed in *O. angasi* from NSW

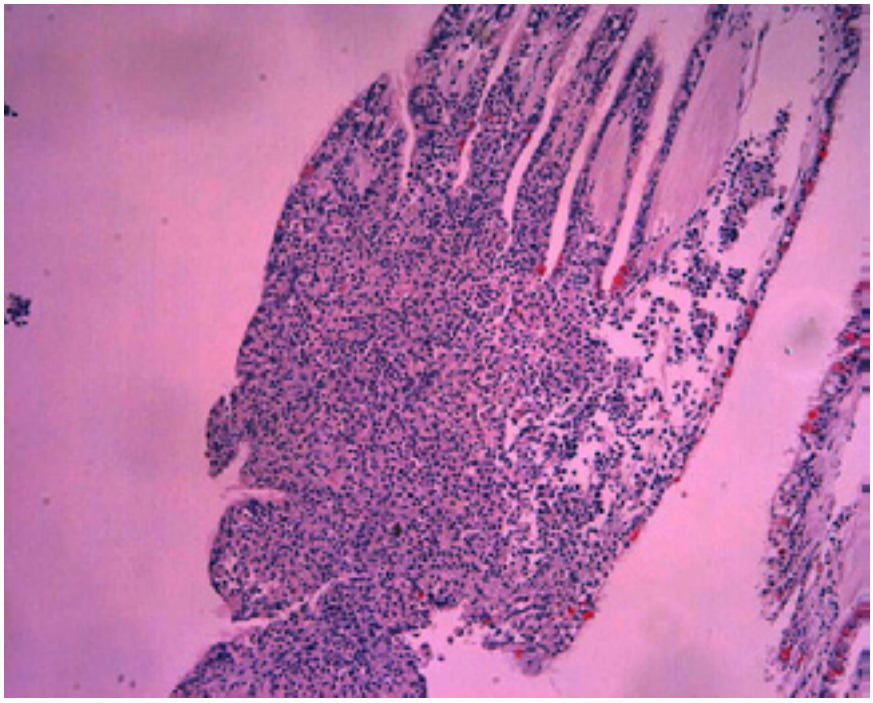


Plate 1. A focal area of haemocytosis in the gills of an oyster from Merimbula infected with *Bonamia*-like microcells. H&E, 100x magnification.

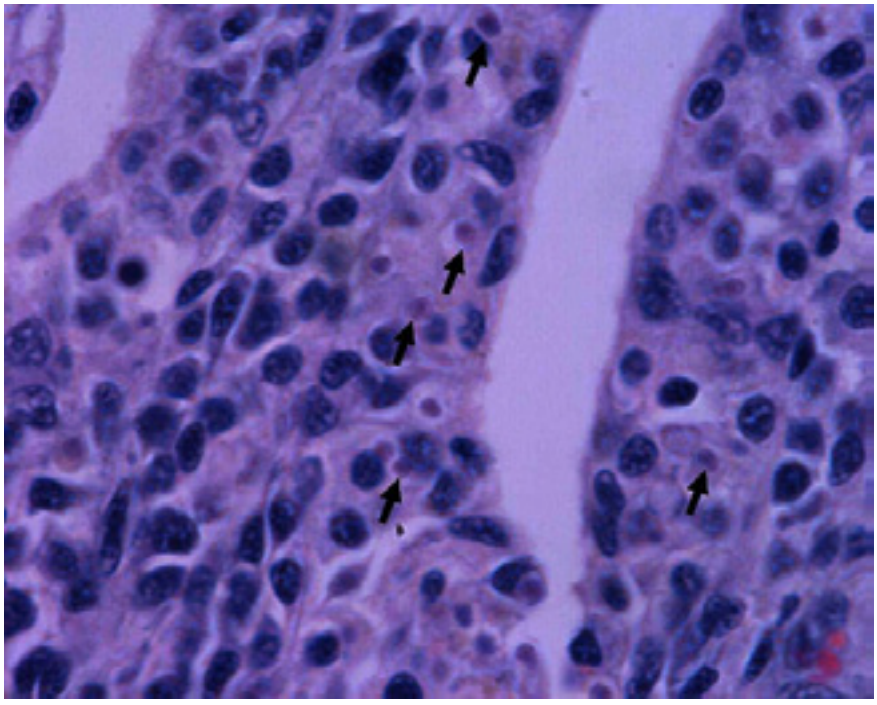


Plate 2. Numerous *Bonamia*-like microcells (arrows) within a focal area of haemocytosis in the gills of an oyster from Merimbula. H&E, 1000x magnification.

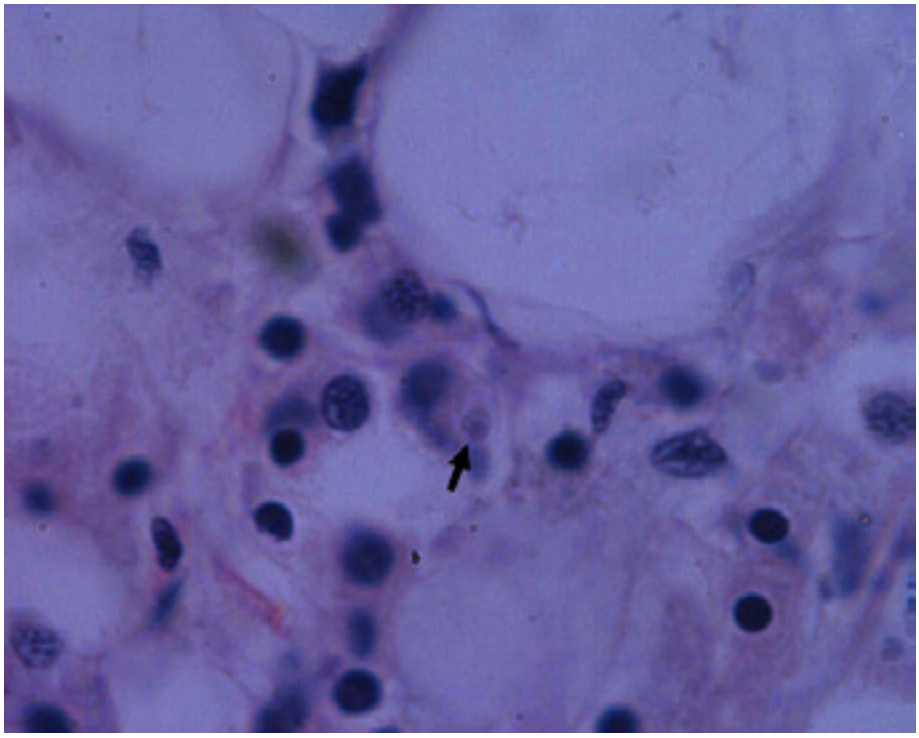


Plate 3. A single *Bonamia*- like microcell (arrow) in the vesicular connective tissue of a lightly infected oyster from Merimbula. H&E, 1000x magnification.

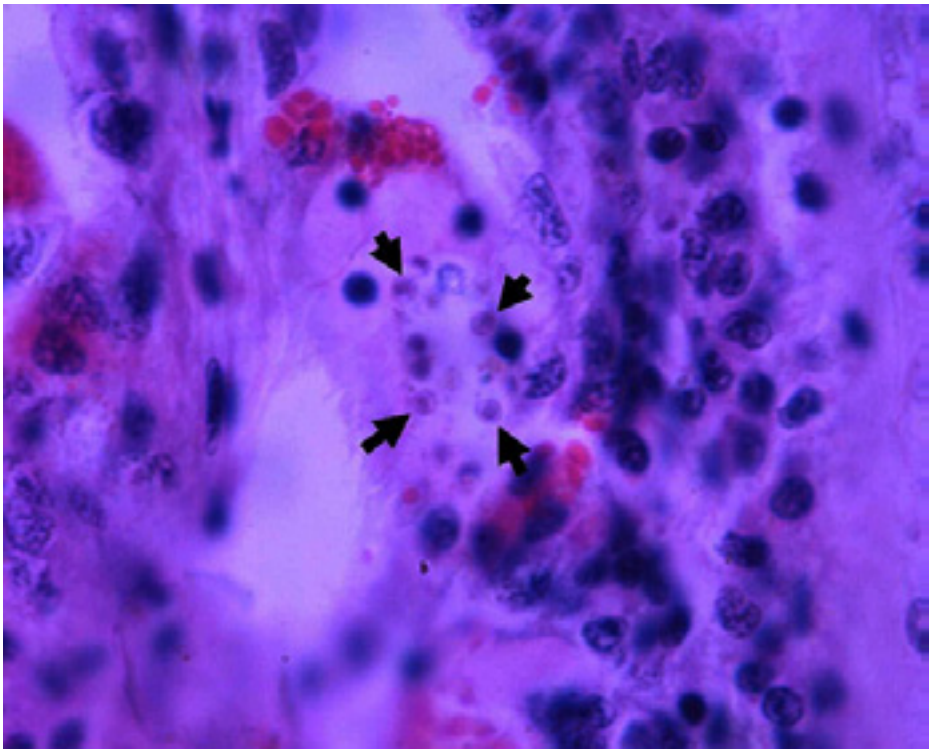


Plate 4. Gills of an oyster from Narooma with 4 *Bonamia*- like microcells (arrows) evident adjacent to a focal area of haemocytosis. H&E. 1000x magnification.

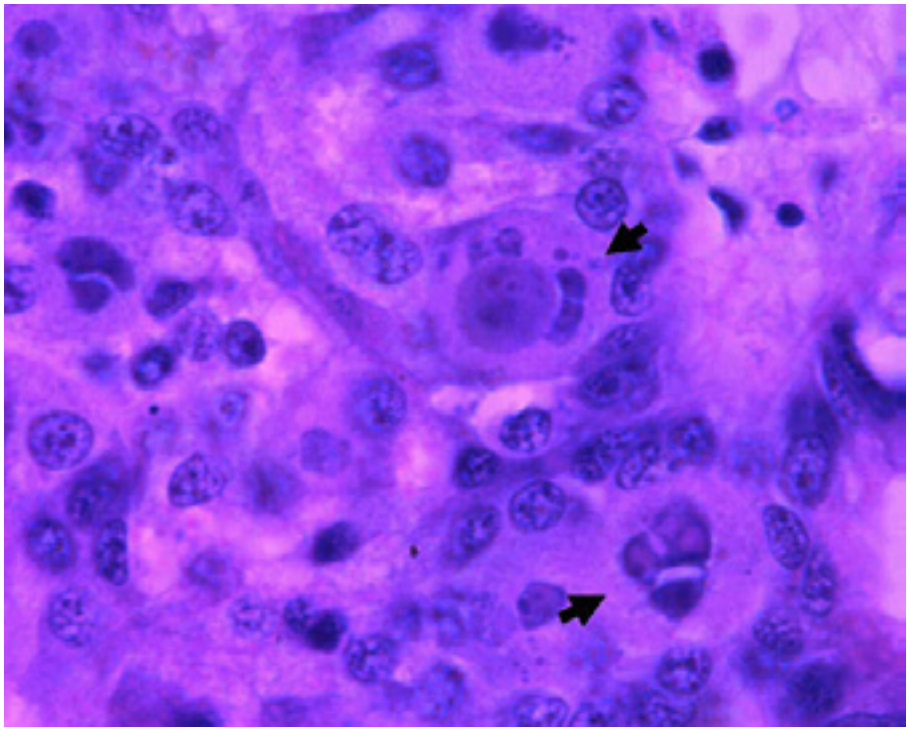


Plate 5. Two nurse cells (arrows) in the digestive gland epithelium of an oyster from Narooma. The cells contain two and three daughter cells of an unidentified *Marteilia* sp. H&E 1000x magnification.

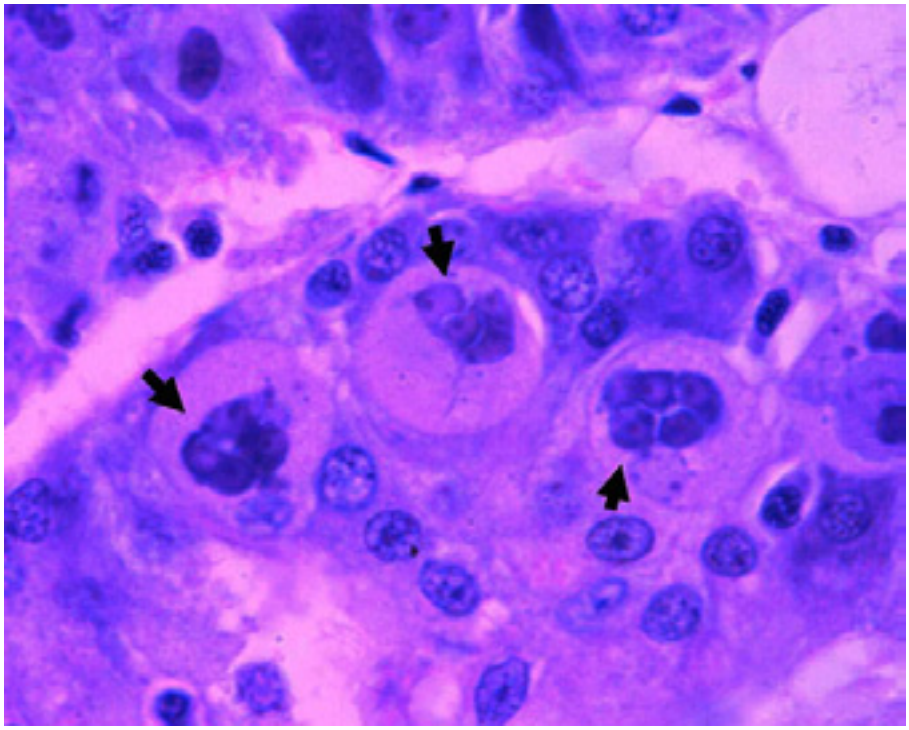


Plate 6. Three adjacent nurse cells (arrows) containing up to 5 *Marteilia* sp. daughter cells. H&E, 1000x magnification.

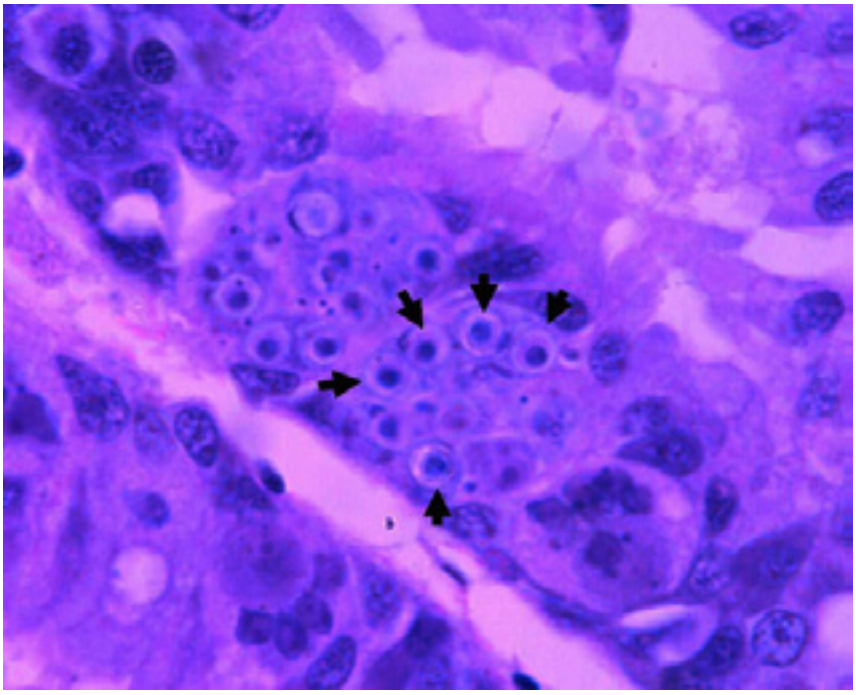


Plate 7. A sporangiosaurus-like structure in the digestive gland epithelium containing at least 5 spore-like bodies (arrows). Adjacent cells are also infected. H&E, 1000x magnification.

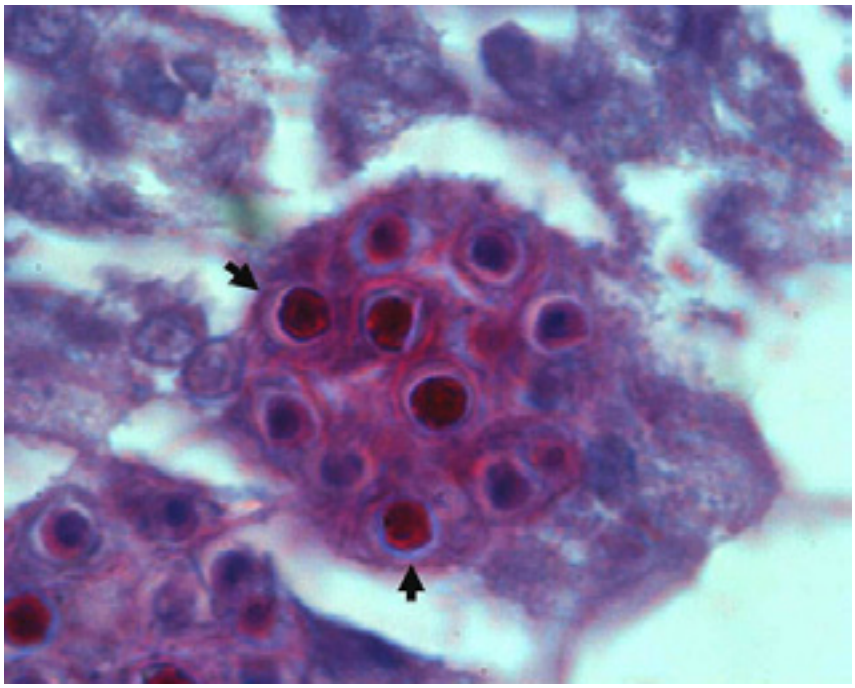


Plate 8. Zeihl Neelsen stain of a sporangiosaurus-like structure showing the spore-like bodies to be acid fast (arrows). ZN stain, 1250x magnification.

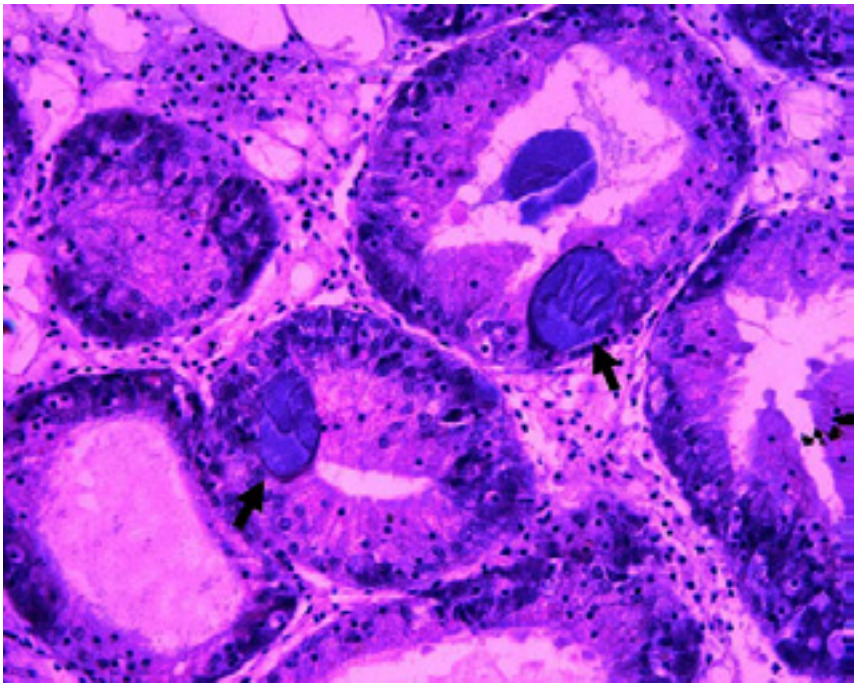


Plate 9. Basophilic chlamydiales-like inclusions (arrows) in the digestive gland epithelium of an oyster from Pambula. The lines through the inclusions are probably due to bacteriophage infection. H&E, 200x magnification.

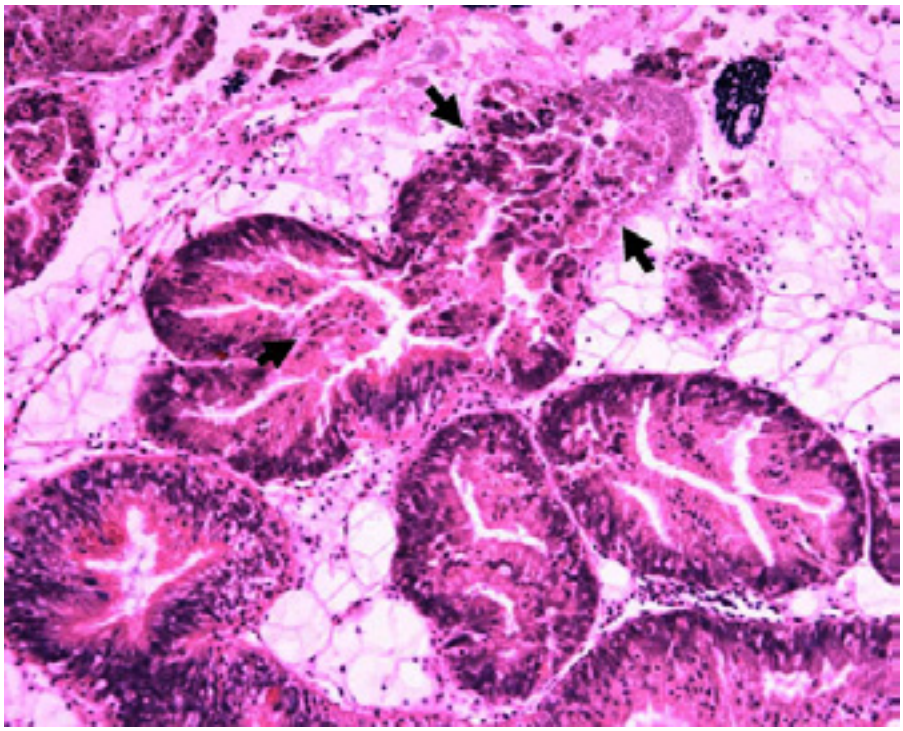


Plate 10. Focal lysis and necrosis (arrows) of the epithelium of a digestive gland tubule in an oyster from Pambula. H&E, 200x magnification.

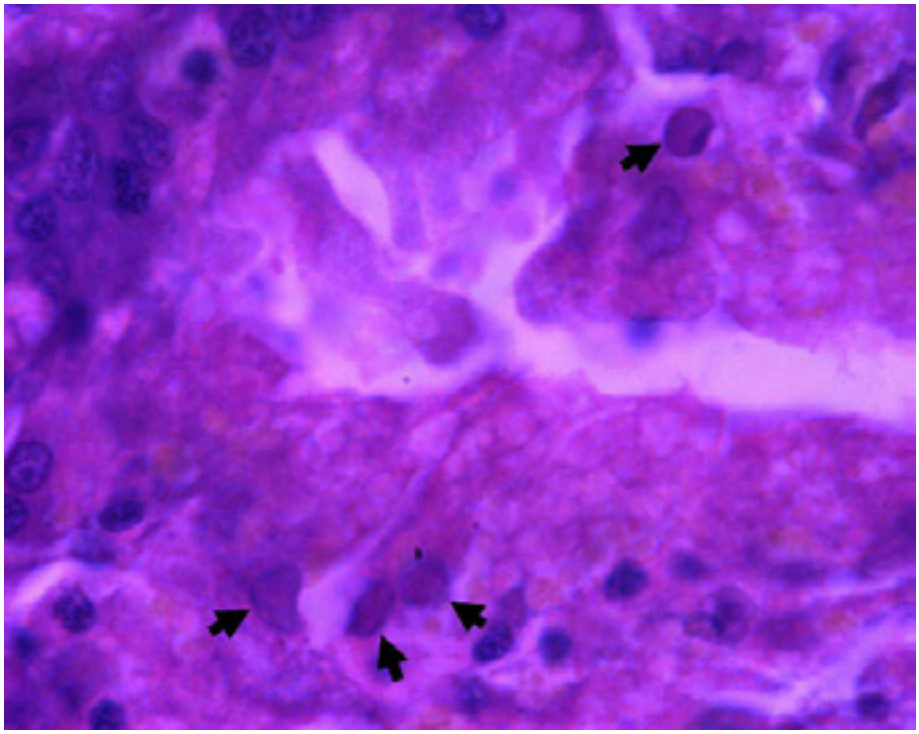


Plate 11. High power view of sloughing digestive gland cells with hypertrophied nuclei containing marginated chromatin and virus-like inclusion bodies. H&E, 1000x magnification.

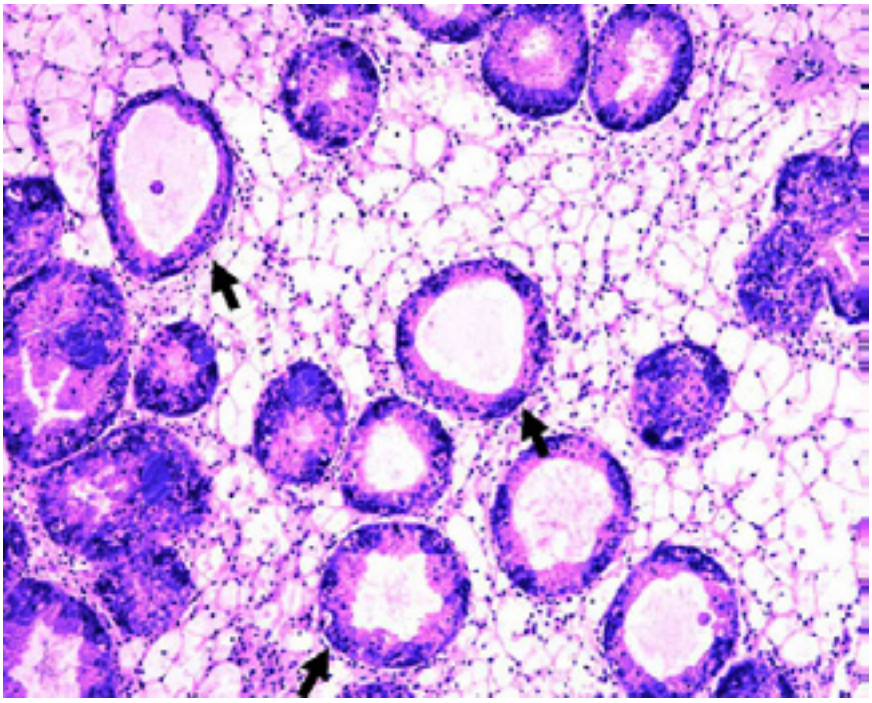


Plate 12. Atrophy of digestive gland tubule epithelia (arrows) in an oyster from Pambula which also is infected by chlamydiales-like organisms and is exhibiting moderate diapedesis across digestive tubule epithelia. H&E, 200x magnification.

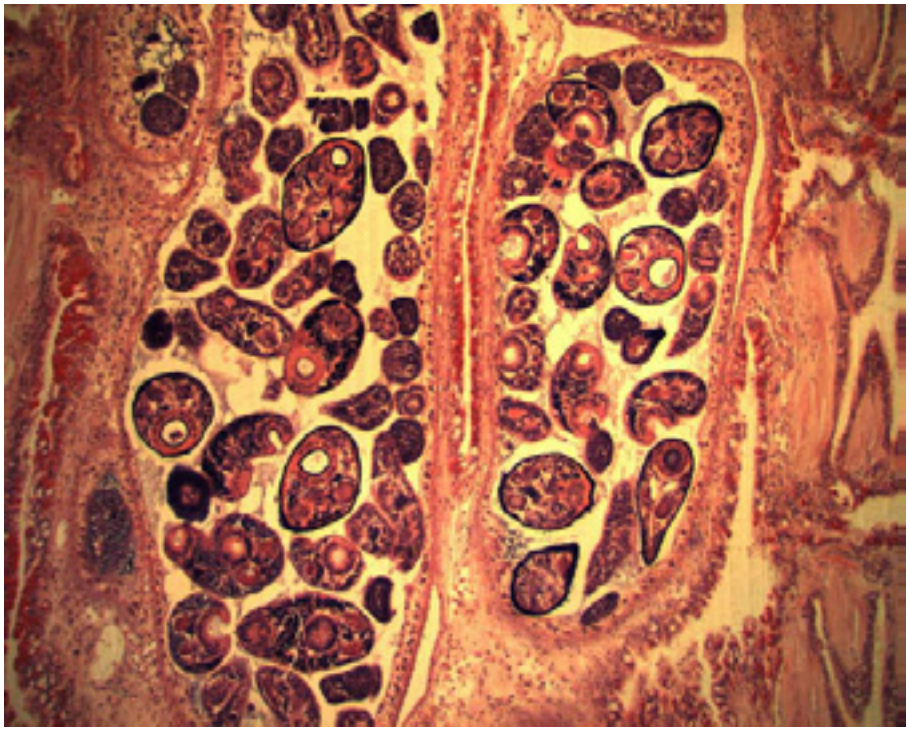


Plate 13. Sporocysts of a digenean in the gills of an oyster from Pambula. 40x magnification.

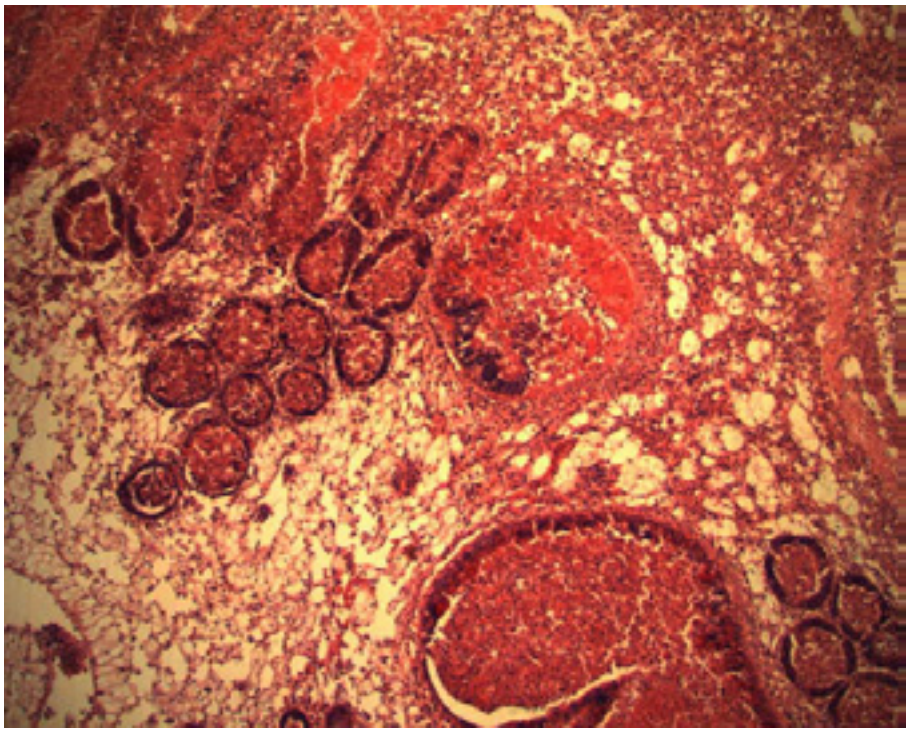


Plate 14. Low power view of an oyster from Pambula with a bacterial infection resulting in acute haemocytosis and massive necrosis and sloughing of digestive gland epithelium. 40 x magnification.

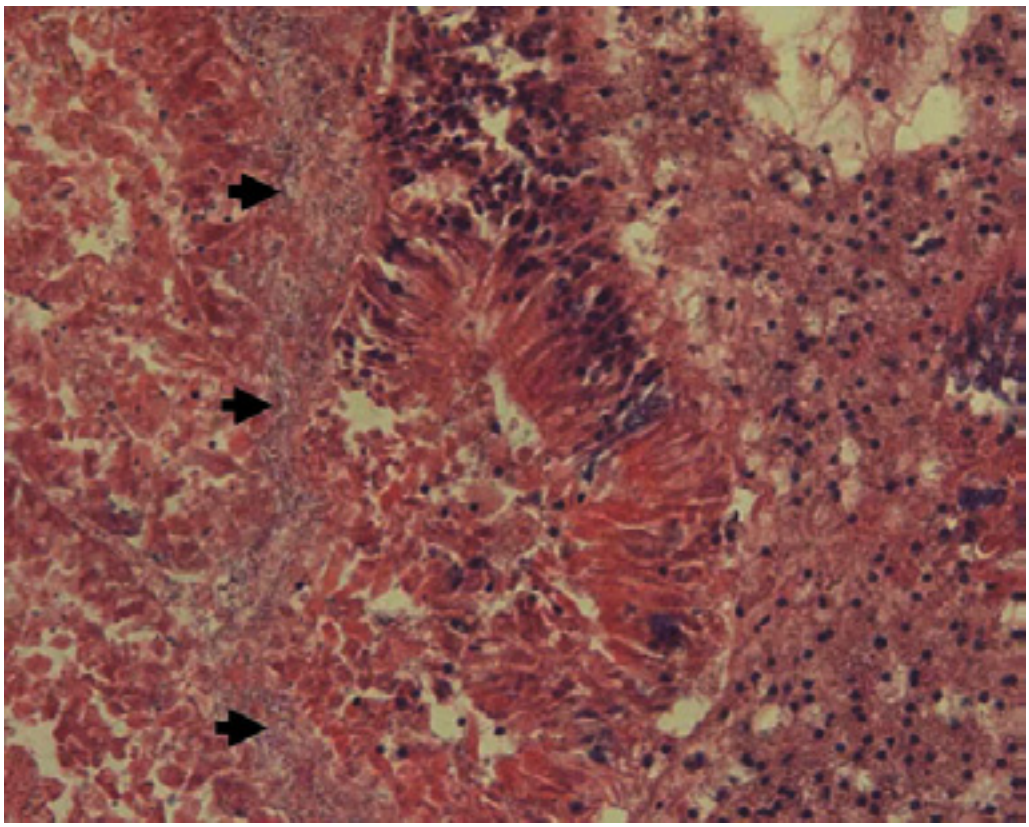


Plate 15. High power view of a bacterial infection in an oyster from Pambula. Note large numbers of rod shaped bacteria in the section (arrows), necrosis of the left side of the digestive gland tubule and a large area of cellular debris to the right of the picture. 400x magnification.

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